

# **IMMUNOLOGY and SEROLOGY**

**BY**

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TO

*Charles A. Stuart*

*THERE is an old imaginary account of men chained from childhood in a cave in such a manner that they cannot even turn their heads. All they see is the shadows of objects cast upon the wall which they face. They perceive neither the depth nor the weight nor the material of these objects and can only speculate about their uses and manner of operation.*

*Until recently antibodies seemed but images with no more substance than the shadows on the wall. Their existence was hardly doubted because only by postulating their activities could the behavior of antiserums be explained. Sixty-five years of research have sharpened our perception so that we can now make shrewd guesses about the properties and mode of action of antibodies. Perhaps the next sixty-five years will remove the chains which restrict our vision and permit us to see their true form and function.*

## PREFACE

IMMUNOLOGY is a hybrid science; its ancestors include bacteriology, physiology, chemistry, and physics. Laymen may reasonably think of it as the study of specific resistance to infectious disease. The physician also is most concerned with this practical aspect of the science. To the biologist, however, immunology is a physiologic phenomenon, an expression of ecologic principles and the laws of natural selection and evolution. The chemist is challenged by the physicochemical peculiarities of the immunologic response and antigen-antibody reactions and may go so far as to think of them completely apart from the animal.

Immunology provides tools which assist in diagnosis of disease, in classification of bacteria, plants and animals, and identification of certain of their components. It is therefore of service to physicians, bacteriologists, botanists, zoologists, criminologists, food inspectors, and many others.

Immunology and serology, the subjects of this book, include the study of resistance to infectious disease and the properties and behavior of anti-serums, or more particularly of the antibodies formed within an animal in response to foreign antigenic substances. Greater emphasis is placed upon serology than upon immunology, partly because more is known about it. Most of the points of view and applications mentioned previously are considered in their proper places.

About fifteen years ago the author was "persuaded" to teach a course in immunity and serology. His principal qualifications were intense interest and eagerness to learn. During the several months of concentrated preparation which preceded the first class meeting he became increasingly aware of a shortage of textbooks in the field, particularly at the advanced undergraduate and early graduate level. Annually thereafter publishers were asked whether they had such a book, until finally the inevitable suggestion was made, "Why don't you write one?"

The outline adopted closely followed that which had evolved during several years of teaching. It soon became apparent why few had attempted a book of this kind. Information is widely scattered and often contradictory, and the field is expanding rapidly, so that it is difficult if not impossible to write a concise and straightforward account. Inevitably some material will be out of date by the time of publication, but the author hopes that sufficient background is presented for understanding current literature. He is also aware that certain phases of the subject are treated

incompletely; the alternative seemed to be considerable expansion without equivalent clarification. Some controversial matter has been presented in the hope that the student will realize that immunology is still young and that its future development demands the best brains available.

At one stage Dr. C. A. Stuart of Brown University planned to be a co-author and worked for untold hours with early drafts of the manuscript. His broad experience, critical judgment and directness of expression appear on nearly every page. The author will never be able to express adequately his appreciation of Dr. Stuart's assistance and regret that the pressure of other duties forced him to withdraw from active participation in the final stages of writing.

The author has also had the benefit of valuable suggestions from Dr. Martin Frobisher, Jr., Dr. Henry P. Treffers, Dr. Merrill W. Chase, and Dr. Johannes Ipsen. Others to whom he is indebted include Mr. Carl R. Woodward, Jr., and Miss Grace A. Catania. He takes special pride in expressing gratitude to his wife, Mrs. Helen E. Carpenter, for her inspiration and encouragement, her patience with a preoccupied husband, and the innumerable hours she spent at a typewriter deciphering deletions, interlineations and other manuscript changes.

Thanks are due to Dr. Adrianus Pijper, Miss Keturah Blakely, the American Instrument Company, and the Specialized Instrument Corporation for certain photographs which they kindly supplied.

It is a pleasure to acknowledge a grant-in-aid by the University of Rhode Island to assist in the collection and cataloguing of literature and a fellowship from the Fund for the Advancement of Education which made possible a year of uninterrupted study.

The author is happy to share credit for any virtues which the book may possess with all who so generously gave their time and constructive criticism. He alone assumes responsibility for any faults which it retains and will appreciate being informed of them.

*Kingston, Rhode Island*

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## CONTENTS

<i>Chapter 1.</i>	
INFECTION AND IMMUNITY .....	1
<i>Chapter 2.</i>	
THE IMMUNE REACTIONS .....	12
<i>Chapter 3.</i>	
ANTIGENS .....	24
<i>Chapter 4.</i>	
SERUM PROTEINS .....	56
<i>Chapter 5.</i>	
PRODUCTION OF ANTIBODY .....	74
<i>Chapter 6.</i>	
THE ANTIGEN-ANTIBODY REACTION .....	96
<i>Chapter 7.</i>	
PRECIPITATION .....	120
<i>Chapter 8.</i>	
AGGLUTINATION .....	141
<i>Chapter 9.</i>	
ISOHEMAGGLUTINATION .....	169
<i>Chapter 10.</i>	
TOXINS AND ANTITOXINS .....	186

<i>Chapter 11.</i>	
PHAGOCYTOSIS .....	210
<i>Chapter 12.</i>	
CYTOLYSIS AND COMPLEMENT FIXATION .....	229
<i>Chapter 13.</i>	
ANTIVIRAL IMMUNITY .....	251
<i>Chapter 14.</i>	
ALLERGY .....	269
<i>Appendix</i>	
EXPERIMENTS IN SEROLOGY .....	299
<i>Index</i> .....	327

## Chapter 1

### INFECTION AND IMMUNITY

ANY DISEASE PROCESS, whether produced by a physical, chemical or biologic agent, is characterized by the abnormal physicochemical behavior of body cells. The obvious signs of disease—malaise, inflammation, fever, pain—are indicative of more deep-seated disturbances such as interference with cellular metabolism and respiration or cell death. The specific response typical of a given disease depends upon the particular physicochemical processes concerned and the location of the affected cells.

#### THE NATURE OF INFECTIOUS DISEASE

Infectious diseases are those caused by bacteria, viruses, rickettsiae, fungi and protozoa. The occurrence of infectious disease is a biologic accident. Any organism can multiply in a living host from which it can secure nutrient materials. A true parasite such as a virus requires living host cells; it inadvertently damages the host by upsetting the normal physiologic balance in the affected cells. Among the Schizomycetes there are no true parasites, but these organisms may invade a living host and secrete toxic substances or liberate materials during autolysis which induce an abnormal physiologic state. Living forms are normally in a condition of delicate physicochemical balance, and any interference like that caused by an infectious agent tends to destroy this balance. The host often reacts strongly to any foreign agent which penetrates its external defenses.

**Pathogenicity or Virulence.** The capacity of an organism to produce disease under the conditions within a particular host is known as its pathogenicity or virulence. Production of infectious disease, in contrast to other kinds of disease, is complicated by the ability of the pathogen to invade the tissues of the host, to multiply and to manufacture substances which provoke a more or less violent response. These processes contrib-

ute to the virulence of the organism and are fundamentally chemical in nature.

**Invasiveness.** Ever since the cause of anthrax was discovered, the various cell components and growth products of bacteria associated with the ability of the organisms to spread among tissues have been subject to investigation. Unknown "spreading factors" were early postulated. One of these may be hyaluronidase, formed by *Clostridium perfringens*, streptococci, pneumococci, certain micrococci and other organisms. This enzyme increases tissue permeability by hydrolyzing hyaluronic acid, a viscous high molecular weight polysaccharide which constitutes part of the intercellular "cement" of many tissues. Injected solutions and suspensions of India ink diffuse more rapidly from the site of injection in the presence of hyaluronidase than in its absence. There is little question that hyaluronidase promotes the diffusion of toxic bacterial products, but its role in the spread of bacteria is still in dispute.

Certain hemolytic streptococci, gas gangrene bacteria and other organisms produce *streptokinase*, formerly called fibrinolysin. This substance activates plasminogen, a proteolytic enzyme in the plasma of certain animals. Activated plasminogen dissolves fibrin clots which normally aid in the localization of various infections. Many proteolytic or other enzymes which participate in the metabolism of a microorganism very likely assist invasion by digesting tissues in the vicinity of a local infection. The *collagenase* of *Cl. perfringens* disintegrates muscles *in vivo* by decomposing the reticular supporting structure of the tissue. *Hemolysins* produced by a variety of pathogenic bacteria dissolve not only erythrocytes but other types of cells and are believed to contribute to invasiveness. Some bacterial components, such as *capsular polysaccharides and polypeptides*, contribute indirectly to invasiveness by preventing normal phagocytic ingestion and destruction of the organisms, thereby permitting continued multiplication and penetration of tissues.

**Toxicity.** Toxic substances contribute greatly to the virulence of a microorganism. *Exotoxins* are excreted by *Cl. botulinum*, *Cl. tetani*, *Cl. perfringens*, *Corynebacterium diphtheriae*, *Shigella dysenteriae*, *Staphylococcus aureus*, some hemolytic streptococci and a few other bacteria. Certain exotoxins are among the most potent poisons known, but with one exception their mode of action has not been learned. The  $\alpha$  toxin of *Cl. perfringens* is a lecithin-hydrolyzing enzyme.

Endotoxins are produced by the cells of bacteria and are released only after death and autolysis. They are found particularly in gram negative bacteria, and are considered by some authorities to be polysaccharide-lipid-protein complexes. They produce nonspecific lesions and symptoms in experimental animals. The most characteristic signs in mice injected intraperitoneally are diarrhea and intestinal congestion, followed in a few hours by death if the dose is sufficiently large. Some relatively

nonpathogenic bacteria like *Escherichia coli* contain materials which produce these symptoms.

The readiness with which an organism undergoes autolysis indirectly influences the course of infection, whether endotoxins or other substances are responsible for the observed signs of disease. The pneumococcus is very susceptible to autolysis; pneumococcal pneumonia is characterized by its sudden onset and rapid course. The typhoid organism, on the other hand, does not readily autolyze, nor is the disease rapid in onset or course. Susceptibility to autolysis is perhaps a more important factor in the pathogenicity of bacteria than has been appreciated.

**Alterations in Virulence of Pathogens.** The virulence of pathogens is subject to change as are most properties of living beings. Natural and induced attenuation of bacterial disease-producing power has been known since Pasteur's early work with immunizing agents for chicken cholera and anthrax, but the chemical basis of decreased virulence is still not clear in most instances.

**Previous History of the Infectious Agent.** Pasteur noted that cultures of the chicken cholera organism which had aged in his laboratory were no longer capable of producing typical disease. *Bacillus anthracis* apparently lost its virulence by cultivation at a high temperature. Many pathogenic bacteria produce severe and typical infection when isolated from active cases of disease and introduced promptly into susceptible experimental animals. Such organisms maintained in the laboratory on artificial media usually lose some or all of their disease-producing power. Cultures of pathogenic bacteria often consist of mixtures of the normal, virulent form and an avirulent variant. Fresh isolates from patients consist chiefly of the virulent organism, but under certain conditions of cultivation the proportion of avirulent cells increases greatly. Strains of some bacteria have apparently completely lost the virulent form after prolonged growth outside an animal host.

**Biologic State of the Pathogen.** Loss of virulence often accompanies the "smooth-rough" transformation, first reported by Arkwright in 1921.<sup>1</sup> Many bacteria, particularly gram negative rods, dissociate into two forms: the smooth form produces small, raised, entire, moist colonies characteristic of fresh isolates; the rough variant gives large, flat, irregular, dry colonies. Rough forms can appear spontaneously at any time, either in cultures or within the body during the course of disease. *Shigella sonnei* isolated from convalescent patients is frequently in the rough state. Although less virulent, rough forms do not necessarily lose toxigenicity; for example, a rough strain of *Sh. dysenteriae* has been used for several years as a potent source of neurotoxin. Loss of virulence accompanying smooth-rough dissociation may therefore be caused by decreased invasiveness.

Capsulated bacteria are in general more highly virulent than non-capsulated forms of the same species. The pneumococcus retains its

disease-producing power only as long as it possesses a capsule. Mice may be killed by one to five viable capsulated organisms injected intraperitoneally, whereas 100,000,000 to 200,000,000 noncapsulated cells of the same serologic type are required to produce the same result. Experimental infections by the type 3 pneumococcus in mice, rabbits and monkeys have been successfully treated with an enzyme which hydrolyzes the capsular polysaccharides. The capsule protects the microorganism against ingestion by the various phagocytic cells of the body.

Our knowledge of virulence is so fragmentary that it is impossible to generalize regarding the chemical characteristics which distinguish pathogenic from nonpathogenic bacteria. Much investigation has been devoted to the effects of parasitic microorganisms upon their hosts but relatively little to the effects of the host upon its parasites. Exploration of this field may be expected to yield valuable information concerning the nature of virulence, the relative virulence of different organisms, the comparative susceptibilities of various hosts and the mechanisms of resistance to and recovery from infectious disease.

#### NORMAL PROTECTION AGAINST INFECTIOUS DISEASE

All individuals possess three types of protection against infectious disease. (1) *Nonsusceptibility* represents an absolute protection against particular diseases and is associated with species characteristics. (2) *Natural immunity* varies in degree and is directed against specific diseases. It depends upon the presence of "natural" antibodies in the blood. Antibodies are proteins associated with the globulin fraction of blood serum. (3) *Resistance* is relatively nonspecific and variable and is determined by physiologic conditions which are subject to variation from one individual to another and within a single individual at different times. Hence, resistance is an individual matter.

**Nonsusceptibility.** Nonsusceptibility is determined by physiologic and anatomic factors associated with the species of animal and is not dependent upon detectable antibodies. It is inherited to the same extent as any other species characteristic. Nonsusceptibility and natural immunity have been poorly defined in much of the literature, and therefore many observations or experiments have been incorrectly interpreted. It is misleading to designate as natural immunity those instances of resistance to disease in which natural antibodies have not been demonstrated on the ground that some kind of antibody may eventually be found.

Nonsusceptibility is illustrated by the characteristic occurrence of infections in certain species but not in others. Man is nonsusceptible to spontaneous infection by a large number of animal pathogens, such as chicken cholera, canine distemper, hog cholera and cattle plague. Lower animals are nonsusceptible to many human diseases, including Asiatic

cholera, dysentery, measles, gonorrhea, syphilis, influenza, mumps, typhoid fever and whooping cough. Cold-blooded animals are nonsusceptible to tetanus toxin.

Certain physiologic factors such as body temperature and diet contribute to nonsusceptibility. The influence of body temperature is shown by the classic early experiments with anthrax infections of frogs and chickens. These animals are normally nonsusceptible to anthrax. Frogs inoculated with *Bacillus anthracis* and warmed to 35° C. succumbed to the infection, and chickens were killed by anthrax when their body temperature was artificially reduced from its normal of about 41° C.

Nonsusceptibility which depends upon a physiologic factor like body temperature is more apparent than real, because infection may be possible when physiologic conditions are altered, thus permitting multiplication of the pathogen. The requirement by an organism for a particular accessory growth factor such as tryptophane or cystine may limit its ability to multiply. Only those hosts which provide the growth factor in available form will support multiplication of the organism. Availability of the growth factor sometimes depends upon the host's diet, which is subject to change. Dogs are ordinarily nonsusceptible to spontaneous anthrax infection but can successfully be infected when fed an herbivorous diet. The turtle is nonsusceptible to tetanus. It is completely unaffected by the toxin of this organism and can tolerate tremendous doses but does not produce antitoxin.

The rabbit never contracts typhoid fever, nor does it display typical symptoms when the organisms are injected because they do not grow extensively within the animal. Such an animal, however, will produce antibodies against the typhoid protein. Injection of a sufficient number of typhoid bacteria, either living or dead, produces fatal endotoxemia.

**Natural Immunity.** Natural immunity is attributed to antibodies present or appearing without obvious external stimulus. The concentration of natural antibodies is usually low. "Immune" antibodies result from actual infection or artificial immunization and sometimes reach high concentration, but this high concentration rarely persists.

The origin of natural antibodies against infectious agents is by no means certain. They are frequently present in the blood of the newborn, tend to decrease to a very low concentration in the infant and return during childhood to a moderate level which is maintained throughout early adulthood. Highest amounts of pneumococcal antibodies, for example, are found in young adults; it may be coincidental that pneumonia mortality is lowest at this period. It is difficult to know what actually constitute normal antibodies because there is great variation between individuals and because antibodies reacting with a given organism may be actively acquired by undiagnosed or subclinical infection with the same organism or by infection with a related organism.

Normal antibodies for *Diplococcus pneumoniae*, *Salmonella typhosa* and other organisms are found in human serums, occasionally in considerable amount. Most animals possess normal antibodies for some bacteria. Natural typhoid antibodies have been found in rabbits and guinea pigs; typhoid, cholera and dysentery antibodies in horses; and pneumonia and dysentery antibodies in rabbits.

Formation of the blood group antibodies (isohemagglutinins) of man is definitely under genetic control. These antibodies, which appear within a few months after birth, react with the red blood cells of some other human individuals. They attain peak concentration by the age of ten years and slowly decrease thereafter.

Natural antibodies against foreign erythrocytes are found in the blood of various animal species. Swine possess antibodies for red cells of the sheep, goat, rabbit and human. Rabbits possess antibodies which react with erythrocytes of the guinea pig, horse, sheep and human. Human serums contain natural antibodies for rabbit, sheep, ox, horse, guinea pig and pigeon erythrocytes.

Wheeler found that rabbits of three inbred families possessed unusual amounts of antibody acting on human red cells at 2° C.<sup>6</sup> Stuart and co-workers reported that all rabbits of one particular family contained normal antibodies for human erythrocytes of group A and were exceptionally vigorous producers of A antibodies when injected with homologous cells.<sup>8</sup>

The genetic origin of hemagglutinins is easily demonstrated, but the role of heredity in natural immunity to disease is difficult to prove. Hirschfeld employed the Schick test to investigate immunity against diphtheria in members of fifty families.<sup>2</sup> In general, children of immune parents were immune, and children of nonimmune parents were non-immune. If only one parent was immune, children who inherited the blood group of the immune parent were usually immune to diphtheria; children of the same blood group as the nonimmune parent were non-immune. This was true whether the immune parent was the father or the mother. These observations, together with the increase of normal antitoxin at some time after birth, suggested a process of genetically controlled serologic maturation. This interesting hypothesis is still subject to confirmation.

**Resistance.** The normal body possesses remarkable nonspecific resistance to infection. The first line of defense consists of the physical and chemical barriers presented by epithelial tissues. The relative impenetrability of the skin, the stickiness of mucous membranes, ciliation of the upper respiratory tract, and the acidity or alkalinity of various parts of the digestive system are more or less effective against numerous pathogens or potential pathogens.

Certain other physiologic or pathologic characteristics of the host,

such as its general state of nutrition, debilitation resulting from aging, fatigue, exposure to extreme temperatures, alcoholism or disease, also play an important role in determining the likelihood of infection and in deciding the outcome of established disease. Environmental factors including climate and geographic location, population density, community sanitation and other socio-economic conditions influence the probability of infection in any given individual. These factors lie outside the scope of immunology *per se*, although they indirectly determine the opportunity for infection and development of immunity.

Resistance to certain pathogens is genetically controlled. Webster found that orally administered *Salmonella enteritidis* killed 37.4 per cent of normal Rockefeller Institute albino mice.<sup>4</sup> Selective breeding yielded lines of mice of which 85 and 15 per cent, respectively, succumbed to experimental oral infection. These percentages were not altered by continued selection. Feces of these animals were repeatedly examined to exclude accidental infection with *S. enteritidis*, but unfortunately the presence or absence of antibodies in the blood was not determined. Since 37.4 per cent of the unselected mice were killed by oral infection, the parent strain was susceptible to the infectious agent, and the increased and decreased mortality in the two lines appeared to indicate inheritance of resistance apart from immunity. Cross breeding with other inbred strains of mice demonstrated that resistance factors were dominant and were not sex linked. Further tests showed that *S. enteritidis* given orally appeared in the blood stream of nonresistant mice more promptly, in larger numbers and in a greater percentage of cases than in the blood of resistant mice. Moreover, the organisms persisted in the feces in larger numbers and for a longer period than in resistsants. Fifty per cent of surviving resistant mice harbored typical *S. enteritidis* in their spleens fifty days after infection. A test for antibody in the blood of these surviving resistant animals indicated that their resistance was not dependent upon the presence of circulating antibody.

The evolution of resistance in human populations is shown by the contrasting response of "civilized" and primitive groups to tuberculous infection. Tuberculosis occurs in chronic form with frequent recovery among civilized people, in whom it has been present for many generations. The previously unexposed native population of Tasmania rapidly died when tuberculosis was introduced. Nine thousand Kaffirs brought to Ceylon by the Dutch were completely wiped out by the same disease. The history of tuberculosis has been similar in other primitive peoples. All are susceptible and lack resistance when they first encounter the tuberculosis organism. A population which escapes extermination, however, undergoes a process of "tuberculization" through decades and centuries, and the disease becomes gradually more chronic and less severe. Under favorable conditions a high state of resistance is approached.

Examples of resistance associated with racial differences are drawn chiefly from lower animals, since socio-economic and other environmental factors make it difficult to secure statistically valid data from human populations. Algerian sheep are more resistant than European sheep to anthrax. Likewise, most races of chickens except barred Plymouth Rocks and a few others are resistant to Rous sarcoma.

The erroneous statement is frequently made that *susceptibility* to pneumonia is increased by primary infection with other organisms such as measles and influenza viruses, *S. typhosa* and *Hemophilus pertussis*, or by chronic alcoholism, malignant tumors or surgery. These infections or conditions obviously diminish the *resistance* of the individual. Lowered resistance may result from impaired phagocytic activity together with increased permeability of mucous membranes and other tissues. The causative organisms of pneumonia therefore become established and multiply, with consequent development of typical pneumonia.

#### ACQUIRED IMMUNITY AGAINST INFECTIOUS DISEASE

An individual who recovers from plague, cholera, yellow fever or various other diseases is usually immune to second attacks of the same disease. Immunity against one infectious agent may contribute marked, little or no immunity against other infectious agents, depending upon the relationships of the causative organisms.

Immunity is never absolute. The size of the infecting dose of organisms is important in determining whether or not a second attack occurs. An individual may possess sufficient immunity to protect against ordinary contact but not enough to overcome massive exposure. The immunity which develops from typhoid fever, smallpox, chickenpox and mumps is often sufficient to protect against reinfection. On the other hand, little if any immunity is conferred by cases of gonorrhea, pneumonia, influenza and some other diseases.

Immunity depends upon the presence of antibodies within an animal or upon the animal's ability to produce antibodies quickly in the presence of the infectious agent or certain of its components. Actual infection is not the only means of acquiring antibody or of stimulating the antibody-producing mechanism. Immunity against many pathogenic agents can be engendered or transmitted artificially.

**Active Immunity.** Active immunity develops following natural or artificial stimulation of the antibody-producing mechanism. In addition to obvious, clinical cases of disease, unrecognized or subclinical infections may also induce immunity. Many persons evidently suffer mild attacks of scarlet fever, poliomyelitis or diphtheria which are never diagnosed as such but which nevertheless produce strong immunity.

The antibody-producing mechanism is stimulated artificially by intro-

ducing into the body the microorganisms which cause disease or some of their components or products. Nearly all pathogenic agents produce fatal disease only when a susceptible individual receives a sufficiently large dose, usually by a particular route. Smaller amounts, or organisms introduced by unnatural routes, may cause illness of short duration or only local irritation without clinical disease. Sublethal doses of virulent microorganisms are not considered sufficiently safe for human immunization but are employed in veterinary practice.

Attenuated organisms are safer immunizing agents. The virulence of microorganisms may be reduced by many methods. Pasteur accidentally discovered one method when he inoculated fowl with old cultures of chicken cholera bacteria. The inoculated chickens survived later introduction of many lethal doses of virulent chicken cholera organisms. He also discovered that the anthrax bacillus cultivated at an unusually high temperature ( $42^{\circ}$  to  $43^{\circ}$  C.) lost its ability to produce more than a transitory illness but induced immunity against virulent bacilli. A third method of attenuation was effective with the virus of hydrophobia. Dried spinal cords of infected rabbits lost their pathogenicity in proportion to the duration of desiccation, while retaining their immunizing capacity. Another method of attenuating viruses consists of cultivating them in unnatural hosts. The French neurotropic strain of yellow fever virus was attenuated for man by serial passage through mice and has been used for human immunization. A second yellow fever virus strain, after many transfers through tissue cultures, became sufficiently attenuated for human inoculation and is in wide use at present.

Injection of killed microorganisms is the preferred means of stimulating active immunity against many diseases. Much larger numbers of killed cells may be administered than is possible with viable organisms. Phenol, tricresol, formalin, acetone or Merthiolate are widely employed killing agents. Heat is sometimes used but is likely to diminish the immunizing power of a bacterial suspension even though the temperature is kept at the minimum consistent with sterilization (e.g.,  $56^{\circ}$  C. for one hour). Chemical disinfectants must be carefully selected because some chemicals combine with bacterial proteins to form complexes having different specificity from that of the living cells. Killed suspensions are always thoroughly tested for sterility before release for human use. The final preparations are known as *bacterins* or *vaccines*. The latter term is more properly reserved for the specific immunizing agent of smallpox.

Chemical extraction has been employed in attempts to secure immunizing substances from the cells of dysentery, typhoid and other bacteria. Some of these, such as the typhoid "Vi antigen," have given indication of considerable effectiveness.

Bacterial exotoxins are potent immunizing agents. The immunity produced is generally strong and relatively permanent. Sublethal amounts of

unmodified toxins were originally employed but were soon found impractical on account of their toxicity and the number of injections necessary for satisfactory immunization. These difficulties were largely overcome by combining antitoxin produced in horses with the toxin in such proportions that the toxin was nearly neutralized. The toxin was apparently released slowly within the body and stimulated active antibody formation over a considerable period. Toxin-antitoxin has been a highly successful immunizing agent and is still employed, particularly in protecting adults against diphtheria.

Heat or formaldehyde converts most toxins into nontoxic but antigenic *toxoids*. Toxoids are so harmless that comparatively large doses may be administered with no risk of tissue damage. They are commonly used in immunization of children against diphtheria and tetanus. Toxoid is employed in the natural fluid form or precipitated by alum. Alum-precipitated immunizing substances are released slowly from the subcutaneous tissues and thereby accomplish about the same result as multiple injections of unprecipitated materials.

**Passive Immunity.** Passive immunization consists of the acquisition of antibodies which have been formed in another individual. It is usually only temporary, lasting a few weeks to a few months, whereas active immunity is ordinarily more durable.

The first months of an animal's life are relatively free from certain infectious diseases. This is attributed to antibody acquired from the mother, either by passage through the placenta or in the first milk after birth (colostrum). Antibody passage through the placenta occurs in animals such as man in which a single layer of cells separates the maternal and fetal circulations. Ruminants, on the other hand, possess four layers of cells separating the two circulatory systems, which prevent antibody from passing into the fetal blood. These animals secrete antibodies in the colostrum which the young ingest within a few hours after birth; the antibodies are absorbed through the wall of the digestive tract. An animal soon loses the immunity which it possessed at birth. This phenomenon is an interesting natural protective mechanism, because very young animals usually possess lower ability to produce antibody than more mature animals.

The widest use of artificial passive immunization is in the prevention of diphtheria, tetanus, gas gangrene, botulism and scarlet fever. Antitoxin is manufactured by inoculation of animals, often horses, with the respective toxins or toxoids. Prophylactic doses of antitoxin administered promptly to exposed persons prevent or modify the course of the various diseases. The temporary protection afforded by antitoxin is usually fortified by active immunization as soon as possible. Antitoxins are also administered in larger doses to individuals with active cases of disease and if given sufficiently early bring about dramatically rapid recovery.

Passive protection is sometimes provided by transfusion of blood from a convalescent individual or by introduction of human immune globulin. This processed material, first prepared on a large scale during World War II, is one of the protein fractions obtained from blood and contains antibodies protective against measles, infectious hepatitis, poliomyelitis and probably other diseases.

Antibacterial serums for treatment of pneumococcal pneumonia and cerebrospinal meningitis were formerly employed to help combat infection early in the disease, before the individual's antibody-producing mechanism had time to manufacture antibody. These antiserums were also prepared in horses or other animals. Antipneumococcal serum was credited with reducing pneumonia mortality as much as 50 per cent. Antibacterial serums have now been almost entirely superseded by sulfonamides and antibiotics.

#### *References*

1. Arkwright, 1921. *Jour. Path. Baer.* *34*, 36.
2. Hirsfeld, 1924. *Klin. Wschr.* *3*, 2084.
3. Stuart, Sawin, Griffin and Wheeler, 1936. *Jour. Immunol.* *37*, 31.
4. Webster, 1933. *Jour. Exp. Med.* *57*, 793.
5. Wheeler, 1938. *Jour. Immunol.* *37*, 409.

## Chapter 2

### THE IMMUNE REACTIONS

**ANTIBODIES**, acting either alone or in conjunction with certain blood and tissue cells, assist in overcoming infection and in preventing reinfection by the same pathogen. Antibody formation apparently occurs in various organs, particularly the spleen, liver, bone marrow and lymph nodes. Antibodies are proteins associated with the globulin fraction of blood serum.

#### THE COMPOSITION OF BLOOD

**Blood, Plasma and Serum.** Blood consists of a fluid containing the red and white blood cells and the platelets. The fluid is an aqueous solution of salts, carbohydrates and proteins. The total amount of blood in the animal body is normally one-twelfth of the body weight. The average man possesses twelve to fourteen pints. The cells comprise slightly less than one-half the volume of whole blood.

Blood, plasma and serum are related as follows:

Blood minus cells = plasma.

Plasma minus fibrin = serum.

Freshly drawn blood clots within a few minutes. Formation of the clot is a complex process in which a protein, fibrinogen, is converted into insoluble fibrin. Most of the blood cells are enmeshed in the fibrin clot, which shrinks after a few hours at low temperature and expels the serum, a clear, straw-colored fluid. Clotting is prevented by mixing the blood with sodium citrate, potassium oxalate, heparin or other chemicals. When blood treated in this manner is allowed to stand a few hours or is centrifuged, the cells settle and leave a clear supernate, plasma, which still contains fibrinogen.

**Serum Proteins.** The serum proteins constitute 6 to 7 per cent of the weight of serum. Normal serum contains several proteins distinguishable

by their "salting out" properties with sodium or ammonium sulfate, their precipitability with alcohol under varying conditions of pH and electrolyte concentration, their electric charges and their molecular weights.

Sodium sulfate in 21.5 per cent concentration precipitates from serum a crude fraction containing globulins; albumins remain in solution. The globulins account for about 50 per cent of the total protein of normal serum. Their exact chemical composition is not known. The predominant globulins in certain animals such as man, monkey and rabbit appear to have a molecular weight of between 150,000 and 160,000, whereas the principal globulins in the serum of the horse, cow and pig possess a molecular weight of approximately 900,000. There is some evidence that these globulins are composed of units having a molecular weight of about 40,000, four of these units comprising the small globulin of man, monkey and rabbit, and twenty-four comprising the major globulin of the horse, cow and pig.

Normal globulins assist in the maintenance of blood and tissue osmotic relations and take part in cell nutrition.<sup>3</sup> Utilization of blood globulin in the manufacture of protoplasm is indicated by experiments with animals fed a protein deficient diet, in which the circulating globulin decreased to a constant level. Furthermore, dogs from which plasma was regularly removed at short intervals replaced more than twenty times their normal blood protein content during a period of sixteen weeks.<sup>4</sup> It seems reasonable to assume that this high protein regenerating capacity is in fairly normal operation and that a certain percentage of the new protein consists of globulin, thus maintaining the customary balance of proteins in the plasma. The use of plasma protein in cell nutrition is confirmed by the observation that animals could be maintained in nitrogen equilibrium on a nitrogen free diet when plasma from the same species was given intravenously as the only source of nitrogen.

Antibodies or *immune globulins* are produced in response to infection or to the injection of appropriate materials, such as killed bacteria or some of their components or products. They are believed to represent a slightly modified form of the normal globulins and are able to combine specifically with the substances which induced their formation. Combination with antibody sometimes results in death of the infectious agent or neutralization of its toxicity.

**Blood Cells.** Normal human blood contains between 4,500,000 and 5,500,000 red cells, or erythrocytes, per cubic millimeter. These cells are about  $7.5\mu$  in diameter and  $2\mu$  in thickness. They are formed in the bone marrow and have an average life in the circulation of 80 to 120 days.

The white blood cells or leukocytes are an important defense against infection. Their total number varies normally between 5,000 and 8,000 per cubic millimeter. The several kinds of leukocytes are classified according to their size ( $7\mu$  to  $20\mu$ ), presence and type of granules, shape

of nucleus and character of cytoplasm, and are usually present in fairly constant percentages:

polymorphonuclear neutrophiles	.....	50-70%
basophiles	.....	0.5-1.0%
eosinophiles	.....	1-5%
lymphocytes	.....	20-30%
monocytes	.....	2-6%

The so-called granulocytes (polymorphonuclear neutrophiles, basophiles, and eosinophiles) are formed chiefly in the marrow of the flat bones; the lymphocytes are believed to be formed in the lymph follicles; and the monocytes are derived from reticulum cells, particularly in the spleen. The average life of the leukocytes in the blood is short, probably from a few hours to four days.

The principal function of the granulocytes, particularly the neutrophiles, and possibly of the lymphocytes, appears to be phagocytosis. These cells can engulf particles such as certain bacteria and may eventually digest them, presumably by the action of intracellular proteases and other enzymes. A few bacteria such as *Mycobacterium tuberculosis* are ingested but not digested, the organisms often being transported by the phagocytic cells to other regions of the body where they establish secondary sites of infection after the phagocytes have died and autolyzed.

#### THE RETICULOENDOTHELIAL SYSTEM

Phagocytosis is a function of certain tissue cells as well as of the blood leukocytes. These cells, which are parts of numerous organs widely distributed over the body, comprise the *reticuloendothelial system*. Various dyes, injected intravenously into animals, are not eliminated in the urine or bile but are deposited in the liver, spleen, bone marrow and lymph nodes, where they remain for several weeks. These dyes are stored within cells which the immunologist calls *macrophages* and which possess the ability to ingest particulate matter. These cells are either *fixed* or *wandering*. The most actively phagocytic fixed macrophages are found in the endothelium of the liver capillaries (Kupffer's cells), the sinuses of the spleen, lymph, and marrow, and the adrenal and pituitary capillaries. Less active fixed macrophages occur among the reticular cells of the spleen, the lymphatic glands and tissues and the thymus. Wandering macrophages are found in the tissue spaces and occasionally gain access to the blood.

The reticuloendothelial cells perform two important functions. They remove worn out or damaged cells such as erythrocytes and leukocytes from the circulation, and they dispose of foreign organic matter which has penetrated the tissues. Removal from the blood of leukocytes dam-

aged by ingesting bacteria simultaneously disposes of the ingested organisms. In addition, bacteria which escape leukocytic phagocytosis and enter the blood stream may be ingested by reticuloendothelial cells in the various organs through which the blood passes.

The effectiveness of phagocytosis in removing microorganisms from the blood has been demonstrated experimentally. Bull reported that the blood of dogs intravenously injected with typhoid bacteria contained 10,000,000 organisms per milliliter one minute after injection and only 40 per milliliter fourteen minutes later.<sup>1</sup> Nogueira found that *Streptococcus viridans* disappeared completely from the blood of rabbits within six hours after intravenous injection.<sup>5</sup> The liver and spleen contained a large percentage of the organisms. There is no way of knowing how frequently infections terminate by phagocytosis of the pathogen. Probably many inapparent or mild infections progress to the stage at which some bacteria enter the blood stream, are removed and destroyed, and the patient never realizes that anything spectacular has happened.

The cells which synthesize antibody globulin and other blood proteins are probably closely associated with those which carry on phagocytosis. Reticuloendothelial cells are widely believed to contribute in some manner to this process. Lymphocytes and plasma cells have also been suggested as possible sites of plasma protein synthesis.

#### THE REAGENTS IN IMMUNE REACTIONS

**Antigens.** An antigen is a substance which stimulates formation of antibody within an animal and which can react observably with that antibody. Ordinarily antigens are introduced parenterally—that is, by some route other than the alimentary tract. Otherwise, the antigen might be digested before reaching the antibody-producing tissues. There is evidence that some antigenic materials pass undigested through the mucous membranes of certain individuals. Hypersensitivity to these substances often ensues, as in hay fever, food allergies and similar disorders. The most effective antigenic stimuli, however, are obtained by injection into the skin or the underlying tissues, body cavities, the blood stream, or by actual infection.

The word *antigenicity* is usually employed to indicate the ability of a substance to stimulate antibody formation. There is no single word to denote the ability of an antigen to react with antibody. The term *in vitro antigenicity* may be employed for this purpose when the reaction has a visible result such as formation of a precipitate, and *combining power* when the reaction can be detected only indirectly.

**Haptens.** Some bacteria possess carbohydrate-like components which cannot by themselves stimulate antibody formation but which can react demonstrably with antibodies produced against the whole organism.

These substances are known as *haptenes*; they are also sometimes called *partial antigens* because they perform only one of the functions of an antigen.

**Antibodies.** An antibody is a modified blood globulin formed in response to an antigenic stimulus; it is capable of combining specifically with the corresponding antigen. The word "specifically" implies that the antibody can react only with the substance which engendered its production. Specificity depends upon the physicochemical structure of the reacting substances, not upon their source. The same substance may be found in widely divergent forms of life; for example, certain human red blood cells contain an antigen which appears to be chemically identical with a substance in hog gastric mucin, horse saliva, pneumococci and various plants. Antibody for the blood cell substance reacts equally well with the same antigen from the various sources mentioned. Some investigators tend to call such an antigen nonspecific because of its widespread distribution; actually distribution has nothing to do with specificity, which is a chemical matter.

#### LABORATORY PRODUCTION OF ANTISERUMS

Antisera (i.e., serums containing antibodies) for experimental purposes are usually produced in laboratory animals, occasionally in humans. Rabbits are most often employed because they are inexpensive and easy to maintain, inject and bleed. Guinea pigs are also used, and chickens, rats and mice to a lesser extent. Small animals possess the disadvantage that they yield little serum. Large animals such as horses, sheep, goats and cattle are expensive but provide large quantities of serum. They are employed in the commercial production of antitoxins and antibacterial serums.

Injections are usually made by the intravenous, intraperitoneal or subcutaneous routes. Material enters the blood stream slowly from a subcutaneous depot but quite rapidly from the peritoneal cavity. A series of injections is often given, varying from daily to weekly. The sizes of the doses depend upon the nature of the antigen. Highly toxic materials are given cautiously, beginning with small amounts and gradually increasing. The first injection of a tetanus culture filtrate into a rabbit might be 0.1 milliliter of 1:1000 dilution. Fairly large amounts of non-toxic agents may be given: 1.0 milliliter of 1 per cent protein solution or foreign blood serum diluted 1:5.

Periodic trial bleedings of a few milliliters permit titration of the antibody content of the serum. Final bleeding is performed when sufficient antibody has been produced, usually three to five days after the last injection. The serum is separated from the clotted blood, centrifuged if necessary until clear and stored at a low temperature. Preservatives are

customarily added to prevent growth of contaminants, especially if the bottle is to be entered frequently. Phenol, tricresol and Merthiolate are among the chemicals valuable for this purpose.

Parenteral injection of an organism into an animal stimulates production of antibodies in equal or greater concentration than is obtained in a naturally infected animal. The antibodies in a surviving susceptible animal protect against both natural infection and experimental injection; antibodies formed in an inoculated nonsusceptible animal protect only against parenteral injection of the organism. Mice, for example, are naturally susceptible to *Salmonella typhimurium*, and when immunized against this organism they develop antibodies which will protect against small natural or parenteral exposure. On the other hand, mice and rabbits injected with *S. typhosa* acquire immunity only against parenteral inoculation of the same organism, since these species are not susceptible to typhoid fever.

The term *immunization* is loosely applied by laboratory workers to the process of antibody production for experimental or therapeutic use. Immunizing antigens need not be pathogenic; in fact, most theoretic investigations of the antigen-antibody reaction employ nonpathogenic agents such as erythrocytes, egg albumin and serum proteins. These substances are highly antigenic for most laboratory animals and yield potent antiserums.

#### DEMONSTRATION OF ANTIBODIES

The techniques by which reactions between antigens and antibodies are studied in the laboratory constitute the methods of *serology* ("the study of serums"). Serology is a tool in the investigation of immunity.

Most serologic methods, as currently practiced, are subject to serious quantitative limitations because the principal reagents are of unknown composition, that is, proteins. Methods having the accuracy of quantitative chemical procedures have been recently introduced but are not yet in wide application outside the research laboratory because some of the apparatus required is expensive, and exceedingly careful technique is essential. Serology probably will become another branch of chemistry, closely allied to physiologic chemistry. Several institutions already have departments of immunochemistry.

**Agglutination.** Agglutination is the phenomenon observed when a cellular antigen, such as a saline suspension of bacteria or erythrocytes, is mixed with a small amount of homologous antiserum. The cells clump and gradually settle to the bottom of the fluid. Flocculent masses, compact granules or thin sheets form in test tube mixtures and settle to the bottom, from which they may be dislodged by gentle agitation. In some instances microscopic examination reveals that the particles of antigen

behave as though they are sticky and cling tenaciously together, whereas in other instances the clumps look very similar but can be broken up by shaking.

The agglutinating antibody content or *titer* of an antiserum is determined by preparing dilutions (1:40, 1:80, 1:160, etc.) of the serum in saline (0.85 per cent NaCl) and adding a suspension of the appropriate antigen. The test tubes are incubated in a waterbath at a suitable temperature (e.g. 37° C., 55° C.) and agglutination is read after a period determined by the nature of the antigen. A tube in which all cells have agglutinated is graded ++++. Absence of agglutination is designated -, and intermediate degrees of agglutination are indicated +, ++, or +++.

The results of a typical test might appear as recorded in Table 1. The titer of an antiserum is the reciprocal of the last dilution showing

*Table 1. Results of an Agglutination Experiment*

ANTIBACTERIAL SERUM DILUTIONS									CONTROL (saline)
1:40	1.80	1 160	1,320	1,640	1 1280	1 2560	1.5120	1:10240	
++++*	+++	+++	++	++	++	++	+	-	-

\* ++++ = complete agglutination (all cells clumped)

- = no agglutination

definite agglutination (+), so the titer illustrated is 5,120. The control tube, containing saline instead of antiserum dilution, is necessary because occasionally cells clump spontaneously in the presence of NaCl.

**Precipitation.** Solutions of proteins or certain polysaccharides constitute the antigens in precipitation tests with appropriate antisera. Antigens suitable for these tests are derived from all forms of life: bacteria, plants, and animals.

Most serologic tests are set up by diluting the immune serum and adding a constant quantity of antigen, but the reverse is true in precipitin tests: the antigen is diluted and added to undiluted antiserum. Mixtures of antigen and antibody are incubated at 37° C. for two hours. Cloudiness appears within a few minutes and a precipitate gradually settles out. Although maximum titers are obtained by using undiluted antiserum, the cost of the immune serum generally necessitates a twofold to fivefold dilution of the antiserum at the expense of considerable loss in titer. Such a procedure should never be employed with weak antisera, as in attempts to determine normal antibodies.

Precipitation is also demonstrated by the ring or interfacial test. A layer of antigen dilution is placed over the antiserum in a test tube or capillary. A fine line of precipitate appears at the interface within a few minutes and gradually increases in amount. The titer is expressed as the

reciprocal of the greatest antigen dilution yielding a positive result. The amount of precipitating antibody in an antiserum can be determined quantitatively by micro-Kjeldahl nitrogen analyses or by other sensitive methods.

**Neutralization of Toxins.** Exotoxins are neutralized by homologous antibodies (i.e., antitoxins) in proper proportions. Neutralization of toxicity is determined by inoculation of suitable experimental animals. Various amounts of toxin are mixed with a constant amount of antitoxin, incubated a short time to allow the reagents to combine chemically and injected. Small amounts of most antitoxins neutralize many lethal doses of the corresponding exotoxins, and the potency of an antitoxin is indicated by the amount of toxin which it neutralizes.

**Phagocytosis.** Phagocytosis of bacteria is greatly enhanced by homologous antibacterial antibody. This can be demonstrated *in vitro* with an organism such as *Staphylococcus aureus*. Equal parts of whole blood, bacterial suspension and antiserum are mixed and incubated at 37° C. for thirty minutes. A stained smear is prepared and the average number of bacteria ingested by the polymorphonuclear leukocytes is determined. A similar preparation in which normal serum is substituted for antiserum indicates the "normal" phagocytosis by the leukocytes. The ratio obtained by dividing the number of bacteria ingested in the presence of antiserum by the number ingested in the presence of normal serum is called the *opsonic index*. This is a measure of antibody involved in phagocytosis.

Antibody appears to assist phagocytosis by altering the surface properties of the bacteria. Some organisms, such as pneumococci, possess a capsular coating which is resistant to phagocytosis except when the capsule has combined with antibody.

**Lysis and Complement Fixation.** The reaction between certain cellular antigens and their antibodies in the presence of complement, a normal serum component, is followed by dissolution of the antigen. This phenomenon is known as *lysis* or *cytolysis*. *Bacteriolysis* and *hemolysis* are specific terms applying to the dissolving of bacteria and erythrocytes, respectively. The theoretic aspects of this reaction have been determined principally by study of hemolysis, in which a positive result is readily detected by macroscopic inspection.

Anti-erythrocyte serum is prepared by inoculating rabbits or other animals with foreign red blood cells. The test antigen employed to demonstrate hemolysis is a saline suspension of erythrocytes washed several times with saline to remove normal serum proteins. Erythrocytes and complement (fresh guinea pig serum) are added to serial dilutions of antiserum. Hemolysis takes place within fifteen to sixty minutes at 37° C. and is shown by complete dissolving of the cells leaving a clear, red solution. Unhemolyzed cells settle to the bottoms of the tubes.

Bacteriolysis can best be demonstrated with *S. typhosa*, *Vibrio cholerae* and a few other bacteria. Dissolution of the cells is sometimes macroscopically evident and can also be observed in stained preparations examined with the microscope in comparison with suitable controls.

Complement is "fixed" by specific precipitates. Egg albumin and anti-egg albumin serum, although reacting in proportions which do not yield a trace of precipitate, will nevertheless combine with complement to form a three-component complex (Figure 1). The complement becomes "fixed" and is no longer able to react with other antigen-antibody systems subsequently added, such as red blood cells "sensitized" by

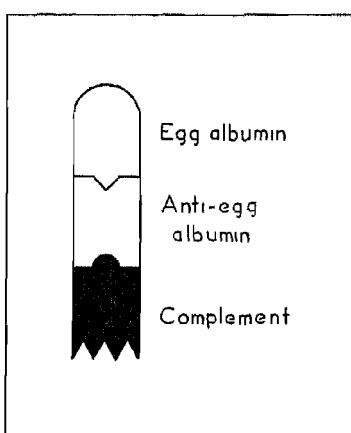


FIGURE 1. Diagrammatic representation of a complex consisting of antigen, antibody and complement.

contact with their homologous antibody (hemolysin). In the absence of antibody which can combine with the test antigen, egg albumin, complement hemolyses the sensitized red cells.

An experiment illustrating *complement fixation* is shown in Table 2. Lack of hemolysis in mixtures containing low dilutions of egg albumin showed that no complement remained uncombined, whereas complete hemolysis in the mixture containing 1:1,000,000 egg albumin indicated insufficient antigen to fix complement in the presence of anti-egg albumin serum. The free complement lysed the erythrocytes sensitized by anti-erythrocyte serum, as also occurred in the saline control tube. The complement fixation titer of the anti-egg albumin serum is approximately 100,000 because this dilution of antigen with its antibody partially inhibited hemolysis.

The complement fixation test is exceedingly sensitive and can be used to detect traces of either antigen or antibody, depending upon the

manner of setting up the test. It is the basis for the Wassermann test for syphilis, in which syphilis "antibody" or *reagent* is the unknown quantity.

**Anaphylaxis.** The preceding antigen-antibody reactions are readily demonstrable in the test tube. Anaphylaxis can be shown only in an animal or with living animal tissue because it results from the reaction of antigen and antibody in or upon body cells.

Anaphylaxis is conveniently demonstrated in a guinea pig. The animal is given a small injection of a foreign protein such as egg albumin or horse serum, and about twenty-one days later is reinjected intravenously with a larger dose of the same antigen. The animal begins to cough within a few moments, gasps for breath, has convulsions and dies.

Table 2. Complement Fixation by a Soluble Antigen and Its Antibody

	1:100	1:1000	1:10,000	1:100,000	1:1,000,000	Saline
Egg albumin (0.1 ml.)						
Anti-egg albumin serum (ml. 1:5)	0 1	0 1	0 1	0 1	0 1	0 1
Complement (ml. 1:25)	0 1	0 1	0 1	0 1	0 1	0 1
Incubation at 37° C. for 30 minutes						
Anti-erythrocyte serum (ml. 1:500)	0 1	0 1	0 1	0 1	0 1	0 1
Erythrocytes (ml. 2%)	0 1	0 1	0 1	0 1	0 1	0 1
Incubation at 37° C. for 30 minutes						
Results: Hemolysis	--	--	--	+++	+++	+++
Complement fixation	++++	++++	+++	-	-	-

This reaction involves an antibody produced in small amounts by the animal within three weeks after first injection. Thereafter the animal is hypersensitive to a second or "shocking" injection of the same antigen. A guinea pig can also be sensitized passively by injecting serum from a hypersensitive animal or by giving it a small amount of antiserum from an immunized animal, even of another species.

The anaphylactic reaction is a very sensitive indicator of antigenicity, particularly in the guinea pig. The ease of sensitization and severity of anaphylaxis differ in other animals. Anaphylactic manifestations vary in part with the distribution of smooth muscle, which contracts strongly in a typical reaction.

**Protection Tests.** Protective antibodies are detected and titrated by "challenging" actively or passively immunized animals with multiple lethal doses of the pathogenic agent. Immune animals withstand a greater challenge dose than normal animals. The test is made more or less quantitative by varying the dosage of the immunizing agent or of

the challenge; the virulence of the pathogenic agent is determined by simultaneous tests in nonimmune animals.

The term *immunogenicity* is used to indicate the ability of an antigen to induce specific resistance demonstrable by *in vivo* test. The methods employed in immunization and challenge are always stated in describing the immunogenicity of a particular material.

**The Unitarian Concept of Antibodies.** The various antigen-antibody reactions were originally believed to be caused by different kinds of antibodies. The antibodies were named agglutinin, precipitin, antitoxin, opsonin and lysin, corresponding to the respective phenomena of agglutination, precipitation, neutralization of toxins, phagocytosis and lysis.

It soon became evident that two or more types of antigen-antibody reaction could be demonstrated with a single antigen and its antiserum. Antitoxins react with homologous exotoxins and neutralize their toxicity for animals; precipitates are also formed when the two reagents are combined in suitable proportions. Similarly, antiprotein serum precipitates the protein and fixes complement. Such observations led Dean<sup>2</sup> in 1917 and Zinsser<sup>6</sup> in 1921 to formulate the *unitarian hypothesis*, which states that a single antigen induces the formation of only one kind of antibody. The antibody is capable of reacting with or sensitizing the corresponding antigen. A sensitized antigen may participate in various detectable reactions depending upon the experimental conditions.

The variety of reactions obtainable is illustrated by the behavior of antibody against the specific capsular polysaccharide of pneumococci. This antibody sensitizes the intact cocci so that they agglutinate under appropriate conditions of electrolyte concentration and temperature. Phagocytosis of the sensitized cocci may also be demonstrated in the presence of living leukocytes. The capsular polysaccharide itself, when removed from the organisms and purified, is precipitated by the antibody, and the combination fixes complement. The polysaccharide can also be used to elicit allergic skin reactions or anaphylactic shock in sensitized animals.

The pneumococcal polysaccharide-antibody system is perhaps an unfair illustration of the unitarian concept. Few other bacteria have yet been found to display as striking a parallel between the various demonstrations of antigen-antibody reaction. The hypothesis has been useful, however, in directing attention to the essential similarities between the various antigen-antibody reactions.

It should be emphasized that the unitarian concept refers to *single* antigens and their antibodies. A whole bacterial cell is a complex collection of many antigens. Each antigen induces the formation of a specific antibody and can react only with that antibody or with antibodies for closely related antigens. Some antigens, because of their situation, may be demonstrable only by particular reactions. Antigens associated with

bacterial flagella, for example, participate in agglutination but apparently not in other reactions.

#### *References*

1. Bull, 1915. *Jour. Exp. Med.* **22**, 475.
2. Dean, 1917. *Lancet I*, 45.
3. Holman, Mahoney and Whipple, 1934. *Jour. Expt. Med.* **59**, 251.
4. Melnick and Cowgill, 1937. *Jour. Expt. Med.* **56**, 493.
5. Nogueira, 1942. *Arq. Inst. bact. Câmara Pestana* **8**, 145.
6. Zinsser, 1921. *Jour. Immunol.* **6**, 289.

## Chapter 3

### ANTIGENS

#### GENERAL PROPERTIES OF ANTIGENS

**Detection of Antigens.** The antigenicity of a substance is ascertained by inoculating it into a suitable experimental animal. Serum from the animal is tested with the substance by one or more of the antigen-antibody reactions outlined in the preceding chapter. A positive result shows that the material is antigenic.

**Classes of Antigens.** Antigens have previously been defined as substances which stimulate the formation of antibody within an animal and which can react observably with that antibody. Landsteiner in 1921 proposed the term *haptenes* to refer to substances which are serologically active in the test tube but do not cause formation of antibodies when injected into laboratory animals.<sup>36</sup> Some lipids and polysaccharides of animal and bacterial origin were found to behave in this manner. Further work necessitated redefinition of the word hapten. Topley and Wilson designated as "complex haptene" those substances which combine specifically with homologous antibody to yield a precipitate or other visible reaction but do not stimulate antibody formation.<sup>37</sup> Alcoholic extracts of guinea pig kidney, for example, do not produce antibodies in a rabbit but react with the antibodies produced by immunization of rabbits with saline kidney emulsions. "Simple haptene" were defined as substances which do not stimulate antibody formation nor react visibly with homologous antibody but combine with such antibody and prevent precipitation of the corresponding complete antigen or complex hapten. Partial hydrolysis of pneumococcal polysaccharide yields a product which fails to precipitate with rabbit antipneumococcal serum but which nevertheless still possesses specific properties. The partial hydrolysate combines with the antibody, blocking precipitation of the polysaccharide. This is the "inhibition test." Blocking antigens may be very simple substances, such as tartaric acid or benzoic acid.

More recent work has shown that a complex haptene may be antigenic in one animal but not in another.<sup>27</sup> Pneumococcal polysaccharides have been isolated which produce antibodies in mice, horses and humans, but not in rabbits.

**Factors Determining Antigenicity.** Antigens generally possess a molecular weight of 10,000 or greater. Blood proteins having molecular weights upward of 60,000 are excellent antigens, and substances of very high molecular weight, such as hemocyanins (M. W. about 6,700,000) and tobacco mosaic virus (M. W. about 17,000,000) are also excellent antigens. Egg albumin, with a molecular weight of slightly over 40,000, is a good antigen. Production of precipitins has been reported with ribonuclease, which possesses a molecular weight of about 15,000, and

Table 3. Precipitation of Ocular Lens Proteins by Homologous and Heterologous Rabbit Antiseraums

ANTISERAUMS AGAINST	ANTIGENS (1.4000)			
	Sheep lens	Swine lens	Chicken lens	Fish lens
Sheep lens	+++	++ +	+	-
Swine lens	++ +	++ ++	+	-
Chicken lens	+ ±	++	++ + +	-
Fish lens	-	-	++	++ +

(From Ecker and Pillemer,<sup>18</sup> by permission.)

with a phenylisocyanate of clupein having a molecular weight of approximately 5,000.<sup>24</sup>

Antigens usually possess a large molecular surface. The role of surface area in antigenicity is indicated by the observation that some nonantigenic low molecular weight substances become antigenic when adsorbed onto inert particles like charcoal, quartz, aluminum hydroxide or collodion.

Most antigens are foreign to the animal in which they induce antibody formation. A few kinds of cells or substances are antigenic in the animal from which they are derived. Gonadal tissues and proteins from the crystalline lens of the eye may induce antibodies in other members of the same species or, under normal conditions, in the same individual. Guinea pigs injected with guinea pig sperm produce "spermocidin," an antibody which immobilizes homologous sperm *in vitro*. Furthermore, guinea pigs can be sensitized with lens protein from one eye and anaphylactically shocked by injection of lens protein from the other eye. Ecker and Pillemer found considerable antigenic similarity between the ocular lens proteins of sheep, swine and chickens (Table 3).<sup>18</sup> Chicken lens protein even reacted with antiserum against fish lens. Formation of antibodies against substances from the same individual is known as autoimmunization. Antigens producing auto-antibodies are normally confined

to certain cells or tissues and do not gain access to antibody-producing cells. It is significant that auto-antigenic substances are serologically similar or identical in many phylogenetically unrelated species.

The erythrocytes of one individual (human or otherwise) may contain antigens which cause the production of antibodies in another individual of the same species. This process, called *isoimmunization*, will be discussed more fully in Chapter 9.

An antigen must ordinarily be introduced beyond the epithelial tissues in order to stimulate antibody formation. Intravenous and intraperitoneal injections almost exclusively are employed for antibody production in experimental animals. Each route gives excellent antisera. Intravenous inoculations are often preferred because the technique is simpler and the results are comparable in the speed of antibody production and the final titers attained. The dosage of toxic antigens is more critical by the intravenous method than by the intraperitoneal route. Furthermore, anaphylactic reactions are more likely to follow second or subsequent intravenous inoculations than intraperitoneal injections. Intramuscular injections yield fair titers. Subcutaneous inoculations induce slow antibody formation, and many injections must be given to obtain potent antisera. The intracutaneous route is rarely employed with animals and usually yields low titers. Primary immunization of man is almost exclusively by the intramuscular route. For reimmunizing previously inoculated individuals a common practice is to inject intracutaneously. Vaccines containing "adjuvants" such as mineral oil have recently been employed to increase the effectiveness of subcutaneous injections. These preparations provide reservoirs of antigen from which the active material is slowly but continuously released.

**Specificity of Antigens.** The specificity of antigenic complexes like those which occur in bacterial cells or body fluids should be considered separately from that of relatively pure isolated proteins and artificially modified antigens, although it must be emphasized that the same fundamental laws govern the specificity of all antigens.

Bacterial cells contain a heterogeneous collection of antigenic materials, principally proteins. Some are associated with the flagella, others with the cell bodies and still others with the capsules and "envelopes." Each antigenic component induces formation of homologous antibody. The whole cell, when mixed with its antiserum, reacts with all the antibodies. Any other cell possessing one or more of the same antigens will also react with that antiserum to the same or a lesser degree.

Three bacteria, *1*, *2* and *3*, possessing respectively antigens *A* and *B*, *B* and *C*, *C* and *D*, cause the formation of antibodies *a* and *b*, *b* and *c*, *c* and *d* (Figure 2). If Bacterium *1* is mixed with Antiserum *1*, antibody *a* combines with antigen *A* and antibody *b* combines with antigen *B*, and agglutination occurs under appropriate conditions. When the same

organism is mixed with Antiserum 2, agglutination occurs by action of antibody *b*. Bacterium 1 does not agglutinate with Antiserum 3 because this antiserum contains neither antibody *a* nor antibody *b*.

Antigen *B* may constitute a minor component of Bacterium 1 and cause the formation of antibody *b* in low titer. Antiserum 1 will therefore agglutinate Bacterium 2 in only a low dilution, regardless of the amount of *B* present.

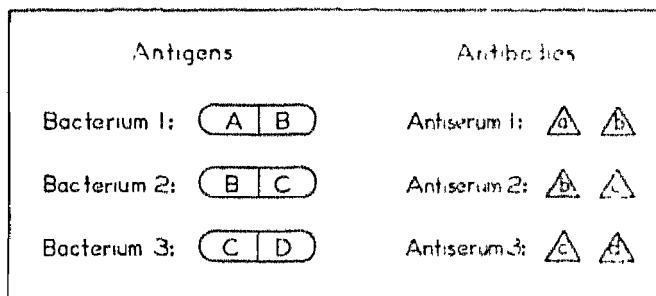


FIGURE 2. Diagrammatic comparison of the antigenic components of three bacteria and the antibodies contained in homologous antisera.

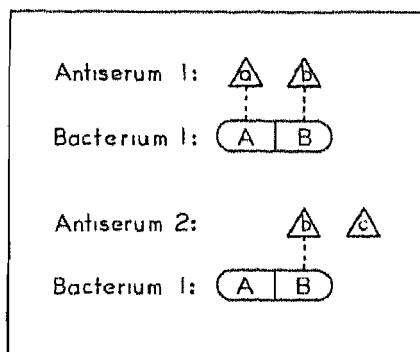


FIGURE 3. Adsorption of antibodies from homologous (Antiserum 1) and heterologous (Antiserum 2) antisera (diagrammatic).

Bacteria mixed with homologous antiserum combine with (adsorb) the antibody molecules in the antiserum, each antibody reacting with its corresponding antigen. A heterologous organism combines with only those antibodies for which it possesses antigens, and any other antibodies remain uncombined. If the concentration of homologous or heterologous antigen is sufficiently great or the dilution of antiserum is sufficiently high, all antibodies will be adsorbed onto the cells. The cells may be removed by centrifugation and the absence of antibody demonstrated in the

supernatant fluid. The *adsorption* test constitutes a most important means of determining the relationships between different bacteria containing one or more common antigenic components.

The principle of adsorption is illustrated in Figure 3. Adsorption of Antiserum 1 with Bacterium 1 removes both antibodies, *a* and *b*, which combine with the bacterial cells. Adsorption of Antiserum 2 with Bacterium 1 results in union of antibody *b* with antigen *B*, leaving antibody *c* in the supernatant fluid following centrifugation. Antigen *C* is a common component of organisms 2 and 3 (see Figure 2), so antibody *c* can be used to distinguish them from organism 1. In this example antigen *A* is specific for Bacterium 1 and antigen *D* is specific for Bacterium 3.

Agglutination tests in which one organism is mixed with antiserum against another organism are known as *cross-agglutination* tests. The results of a cross-agglutination experiment with three strains of *Shigella* *dispar* are illustrated in Table 4. The three organisms were isolated from

Table 4. Cross-agglutination Titers of Three Strains of *Shigella* *dispar*

BACTERIAL ANTIGENS	AGGLUTINATION BY ANTISERUMS		
	171	167	205
171	5120	640	320
167	640	20,480	10,240
205	1280	20,480	10,240

the feces of patients with mild dysentery and possessed indistinguishable morphologic, cultural and biochemical characteristics. Each organism agglutinated strongly in its homologous antiserum. Heterologous titers (strain 171 tested in antiseraums 167 and 205, etc.) were generally, but not always, lower. The various common antigenic components may therefore exist in different concentrations in cells of the three strains.

Table 4 indicates that all three cultures possessed one or more antigens in common. Strain 171, which cross-agglutinated to titers of only 640 and 320 in antiseraums against 167 and 205, was obviously not identical with these organisms. A similar situation was found when antigens 167 and 205 were tested in antiserum 171. However, organisms 167 and 205 both agglutinated to high titer (10,240 or 20,480) in antiseraums 167 and 205, so adsorption, as previously described (see Figure 3), was necessary to determine whether these strains were identical. The results showed that strain 167 possessed all the antigens of 205 and one additional component of its own.

**Species and Organ Specificity.** Species specificity implies that corresponding antigens, such as serum proteins, of *different* species are

serologically distinguishable. Organ specificity signifies that different organs from the *same* animal are serologically distinguishable. The characteristics inherent in a given species often outweigh organ differences, so organ specificity cannot always be clearly demonstrated.

The specificity of antigens from different animal species or organs is subject to the same conditions as the specificity of bacteria. Antiserum from a rabbit immunized with foreign serum contains a variety of antibodies, each capable of reacting with a single antigen. Some of these antigens are major components of the foreign serum; others are minor constituents.

Despite the complexity of the antigenic material, interesting serologic results have been obtained by the study of serum proteins from different animal species. The relationships among serum proteins of a variety of animals are illustrated in Table 5.<sup>11</sup> Antiserum from a rabbit inoculated

*Table 5. Precipitation of Homologous and Heterologous Serums by Rabbit Anti-sheep Serum<sup>11</sup>*

ANTIGEN SOURCE	TITERS WITH ANTI-SHEEP SERUM
Sheep	20,000
Goat	20,000
Beef	10,000
Pig	2000
Man	100
Horse	100
Hen	< 50

with sheep serum was used in precipitin tests with antigens consisting of serums from several animal species. Goat and sheep serum reacted equally and could be identical; only by adsorption might differences be detected. The other serums reacted in lower titers and obviously contained smaller amounts of the serologically reactive constituents of sheep serum. It will be noted that beef serum reacted in a titer of 10,000. At first glance this appears to indicate a marked difference from sheep serum. However, in serologic tests with doubling dilutions this actually represents a difference of only one tube in the series and is within the range of experimental error in preparing dilutions and adding antigen.

Serologic differences have been found also between milk proteins, hemoglobins, muscle proteins and many other proteins from different animal species.<sup>37</sup> The specificity of serum proteins is associated with the phylogenetic relationships of the animals from which they are derived. The intensity of a serologic cross reaction may be proportional to the degree of biologic relationship between the sources of the two antigens. Certain other antigens are widely distributed among many phylogenetically un-

related species. It should be pointed out that too frequently the distribution of an antigen is confused with its specificity and a widely distributed antigen is thought of as having little or no specificity, whereas an antigen confined to a very limited source, such as diphtheria toxin, is highly specific. The Forssman antigen (see page 47), for example, is found in humans, a great variety of animals, birds, fish and even plants, including some bacteria. This antigen is widely distributed but possesses as great specificity as diphtheria toxin.

**Specificity of Chemically Modified Proteins.** Obermayer and Pick in 1906 investigated specificity by joining extra elements and radicals to proteins.<sup>61</sup> Nitric acid, nitrous acid or iodine deprived proteins of their original specificity to an extent which depended upon the intensity of

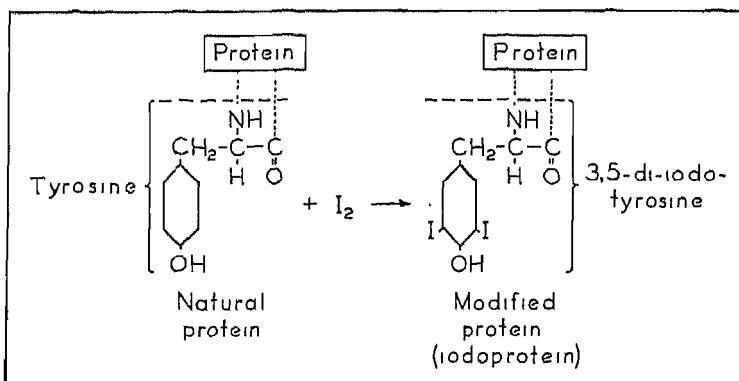


FIGURE 4. Formation of a modified protein by iodination of the tyrosine of a natural protein.

the treatment, and a new specificity determined by the nature of the modifying agent was introduced. Rabbits were immunized with iodoprotein prepared by treating a protein with iodine. The resulting anti-iodoprotein serum precipitated iodoproteins prepared from completely unrelated proteins. Anti-iodoprotein serum might or might not react with the homologous, untreated protein. Even rabbit serum proteins, similarly modified, were capable of stimulating antibody formation in the rabbit and therefore behaved as foreign proteins. More recent experiments showed that nitrate, nitrite and halogen radicals reacted with tyrosine, histidine and probably other aromatic amino acids of proteins.<sup>37</sup> These modified amino acids rather than the introduced groups alone determined the altered specificity of treated proteins. The tyrosine of a protein may combine with iodine to form a disubstituted molecule (see Figure 4). That modification occurs within a protein molecule is indicated by the observation that 3,5-di-iodotyrosine uncoupled to protein prevents precipitation of the homologous iodoprotein by anti-

iodoprotein serum. Other substances containing the 3,5-di-iodo-4-hydroxy group,



such as thyroxine, also inhibit precipitation of iodoproteins.<sup>36</sup>

Further evidence that substitution occurs in the tyrosine of proteins was provided when it was found that the amount of iodine necessary to iodinate a protein completely can be calculated from the tyrosine content of the protein.<sup>7</sup> Complete iodination of serum globulin deprived it of the ability to react with antiserum for native globulin.<sup>35</sup>

**Azoprotein Antigens.** In 1917 Landsteiner and his co-workers began extensive investigations of serologic specificity by joining organic radicals to proteins. A chemical to be tested as a determinant of specificity was attached to an aromatic amine such as aniline, which was then diazotized and coupled to a protein.<sup>39</sup> The resulting product was called a *conjugated antigen* or *azoprotein*.

The added radicals presumably combined with aromatic amino acids of the protein, as in nitration and halogenation. The method may be illustrated by the coupling of p-amino-benzene arsonic acid (atoxyl) to a protein (see Figure 5).

Azoproteins react only weakly with antiseraums against the original proteins.<sup>37</sup> Ordinarily, a foreign source of protein is employed, such as horse or chicken serum or egg albumin, but even rabbit serum azoproteins stimulate the formation of antibodies in rabbits. A conjugated azoprotein contains distinctive radicals which may engender antibodies possessing different specificities. Antibodies may be formed which are capable of reacting with (1) the uncombined azo components, (2) azoproteins made from the same or related proteins, or (3) the homologous natural protein. The relative amounts of the various antibodies in a given antiserum depend upon the extent of chemical modification of the immunizing antigen and often upon the inoculation dosage and number of injections. Prolonged immunization sometimes yields antiseraums with a broader range of reactivity than short immunization. This does not mean that the specificity of each antibody molecule is less but that a succession of antibody molecules is formed against qualitatively and quantitatively different antigenic components. Various antigenic components require different periods of time for antibody formation, with little regard for the quantity injected.<sup>2</sup>

Antibodies reacting with the protein constituent may mask the effect of an added radical. Tests of specificity therefore must be performed in such a manner as to detect only antibodies capable of reacting with uncombined azo radicals or *determinant groups*. Rabbit antiserum

against an azoprotein will precipitate conjugated proteins containing the same azo group combined with a completely unrelated protein. For example, atoxyl azo-horse serum antibodies precipitate a test antigen made by coupling atoxyl to chicken serum proteins. Horse and chicken proteins are so different serologically that any reaction is associated with

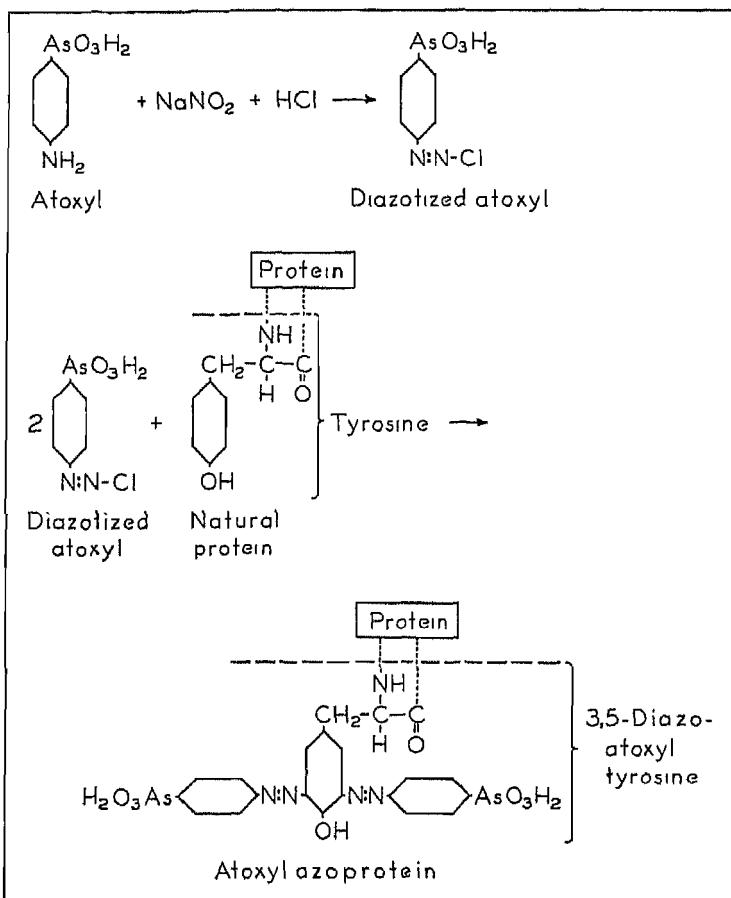


FIGURE 5. Coupling of atoxyl to the tyrosine of a protein.

the atoxyl component, probably combined with the tyrosine or histidine residues of these proteins. A low molecular weight substance containing the azo component, or even the azo component itself, may be used as a simple haptene to prevent precipitation of the usual test antigen. The anaphylactic reaction can also be used to test the effect of an added radical upon specificity; a guinea pig is sensitized with one azoprotein and shocked with an unrelated protein containing the same radical.<sup>87</sup>

Landsteiner immunized rabbits with proteins to which had been coupled aniline, p-aminobenzoic acid, p-aminobenzene sulfonic acid and p-aminophenyl arsonic acid, respectively (Table 6).<sup>37</sup> These determinants differ only in the nature of the acid radicals substituted in the *para* position on the benzene ring attached to protein by the —N—N— linkage. Cross-precipitation tests employing the four antigens with all four antisera demonstrated complete specificity: aniline azoprotein antiserum reacted only with the aniline azoprotein; p-aminobenzoic acid azoprotein

*Table 6. Effect of Strong Acid Radicals on the Specificity of Azoproteins*

ANTISERUMS FOR AZOPROTEINS CONTAINING:	TEST ANTIGENS: AZOPROTEINS CONTAINING			
	Aniline	p-Amino-benzoic acid	p-Amino-benzene sulfonic acid	p-Amino-phenyl arsonic acid
Aniline	+++	-	-	-
p-Amino-benzoic acid	--	++ ±	-	-
p-Aminobenzene sulfonic acid	-	-	++ ±	-
p-Aminophenyl arsonic acid	-	-	-	+++

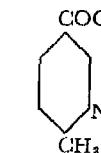
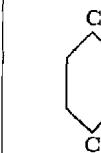
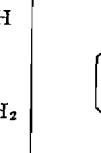
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antiserum precipitated only its homologous antigen, and so forth. In these experiments strong acid radicals exerted a striking effect on the specificity of antigens.

Methyl and halogen radicals were not as effective determinants of specificity as acid radicals. Compounds differing only in the presence of methyl, chlorine or bromine in the 4 position of 3-aminobenzoic acid showed considerable overlapping in precipitation tests (Table 7).<sup>37</sup> Antiserum against 3-amino-4-chlorobenzoic acid azoprotein reacted strongly with the other test antigens. Aniline substituted by addition of chloro, methyl or nitro groups exerted very little effect upon the specificity of azoprotein (Table 8).<sup>37</sup>

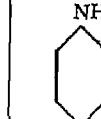
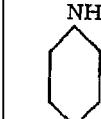
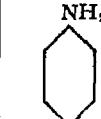
The spatial arrangement of coupled acid radicals is an important factor in the specificity of azoproteins (Table 9).<sup>37</sup> Antigens conjugated with

Table 7. Effect of Halogen and Methyl Radicals on the Specificity of Azoproteins

ANTISERUMS FOR AZOPROTEINS CONTAINING:	TEST ANTIGENS: AZOPROTEINS CONTAINING			
	3-Amino-benzoic acid 	3-Amino-4-methyl benzoic acid 	3-Amino-4-chlorobenzoic acid 	3-Amino-4-bromobenzoic acid 
3-Amino-benzoic acid	++++	--	--	--
3-Amino-4-methylbenzoic acid	-	++	+±	±
3-Amino-4-chlorobenzoic acid	+++	+++	++++	+++-
3-Amino-4-bromobenzoic acid	-	±	++±	++

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Table 8. Effect of Chloro, Methyl, and Nitro Groups on the Specificity of Azoproteins

ANTISERUMS FOR AZOPROTEINS CONTAINING:	TEST ANTIGENS: AZOPROTEINS CONTAINING				
	Aniline 	o-Chloro-aniline 	p-Chloro-aniline 	p-Toluidine 	p-Nitro-aniline 
Aniline	++±	+±	++±	+±	+
o-Chloroaniline	++	++±	+	+	tr.*
p-Chloroaniline	+	tr.	++	++	++
p-Toluidine	+±	+	++	++	+±
p-Nitroaniline	+	±	+±	+	++

\* tr. = trace

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aniline derivatives containing carboxyl or sulfonic radicals in the *ortho*, *meta* and *para* positions were highly specific. There were only four cross reactions. Spatial configuration dominated the effect of the acid substituent in one of these; the o-aminobenzene sulfonic acid antigen was precipitated by the o-aminobenzoic acid antiserum. The other three cross reactions involved determinants which differed in the position of the sulfonic radical by only one carbon atom: o- and m-aminobenzene sulfonic acid antigens and antiseraums cross reacted strongly and antibody

*Table 9. Effect of Spatial Distribution of Acid Radicals on the Specificity of Azoproteins*

ANTISERAUMS FOR AZOPROTEINS CONTAINING:	TEST ANTIGENS: AZOPROTINA CONTAINING							
	Aniline	Aminobenzoic acids			Aminobenzene sulfonic acids			
		<i>ortho</i> -NH <sub>2</sub>	<i>meta</i> -NH <sub>2</sub>	<i>para</i> -NH <sub>2</sub>	<i>ortho</i> -SO <sub>3</sub> H	<i>meta</i> -SO <sub>3</sub> H	<i>para</i> -SO <sub>3</sub> H	
Aniline	+++	-	-	-	-	-	-	-
Aminobenzoic acids	<i>ortho</i> -	-	+++	-	-	+++	-	-
	<i>meta</i> -	-	-	+++	-	-	-	-
	<i>para</i> -	-	-	-	+++	-	-	-
Aminobenzene sulfonic acids	<i>ortho</i> -	-	-	-	-	++++	+++	-
	<i>meta</i> -	-	-	-	-	+++	+++	-
	<i>para</i> -	-	-	-	-	-	++	+++-

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against the p-aminobenzene sulfonic compound precipitated the *meta* antigen weakly. In no case did *ortho* and *para* reagents cross react. Cross reactions between heterologous azoproteins and antiseraums therefore occurred when the determinant configurations were identical or not very dissimilar.

Experiments with levo-, meso- and dextro-tartaric acids emphasized the significance of spatial arrangement (Table 10).<sup>37</sup> The acids were converted into aminotartranilic acids before diazotization and coupling to proteins. Specificity was indicated by the strong homologous reactions. Three of the six heterologous combinations gave trace reactions and three were negative. The three trace reactions involved either the m-tartaric acid antigen or antiserum. Meso-tartaric acid differs from the

levo- and dextro- forms in the arrangement of hydrogen and hydroxyl about a single asymmetric carbon atom, whereas the levo- and dextro-compounds differ from each other in the arrangement of hydrogen and hydroxyl about both asymmetric carbon atoms.

Landsteiner also demonstrated the influence of the length of aliphatic chains coupled to protein (Table 11).<sup>37</sup> Antisera against short chain

*Table 10. Effect of Stereoisomerism of Determinant Radicals on the Specificity of Azoproteins*

ANTISERUMS FOR AZOPROTEINS CONTAINING:	TEST ANTIGENS AZOPROTEINS CONTAINING		
	Tartaric Acid		
	levo-	meso-	dextro-
	$\begin{array}{c} \text{COOH} \\   \\ \text{HOCH} \\   \\ \text{HCOH} \\   \\ \text{COOH} \end{array}$	$\begin{array}{c} \text{COOH} \\   \\ \text{HCOH} \\   \\ \text{HCOH} \\   \\ \text{COOH} \end{array}$	$\begin{array}{c} \text{COOH} \\   \\ \text{HCOH} \\   \\ \text{HOCH} \\   \\ \text{COOH} \end{array}$
l-tartaric acid	++±	tr.*	—
m-tartaric acid	tr.	+++	—
d-tartaric acid	—	tr.	++±

\* tr. = trace

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acids (oxanilic and succinanilic) were highly specific, but antisera for the longer acids reacted not only with the homologous antigens but also with those of somewhat greater or lesser chain length. A difference of one or two carbon atoms completely changed the specificity of the shorter chains, but such a difference had relatively little effect on the specificity of the longer chains. Other observations also indicated that, like acid groups, the polar —CONH— linkage enhanced antigenicity in the animal.

The importance of terminal amino acids in the specificity of proteins was shown. Glycyl-leucine, for example,

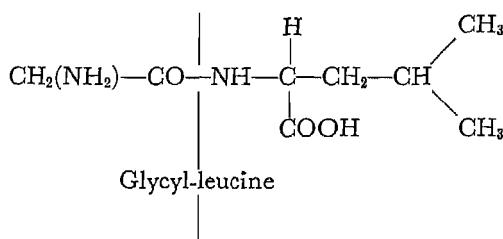
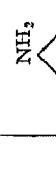
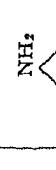
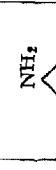
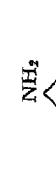
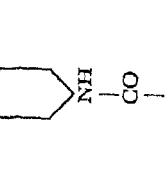


Table 11. Effect of Chain Length on the Specificity of Azoproteins Containing Aliphatic Acids

TEST ANTIGENS. AZOPROTEINS CONTAINING				
	p-Amino-malonanilic acid	p-Amino-succinilic acid	p-Amino-glutarilic acid	p-Amino-dip-amic acid
ANTISERA FOR AZOPROTEINS CONTAINING:	  	 		
p-Aminooxanilic acid	++	-	-	-
p-Aminosuccanilic acid	-	-	+	-
p-Amino adipanilic acid	-	-	+	++
p-Aminosuberanilic acid	-	-	±	++±

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was prepared by combining the carboxyl group of glycine with the amino group of leucine and was attached to protein. Serologic cross reactions occurred only (but not always) when the immunizing and test antigens contained identical terminal amino acids (Table 12).<sup>37</sup>

*Table 12. Effect of Amino Acid Arrangement on the Specificity of Peptide Azoproteins*

ANTISERUMS FOR AZOPROTEINS CONTAINING*	TEST ANTIGENS' AZOPROTEINS CONTAINING			
	Glycyl-glycine	Glycyl-leucine	Leucyl-glycine	Leucyl-leucine
Glycyl-glycine	++±	-	-	-
Glycyl-leucine	-	++±	-	tr.*
Leucyl-glycine	+	-	+++	-
Leucyl-leucine	-	+	-	++

\* tr. = trace

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Further experiments with tri-, tetra- and penta-peptides gave the same general results.

#### ANTIGENICITY AND SPECIFICITY OF PROTEINS, POLYSACCHARIDES AND LIPIDS

Most proteins are good antigens; they possess many reactive chemical groups or radicals and tend to differ markedly from one species to another. A few polysaccharides are excellent antigens in certain animals, others are poorly antigenic, and some are nonantigenic. Lipids are nonantigenic, probably because they are chemically similar if not identical in all species, but many of them function as haptens.

**Native Proteins.** Proteins are exceedingly difficult to isolate in relatively pure form; even when isolated there is no assurance that the chemical and physical manipulations of purification yield products identical with the original substances in their natural state.

A few exceptional proteins do not stimulate antibody formation in any animal so far tested. Histones and protamines, which are relatively low in molecular weight and are among the simplest proteins, possess little or no antigenicity. Gelatin, a derived protein resulting from the hydrolysis of collagen, is nonantigenic. Several explanations have been offered for its lack of antigenicity: (1) it is denatured and possesses no definite structural arrangement; (2) it is easily digested or upon injection is rapidly excreted in the urine; (3) it lacks cystine containing -S:S- bridges which help to maintain proteins in a stable configuration.<sup>28</sup> It also lacks tryptophane and tyrosine, aromatic amino acids which are important in the specificity of proteins and may contribute to anti-

genicity. Gelatin acquires limited ability to induce antibody formation when coupled with tyrosine and certain other aromatic compounds. Landsteiner summarized the state of knowledge regarding the chemical composition of antigenic proteins by stating, ". . . there is as yet no consistent theory embracing the experimental facts."<sup>37</sup> This statement is true today.

The antigenicity of animal proteins usually varies indirectly with the degree of biologic relationship between the protein source and the animal immunized. For example, duck proteins are poor antigens in chickens but good antigens in rabbits; conversely, rodent proteins are relatively poor antigens in rabbits.

The specificity of antibodies against proteins is directly proportional to the biologic similarity of the protein source and the animal inoculated. Rabbits and hares, as members of the same family, Leporidae, are closely related physiologically and morphologically. Few persons can distinguish between adults of these animals. However, rabbits are born naked and are blind for a time after birth, whereas the young of the hare possess fur and open their eyes immediately. Antiserum produced in fowls by injecting rabbit or hare serum reacts strongly with both antigens.<sup>46</sup> Injection of hare serum into rabbits yields antibodies which precipitate only hare serum, thus permitting clear-cut distinction between proteins of these related species.<sup>50</sup>

The same principle was also shown by immunogenetic studies of muscovy and mallard ducks and their F<sub>1</sub> hybrid.<sup>44</sup> Rabbit antiserums against red cells of the three birds agglutinated the homologous and heterologous erythrocytes in high titer. However, antiserums produced by injecting the muscovy or mallard parents with hybrid red cells sharply differentiated erythrocytes of the parent species. Antibody titers developed in the ducks were only about 10 per cent of those produced in rabbits, which confirms the inverse correlation between zoologic relationship and antigenicity.

The effect of phylogenetic relationships upon serologic cross reactions is further illustrated by the precipitation of ovalbumins from a variety of domestic fowl by rabbit antiserums against hen ovalbumin (Table 13).<sup>41</sup> The homologous antigen reacted most strongly with the antiserums; albumins from the related fowls, turkey and guinea hen, gave intermediate reactions; those from the duck and goose yielded the smallest precipitates. Reading horizontally, these results parallel the general systematic relationships between the birds and presumably reflect slight chemical differences between the various albumins. The vertical columns, except for the homologous reactions, show variation in antibody titers produced by animals A, B and C. Individual differences in antibody-producing capacity may be observed when several animals of the same species are inoculated with identical antigens. Even siblings occasionally

produce antiserums of markedly different titers. Furthermore, certain breeds or "families" of animals are exceptionally potent producers of particular antibodies.

At least six proteins from the hen's egg, five from the white and one from the yolk, have been distinguished chemically and shown to be different by the precipitin test.<sup>29</sup> Four chemically and serologically distinct proteins have been demonstrated in milk. Blood fibrinogen, albumin and various globulins are readily distinguished. A rabbit inoculated with whole horse plasma produces antibodies which precipitate all the proteins of horse plasma. Removal of the precipitate formed by

*Table 13. Effect of the Source of Ovalbumin on the Intensity of Precipitation with Antisera for Hen Ovalbumin*

RABBIT ANTI-HEN OVALBUMIN SERUM	SOURCES OF OVALBUMIN ANTIGENS				
	Galliformes			Anseriformes	
	Hen	Turkey	Guinea hen	Duck	Goose
Relative volumes of precipitates					
A	100%	35%	26%	9%	9%
B	100	67	57	42	31
C	100	61	51	30	18

(From Landsteiner and van der Scheer,<sup>31</sup> by permission.)

mixing horse serum globulins with rabbit anti-horse serum leaves antibodies capable of precipitating horse albumin and fibrinogen.

The chemical basis of specificity is supported by serologic studies of chemically distinguishable proteins. Wells, forty years ago, investigated storage proteins of seeds, which occur in relatively pure form, and found that chemically distinct proteins were serologically different, whereas chemically like proteins from a variety of plants were serologically alike.<sup>65</sup>

Chemically similar or identical antigenic or haptenic substances are widely distributed throughout nature, and these substances may be responsible for many serologic cross reactions between antigens of diverse biologic origin.

**Denatured, Hydrolyzed and Racemized Proteins.** Denaturation is an incompletely understood process peculiar to proteins, characterized by partial or complete loss of solubility (according to the degree of denaturation) at the isoelectric point in water or dilute electrolyte. Denaturation is accomplished by numerous agents including heat, vigorous agitation, adsorption onto surfaces, strong alkalies, heavy metal salts, alcohol, ether and urea. The changes were first thought to be purely physical and irreversible, but recently reversibility has been indicated in some in-

stances. Chemical changes have been reported, particularly liberation of sulphydryl and other reducing groups. A current view describes denaturation as the breaking of relatively unstable bonds which normally maintain the spatial configuration of a peptide chain, followed by formation of new bonds in an irregular rearrangement.

Heated proteins do not react, or react only weakly, with antiseraums against the native proteins. The antigenicity of heated proteins may be diminished or completely destroyed. When antibodies are formed they do not always react strongly with the native proteins but often react with other heated proteins. Cross reactions of heated serum proteins

*Table 14. Precipitation of Native and Heated Serums by Antiserum for Heated (100° C.) Beef Serum*

	TEST ANTIGENS	BEEF SERUM	SHEEP SERUM	HORSE SERUM	HUMAN SERUM	RABBIT SERUM	GUINEA PIG SERUM
Rabbit anti-serum for heated (100° C.) beef serum	Native (unheated)	+++	++	-	-	-	-
	Heated (100° C.)	+++	+++	++	++	+	±

(From Furth,<sup>22</sup> by permission.)

are illustrated in Table 14.<sup>22</sup> Antiserum against heated beef serum precipitated both heated and unheated sheep serum. Heat altered the chemical composition or structure of the antigenic constituents of horse, human, rabbit and guinea pig serums as shown by their precipitation with antibodies against heated beef proteins. Precipitation of heated rabbit serum is especially noteworthy because the antiserum employed was obtained from rabbits. Heat or denaturation evidently diminished the specificity of a variety of otherwise easily differentiated proteins.

It is interesting that rabbits also can be immunized with rabbit serum which has been heated to 120° C. (i.e., autoclaved) for thirty minutes and that the antiserum produced precipitates autoclaved serum proteins of this and a number of other mammalian species.<sup>60</sup>

Proteins lose antigenicity when completely coagulated but may regain the ability to stimulate antibody formation if coagulation is reversed. Relatively few proteins completely coagulate upon heating; hence, they retain antigenicity because the amount of protein necessary for immunization may be extremely small. Somatic protein antigens of bacteria do not always possess the same heat lability as serum proteins. Boiled antigens of some nonmotile and noncapsulated organisms produce just as specific agglutinating serums as do the unheated antigens.

*Hydrolysis* of proteins yields compounds of progressively shorter

peptide chain length and hence of lower molecular weight. Intermediate stages are designated proteoses, peptones and polypeptides, although it is impossible to define these various substances accurately. Amino acids are the final hydrolytic products. They may be split off throughout the process of hydrolysis, depending upon whether the hydrolytic agent attacks terminal or internal peptide linkages. Completely hydrolyzed proteins consisting of a mixture of amino acids are nonantigenic. The stage of hydrolysis at which antigenicity is lost is not known with certainty. Many attempts to immunize with proteoses have been unsuccessful, but some investigators have reported formation of antibodies which reacted with the immunizing material and also with the original protein. These discrepancies may be attributed to lack of methods for separating proteoses and distinguishing them from unhydrolyzed proteins.

The antigenicity of proteins is decreased or destroyed by strong acids and strong alkalies.<sup>64</sup> Alkalics appear to be more effective than acids. Landsteiner and Barron found that some antigenic activity remained after treatment with 5N hydrochloric acid for fifteen hours at room temperature, whereas 1N alkali completely deprived serum proteins of antigenicity in sixteen hours.<sup>38</sup> The alkali-treated proteins regained partial antigenicity when converted into xanthoproteins by nitric acid but possessed altered specificity. Dakin and Dudley reported that alkali treated proteins were resistant to enzymes and were excreted unchanged in the feces when ingested by animals and in the urine when injected subcutaneously.<sup>10</sup> Dakin believed that decreased antigenicity was caused by racemization (loss of optical activity). Later evidence, however, indicated that loss of antigenicity precedes loss of optical activity. Landsteiner suggested that alkali destroys certain chemical structures necessary for antigenicity,<sup>37</sup> and Boyd postulated that alkali splits proteins into molecules of small size.<sup>6</sup>

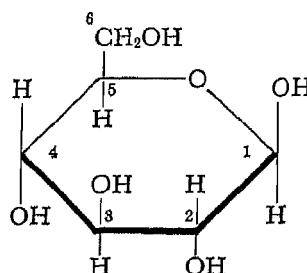
*Racemization* has been useful in demonstrating structural differences between proteins otherwise chemically indistinguishable. Alkali destroys the optical activity of all except the terminal amino acids in a peptide chain. The identity of the remaining optically active amino acids can then be determined in the products of hydrolysis. Different amino acids were detected at the chain ends of hen and duck egg albumin and cow and sheep caseinogen; these proteins have been found serologically different.<sup>9, 12</sup>

**Polysaccharides.** Polysaccharides are serologically important components of most cells. Many are not antigenic but may dominate the serologic specificity of proteins with which they are combined.

Starches, dextrans, glycogens and similar substances have generally been reported nonantigenic under ordinary conditions. Glycogen adsorbed onto a colloidal carrier such as aluminum hydroxide has been said to stimulate weak formation of antibody.

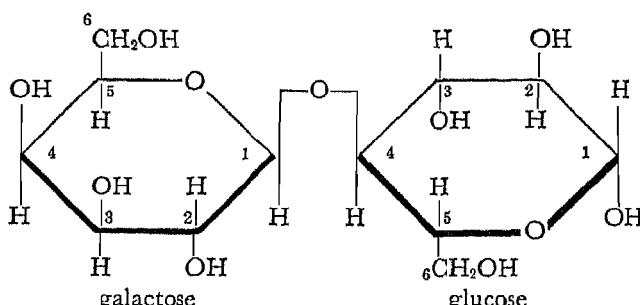
The serologically active polysaccharides are chiefly substances isolated from microorganisms. Bacterial polysaccharides were discovered by Zinsser and others and were called *residue antigens*.<sup>68</sup> Goebel, and Heidelberger and Avery<sup>1, 27, 28</sup> found that the specificity of the pneumococcus types was dependent upon their capsular polysaccharides. These substances were precipitated by antiserums for the respective whole bacteria. Certain polysaccharide-protein complexes, either with or without lipids, are also important bacterial constituents because of their toxicity.<sup>5</sup>

A typical polysaccharide is composed of a number of monosaccharide molecules joined by the  $\equiv\text{C}-\text{O}-\text{C}\equiv$  linkage. The monosaccharides most frequently found in serologically important polysaccharides are pentoses or hexoses. The respective empiric formulas,  $\text{C}_5\text{H}_{10}\text{O}_5$  and  $\text{C}_6\text{H}_{12}\text{O}_6$ , give no indication of the actual structures of monosaccharide molecules, which are pictured as rings. A hexose such as glucose is represented as a hexagonal ring in which an atom of oxygen is one of the members:



The ring is supposed to be at a right angle to the plane of the paper, with the heavy lines in front and the light lines in back.

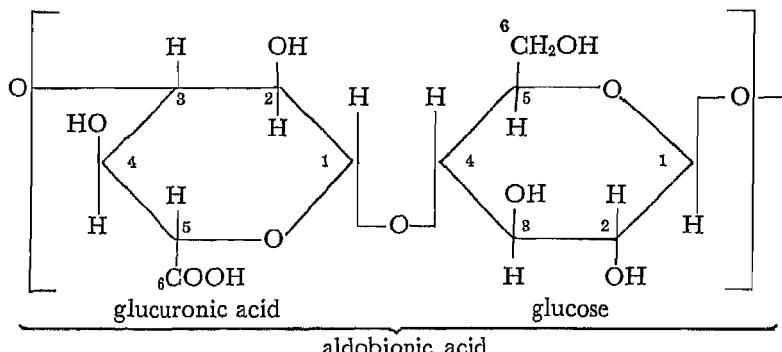
Hexoses can combine with alcohols by elimination of a molecule of water. The alcohol may be another monosaccharide, either of the same or of a different kind. Lactose, for example, is a glucose-galactoside in which glucose has been rotated 180 degrees around its horizontal axis:



Several hundred hexose and other simple sugar molecules of various kinds may be connected to form a long chain. Oxidation to -COOH at

the number 6 carbon atom of a hexose yields a hexuronic acid (e.g., glucuronic, galacturonic). Radicals such as methyl and acetyl may be attached at various positions around the sugar ring. Such derived and substituted monosaccharides are found in naturally occurring polysaccharides. It is obvious that a vast number of configurations and antigenic specificities is possible.

The antigenic pneumococcus 3 polysaccharide is composed of aldobionic acid molecules joined by glucoside linkages. These aldobionic acid molecules contain glucuronic acid and glucose. The postulated structure of the units comprising the polysaccharide chain is as follows:



The minimum molecular weight of this polysaccharide has been calculated to be about 62,000, which corresponds to about 180 aldobionic acid residues.

Polysaccharides used in early work were considerably altered in the process of purification. Type 1 pneumococcal polysaccharide, for example, lost acetyl groups as a result of drastic methods of isolation. Procedures which avoided heat and strong acids or alkalies permitted separation of this substance from cells or culture filtrates in a form which proved to be antigenic for mice, man, horses and a few other animals,<sup>20</sup> but not for rabbits.<sup>21</sup> This polysaccharide, artificially combined with protein, is a better immunizing agent than the pure substance. The polysaccharide-protein complex, unlike the polysaccharide itself, immunizes rabbits against challenge with capsulated pneumococci, and the animals produce antibody demonstrable by passive protection in other rabbits, by agglutination of homologous pneumococci and by precipitation of the polysaccharide haptene. Small amounts of protein may suffice to render a polysaccharide antigenic in the animal body. This fact was not appreciated at first, and conflicting reports of the antigenicity of polysaccharides are common.

Cellular extracts containing "Vi antigen" in varying degrees of purity have been obtained by several investigators from *Salmonella typhosa*, *Escherichia coli* and other organisms. This substance appears to be a

water-soluble polysaccharide-like antigen containing amino nitrogen.<sup>42</sup> Felix and others stated that the Vi antigen was destroyed at 60° C. in thirty minutes or at 100° C. in five minutes.<sup>15</sup> However, it can be extracted from saline suspensions of Vi containing bacteria by boiling for one hour and is present in active form in the supernatant liquid. Recently Felix has shown that certain strains of *S. typhosa* boiled two hours can still adsorb Vi antibodies, whereas other strains boiled one hour cannot.<sup>14</sup> The supernatant fluid from boiled cultures of *E. coli* and *Paracolobactrum ballerup* is capable of inducing formation of Vi antibodies.

Capsular materials from *Klebsiella pneumoniae* and substances from mucoid strains of streptococci are also serologically active. The capsules of *Bacillus anibracis* do not contain carbohydrate, but the cell bodies contain a specific polysaccharide haptene composed of glucosamine (i.e., glucose in which an amino group replaces an hydroxyl radical), galactose and acetic acid. Most of the noncapsulated gram negative rods studied possess carbohydrates associated with some of their antigenic constituents. *S. typhosa* contains a specific polysaccharide haptene consisting of glucose, mannose, galactose, nitrogen, phosphorus and acetyl radicals, and having a minimum molecular weight of 10,000. *Shigella dysenteriae* also possesses a polysaccharide haptene composed of an acetylated amino sugar, galactose, rhamnose and a phosphorus compound, with a minimum molecular weight of 5,000.

The type specific human "A" substance found in horse saliva, pepsin and urine and in some vegetable gums contains serologically active polysaccharides. Some of these materials may cross react with antiserum against pneumococcal polysaccharides.

There are numerous serologic cross reactions among materials of animal, plant and bacterial origin in which polysaccharides are concerned. Therefore it seems likely that only a limited variety of polysaccharides is widely distributed in nature.

**Lipids.** Lipids are poorly defined substances. They are considered to be esters of organic acids with various alcohols, plus other radicals such as phosphate and sulfate, nitrogenous bases, etc. It has been assumed in immunologic literature that substances extracted by solvents like alcohol, ether and chloroform are confined to lipids, but it is now known that an amount of protein sufficient to cause antibody formation may be found in these extracts.

There are relatively few lipids and all are widely distributed in nature. Therefore, they should lack the ability to stimulate antibody production and, with few exceptions, this is true.

Sachs and Klopstock reported that cholesterol or lecithin in combination with swine serum induced in rabbits formation of antibodies which fixed complement and flocculated with the respective lipids.<sup>53</sup> Other authors maintained that such results, although capable of duplication,

were caused by traces of impurities in the lipid preparations. On the other hand, antibody formation was obtained against a synthetic distearyl-lecithin by Weil and Besser<sup>42</sup> and by Maier,<sup>45</sup> and against several times recrystallized cholesterol by Berger and Scholer.<sup>3</sup> Obviously the conflicting results and interpretations of such experiments with lipids necessitate further work with chemically well defined preparations.

Alcoholic extracts of animal organs which alone do not cause antibody formation may be capable of stimulating antibody production when mixed with foreign protein such as swine serum. This procedure has repeatedly been followed with successful results and is called "combination immunization." Landsteiner thought the lipid might form a loose union with serum proteins.<sup>40</sup> Sachs, Klopstock and Weil suggested that the protein merely served as an "envelope" which permitted entry of the nonprotein matter into the cells and referred to the protein as a "schlepper" or "conveyer."<sup>54</sup> Combination immunization has been employed with extracts of brain, testicle and other organs. Antibodies have even been induced in an animal against its own tissues. Lewis produced antibodies in rabbits by injecting alcoholic extracts of rabbit brain mixed with normal horse serum.<sup>43</sup> These antibodies completely fixed complement when reacting with as little as 0.02 milligram of the lipoid (alcohol soluble) material from rabbit brain.

**Serologic Role of Lipids.** Lipids appear to take definite part in certain serologic reactions. The antigen used in the Wassermann test for syphilis is an alcoholic extract of beef heart. This antigen can be made more sensitive by addition of pure lipids, which may function by increasing the dispersion of the haptenic antigen. Hartley reported that if a protein antigen such as horse serum and homologous immune serum were extracted with ether, they did not flocculate when mixed, although antigen and antibody apparently combined and the mixture became opalescent.<sup>25</sup> Horsfall and Goodner removed the agglutinating and precipitating activity from antipneumococcal *horse* serum by extraction with alcohol and ethyl ether and greatly reduced the agglutinating and precipitating power of antipneumococcal *rabbit* serum by extraction with similar solvents.<sup>31</sup> Extracted horse antiserum did not regain its agglutinating and precipitating activity when recombined with its lipid extract, but such activity was restored by adding a small amount of purified lecithin. The activity of rabbit antiserum was restored by cephalin. Human, mouse, dog, cat and goat antipneumococcal serums behaved like horse antiserum, and guinea pig, rat and sheep antiserums resembled rabbit antiserum.<sup>32</sup> Removal of lipids did not seem to affect the combining power of the antiserums with homologous pneumococcal antigen. Similarly, Hartley found that diphtheria antitoxin, extracted with alcohol and ether, retained its ability to neutralize toxin, as indicated by skin tests in guinea pigs, but no longer flocculated toxin *in vitro*.<sup>25</sup> No reference

was made to the restoration of flocculating power by the extracted or other lipids.

#### HETEROPHILE ANTIGENS

Heterophile antigens are substances which stimulate production of antibodies capable of reacting with tissues from other animals, fish or even plants.

Forssman discovered in 1911 that emulsions of guinea pig liver, kidney, adrenals, testicles and brain produced in rabbits high concentrations of antibodies which lysed sheep red blood cells in the presence of complement.<sup>18</sup> Guinea pig erythrocytes and serum did not cause production of these antibodies. The antigenic substance was later called the Forssman antigen, and the hemolysin was designated the Forssman antibody. Forssman antigen free from protein can be extracted by alcohol from tissues of certain animals. This material combines with Forssman antibody, but when properly extracted has no immunizing power unless artificially combined with a protein such as swine serum.

Forssman antigens can be boiled or autoclaved for several hours without being completely destroyed. At the present time the composition of the Forssman antigen is not definitely known, but it seems to contain nitrogen and upon hydrolysis liberates a reducing sugar which may be glucosamine.<sup>37</sup> There is also evidence of fatty acids and other components, but apparently no sulfur or phosphorus.

The Forssman antigen has been found in the horse, dog, cat, mouse, fowl, tortoise and many other animals. It is present either in the organs or in the erythrocytes but usually not in both. It is found in erythrocytes of sheep and man (blood groups A and AB) but is absent from both organs and erythrocytes of the rabbit, cow, pig and rat. The Forssman antigen is also present in certain strains of *Salmonella* and *Pasteurella*, *Sh. dysenteriae*, *Diplococcus pneumoniae* and *B. anthracis*. Its occurrence in parasitic bacteria and their animal hosts and its virtual absence from saprophytes suggested to Holtzman that the antigen was originally implanted in the microorganisms by intimate contact with the host.<sup>38</sup> He cultivated *S. typhosa* and *S. paratyphi* in collodion sacs within the peritoneal cavity of guinea pigs and found that the bacteria acquired Forssman antigen which persisted through fifty daily transfers on a synthetic agar but was lost by the seventy-seventh transfer. Forssman antigen was also acquired by the organisms when cultivated on horse serum agar. The "implanted antigen" hypothesis seems plausible in view of the successful addition of other specific antigens to certain pneumococci and *Salmonellas* (see page 165).

Forssman antibody can be produced by injecting rabbits with sheep erythrocytes, saline suspensions of guinea pig kidney and other tissues

containing Forssman antigen. Rabbits injected with sheep cells produce, in addition, antibodies against the species-specific substances of the cells. These are called *isophile* antibodies. The heterophile titers are invariably much greater than the isophile titers. Anti-sheep cell serums having titers of 20,480 possess isophile titers of only 320 to 640, as shown by adsorption with boiled sheep erythrocytes.

The incompatibility between Forssman antigen and antibody can be demonstrated dramatically *in vivo* by intravenously injecting a guinea pig, whose tissues contain the antigen, with normal or immune serum containing sufficient of the antibody. The animal immediately dies with symptoms resembling acute anaphylactic shock.

There are many heterophile systems, of which the Forssman antigen is only one. Unfortunately the terms *Forssman antigen* and *heterophile antigen* have frequently been used synonymously. The designation *Forssman antigen* should be limited to the antigen discovered by Forssman in guinea pig tissues, and *heterophile antigen* should be used to denote a broad group of antigens present in various plants and animals and possessing characteristics similar to those of the Forssman antigen.

Buchbinder found a heterophile antigen in bacteria of the hemorrhagic septicemia group and in the erythrocytes of a wide variety of birds.<sup>8</sup> Rabbit antiserums against these organisms agglutinated and lysed bird erythrocytes. The Buchbinder antigen was distinct from the Forssman antigen; both were present in some of the organisms and birds.

Another heterophile antigen is shared by human erythrocytes and type 14 pneumococci. Finland and Curnen found that horse antiserums against type 14 pneumococci agglutinated human red cells of any blood group in titers of 20 to 2560.<sup>16, 17</sup> Weil and Sherman did not find the antigen in alcoholic extracts of erythrocytes, but detected it in saline suspensions of human heart, kidney and liver tissue and in rabbit erythrocytes.<sup>83</sup> It is of interest in this connection that *rabbit antipneumococcus 14* serums, in contrast to corresponding *horse* antiserums, failed to agglutinate human red cells of groups O and B and agglutinated cells of groups A and AB and sheep cells in low titers. This would indicate that type 14 pneumococci contain not only the antigen described by Finland and Curnen, but also a low concentration of the Forssman antigen.

Goebel and co-workers obtained a lipopolysaccharide from the cellular debris of autolyzed rough type 1 pneumococci which was antigenic in rabbits, giving rise to sheep hemolysins and to precipitins for the lipopolysaccharide.<sup>28</sup>

The heterophile systems which involve sheep hemolysins or hemagglutinins constitute a confusing example of serologic complexity. The serums of many normal humans contain sheep hemagglutinins in titers as high as 320. These normal antibodies are completely or almost completely removed by adsorption with guinea pig kidney but not by beef red blood

cells, and are therefore for the most part Forssman antibodies. Forssman serums prepared by immunizing rabbits with saline emulsions of guinea pig kidney contain high titers of sheep hemolysin. Adsorption of such antiserums with guinea pig kidney removes all sheep cell antibodies, but adsorption with beef erythrocytes does not affect the sheep cell titers.

Human serums in infectious mononucleosis contain moderate amounts of both sheep hemolysins and hemagglutinins. They also agglutinate beef erythrocytes. The sheep agglutinins are reduced slightly if at all by adsorption with guinea pig kidney but are almost completely removed by beef red blood cells. These heterophile antibodies therefore appear to be non-Forssman in nature.

Serums from patients with serum sickness (produced by horse serum injection) contain moderate titers of sheep hemagglutinins which are partially removed by adsorption with guinea pig kidney and completely removed by beef red cells. Rabbit erythrocytes, which lack Forssman antigen, also adsorb sheep agglutinins from some serums. These observations indicate that horse serum probably contains more than one heterophile antigen capable of inducing increased sheep hemagglutinin titers, only one of which is the Forssman antigen.

The picture is further complicated by the observation that human blood cells of groups A and AB possess the Forssman antigen. However, sheep hemagglutinins and hemolysins in infectious mononucleosis and serum sickness do not produce apparent reactions *in vivo* with such blood cells, which indicates that the heterophile antigen in human A and AB cells is not identical with that of infectious mononucleosis or serum sickness.

Early investigators noted that Forssman serums containing sheep hemolysins in high titer failed to agglutinate sheep erythrocytes. This phenomenon appeared so consistently that it was stated as a characteristic of such serums.<sup>19</sup> It is true that injection of saline extracts of guinea pig kidney into rabbits causes rapid production of sheep hemolysins in high titers (e.g., 10,240 or 20,480). However, low titers (160 to 640) of sheep hemagglutinins may also be produced. Serums from infectious mononucleosis and serum sickness appear to contain both agglutinins and lysins for sheep red cells, but only the agglutinins are ordinarily determined in routine tests.

Tomcsik and Schwarzweiss recently obtained the infectious mononucleosis and serum sickness antigens in highly active form from beef erythrocytes.<sup>56, 58</sup> The infectious mononucleosis antigen was secured by extraction with boiling 80 per cent alcohol. The dried extract was obtained as a brownish red powder which, diluted 1:2,400,000, inhibited agglutination of sheep red cells by infectious mononucleosis serum. The serum sickness antigen was extracted from beef cells by boiling absolute alcohol; the product in a dilution of 1:500,000 inhibited sheep cell agglu-

tination by serum sickness serums. On the other hand, serum sickness antigen of only low potency was extracted from guinea pig kidney and from horse serum by the same method. Also, it has long been known that the Forssman antigen can be secured from guinea pig kidney or sheep erythrocytes by extraction with absolute alcohol at room temperature. These observations strongly indicate that there are some definite chemical differences between the three heterophile antigens.

It can be concluded at this time only that the heterophile antigen-antibody relations are extremely intricate and in need of further clarification.

#### POLYSACCHARIDE-PHOSPHOLIPID COMPLEXES OF BACTERIA

The search for toxic bacterial components led to discovery of complex substances which have been termed *Boivin antigens*, after one of their discoverers. Boivin and his co-workers,<sup>4</sup> and Raistrick and Topley,<sup>52</sup> Morgan and Partridge<sup>49</sup> and others extracted bacterial cells with trichloroacetic acid or diethylene glycol or digested them with trypsin. Toxic antigenic substances were secured from smooth gram negative bacteria, particularly *Salmonella* and *Shigella* species. These substances represented 5 to 20 per cent of the weight of the dried organisms. Boivin's trichloroacetic acid extract of *S. typhosa* was lethal for mice in doses of 0.05 to 0.1 milligram intraperitoneally or intravenously. The minimal lethal dose for mice of similar material from *Sh. dysenteriae* was also about 0.1 milligram. These toxic agents were believed to be endotoxins. They consist of a *loose* complex of polysaccharide, phospholipid and often protein.

The pathologic effects of endotoxins from all the *Salmonella*-dysentery organisms studied are similar. Characteristic symptoms following injection into experimental animals include diarrhea, extreme loss of weight and death. Autopsy reveals intestinal hemorrhage with swelling and necrosis of Peyer's patches and the underlying mucosa; degeneration occurs in the liver, spleen, kidney and heart. Other physiologic changes preceding death include increased and later decreased blood sugar, initial leukopenia and subsequent leukocytosis, and either hyperthermia or hypothermia according to the dose and other circumstances. Many of the same reactions occur in man infected by *Salmonella* or *Shigella*. Comparable reactions can be produced in laboratory animals by injection of similar materials from *E. coli*, *Neisseria* and other gram negative bacteria.

Sublethal doses of the polysaccharide-phospholipid-protein complex incite the formation of agglutinating antibody. The serums of animals immunized against Boivin endotoxins neutralize a very few minimal lethal doses of the endotoxins. Boivin antigen is evidently contained in the cell bodies and is considered to be a *somatic antigen*, along with other

proteins and cellular components. Frequently the total somatic antigens of the cell are considered to be its endotoxins. However, the work of Boivin, in which a large part of the somatic protein was eliminated, tends to refute this because the Boivin antigens possess considerable toxicity but are only weakly immunogenic.

Morgan and Partridge isolated antigenic materials from gram negative bacteria by diethylene glycol extraction. They subjected the purified somatic antigen of *S. typhosa* to acid hydrolysis and partially determined its composition (Table 15).<sup>48</sup> The polysaccharide-protein complex freed from phospholipid by precipitation with alcohol was strongly antigenic but was less toxic for mice than the complete antigen. The polysaccharide alone possessed slight, if any, antigenicity but regained antigenicity when

*Table 15. Components of a Purified Somatic Antigen of Salmonella typhosa (Products of Acid Hydrolysis)*

FRACTION	PER CENT OF ANTIgenic EXTRACT	CHEMICAL NATURE	SEROLOGIC NATURE
1. Ether soluble	5-7	Phospholipid	Not antigenic
2. Acid insoluble, soluble in dilute alkaline solution	20	Conjugated protein	Strongly antigenic, not specific (indistinguishable from protein of <i>Shigella dysenteriae</i> )
3. Water soluble	50-55	"Degraded polysaccharide"	Nontoxic haptene

(From Morgan and Partridge,<sup>48</sup> by permission.)

recombined with the protein. The protein therefore conferred antigenicity upon the complex.

The phospholipid-protein-polysaccharide complex extracted from *Sh. dysenteriae* by Morgan and his co-workers also was a complete antigen. The phospholipid component contained palmitic, oleic and  $\alpha$ -glycerophosphoric acids and was nonantigenic. The polysaccharide component dominated the specificity of the complex and behaved as a haptene. It contained acetylglucosamine, galactose and rhamnose. Rough bacterial forms lacked this polysaccharide. The protein fraction of the complex was antigenic and was chemically and serologically indistinguishable from the corresponding protein of *S. typhosa*.

The relationship between the so-called Boivin antigens and the somatic antigens of bacteria is not clear. Materials isolated from a given organism by a variety of methods have been found to behave like somatic antigens, but it does not appear to have been shown that these various materials are identical. In the case of diethylene glycol extracts, Morgan reported that an extract of *Sh. dysenteriae* precipitated all the O (i.e., somatic) antibody from antisera against living or dead bacteria of this species.<sup>47</sup>

Furthermore, the specific agglutinin for *Sb. dysenteriae* induced by immunization with the extract was completely adsorbed by the homologous organisms. These results indicate that the diethylene glycol extract contained the complete O antigens of this organism. Similar reciprocal adsorption experiments seem not to have been performed with antigenic material extracted by other methods. It would be interesting to learn the effect of further purification of the various extracts on such adsorption tests; the pure material might conceivably be unable to remove more than a small percentage of the antibodies in an antibacterial serum.

Study of the endotoxins and somatic antigens of bacteria is not entirely academic. The practical aspect of these investigations lies in the attempt, so far not completely successful, to prepare potent but nontoxic antigens for human immunization. Typhoid, paratyphoid, and other bacterins may produce considerable inflammation at the site of inoculation, general malaise, fever, nausea or other unpleasant reaction. It was hoped that chemical extraction would yield antigens producing better protective antibodies with no undesirable side reactions, but at present typhoid and paratyphoid immunization is still performed with killed whole bacteria.

A series of observations which date from early in the present century and which have been neglected in more recent literature may profitably be reviewed in connection with the discussion of bacterial toxicity.

Vaughan showed that almost all proteins contain toxic constituents which are released by appropriate chemical treatment.<sup>21</sup> Cells of even nonpathogenic bacteria liberated such substances when digested with 2 per cent NaOH in absolute alcohol. The poisonous material was a protein. So-called "protein poisons" derived from a variety of bacteria appeared to be identical and produced similar effects. Vaughan considered that the poison was not a toxin and was nonspecific but occurred commonly in all proteins. He proposed that body cells to which bacteria or foreign protein have gained access elaborate a specific "ferment" or enzyme which digests the foreign material and liberates toxic protein. The pathogenicity of bacteria is determined by their ability to multiply within the animal body, and the nature of the disease produced depends upon the site in which they can grow.

In the absence of satisfactory demonstration of Vaughan's "ferments," Friedberger postulated that a toxic substance, anaphylatoxin, is formed from the products of an antigen-antibody reaction by the supposed proteolytic action of complement.<sup>21</sup> This toxic substance causes local inflammation, fever, injury to the central nervous system and other symptoms. Any bacteria may furnish the substrate from which this substance is made, with the cooperation of specific antibodies and complement.

Thiele and Embleton also proposed that cleavage of proteins within the body, either by complement and antibody or by autolysis or other

enzyme action, yields nonspecific "endotoxins" to which the symptoms of disease are attributed.<sup>57</sup>

Keysser and Wassermann found that poisons resembling Friedberger's toxic material were produced in the absence of bacterial antigen by mixing kaolin with horse serum and then treating the kaolin with guinea pig serum or complement.<sup>54</sup> They concluded that the poisons were formed by complement acting on horse serum constituents adsorbed to the kaolin. Jobling and Peterson also found that similar poisons were produced *in vitro* by mixing fresh serum with bacteria, kaolin, agar or chloroform.<sup>55</sup> They suggested that serum normally contains anti-enzymes which prevent the action of serum enzymes on blood proteins, thus maintaining a condition of delicate balance. The anti-enzymes, however, are adsorbed by bacteria, kaolin or agar, or are inactivated by chloroform. Serum treated with these agents therefore undergoes proteolysis and becomes toxic. Novy and DeKruif likewise postulated that bacterial protein catalyzes the formation of toxic substances from blood constituents.<sup>56</sup>

These various suggestions fall into two principal categories. First are the hypotheses of Vaughan, Friedberger and Thiele and Embleton, which state that bacterial toxicity is the result of liberation within body cells of "protein poisons" or "endotoxins" from bacteria as a result of specific enzyme or complement and antibody action. Second is the hypothesis of Keysser and Wassermann, supported by Jobling and Peterson and by Novy and DeKruif, that similar toxic proteins are formed from serum components by the action of enzymes normally present in blood, such proteolytic action being initiated by the presence of bacteria which adsorb anti-enzymes.

Present attempts to isolate endotoxins and somatic antigens responsible for the toxicity and some of the specific properties of various bacteria seem to represent a continuation of the line of investigation begun by Vaughan, aided by somewhat more advanced chemical and physical methods. It is obvious, however, that the complete story of bacterial toxicity is not yet known.

#### *Summary of the Properties of Antigens*

1. *Antigens* are high molecular weight foreign substances possessing many "active" chemical groups, which induce antibody formation only when injected into or beyond the epithelial tissue of an animal. *Complex haptenes* cannot incite antibody formation but may react with antibodies. *Simple haptenes* can neither cause antibody production nor react visibly with antibodies but may inhibit antigen-antibody combination.

2. The specificity of protein antigens depends upon their chemical composition and spatial configuration and may be altered by introducing

various chemical groups. Most effective modifiers of specificity are strongly polar radicals such as acids and bases; neutral radicals are less effective. The size and stereochemical configuration of determinant radicals affect the specificity of an antigen. There is evidence that the arrangement of amino acids in a protein chain, particularly the identity of the terminal amino acid, determines the serologic character of the protein.

3. Certain polysaccharides stimulate antibody formation in some animals, but most of them behave as haptens, contributing to the specificity of the proteins with which they are associated in nature.
4. Lipids, if antigenic, act as haptens but when combined with protein will stimulate antibody formation.

### *References*

1. Avery and Goebel, 1933. *Jour. Exp. Med.* **58**, 731.
2. Battey, Stuart and Wheeler, 1938. *Jour. Immunol.* **35**, 75.
3. Berger and Scholer, 1932. *Ztschr. f. Immunitätsforsch.* **76**, 16.
4. Boivin and Mesrobeanu, 1933. *Compt. rend. Soc. de biol.* **113**, 490.
5. Boivin and Mesrobeanu, 1935. *Rev. d'Immunol.* **1**, 553.
6. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
7. Boyd and Hooker, 1934. *Jour. Biol. Chem.* **104**, 329.
8. Buchbinder, 1935. *Arch. Path.* **19**, 841.
9. Dakin and Dale, 1919. *Biochem. Jour.* **13**, 248.
10. Dakin and Dudley, 1913. *Jour. Biol. Chem.* **15**, 263, 271.
11. Doerr and Russ, 1909. *Ztschr. f. Immunitätsforsch.* **3**, 181.
12. Dudley and Woodman, 1915. *Biochem. Jour.* **9**, 97.
13. Ecker and Pillemer, 1940. *Jour. Exp. Med.* **71**, 585.
14. Felix, 1932. *Jour. Hyg.* **50**, 515.
15. Felix and Pitt, 1934. *Jour. Path. Bact.* **38**, 409.
16. Finland and Curnen, 1938. *Science* **87**, 417.
17. Finland and Curnen, 1940. *Jour. Immunol.* **38**, 457.
18. Forssman, 1911. *Biochem. Ztschr.* **37**, 78.
19. Forssman, 1931. In Kolle, Kraus and Uhlenhuth, *Handbuch der pathogenen Mikroorganismen*, 3rd ed., Fischer, Jena.
20. Francis and Tillett, 1930. *Jour. Exp. Med.* **52**, 573.
21. Friedberger, 1910. *Ztschr. f. Immunitätsforsch.* **6**, 179, 299; **7**, 94, 665, 748.
22. Furth, 1925. *Jour. Immunol.* **10**, 777.
23. Goebel and Adams, 1943. *Jour. Exp. Med.* **77**, 435.
24. Gutman, 1938. *Rev. d'Immunol.* **4**, 111.
25. Hartley, 1925. *Brit. Jour. Exp. Path.* **6**, 180.
26. Haurowitz, 1952. *Biol. Rev.* **27**, 247.
27. Heidelberger and Avery, 1923. *Jour. Exp. Med.* **32**, 73.
28. Heidelberger and Avery, 1924. *Jour. Exp. Med.* **40**, 301.
29. Hektoen and Cole, 1928. *Jour. Infect. Dis.* **42**, 1.
30. Holtzman, 1939. *Jour. Immunol.* **36**, 413.
31. Horsfall and Goodner, 1935. *Jour. Exp. Med.* **62**, 485.
32. Horsfall and Goodner, 1936. *Jour. Immunol.* **31**, 135.
33. Jobling and Peterson, 1914. *Jour. Exp. Med.* **19**, 485.
34. Keysser and Wassermann, 1911. *Ztschr. Hyg. Infektkr.* **68**, 535.
35. Kleczkowski, 1940. *Brit. Jour. Exp. Path.* **21**, 98.
36. Landsteiner, 1921. *Biochem. Ztschr.* **119**, 294.

37. Landsteiner, 1945. *The Specificity of Serological Reactions*, rev. ed., Harvard University Press, Cambridge, Mass.
38. Landsteiner and Barron, 1917. Ztschr. f. Immunitätsforsch. 26, 142.
39. Landsteiner and Lampl, 1917. Biochem. Ztschr. 86, 343.
40. Landsteiner and Simms, 1923. Jour. Exp. Med. 38, 127.
41. Landsteiner and van der Scheer, 1940. Jour. Exp. Med. 71, 445.
42. Landy, Webster, Freeman and Batson, 1951. Fed. Proc. 10, 413.
43. Lewis, 1941. Jour. Immunol. 41, 397.
44. McGibbon, 1944. Genetics 29, 407.
45. Maier, 1933. Ztschr. f. Immunitätsforsch. 78, 1.
46. Moody, Cochran and Drugg, 1949. Evolution 3, 25.
47. Morgan, 1949. In Miles and Pirie, *The Nature of the Bacterial Surface*, Basil Blackwell & Mott, Ltd., Oxford.
48. Morgan and Partridge, from Bornstein, 1943. Jour. Immunol. 46, 439.
49. Morgan and Partridge, 1940. Biochem. Jour. 34, 169.
50. Novy and DeKruif, 1917. Jour. Infect. Dis. 20, 449.
51. Obermayer and Pick, 1906. Wien. klin. Wschr. 19, 327.
52. Raistrick and Topley, 1934. Brit. Jour. Exp. Path. 15, 113.
53. Sachs and Klopstock, 1925. Biochem. Ztschr. 159, 491.
54. Sachs, Klopstock and Weil, 1925. Dtsch. med. Wschr. 51, 589.
55. Schwarzwäiss and Tomcsik, 1948. Proc. Soc. Exp. Biol. 69, 558.
56. Snapper and Grunbaum, 1936. Brit. Jour. Exp. Path. 17, 361.
57. Thiele and Embleton, 1913. Ztschr. f. Immunitätsforsch. 19, 643, 666.
58. Tomcsik and Schwarzwäiss, 1948. Proc. Soc. Exp. Biol. 69, 562.
59. Uhlenhuth, 1905. Dtsch. med. Wschr. 1673.
60. Uwazumi, 1934. Arb. med. Fak. Okayama 4, 53.
61. Vaughan, 1913. *Protein Split Products*, Lea and Febiger, Philadelphia.
62. Weil and Besser, 1932. Ztschr. f. Immunitätsforsch. 76, 76.
63. Weil and Sherman, 1939. Jour. Immunol. 36, 139.
64. Wells, 1909. Jour. Infect. Dis. 6, 506.
65. Wells, 1929. *The Chemical Aspects of Immunity*, rev. ed., Chemical Rubber Co., New York.
66. White, 1938. *The Biology of Pneumococcus*, Commonwealth Fund, New York.
67. Wilson and Miles, 1946. *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., Edward Arnold & Co., London.
68. Zinsser and Parker, 1923. Jour. Exp. Med. 37, 275.

## Chapter 4

### SERUM PROTEINS

#### CHEMICAL NATURE OF ANTIBODIES

ANTIBODIES are considered to be protein or inseparably associated with protein. Heat destroys antibody activity and denatures proteins in antiserum at approximately equal rates; both processes are generally minimal at pH 7 and increase in acid and alkaline media. High concentrations of salts, glycerol or sugar delay thermal denaturation of proteins and protect antibody against destruction by heat. Antibodies may differ in their heat resistance: flagellar antibodies are often somewhat more thermostable than the corresponding somatic antibodies present in the same antiserum, an observation which indicates a difference in the physicochemical structure of the two kinds of antibodies.

Antibodies are removed from antiserum by precipitation of proteins and can often be recovered in active form from the precipitate. The disappearance of antibodies from an immune serum acted upon by proteolytic enzymes depends upon the extent to which hydrolysis is allowed to proceed. For example, trypsin has been found to destroy partially purified pneumococcus type 1 antibody, the rate of destruction being approximately the same as the rate of increase of amino nitrogen.<sup>4</sup>

Further evidence for the protein nature of antibody is afforded by analysis of the antigen-antibody complex formed when antiserum reacts with its homologous antigen. The precipitate or agglutinate contains considerably more protein than the antigen itself, even though thoroughly washed to remove nonantibody proteins before analysis. These observations are especially convincing when protein-free antigens or haptens are employed. Felton and Bailey reported that 2.5 milligrams of pneumococcus polysaccharide treated with homologous antiserum yielded a precipitate containing thirty-seven milligrams of protein, which could have come only from the antiserum.<sup>10</sup>

Dean, Taylor and Adair produced a polyvalent rabbit antiserum by

immunizing with two different antigens, egg albumin and horse serum albumin, simultaneously.<sup>7</sup> They found (Table 16) that either antigen alone yielded the same amount of precipitate from equivalent amounts of antiserum, whether or not antibody corresponding to the other antigen had been previously removed by precipitation. Moreover, the totals of the precipitates obtained with the individual antigens separately were the same as that formed when the antiserum was treated with the two antigens together. This experiment performed in the presence of heter-

*Table 16. Precipitation of Two Different Antigens by a Polyvalent Rabbit Antiserum*

TUBE	ANTISERUM VS. EGG ALBUMIN + SERUM ALBUMIN	ANTIGEN		PRECIPITATE	TOTAL PRECIPITATE WITH EGG ALBUMIN AND SERUM ALBUMIN ANTIGENS
		Egg albumin	Serum albumin		
1	1 ml.	0.57 mg.	—	5.9 mg.	
2	Supernate from 1	—	1.14 mg.	9.9 mg.	15.8 mg.
3	1 ml.	—	1.14 mg.	9.7 mg.	
4	Supernate from 3	0.57 mg.	—	5.9 mg.	15.6 mg.
5	1 ml.	0.57 mg	1.14 mg.	15.1 mg.	15.1 mg.

(Data from Dean, Taylor and Adair: *Precipitation reaction; experiments with antiserum containing two antibodies (1935)*,<sup>7</sup> recalculated on the basis of 1 ml. antiserum. By permission of Cambridge University Press.)

ologous antiserum or normal serum would have yielded the same result, because the amount of protein precipitated is completely uninfluenced by other proteins, including heterologous antibodies which may be present.

#### PHYSICAL PROPERTIES OF SERUM PROTEINS

Before further considering the nature of antibodies, it is necessary to discuss the physical characteristics of serum proteins.

**Electrophoretic Mobility. Electrophoresis.** Electrophoresis is the migration of charged particles in an electric field. The rate of migration depends upon the magnitude of the charge upon the particles, the viscosity of the medium, the voltage of the electric field and other factors. The particle charge is affected by pH and by the electrolyte and its concentration in the suspending medium. It is determined experimentally by measuring the rate of migration when all other factors are known. The direction and rate of migration of microscopic particles such as bacteria and blood cells are observed in a chamber mounted on the stage of a microscope. Electric current is applied through two electrodes and

the distance which individual cells traverse per unit of time is noted. The net charge on the cells is calculated by means of a formula.

All proteins are amphoteric; that is, they contain radicals which dissociate to give positive and negative ions (e.g.,  $\text{—COO}^-$  and  $\text{—NH}_3^+$ ).

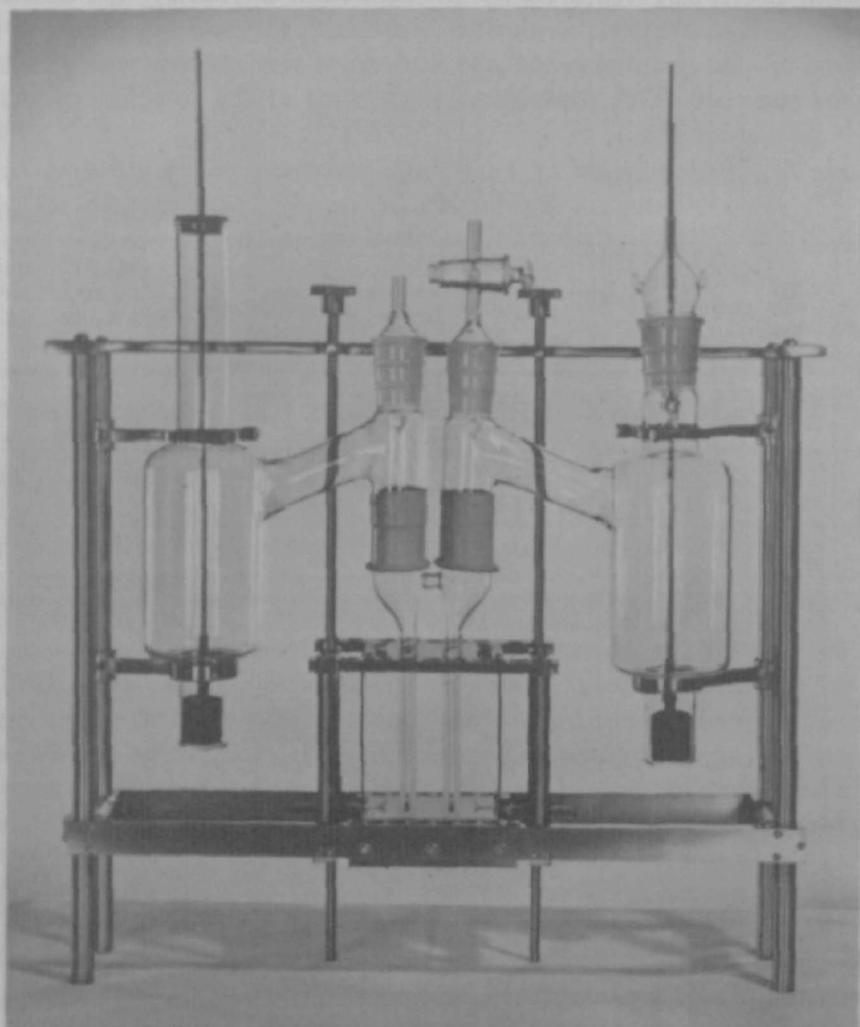


FIGURE 6. Electrophoresis cell. The U-tube is at bottom center and electrodes are in glass columns at left and right. (From Gray, *Sci. Amer.* 185, 45, 1951, by permission of the author and the editors of *Scientific American*. Photograph by Keturah Blakely.)

The relative and absolute numbers of positive and negative ions determine the sign and magnitude of the charge on the surface of the molecule or protein-containing particle. The charge is negative when negative ions predominate. A negatively charged particle migrates toward the positive electrode in an electric field. If such a suspension is made more acid, additional basic radicals dissociate and their positive charges reduce the

net negative charge on the protein. At a certain reaction (pH) known as the *isoelectric point* the protein is maximally ionized, although the net charge is zero since positive and negative charges are equal. The particle does not migrate in either direction in an electric field, but may precipitate out of solution. The isoelectric point of one protein usually differs from that of another. Below the isoelectric point the particles possess a net positive charge and migrate toward the negative pole of the electrophoresis cell.

The electrophoretic migration of submicroscopic or macromolecular particles such as proteins in solution can be determined by use of appropriate apparatus. A protein solution is placed in the bottom half of a U-shaped cell (Fig. 6)<sup>11</sup> and covered with a buffer of proper pH. Elec-

Table 17. Electrophoretic Mobility and Isoelectric Points of the Four Principal Protein Fractions of a Normal Serum

pH	MOBILITY IN CMS. <sup>2</sup> /VOLT/SEC. $\times 10^5$			
	Albumin	$\alpha$ -globulin	$\beta$ -globulin	$\gamma$ -globulin
6.02	-4.60	-3.34	-2.55	+0.01
8.03	-7.15	-6.16	-4.20	-1.51
Isoelectric point: pH	4.64	5.06	5.12	6.0

(From Tiselius: Electrophoresis of serum globulin,<sup>20</sup> By permission of Cambridge University Press.)

trical connections are made to the buffer in both arms of the cell. The protein migrates down one arm of the U-tube and up the other, through the buffer solution. The migrating faces of a pure protein form sharp boundaries because each molecule of a protein moves at the same rate in the same direction. In a solution containing a mixture of proteins, the more highly charged molecules migrate faster and form separate boundaries. Slowly moving molecules are sorted out according to their respective rates of migration. The distance between boundaries constantly increases as long as the current is applied. Boundaries in solutions containing proteins of nearly the same charge are diffuse as a result of concentration gradients.

Optical methods of observation are employed because the boundaries are usually not distinguishable by visual inspection. Most of these methods depend upon the principle that a solution containing a substance such as protein has an index of refraction different from the pure solvent. A narrow beam of light passing through an electrophoretic U-tube containing a boundary or concentration gradient of protein is refracted at an angle dependent upon the protein concentration. Optical scanning of the entire contents of the U-tube yields a series of crests and troughs,

The number of crests corresponds to the number of electrically distinct proteins, and the areas under the crests are proportional to their concentrations. The most rapidly migrating fraction (i.e., that with the greatest net charge) forms the highest boundary in the so-called ascending limb of the U-tube and the lowest boundary in the descending limb; slower fractions form succeeding boundaries.

*Electrophoretic Fractions of Normal Serum.* Electrophoretic analysis of normal serum reveals at least four major fractions (Table 17).<sup>25</sup> At pH 6.02, the albumin in a normal specimen migrated toward the anode at a rate of  $4.60 \times 10^{-5}$  cm.<sup>2</sup>/volt/second, and was followed in order by the alpha-, beta-, and gamma-globulins. The molecular charge

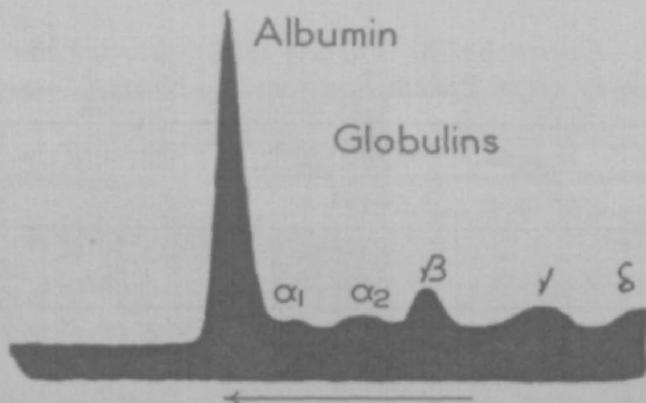


FIGURE 7. Electrophoretic diagram of normal pooled human serum diluted 1:1.5 in barbiturate buffer, pH 8.54, ionic strength 0.1. Total protein concentration, 3.3%. The descending boundary after 121 minutes is shown, and direction of migration is toward the left. (From Bulletin 2175, 1949, by permission of the American Instrument Company.)

and rate of migration of a protein clearly depend upon the pH of the solution.

Figure 7 illustrates the results obtained with normal human serum in the descending limb of an electrophoresis cell after 121 minutes.<sup>1</sup> The buffer solution was at pH 8.54. The direction of migration of boundaries, as indicated by the arrow, was toward the left in this diagram. The  $\alpha$ -globulin consisted of two components, designated  $\alpha_1$  and  $\alpha_2$ . The so-called  $\delta$ -anomaly at the extreme right of the diagram does not represent another protein fraction but was probably caused by interaction between the proteins of the serum and the buffer solution. The concentrations of the protein fractions, determined from the areas under the respective peaks, were as follows: albumin, 59.1 per cent;  $\alpha_1$ -globulin, 4.5 per cent;  $\alpha_2$ -globulin, 8.6 per cent;  $\beta$ -globulin, 12.6 per cent;  $\gamma$ -globulin, 15.2 per cent. Further refinements in technique have indicated that some serums contain two  $\beta$ -globulins,  $\beta_1$  and  $\beta_2$ . Whole plasma also contains fibrinogen, migrating between the  $\beta$ -globulin and  $\gamma$ -globulin.

The shape of a single crest or peak obtained by electrophoretic analysis reflects the homogeneity of the test substance or fraction. A narrow, steep peak is obtained with a highly homogeneous material.<sup>13</sup> Most electrophoretic components of serum give broad curves which indicate that the electric charges of the proteins grade into one another.

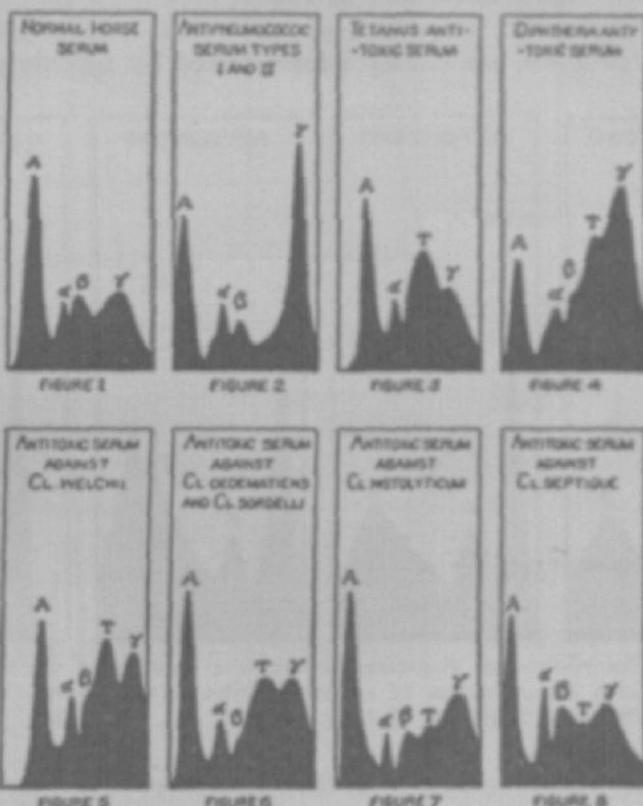


FIGURE 8. Electrophoretic diagrams of normal, antipneumococcal and antitoxic horse serums. (A = albumin;  $\alpha$ ,  $\beta$  and  $\gamma$  refer to the respective globulins; T = "T" component.) (From Van der Scheer *et al.*, 1940. Jour. Immunol. 39, 65.)

**Electrophoretic Properties of Antibodies.** Antibodies usually migrate with the  $\gamma$ -globulin fraction of serum, or as a distinguishable component between the  $\beta$ - and  $\gamma$ -globulins. The antibody activity of rabbit immune serums appears to be associated with  $\gamma$ -globulin, whereas the antibody-like component of human serums which reacts in serologic tests for syphilis migrates between the  $\beta$ - and  $\gamma$ -globulins. Both electrophoretic types of antibody are found in certain animals, such as the horse. The antibody in most antibacterial horse serums is associated with  $\gamma$ -globulin. However, pneumococcal antibody produced during early stages of immunization is intermediate between the  $\beta$ - and  $\gamma$ -globulins, whereas that formed later is almost entirely  $\gamma$ -globulin. The reverse situation is true of

*antitoxic* antibodies produced in the horse; in this case the fraction which appears during late stages of immunization is sometimes designated the "T" component. Electrophoretic diagrams of normal, antipneumococcal and antitoxic horse serums are shown in Figure 8.<sup>27</sup> The increase of  $\gamma$ -globulin and the "T" component during immunization of a horse against tetanous toxin is illustrated by Figure 9.<sup>28</sup>

The electrophoretic properties of antibodies, as distinguished from other serum proteins, are clearly demonstrated by specific precipitation

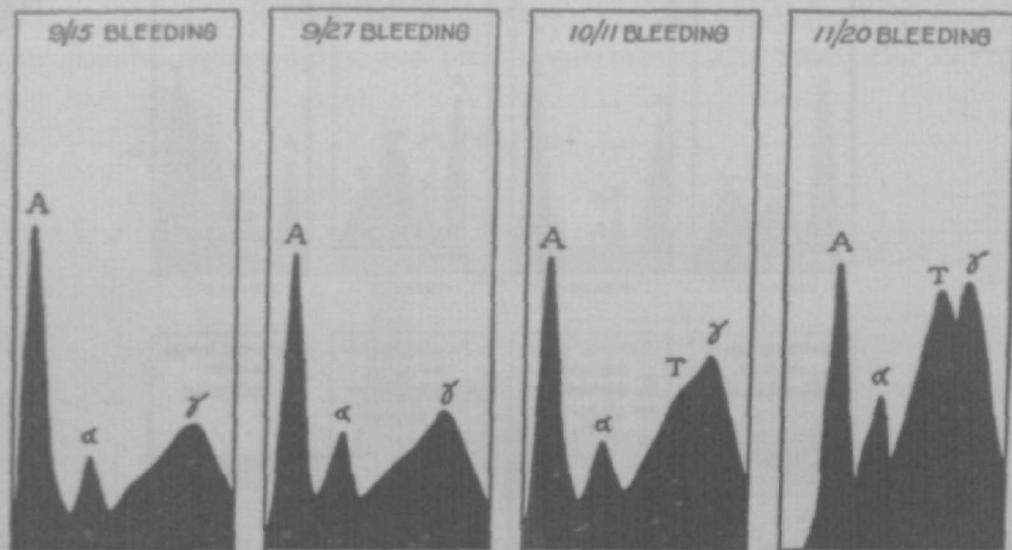
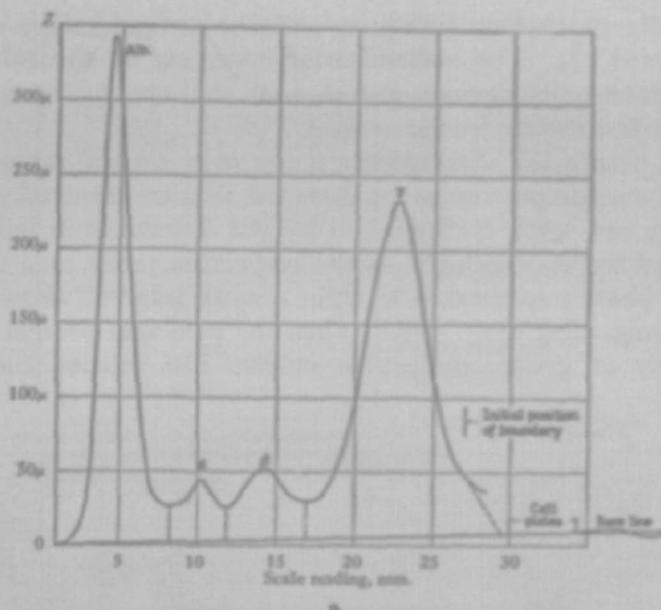


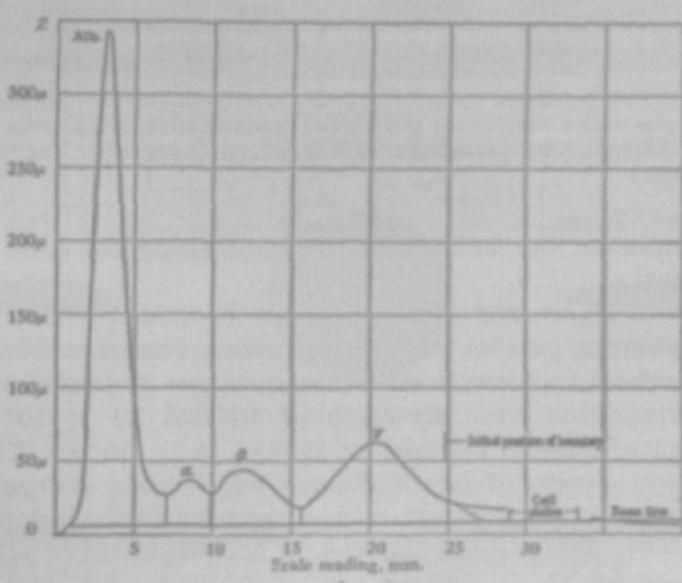
FIGURE 9. Electrophoretic diagrams showing the increase of  $\gamma$ -globulin and "T" component during immunization of a horse against tetanous toxin. (From Van der Scheer *et al.*, 1941. *Jour. Immunol.* 40, 173.)

of antisera with homologous antigen. Removal of antibodies from an anti-egg albumin rabbit serum by precipitation with egg albumin did not greatly affect the  $\alpha$ - and  $\beta$ -globulins (Figure 10),<sup>26</sup> but decreased the  $\gamma$ -globulin approximately 75 per cent. This indicates that antibody is composed of  $\gamma$ -globulin, but that  $\gamma$ -globulin is not entirely antibody.

**Molecular Weight Determinations. Ultracentrifugation.** Information concerning the molecular weights of proteins is obtained by centrifugation at speeds of 60,000 to 70,000 revolutions per minute in special centrifuges driven by air, electric motors or oil turbines. Sedimentation forces as high as 750,000 times gravity have been obtained.<sup>14</sup> A transparent analytic cell (quartz or lucite) containing the protein solution is placed in a rotor so that a beam of light can pass through it every revolution. Molecules of different weights are sorted into layers or boundaries, the heaviest molecules falling the greatest distance from the center of rotation. Observation of the boundaries is made in the same manner as in electrophoresis. The sedimentation rate is determined from



a



b

FIGURE 10. Electrophoretic scale diagrams of rabbit anti-egg albumin serum (a) before and (b) after adsorption of the antibody. The  $\gamma$ -globulin decreased greatly. (From Tiselius and Kabar, 1939. Jour. Exp. Med. 69, 119.)

consecutive observations at short intervals by measuring the distance migrated per unit of time. The sedimentation constant is derived by dividing the observed velocity of migration by the acceleration caused by centrifugal force. The rate of sedimentation depends in part upon the temperature, so values are either determined at 20° C. or are corrected

mathematically to this temperature, and the sedimentation constant is then designated  $s_{20}$ . The sedimentation constant of the substance, its diffusion constant, its density and that of the suspending medium are used to calculate the molecular weight.

Figure 11 illustrates observations made in a typical ultracentrifugal analysis.<sup>24</sup> Two components were detected in a commercial preparation of  $\gamma$ -globulin and were distinguished by the difference in sedimentation rate indicated by displacement of the respective peaks as a function of time. These photographs taken at eight minute intervals show the minor component migrating more rapidly than the principal protein and therefore probably of greater molecular weight. The relative concentration

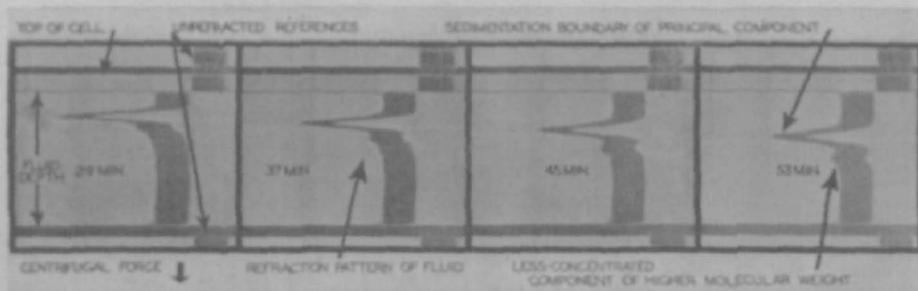


FIGURE 11. Ultracentrifugal study of a commercial  $\gamma$ -globulin (1 per cent) preparation, showing one major component and a small concentration of a heavier component. (From Form 1S1148U8 by permission of Specialized Instruments Corporation, Belmont, California.)

of each component can be evaluated by comparing the areas under the respective peaks.

*Molecular Weights and Sizes of Serum Proteins.* The molecular weights of proteins parallel their sedimentation constants. Normal horse serum yields three fractions in the ultracentrifuge: two main components having sedimentation constants ( $s_{20}$ ) of  $4.5$  and  $7.1 \times 10^{-13}$ , and a small amount of heavier material of  $s_{20} = 18 \times 10^{-13}$ . The lightest (first) fraction consists of serum albumin which has a molecular weight of about 70,000. The intermediate material is the principal globulin of normal horse serum; its molecular weight is approximately 167,000. The heavy component ( $s_{20} = 18 \times 10^{-13}$ ) is also a globulin, present in small amount. Its molecular weight is 900,000 or slightly more (Table 18).<sup>14</sup>

Antibodies in various animal species appear to consist of two types of globulin. The first has approximately the same molecular weight (150,000 to 200,000) as the principal normal serum globulins and is represented by pneumococcal antibodies produced in man, monkey and rabbit and by diphtheria antitoxin in the horse. The second type is heavier (M.W. about 900,000) and is illustrated by antibacterial antibodies from the

Table 18. Molecular Weights of Globulins in Normal and Immune Serums

SPECIES	NORMAL GLOBULIN	MOLECULAR WEIGHT	IMMUNE GLOBULIN	MOLECULAR WEIGHT
Horse	Major	167,000	Diphtheria antitoxin	184,000
	Minor	900,000	Pneumococcus	920,000
Man		156,000	Pneumococcus	195,000
Monkey			Pneumococcus	157,000
Cow			Pneumococcus	910,000
Pig			Pneumococcus	930,000
Rabbit			Pneumococcus	157,000
			Egg albumin	163,000

(From Kabat and Mayer,<sup>14</sup> by permission.)

horse, cow and pig. Human isoagglutinins are also of high molecular weight. It should be emphasized that the molecular weights of antibodies do not differ significantly from the molecular weights of globulins in normal serums of the various species.

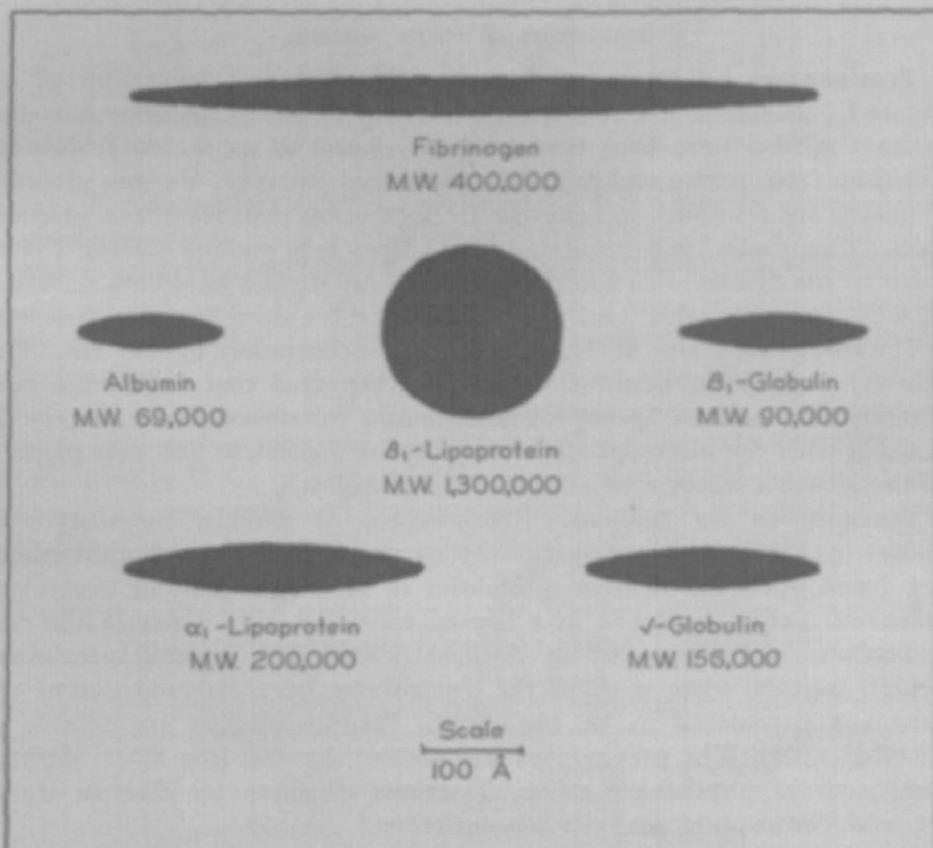


FIGURE 12. Relative sizes and shapes of some blood protein molecules. (From data of Oncley, Scatchard and Brown, 1947. Jour. Phys. Coll. Chem. 51, 184.)

Relative sizes and shapes of some blood protein molecules are pictured in Figure 12.<sup>2</sup> Neurath calculated that certain antibodies produced in the rabbit have a major axis of 274 Ångström units and a minor axis of 37 Ångström units and that corresponding values for horse antibody are 950 and 47 Ångström units.<sup>16</sup> Relative molecular volumes calculated from these dimensions agree closely with the comparative molecular weights determined by centrifugal methods. It is of interest that the length of the horse antibody molecule ( $950\text{Å} = 0.095\mu$ ) is great enough to be resolved by the optical microscope, but the diameter is too little to be resolved. Pappenheimer obtained evidence by ultracentrifugal analysis that horse antibody against pneumococcus type 2 may consist of five or six small units joined in a large molecule of molecular weight slightly under 1,000,000.<sup>20</sup> His results would indicate that there may be no difference between high and low molecular weight antibodies except that the former is a polymer of the latter. Both types of antibody have been effectively used in the treatment of pneumonia.

#### SOLUBILITIES OF SERUM PROTEINS

**Precipitation by Neutral Salts.** Identification and separation of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins is a relatively recent development. Ammonium and sodium sulfates have been used for many years to secure crude protein fractions from serum and to purify antibodies partially. Various globulin fractions are removed successively by increasing concentrations of these salts. "Euglobulin" is precipitated by 13.5 per cent sodium sulfate. Treatment of the filtrate with additional sodium sulfate in a final concentration of 17.4 per cent yields "pseudoglobulin 1," and a third fraction, "pseudoglobulin 2," separates at 21.5 per cent concentration of the salt. The albumin remains in solution. Cohn *et al.* reported that euglobulin precipitated from horse serum by ammonium sulfate consisted largely of protein with the electrophoretic mobility of  $\gamma$ -globulin and that pseudoglobulin was a mixture of  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins.<sup>5</sup>

**Precipitation by Dialysis.** Precipitation of protein by dialysis is somewhat similar to precipitation by neutral salts because both methods are based upon the relative solubilities of serum proteins in electrolyte solutions. Serum is placed in a bag of semipermeable material like thin cellophane and suspended in distilled water. The protein precipitate which appears when most of the electrolytes have dialyzed out of the serum is considered to be euglobulin. Pseudoglobulins are soluble in distilled water. The precipitated euglobulins dissolve in a dilute electrolyte such as physiologic saline. Fractions obtained by dialysis are in general electrophoretically inhomogeneous.<sup>5</sup>

**Precipitation by Alcohol.** A method of separating blood proteins which is adaptable to the concentration and partial purification of anti-

bodies is based upon Felton's observation that antibody can be precipitated from antiserum by use of alcohol if precautions are taken to prevent denaturation of the protein.<sup>6</sup> Denaturation is kept to a minimum by conducting all operations at temperatures very close to the freezing point of the serum-alcohol mixtures.

This method was further developed by Cohn and his co-workers during and after World War II. Several fractions were obtained by varying the protein and ethyl alcohol concentrations, pH and other factors.

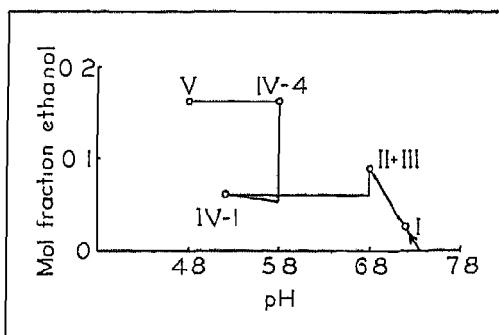


FIGURE 13. Flow sheet indicating the fractionation of plasma proteins by Cohn's Method 6. (From Cohn *et al.*, 1946. *Jour. Amer. Chem. Soc.* 68, 459.)

Some of these plasma fractions were purified for specific prophylactic or therapeutic uses.

The steps in one of Cohn's methods of separating plasma fractions are illustrated in Figure 13.<sup>6</sup> Normal plasma, which has a reaction about pH 7.4, is chilled to  $-2.5^{\circ}$  C. A buffer solution containing alcohol is gradually added until pH 7.2 and a concentration of 8 per cent alcohol is reached, whereupon precipitate I forms. This contains most of the fibrinogen (Table 19)<sup>6</sup> and is removed by centrifugation. The next precipitate is produced at  $-5^{\circ}$  C. by adding buffer and alcohol to pH 6.8 and 25 per cent alcohol concentration. This precipitate, II + III, consists of  $\beta$ - and  $\gamma$ -globulins with prothrombin, cholesterol and other substances. The

*Table 19. Protein Composition of the Principal Fractions Separated from Normal Human Plasma by Cohn's "Method 6"*

FRACTION	ALBUMIN	$\alpha$ -GLOBULIN	$\beta$ -GLOBULIN	$\gamma$ -GLOBULIN	FIBRINOGEN	TO TAL
I	7%	8%	15%	9%	61%	100%
II+ III	4	6	48	37	5	100
IV-1	—	89	10	1	0	100
IV-4	16	46	38	0	0	100
V	95	4	1	0	0	100

(From Cohn *et al.*,<sup>6</sup> by permission.)

supernate is diluted with water to an alcohol concentration of 18 per cent and is acidified to pH 5.2. Precipitate IV-1 consists chiefly of  $\alpha$ -globulins, together with some lipoid material. The supernatant liquid is adjusted to pH 5.8 and the alcohol concentration to 40 per cent. Precipitate IV-4 separates and consists chiefly of  $\alpha$ - and  $\beta$ -globulins. The

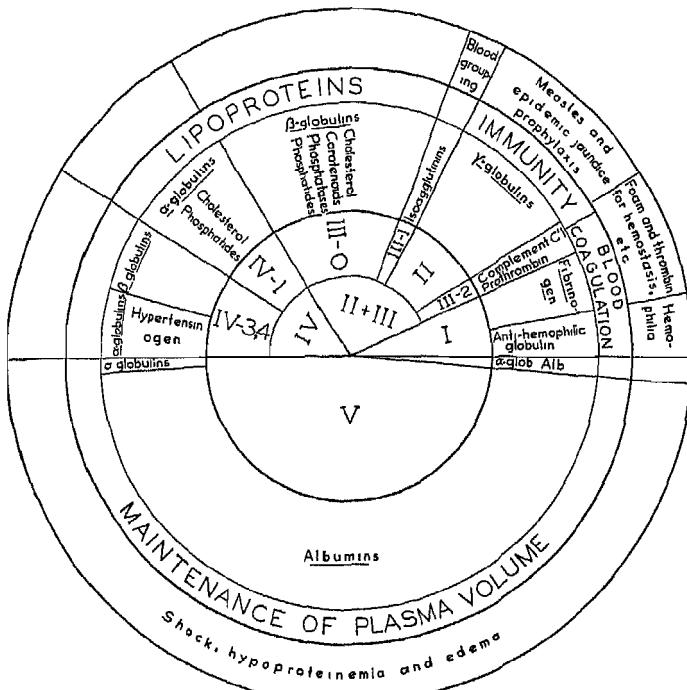


FIGURE 14. Relative proportions and some properties, natural functions and uses of plasma fractions secured by alcohol precipitation. (From Oncley *et al.*, 1946. Ann. N. Y. Acad. Sc. 46, 899.)

supernate is brought to pH 4.8 and precipitate V forms. This contains most of the plasma albumin.

The major fractions can be further purified and subfractionated by alcohol precipitation. Fraction II + III contains at least seven components: II-1, II-2, II-3, III-0, III-1, III-2, and III-3. The subfractions of II are principally  $\gamma$ -globulin, whereas the subfractions of III include  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulins.

The conditions employed in Cohn's method were selected to separate electrophoretic fractions. About 80 per cent of the fibrinogen is concentrated in fraction I; 97 per cent of the  $\gamma$ -globulin and 87 per cent of the  $\beta$ -globulin are in II+III; nearly 50 per cent of the  $\alpha$ -globulin is in

fraction IV-1 and 30 per cent in IV-4; and 83 per cent of the albumin is found in V.

The relative proportions of the various fractions of human plasma are indicated graphically in Figure 14, together with their electrophoretic nature and some of their natural functions and clinical uses.<sup>18</sup> Antibodies are confined to fraction II+III and are chiefly  $\gamma$ -globulins.

Table 20 compares the content of certain antibodies in subfractions II-1,2, II-3, and III-1, with that of equal weights of whole plasma

*Table 20. Average Antibody Contents of Certain Fractions of Human Serum Compared with Equal Weights of Plasma Protein  
(Concentration in whole plasma protein = 1.0)*

ANTIBODIES	SERUM FRACTION		
	II-1, 2	II-3	III-1
Isohemagglutinins	(0.2)	(0.4)	16
Typhoid somatic agglutinin	0.4	1.2	16
Typhoid flagellar agglutinin	8	8	4
Influenza A, Hirst test	4	4	3
Influenza A, mouse protection	9	12	4
Influenza A, complement fixation	7	(7)	.
Mumps, complement fixation	8	(8)	..
Diphtheria antitoxin	10	7	4
Streptococcal antitoxin	9	9	..

(From Oncley et al.,<sup>19</sup> by permission.)

protein.<sup>19</sup> Isohemagglutinins, including anti-A, anti-B and various Rh antibodies, are almost entirely confined to fraction III-1, whereas typhoid flagellar antibodies are present in the subfractions of II. Influenza and mumps viral antibodies and diphtheria and streptococcus antitoxins also appear in greatest concentration in fraction II.

Precipitation by neutral salts or by dialysis is still widely used commercially for the concentration and partial purification of antibodies. Some antibodies, such as various horse antitoxins, are principally pseudoglobulins. Horse antibodies against pneumococci are found in euglobulin precipitates, whereas pneumococcal antibodies in rabbit serums are usually pseudoglobulins.

#### DIFFICULTIES IN THE STUDY OF SERUM PROTEINS

The terminology of protein chemistry is a source of great difficulty. Names have been coined for components detected by various procedures in complex mixtures such as serum without exact knowledge of the nature of the substances. In consequence, a massive and confusing termin-

ology has evolved in which there is often little correlation between the different systems of nomenclature. Fractions secured by one method rarely duplicate those obtained by another. Approximately 70 per cent of the "euglobulins" and 80 per cent of the "pseudoglobulins" precipitated by ammonium sulfate are water soluble. "Euglobulins" are principally  $\gamma$ -globulin, and "pseudoglobulins" are a mixture of  $\alpha$ -,  $\beta$ - and  $\gamma$ -globulins.

Part of the difficulty may be attributed to dissociation or association of proteins during the process of separation: certain fractions may contain complexes not existing as such in native serum or may consist of fragments of the natural molecules. There is some experimental evidence to substantiate this view. Furthermore, the various physical methods of separation and characterization are based upon properties which might not necessarily be expected to give concordant results. Electrophoretic charge need not be correlated with solubility. Solubility may reflect the number of ionizable radicals, such as  $-COOH$ ,  $-NH_2$ , and  $-OH$ , but may be unaffected by the net charge, which determines the rate and direction of electrophoretic migration. Similarly, there is no necessary parallel between molecular size and electric charge or character of ionizable radicals.

Serum seems to contain a series of proteins of intergrading properties. Each new technique for characterizing proteins at first yielded a few well defined fractions, but further study showed that sharp lines of demarcation did not exist and only served to reveal similarities between proteins. For example, ultracentrifugation of human plasma showed a series of ten lipoproteins whose molecular weights ranged from 70,000 to 5,900,000.<sup>17</sup> Refinement and extension of the alcohol precipitation procedure yielded numerous fractions not originally detected.

It has long been known that among bacteria and higher forms intergradations between strains, species, and even genera cause considerable confusion in classification. It is apparent from the work of Cohn and others that intergrading actually starts at the molecular level.

#### PURIFICATION OF ANTIBODIES

Purification of antibodies has been accomplished in a few cases by dissociation of antigen-antibody complexes. Antiserum is mixed with homologous antigen in proper proportions for maximum precipitation or agglutination. The aggregate, washed free from nonreacting serum constituents, is then dissociated by means which permit recovery of the antibodies.

Antibodies may be dissociated from agglutinated cells of certain Enterobacteriaceae by mild heating. Removal of the cells in a heated centrifuge

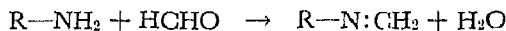
leaves a supernatant liquid which contains the original concentration of antibody.

Heidelberger and his co-workers precipitated pneumococcal polysaccharide by homologous antiserum in physiologic saline.<sup>12</sup> The washed precipitate was resuspended in 10 to 15 per cent sodium chloride and incubated for one hour at 37° C. Between 10 and 20 per cent of the bound antibody was released from the precipitate, which was then removed by centrifugation. Dialysis of the supernatant liquid against 0.9 per cent sodium chloride yielded antibody preparations from which as much as 80 to 100 per cent of the nitrogen was specifically precipitable by pneumococcal polysaccharide. Antibody solutions were obtained which were homogeneous electrophoretically and ultracentrifugally. Satisfactory results were obtained with horse, cow, pig, rabbit, monkey and human antipneumococcal serums. This procedure is applicable chiefly to carbohydrate-anticarbohydrate systems. Specific precipitates can also be dissociated with barium hydroxide and barium chloride and with dilute alkali.<sup>14</sup>

Pope<sup>22</sup> and Petermann and Pappenheimer<sup>21</sup> purified diphtheria antitoxin by peptic digestion of toxin-antitoxin flocs. Northrop employed trypsin, digestion being followed by precipitation of the antitoxin with ammonium sulfate.<sup>16</sup> Very low yields were obtained, but the product was apparently pure antitoxin.

#### RELATION OF ANTIBODY GLOBULIN TO NORMAL GLOBULIN

Chemical analysis reveals no uniform difference between antibody globulin and normal globulin. The association of particular radicals with the antibody function has been suggested in specific cases but has not been confirmed for all antibodies.<sup>3</sup> Certain horse antipneumococcal precipitins possess an unusually alkaline isoelectric point (pH 7.6), which could be attributed either to an increased percentage of basic amino acids such as lysine and arginine or to a high ratio of amino to carboxyl groups. However, the distribution of basic amino acids in normal and immune globulin is essentially the same, so the alkaline property of the immune globulin may be associated with free amino groups. These, if properly oriented, can react with carboxyl radicals of antigens or haptens. This suggestion is supported by the observation that formalized antisera often fail to precipitate or agglutinate homologous antigens, possibly because of a reaction such as the following:



Repeated failure to find consistent chemical or physical differences between antibody globulin and normal globulin has resulted in the

suggestion that immunization does not lead to formation of a new protein but merely to increased production of a protein normally present, which may in some cases be natural antibody.

Numerous experiments have indicated that the molecules of immune globulin possess at least two kinds of serologic specificity. One kind of specificity is peculiar to immune globulin and is shown by the reaction with homologous antigen. It depends upon antibody groups or radicals, which are probably few in number. The other type of specificity is common to both immune and normal globulins as species proteins. Smith and Marrack precipitated antitoxic horse pseudoglobulin with a rabbit antiserum against normal horse pseudoglobulin.<sup>23</sup> After precipitation, less than one-ninth of the antitoxin remained in the supernatant fluid, as shown by tests in guinea pigs. The antitoxin-antipseudoglobulin precipitate was able to combine with and neutralize toxin. Eagle reported similar experiments showing that diphtheria antitoxin, when precipitated with a large excess of rabbit anti-horse serum precipitin, still retained almost its original capacity for neutralizing toxin.<sup>8</sup>

Antibody globulin molecules seem to possess specific reactive groups (e.g., horse) present in normal globulin molecules of the same species as well as the radicals upon which their antibody function (e.g., antitoxin) depends. An antibody may be pictured as a globulin slightly modified in the process of manufacture to possess a few sites capable of combining specifically with certain characteristic chemical configurations of the antigen.

### References

1. American Instrument Co., 1949. Bull. 2175.
2. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
3. Chow and Goebel, 1935. Jour. Exp. Med. 62, 179.
4. Chow, Lee and Wu, 1937. Chin. Jour. Physiol. 11, 175.
5. Cohn, McMeekin, Oncley, Newell and Hughes, 1940. Jour. Amer. Chem. Soc. 62, 3386.
6. Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946. Jour. Amer. Chem. Soc. 68, 459.
7. Dean, Taylor and Adair, 1935. Jour. Hyg. 35, 69.
8. Eagle, 1936. Jour. Immunol. 30, 339.
9. Felton, 1931. Jour. Immunol. 21, 357.
10. Felton and Bailey, 1926. Jour. Infect. Dis. 38, 145.
11. Gray, 1951. Sci. Amer. 185, 45.
12. Heidelberger and Kendall, 1936. Jour. Exp. Med. 64, 161.
13. Hess, 1951. Science 113, 709.
14. Kabat and Mayer, 1948. *Experimental Immunochemistry*, Charles C Thomas, Springfield, Ill.
15. Neurath, 1939. Jour. Amer. Chem. Soc. 61, 1841.
16. Northrop, 1942. Jour. Gen. Physiol. 25, 465.
17. Oncley and Gurd, 1953. In Tullis, *Blood Cells and Plasma Proteins*, Academic Press, New York.
18. Oncley, Melin, Cameron, Richert and Diamond, 1946. Ann. N. Y. Acad. Sc. 46, 899.

19. Oncley, Melin, Richert, Cameron and Gross, 1949. Jour. Amer. Chem. Soc. *71*, 541.
20. Pappenheimer, 1953. In Pappenheimer, *The Nature and Significance of the Antibody Response*, Columbia University Press, New York.
21. Petermann and Pappenheimer, 1941. Jour. Phys. Chem. *45*, 1.
22. Pope, 1939. Brit. Jour. Exp. Path. *20*, 132, 201.
23. Smith and Marrack, 1930. Brit. Jour. Exp. Path. *11*, 494.
24. Specialized Instruments Corporation, Belmont, Calif. Form 1S1148U8.
25. Tiselius, 1937. Biochem. Jour. *31*, 1464.
26. Tiselius and Kabat, 1939. Jour. Exp. Med. *69*, 119.
27. Van der Scheer, Wyckoff and Clarke, 1940. Jour. Immunol. *39*, 65.
28. Van der Scheer, Wyckoff and Clarke, 1941. Jour. Immunol. *40*, 173.

## Chapter 5

### PRODUCTION OF ANTIBODY

#### SITE OF ANTIBODY FORMATION

**Effect of Removing Tissues or Organs.** Possible sites of antibody formation have been investigated by determining the effect on antibody production of removing various tissues or organs.

Blood serum and erythrocytes do not participate directly in antibody production. Hektoen and Carlson injected goat erythrocytes into dogs; before antibody titers in their serums increased, the dogs were almost completely exsanguinated and immediately transfused with blood from normal dogs.<sup>48</sup> Subsequent titrations indicated that antibody formation proceeded unchecked.

Early experiments in which organs were removed before or after injection of antigen were disappointing. Removal of the pancreas, stomach and small intestine had little effect upon antibody-producing ability beyond that which might be expected from the physiologic effects of such operations.<sup>44</sup>

The possible role of the spleen and liver in antibody formation was indicated by experiments in which these organs were extirpated from animals at intervals after an immunizing injection, extracted with saline and the antibody content of the extracts determined. Spleen and liver possessed a somewhat higher content of antibody than other organs or the blood.<sup>16</sup> Deutsch found that guinea pigs from which the spleens had been removed three to five days after injection of typhoid bacteria produced less antibody than control animals.<sup>17</sup> The spleens were ground and injected into the peritoneums of normal animals, whereupon antibody appeared in the serums of the recipients in relatively low titer. Splenectomy twenty-two to twenty-seven days before injection of antigen had no effect on antibody formation. Although the spleen evidently played some part in antibody production, it appeared not to be the only organ involved. Deutsch remarked that the spleen of an injected animal might

store microbial constituents which provoke the formation of agglutinins. The spleen would thus act as a reservoir of antigenic material. It is of interest that a recent immunizing technique consists of injecting antigen emulsified in beeswax or paraffin oil. The antigen is slowly released from its subcutaneous depot and stimulates prolonged antibody formation.

**Participation of the Reticuloendothelial System in Antibody Formation.**

At one time it was believed that cells of the reticuloendothelial system bear the major if not the sole burden of antibody formation. The R-E system has been called the *blood-clearing mechanism* because its cells possess the ability to ingest dyes and particulate materials such as damaged blood cells. They therefore directly or indirectly receive bacteria which find their way into the body. It was presumed likely that the cells which receive antigenic material in this manner would constitute the sites of antibody formation. Many antigens, however, are not in particulate form. Cell-free filtrates of bacterial cultures may produce antibodies more rapidly than the whole cells, probably because they can be more readily absorbed by antibody-producing cells. Recent experiments by the radioactive tracer technique indicate that bovine  $\gamma$ -globulin labeled with  $I^{131}$  may accumulate in the lungs and kidneys of rabbits in greater concentration than in organs containing R-E cells.<sup>18</sup>

Early experiments in which reticuloendothelial cells were "blocked" or injured seemed to confirm the participation of these cells in antibody formation. Animals injected with India ink and subsequently injected with an antigen sometimes produced less antibody than controls not treated with India ink.<sup>5</sup> It was assumed that extensive ingestion of inert carbon particles of the ink blocked the antibody-forming activity of the cells. Less antibody also was produced when R-E cells were damaged by benzene or by x-rays.<sup>41, 58</sup>

Experiments in which organs are removed or cells are blocked or damaged provide no decisive evidence of the site of antibody formation because of the unknown general physiologic injury which such treatment produces. These methods are most useful in eliminating from further consideration organs or cells whose injury does not seem to affect antibody production.

Histologic studies indirectly implicating the reticuloendothelial system in antibody formation were reported in 1939 by Sabin.<sup>59</sup> Animals were injected intradermally or subcutaneously with a colored dye-protein complex precipitated by alum, whereupon the dye-protein appeared in the digestive vacuoles of local macrophages and in the regional lymph nodes. Intravenously injected antigen was seen in the Kupffer cells of the liver, in macrophages of the spleen, and to a lesser extent in the bone marrow. The colored antigen then disappeared, surface films of the macrophages were shed at a rate greater than normal, and simultaneously antibody appeared in the blood.

Experiments of a more direct nature were made possible by development of techniques for cultivation of isolated tissues *in vitro*. Carrel and Ingebrigsten cultivated guinea pig bone marrow and lymph glands in guinea pig plasma.<sup>12</sup> Goat erythrocytes were added to the tissue culture and the solution was tested for antierythrocyte antibodies. Opsonizing and hemolytic antibodies were detected on the third and fourth days, respectively. In tissue cultures of spleen, lymph glands or omental milk spots taken from rabbits inoculated three to five days previously with typhoid bacteria, Meyer and Loewenthal reported agglutinin titers of 320, a fourfold increase above serum titers in the animals from which the tissues were secured.<sup>52</sup> Omental milk spots contain, in addition to fibroblasts, only cells of reticuloendothelial type. Titers produced in tissue cultures are usually low. Most investigators have succeeded in obtaining antibodies *in vitro* only when the tissues were taken from animals previously inoculated with the antigen.

**Role of Lymphoid Tissue and Plasma Cells in Antibody Formation.** Numerous reports indicate that antibody is formed in lymph nodes draining areas of intradermal or subcutaneous injection of killed bacteria or other antigens, and sometimes in the skin at the site of inoculation. McMaster and Hudack injected *Salmonella enteritidis* and *Serratia marcescens* intradermally into mice, one antigen being injected into each ear.<sup>50</sup> The homologous antibody appeared first in the cervical lymph node of the injected side preceding appearance of the same antibody in the serum. This observation strongly implied formation of antibody in regional lymph nodes.

Ehrich injected sheep erythrocytes or killed typhoid bacteria subcutaneously into the foot pads of rabbits.<sup>29</sup> He observed hyperplasia of the popliteal lymph nodes draining the site of injection amounting to five times the normal weight and an increase of three to five times in the number of lymphocytes in the efferent lymph. The antibody content of the efferent lymph was as much as one hundred times greater than that of the afferent lymph. The concentration of antibody in extracts of efferent lymph cells was eight to sixteen times as great as in the lymph fluid.

Absorption of antibody by lymph cells apparently did not occur in Ehrich's experiments.<sup>29</sup> Lymphocytes containing antibodies against sheep erythrocytes were incubated *in vitro* in lymph plasma containing typhoid antibodies. Sheep cell antibodies passed from the cells to the lymph fluid, but typhoid antibodies did not pass from the fluid to the cells. Similarly, when normal lymphocytes were allowed to incubate *in vivo* within ligatured lymph nodes to which sheep cell antiserum had been added, little or no sheep antibody was absorbed by the cells.

Ehrich concluded that lymphocytes are instrumental in antibody formation. He also suggested that polymorphonuclear leukocytes and macro-

phages assist by breaking down bacteria and other particulate antigens, the soluble components being then absorbed by lymphocytes.<sup>23</sup> It is now known that polymorphonuclear leukocytes contain unusually high concentrations of actively digestive enzymes, including pepsin, cathepsin and trypsin. These might be expected to hydrolyze protein antigens to such an extent that they would lose their antigenicity. The relation of polymorphonuclear leukocytes to antibody formation is therefore uncertain.

Burnet also obtained evidence that antibody formation occurs in lymphatic tissue near a site of antigen injection.<sup>9</sup> Formalin-killed dysentery bacteria were injected subcutaneously into the right foot pads of rabbits, and four days later the animals were bled and sacrificed. The popliteal lymph nodes of both right and left legs were carefully removed, weighed, emulsified and extracted with saline. Antibody titers were determined in the extracts and in the animals' serums (Table 21). Injection into the

*Table 21. Response of the Popliteal Lymph Nodes of Rabbits to Subcutaneous Injection of Killed Bacteria into the Right Foot*

Rabbit	WEIGHT OF LYMPH NODE		AGGLUTININ TITERS		
	Right	Left	Right	Left	Serum
42	0.199 gm.	0.102 gm.	1280	80	1280
43	0.219 gm.	0.112 gm.	1280	40	1280

(From Burnet et al.,<sup>9</sup> by permission.)

right foot obviously caused a marked increase of right lymph node tissue, and the antibody titer of this organ was much higher than that of the left node.

The cellular response in lymph nodes draining a site of subcutaneous injection consists chiefly of an increase in the number of lymphocytes and plasma cells. The latter are large cells with abundant, deeply basophilic cytoplasm, regarded generally as lymphoid in origin although not considered to be immature lymphocytes. Injection of an antigen such as ovalbumin into rabbits causes a marked increase of plasma cells in the spleen, and in tissue cultures of spleen fragments from immunized rabbits the capacity of the red pulp to produce antibody is directly correlated with its content of plasma cells.

Burnet tentatively stated that local injection of particulate antigens stimulates lymph nodes to produce antibody, and the antibody passes to the circulation largely in the cytoplasm of lymphocytes and plasma cells in the efferent lymph.<sup>10</sup> The spleen performs much the same function against intravenously administered antigen. Lymphocytes are very short lived, being replaced two or three times a day,<sup>22</sup> and their disintegration may account for rapid release of antibody. There is evidence that adrenal cortical hormones aid in dissolution of these cells.<sup>20</sup>

Chase and other investigators recently demonstrated the participation of cells of the spleen and lymph nodes in antibody formation by transfer of the antibody-producing function from immunized to normal animals by means of cells of these organs.<sup>51</sup> Chase and colleagues inoculated rabbits with diphtheria toxoid. Eight to ten days later, while these rabbits were still completely devoid of antitoxin, the animals were sacrificed, the lymph nodes dissected out, washed, and injected into normal animals. Antitoxin was detected as early as eighteen hours after introduction of the cells, increased until the fifth day and decreased during the next five to nine days. It is especially important to note that antitoxin was not found in extracts obtained by grinding or freezing and thawing the lymph node cells. Moreover, transfer of damaged cells was not followed by production of circulating antibody.

Stavitsky also reported antitoxin formation in rabbits injected intravenously with tissue cells from animals previously inoculated with diphtheria toxoid.<sup>50</sup> Lymph node cells removed six days after *local* injection of toxoid were effective, as were splenic cells of rabbits inoculated *intravenously*. Stavitsky suggested that "the transferred cells either synthesize or cause the synthesis of antitoxin *de novo* in their new host." Harris *et al.*, working with an entirely different kind of antigen, produced antibodies against *bacteria* by transfer of lymph node cells and noted that the time required for the appearance of antibody in animals receiving the lymph cells was inversely related to the interval between injection of antigen and removal of tissue from the donor animals.<sup>50</sup> When the interval was three or four days, antibodies appeared in the recipient on the first day following transfer of the lymph node cells.

Doubtless both reticuloendothelial and lymphoid cells participate in antibody formation. The particular tissues concerned probably differ according to the site and route of inoculation and the nature of the antigen.

#### RESPONSE TO ANTIGENIC STIMULI

The antibody response to injection of antigen varies with the nature of the antigen; the dosages, number, frequency and route of injections; and the species and individual animal.

**The Response to a Series of Injections of Antigen.** Prophylactic immunization of man or laboratory immunization of animals is almost always accomplished by a series of injections of antigen. Closely spaced injections provide constant presence of the antigenic material within the tissues without excessive danger of toxic reactions which might follow introduction of the total quantity at one time. Moreover, even nontoxic antigens are often more effective antibody stimulants if given in divided doses.

The titer of circulating antibody increases more or less rapidly toward a maximum beyond which additional injections have no effect. Titers attained vary widely. It is not difficult to secure agglutinin titers of 40,000 in rabbits injected with members of the Enterobacteriaceae. Titers produced in man by the routine three weekly inoculations are usually somewhat lower. Gram positive bacteria induce relatively low antibody concentrations.

The steep rise in antibody which accompanies and follows immediately after the injections is succeeded by a plateau or interval of months or years during which high titer is maintained. Thereafter antibodies

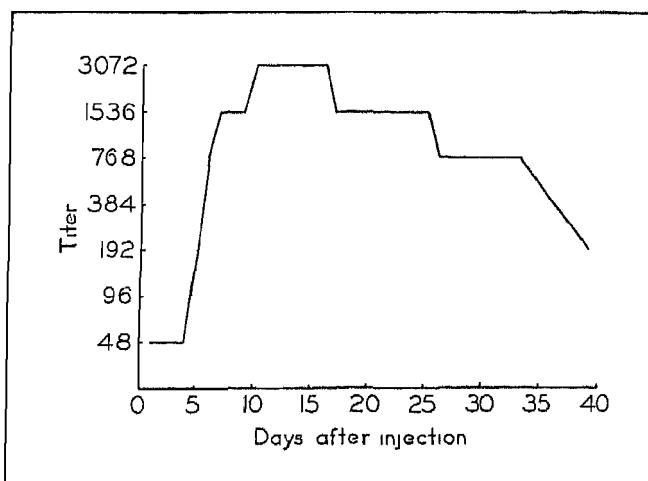


FIGURE 15. Agglutinin titers in a dog injected with rabbit erythrocytes. (Plotted from data in Iektoen and Curtis, 1915. *Jour. Infect. Dis.* 17, 409.)

in the blood slowly diminish and may eventually (but not always) disappear completely. Antibody titers usually persist at a high level for a longer period following several immunizing injections. Barr and Glenny reported that serum taken from a horse thirteen months after the last of forty-one immunizations with tetanus toxoid and toxin still contained an antitoxic titer 88 per cent of the value when injections ceased.<sup>2</sup> Two other horses which had been immunized only once or twice retained about 2 per cent of their highest antitoxic titers after one year.

**The Response to a Single Injection.** The response of the body cells to antigenic stimulation can best be learned by studying the response to a single injection. Three or four phases usually follow injection of antigen into a previously uninoculated animal (Figure 15).<sup>14</sup> There is often a latent period after injection, during which antibody is not

detectable or does not increase in the circulating blood. The latent period varies from a few hours to several days, after which there is a sharp rise in titer. Circulating antibodies increase to a peak or plateau of varying duration and then gradually decrease but may persist in detectable amount for many months.

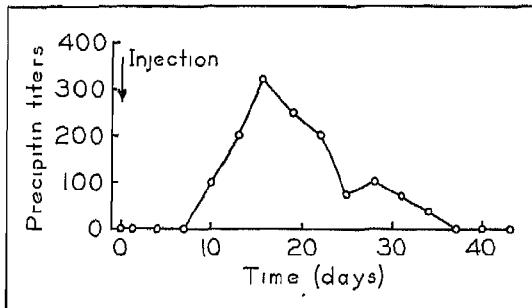


FIGURE 16. Precipitin titers in a rabbit injected with horse serum. (From Dean and Webb, 1928. *Jour. Path. Bact.* 31, 89.)

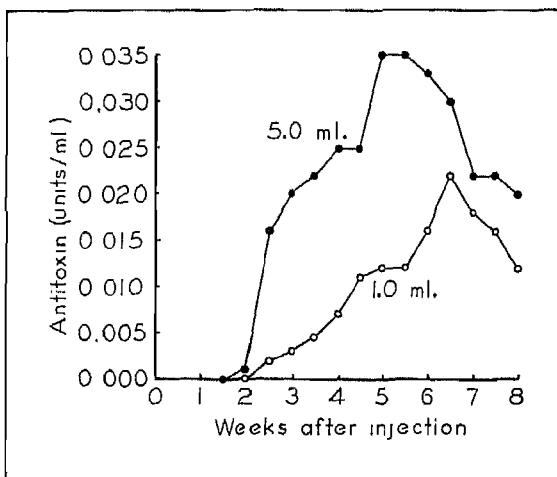


FIGURE 17. Antitoxin titers in rabbits injected intravenously with 1.0 or 5.0 ml. of diphtheria toxin-antitoxin. (Plotted from data in Glenny and Pope, 1925. *Jour. Path. Bact.* 28, 273.)

Antibodies against particulate antigens such as bacteria, Rickettsiae and foreign erythrocytes appear in the blood of rabbits within two to five days after a single intravenous injection. The latent period after injection of soluble antigens is often longer. Dean and Webb reported that eight days elapsed after injecting horse serum into a rabbit before precipitating antibodies were produced (Figure 16).<sup>15</sup> Diphtheria anti-

toxin appears within two to three weeks after administration of large doses of toxoid or toxin-antitoxin to guinea pigs or rabbits (Figure 17).<sup>23</sup> Burnet found that staphylococcus antitoxin appeared in rabbits eight to thirteen days after a single injection of antigen.<sup>9</sup>

Maximum titers may be reached within three or four days or may be delayed a few weeks. Rabbits usually respond more rapidly than guinea pigs or humans, and horses appear to respond most slowly. Unfortunately, few comparable data are available which take into account the body weights of the respective animals. The persistence of high titers varies greatly and depends in part upon the immunizing material. Diphtheria antitoxin in humans inoculated with a single dose of toxoid decreases sharply within two weeks and more slowly thereafter.<sup>49</sup> It should be

*Table 22. Effect of Dosage on the Maximum Titers of Flagellar Agglutinins for *Salmonella schottmüller* in Rabbits Injected Intravenously*

BACTERIA INJECTED PER KILOGRAM OF BODY WEIGHT	NUMBER OF RABBITS	HIGHEST TITER (averages)
100,000,000	3	3540
10,000,000	3	2480
100,000	6	330
10,000	4	< 4

(From Topley,<sup>60</sup> by permission.)

pointed out, however, that such individuals possess increased ability to respond to subsequent antigenic stimulation with toxoid and quickly produce high titers of antitoxin. Type-specific antibodies against pneumococcal polysaccharides are produced slowly in man but remain near peak titer for five to eight months.<sup>39</sup>

The decrease in antibody titer after the peak or plateau varies with the animal, the antigen and other factors. Different individuals of the same species vary greatly with respect to the rate at which their antibody titers drop. Jensen reported that in one child immunized with diphtheria toxoid a 95 per cent drop in titer required two years, whereas in another child only three months was required.<sup>49</sup> There are also wide differences between species, and some justification has been found for the statement that antibody titers diminish more rapidly in animals possessing a high metabolic rate.

Antibody formation is not demonstrable when the dosage of bacterial cells is below a certain minimum (Table 22).<sup>60</sup> Topley noted that increasing amounts of antigen yielded greater antibody production but not in proportion to the increments of antigen. Topley and Wilson concluded that a maximum dosage is eventually reached beyond which no further increase in antibody titer occurs.<sup>63</sup>

*Primary versus Secondary Antigenic Stimuli.* A second or subsequent injection of the same antigen at a considerable interval after the preceding injection usually causes a more rapid rise in titer than the first inoculation, the peak attained is greater, and antibody persists for a longer period. This is the so-called secondary response. It is well illustrated by quantitative data of Dixon *et al.* obtained by immunizing a rabbit with bovine  $\gamma$ -globulin labeled with  $I^{181}$  (Figure 18).<sup>10</sup> In the same experiment the concentration of antigen in the blood was also determined, and it was found that detectable antibody did not appear until antigen had

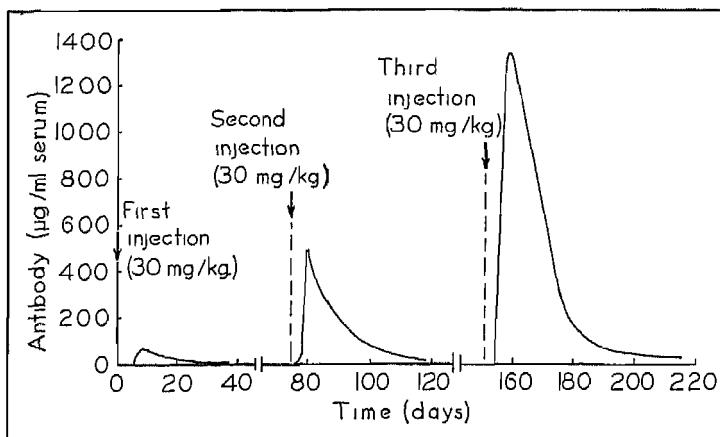


FIGURE 18. Antibody production in a rabbit following three widely spaced injections of bovine  $\gamma$ -globulin labeled with  $I^{181}$ . (Redrawn from Dixon *et al.*, 1954. Jour. Immunol. 72, 179.)

disappeared. This latent period was seven days after the first (primary) antigenic stimulus and four days after each of the other (secondary) stimuli. The greater persistence of antibody in man after a second injection of a bacterial antigen is shown by comparison of Figures 19 and 20.<sup>63</sup>

Certain antigens do not appear to elicit a typical secondary response. Burnet and Freeman reported that Q fever Rickettsiae produced no secondary response in rabbits injected intravenously.<sup>11</sup> Heidelberger and co-workers immunized humans with killed pneumococci or purified pneumococcal polysaccharide and two years later reinjected antigen but obtained no rise in antibody.<sup>38,39</sup> They suggested that the polysaccharide, which is extremely resistant to enzymatic digestion, may have persisted during this time in the antibody-producing cells. The second injection therefore did not provide a new antigenic stimulus.

The characteristic sharp response to another injection of antigen may be elicited months or years after the first injection, at a time when

circulating antibody is not demonstrable. It was noted previously (Table 22) that 10,000 cells of *S. schottmüller* per kilogram of body weight produced no detectable agglutinin in rabbits. However, rabbits inoculated with this number of organisms and subsequently reinjected with

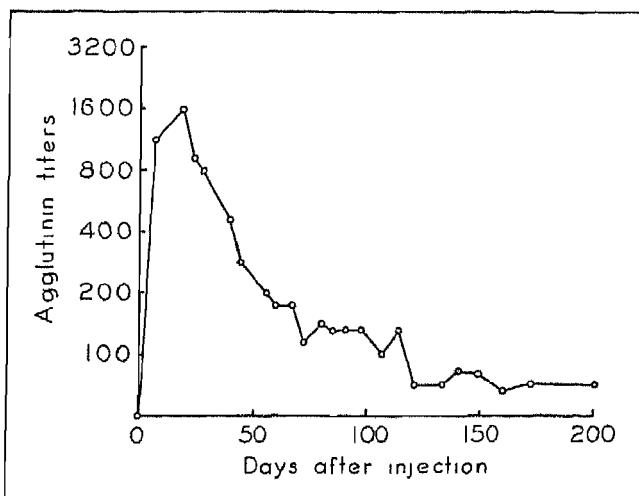


FIGURE 19. Agglutinin titers in an uninoculated person following a single injection of typhoid vaccine. (From Wilson and Miles: *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., 1946, Edward Arnold & Co., London.)

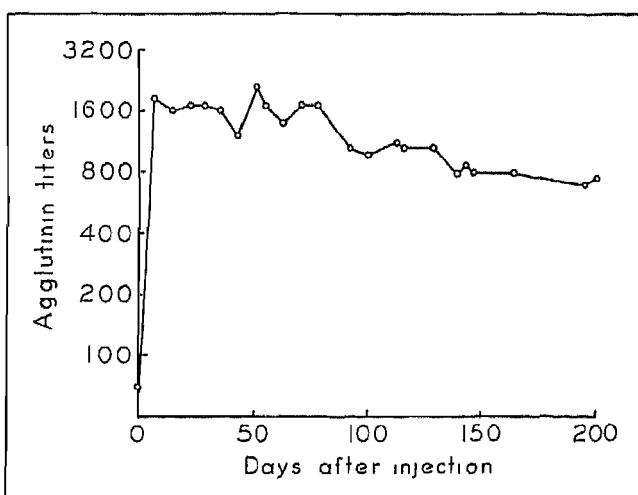


FIGURE 20. Agglutinin titers in a previously inoculated person following an injection of typhoid vaccine. (From Wilson and Miles: *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., 1946, Edward Arnold & Co., London.)

the same dose produced a definite antibody response of the secondary type.

**Response to Reimmunization.** Reimmunization of man is practiced for the purpose of increasing the antibody concentration when the titer has fallen below a protective level. It is illustrated by "booster" inoculations against diphtheria, tetanus and typhoid fever. With certain antigens the usual course of injections is administered. A recent practice in typhoid reimmunization consists of a single intradermal injection of 0.1 milliliter of killed bacteria. This procedure is said to give as good results as the customary three weekly subcutaneous inoculations of 0.5 or 1.0 milliliter.

Antibody titers produced by reimmunization follow the general pattern of the secondary response. A high concentration of antibody is quickly reached and is maintained for many months or years.

*The Anamnestic Reaction.* The sudden secondary rise in antibody titer produced by reimmunization or by a second injection of the same antigen some time after the first will be called the *specific anamnestic reaction*. A similar increase in titer brought about by other stimuli will be referred to as the *nonspecific anamnestic reaction*. Specific anamnestic titers are maintained for long periods, but nonspecific anamnestic titers usually drop rapidly.

Typhus fever, lobar pneumonia, tuberculosis and, particularly, infectious mononucleosis have been found to stimulate reappearance of typhoid agglutinins in persons previously immunized against typhoid fever. However, the nonspecific anamnestic reaction is not limited to obvious prior experience with the corresponding antigen. Infectious mononucleosis frequently produces strongly positive Wassermann reactions in persons showing no history of syphilis. Normal isoagglutinin titers often increase during febrile conditions. Injection of apparently unrelated antigens may also increase circulating antibodies against an earlier immunizing agent. Dreyer and Walker reported that killed staphylococci caused a rise in anticolon titers in rabbits previously inoculated with *E. coli*.<sup>21</sup> Bieling immunized a rabbit with *Sh. dysenteriae* and later injected *S. typhosa*, whereupon antidyserentary agglutinins increased markedly.<sup>4</sup>

Other nonspecific anamnestic stimuli include injections of milk, casein, gelatin and peptone. These and other substances were at one time employed to a considerable extent in *nonspecific protein therapy* of certain chronic infections such as gonococcal arthritis and salpingitis, rheumatoid arthritis and some focal infections. Foreign substances of this sort may induce fever, leukocytosis and a general inflammatory reaction together with renewed formation or release of antibodies. The possible beneficial effects of nonspecific protein treatment have been attributed to these factors.

The foregoing responses occur even in animals no longer possessing

detectable antibody. Repeated bleeding stimulates continued formation or liberation of an antibody which is already present in high titer. Animal serums secured by large bleedings on successive or alternate days often contain a constant or even increasing amount of antibody. The maintenance of antibody titers in spite of bleeding may be caused by liberation of previously formed antibody from various body cells or by continued production of antibody globulin in response to the decreased concentration of blood proteins.

White and Dougherty studied the effect of adrenal cortical hormones on previously immunized animals which no longer contained detectable circulating antibodies.<sup>42</sup> A single injection of hormone or of adrenal cortical extract caused the appearance of antibodies in high titer within a very few hours. These antibodies diminished rapidly and were hardly detectable after twenty-four hours. Changes were also observed in the number of blood lymphocytes and in other lymphatic tissues. It was suggested that the hormones caused dissolution of lymphoid cells, thus releasing stored antibodies. These observations and conclusions have been both supported and denied; the question is subject to further investigation.

**The Response to Multiple Antigens.** Simultaneous injection of several antigens usually induces antibodies for most if not all of the respective antigens. Hektoen and Boor immunized a rabbit with a mixture containing thirty-five albumins, pseudoglobulins, hemoglobins and other proteins and detected precipitins against all except one (Hogan protein, a peculiar protein from urine) in the antiserum, many in high titer.<sup>43</sup>

Glenny stated that active production of antibody against one antigen decreased the antibody response against a second antigen.<sup>27</sup> Working particularly with toxins in horses, he reported failure in attempts to secure antitoxins for more than three toxins simultaneously. When four toxins were injected, only three antitoxins were formed. Moreover, the titers of antitoxin varied according to the number of toxins injected. Groups of horses immunized with tetanus alone or with *Clostridium perfringens* and/or *Cl. septicum* toxins produced the following amounts of tetanus antitoxin:

- 1,000 units when immunized with tetanus toxin alone.
- 600 units when immunized with one other toxin.
- 400 units when immunized with two other toxins.

Björneboe inoculated rabbits every other day for eight months with mixtures containing one to eight types of pneumococcus.<sup>6</sup> Antibody production for each type was the same if no more than three types were included in the mixture but decreased progressively when more than three types were present.

In man, combinations of two or three antigens are reported to yield as good antibody production as the respective single antigens. Children are

commonly immunized with a mixture of diphtheria and tetanus toxoids and pertussis bacterin.

It must be remembered that any immunizing agent consisting of bacterial or tissue cells is composed of many antigenic components and that each such component may cause formation of separate antibodies. These antibodies do not all necessarily appear simultaneously. Human erythrocytes of types A and B contain agglutinogens A and B, respectively, as well as species specific antigens. A single injection of human A red cells into a rabbit frequently produces a high titer (5120) of A

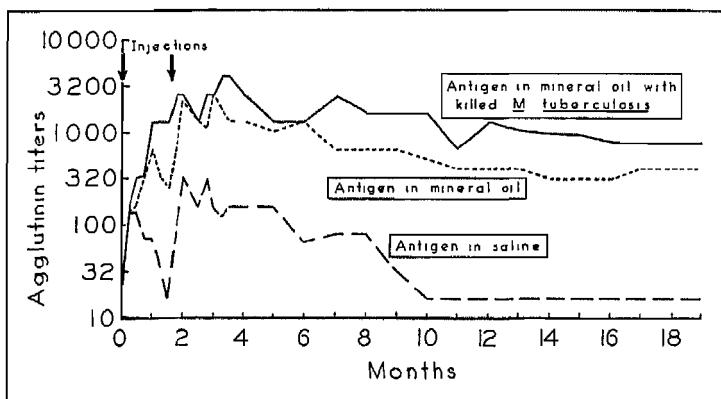


FIGURE 21. Adjuvant effect of mineral oil and killed *Mycobacterium tuberculosis* on agglutinin titers (mean) in rabbits inoculated subcutaneously with formalin-killed *Salmonella typhosa*. (Redrawn from Freund and Bonanto, 1944. Jour. Immunol. 48, 325.)

antibodies before any human species antibodies appear. Subsequent injections cause the appearance of antibodies against the species antigens, and they may eventually equal or slightly exceed the type specific A antibody. On the other hand, a single injection of human B red cells into a rabbit almost always induces formation of human species antibodies without any type specific anti-B fraction. Several injections may be required to develop a satisfactory type specific titer, which nevertheless seldom equals the titer of the human species antibodies.

**Adjuvants.** Numerous substances increase the formation and persistence of antibody when injected with the antigen. Kaolin, charcoal, tapioca, lanolin, and magnesium and calcium salts are more or less effective. Toxoids adsorbed on aluminum phosphate or hydroxide are more efficient immunizing agents than fluid toxoids. Freund recently developed the technique of incorporating antigens in paraffin oil as a water-in-oil emulsion and found that one or two injections induced strong and persistent antibody formation. The antigenicity of such emulsions was sometimes further augmented by addition of a second adjuvant, killed mycobacteria (Figure 21).<sup>26</sup> Acidfast organisms improved typhoid ag-

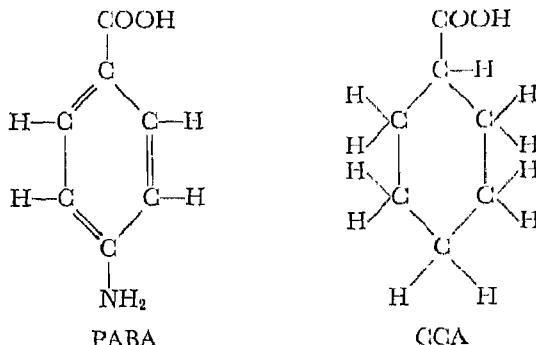
glutinin formation in rabbits but had little effect on production of diphtheria antitoxin. Antitoxin persisted longer following injection of diphtheria toxoid emulsified in oil than following injection of alum-precipitated toxoid. Mineral oil suspensions of poliomyelitis and influenza viruses are currently being investigated as possible immunizing agents in humans.

Paraffin oil and mycobacteria may enhance antibody formation by inciting local inflammation,<sup>25</sup> but the oil also retards absorption, destruction and elimination of the antigen and permits continuous antigenic stimulation for long periods. Herdgen, Halbert and Mudd tested the retention of bacterial antigen in an oil deposit by aspirating material from the site of subcutaneous injection, inoculating it into normal mice and determining the subsequent production of homologous antibodies in these recipient animals.<sup>45</sup> *Sh. paradyssenteriae* persisted in the oil depot as long as twenty-four weeks, the duration of the experiment.

**Heterogeneity of Antibodies for a Single Antigen.** Antibodies produced in response to what is usually considered a single antigen are not necessarily homogeneous, even when formed in the same animal.

The heterogeneity of antibodies may increase with the number of immunizing injections. Heidelberger and co-workers reported quantitative antibody determinations in the serum of a rabbit inoculated with several successive courses of crystalline egg albumin.<sup>37, 40</sup> The antibodies formed after later injections appeared to be "more efficient" in the sense that a given amount combined with more and more egg albumin when an excess of the latter was available. They thus behaved as though they were capable of reacting with an increasing number of radicals on the antigen molecule.

Hooker and Boyd immunized rabbits with hemocyanin-azo-p-aminobenzoic acid (PABA) and employed casein-azo-PABA as the test antigen.<sup>46</sup> Inhibition tests were performed using PABA and cyclohexane carboxylic acid (CCA) alone as simple haptens.



Precipitation of the test antigen by antiserum from an early bleeding was readily inhibited by PABA but not by CCA. Both PABA and CCA

inhibited precipitation of the test antigen by antiserum from a bleeding made seven weeks later. This experiment may indicate that the antibodies formed early possessed relatively simple, specific and presumably small combining groups, and that antibodies produced later contained more complex combining groups which were probably larger and less narrowly specific. On the other hand, the same result would be obtained with a single antibody having strong affinity for PABA and weak affinity for CCA as its concentration increased during immunization. In either case, it is a fairly common observation that the most specific antiserums are produced when adequate titers can be obtained with few injections.

It should not be forgotten that the variety of different antibodies produced by injecting a pure culture of a bacterium such as *S. typhosa* reflects the multiplicity of antigens composing the organism. The fact that antityphoid serum agglutinates certain paratyphoid and other bacteria is a consequence of the distribution of common antigens in the various bacteria and is not necessarily caused by variation in antibodies against particular antigenic fractions.

The physical properties of antibodies for a given antigen produced in the same animal sometimes vary during the course of immunization. Horse antitoxic serums possess two antibody components apparently produced at different rates. The antitoxin after short immunization is associated with  $\gamma$ -globulin, whereas that produced after prolonged immunization is predominantly the "T" component. The situation in antipneumococcal polysaccharide horse serums is the reverse. Moreover, pneumococcal antibody produced first is of high molecular weight and that formed later is of low molecular weight.

It is probably of significance that toxoids and toxins are usually injected into a horse by the subcutaneous route, whereas pneumococci, meningococci and certain other antigens are injected intravenously. The route of injection obviously may determine the particular organs within which antibody formation occurs. Minute amounts of antigen injected locally (i.e., subcutaneously) affect primarily regional lymph nodes, whereas antigen introduced intravenously reaches the spleen, liver and bone marrow rapidly. Large or repeated subcutaneous injections may eventually gain access to the spleen, liver and bone marrow. All antibody globulins share the common property of combining with antigen but because of their possibly varied origins do not necessarily possess identical physical characteristics. This may, in part, explain the heterogeneity of antibodies produced during continued administration of a single antigen.

#### MECHANISM OF ANTIBODY FORMATION

No hypothesis of antibody formation can be universally accepted until it is known whether the persistence of antigen within antibody-forming

cells is a necessary prerequisite for antibody manufacture. Evidence upon this point is contradictory. It has been assumed by some investigators that killed bacteria injected into an animal are quickly and completely digested within macrophages and thereby lose their antigenicity. This assumption, together with the maintenance of high antibody titers for many months after reimmunization, led to the conclusion that antibody formation can continue in the absence of antigen. Moreover, lifetime immunity may follow measles and yellow fever, occasionally with demonstrable humoral antibody, but without detectable virus. However, the possibility that virus is present in unrecognized form has not been excluded. In addition, the nonspecific anamnestic reaction and also the specific anamnestic reaction occurring at a time when there is no circulating antibody are difficult to explain without postulating some permanent change in the antibody-producing cells.

**Early Hypotheses.** The specificity of antibodies strongly impressed early investigators. Buchner (1893) postulated that antibodies might be formed by the union of body proteins with part of the antigen.<sup>8</sup> Doubt was cast upon this hypothesis by repeated failure to detect distinctive components of artificial antigens in homologous antibodies. Animals inoculated with a colored azo-egg albumin antigen produced antibody which contained no colored traces of the antigen.<sup>34</sup> Similarly, antibodies against antigens containing arsenic or iodine possessed no more arsenic or iodine than normal globulin or antibodies against antigens devoid of these elements.<sup>3, 34, 47, 48</sup> Moreover, each milligram of an antigen may call forth the production of as much as 100,000 milligrams of antibody,<sup>18</sup> and since only a few atoms of arsenic or iodine may be present in such an antigen it is obviously impossible for each antibody molecule to receive the respective element from the antigen.

Ehrlich (1897) postulated that body cells possess "side chains" or "receptors" consisting of atom groups or substances having an affinity for toxins and other antigens.<sup>24</sup> Chemically different receptors were presumed to combine with different antigens. Such reactions occurring upon body cells rendered the receptors useless for their normal functions. The cells therefore produced more receptors, some of which were cast off into the blood stream. Circulating receptors protected body cells by reacting with antigen in the blood. Ehrlich's hypothesis placed the specificity of antibodies upon a chemical basis and foreshadowed the conclusions reached by Landsteiner and others. This hypothesis encountered opposition when later investigators produced antibodies against synthetic antigens which did not occur in nature and for which cell receptors would not be presumed to exist.

**The Template Hypothesis.** About 1930 several suggested mechanisms of antibody formation appeared which may be called collectively the "template hypothesis." According to this hypothesis antibodies consist

of globulin having a reverse structural image of the determinant group of the antigen, much as a series of identical coins represent mirror images of the die from which they are struck. This concept is reminiscent of Emil Fischer's lock-and-key idea of enzyme and substrate. Globulin synthesized in cells containing the antigen bears the imprint of the latter. For example, positively and negatively charged radicals such as amino and carboxyl might be arranged in a complementary pattern on the molecules of antigen and antibody. The various authors of these suggestions differed in their explanations of how and where these antibody structures are formed.

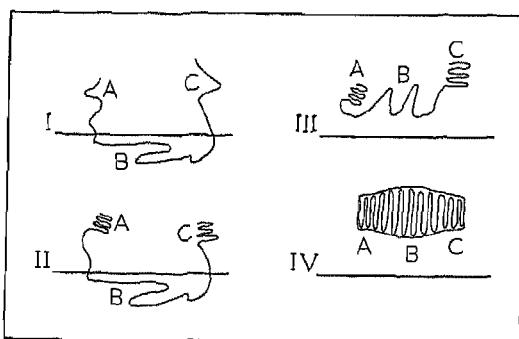


FIGURE 22. Pauling's postulated steps in the folding of a normal globulin chain into its final stable configuration. (Redrawn from Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.)

Breinl and Haurowitz postulated that cells which synthesize globulin possess surfaces containing radicals or groups with residual valences which attract or repulse amino acids and orient them into the globulin configuration characteristic of the species.<sup>7</sup> An antigen alters the residual valences of the surfaces and the cell thereafter produces a modified globulin, as long as the antigen is present.

Mudd emphasized the spatial relationships during synthesis of globulin.<sup>53</sup> It was known that antibodies are capable of distinguishing between stereoisomeric substances coupled to proteins. Mudd proposed that the synthesis of an antibody globulin chain from its constituent amino acids takes place at the interface between antigen and protoplasm, presumably within the cell. This interface constitutes an orienting environment in which a structure is formed having a spatial configuration complementary to that of the antigen. The antibody is synthesized in contact with the antigen and then dissociates from it, leaving the antigen free to act as template for the next synthesis of globulin.

Pauling (1940) believed it unnecessary to assume that antibody globulin molecules contain different amino acids or that the amino acids are

arranged in any different order from those of normal globulin.<sup>54</sup> He postulated only that the *configuration* of the antibody peptide chain in its final stable state differs from that of the normal protein. Formation of normal globulin was postulated to proceed in several steps (Figure 22).<sup>54</sup> The peptide chain is first synthesized and the chain ends are liberated from the place of synthesis in an *unstable* extended form. The chain ends then coil into stable configuration. Thereafter, the central portion of the chain is liberated and the entire molecule assumes a stable folded form. Stability is attributed to hydrogen bonds and other weak bonds between parts of the chain.

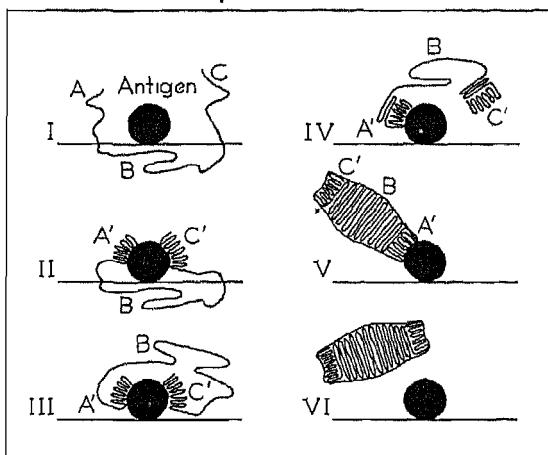


FIGURE 23. Pauling's postulated steps in the formation of antibody globulin, showing how a configuration complementary to the antigen may be produced. (Redrawn from Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.)

Each antibody molecule was assumed to contain two specifically reactive sites or regions capable of combining with antigen. Synthesis of the globulin chain occurs exactly as in the case of normal globulin, but the liberated chain ends fold into a pattern complementary to part of the antigen (Figure 23).<sup>54</sup> One end of the chain dissociates from the antigen and the middle portion folds into a configuration like that of normal globulin. Dissociation of the other end leaves the antigen free to influence the configuration of the next globulin molecule synthesized. The attraction between certain antigen determinant groups and their antibodies may be so strong that dissociation does not occur, with the result that little antibody appears in the body fluids. For example, synthetic antigens containing radicals such as arsonic and sulfonic acids are known to be relatively poor incitants of circulating antibody.

Pauling postulated that it should be possible to prepare antibody *in vitro* by mildly denaturing normal globulin to loosen the chain structure

and renaturing the globulin in the presence of antigen. The globulin should assume a form stable in the presence of the antigen—that is, complementary to active regions of the latter. Successful experiments of this sort were reported,<sup>35</sup> but unfortunately other investigators have been unable to duplicate the results.<sup>38</sup>

Haurowitz recently modified his own hypothesis on the basis of newer knowledge and theories of the mode and site of synthesis of proteins.<sup>31</sup> Plasma proteins are formed in the liver and to a lesser extent in the spleen, bone marrow and other organs. Synthesis probably takes place in mitochondria, cytoplasmic granules which contain ribonucleic acids together with phosphatases and other enzymes capable of providing energy. Protein constituents are selected and arranged by a template consisting of a species specific protein unfolded in a layer of monomolecular thickness. Amino acids or peptide fragments from the cellular fluid are adsorbed to the identical amino acids or peptide fragments of the template. Peptide linkages are formed with the help of a protease, and the expanded peptide layer folds repeatedly to form a globular molecule.

Antibodies differ from normal globulins only by their immunologic specificity for a definite antigen. Antigen therefore interferes with the process of folding the expanded peptide layer so that a structure complementary to the antigenic determinant is formed.

Haurowitz' hypothesis is predicated upon the persistence of antigen within the antibody-producing cells. He and Crampton injected rabbits intravenously with ovalbumin containing radioactive iodine and determined its distribution at various intervals.<sup>32</sup> The antigen quickly disappeared from the blood and appeared in the liver, spleen, kidney and other organs. It then decreased in these organs, rapidly at first and later more slowly. A decrease from 2000 to 200 molecules of antigen per liver cell was calculated to require about 300 days, which indicated that antigen might be retained during the period of antibody formation. The iodo-ovalbumin appeared first within the microsomal fraction of liver and spleen cells and in a few minutes was present within the mitochondrial fraction of cells of the same organs. These granules were suggested as one of the sites of antibody formation.

It is pertinent in this connection to note that McMaster and Kruse detected beef  $\gamma$ -globulin in the liver of the mouse for 101 days after intravenous injection.<sup>51</sup> Moreover, Coons and co-workers, employing a stain consisting of pneumococcal antibodies coupled with a fluorescent dye, detected pneumococcal polysaccharide in R-E cells and connective tissues of mice six months after intravenous injection.<sup>14</sup>

The template hypothesis is widely accepted at present. However, if the body cells which produce antibody have a life span less than the period of active antibody formation, it is necessary to postulate that the

Antigenic template is transmitted intact from parent to daughter cells. The alternative appears to be a cellular alteration which is transmissible to daughter cells. Such an alteration might be genetic or it might consist of enzymes or plasmagenes. Plasmagenes are self-replicating cytoplasmic units probably composed of nucleoprotein. They are subject to modification by conditions in their intracellular environment, such as the nature and concentration of available substrates, and the induced changes are replicated in succeeding cell generations.

Alexander (1931), although apparently subscribing to the template hypothesis, believed that the tremendous yield of antibody per unit of antigen indicates a catalytic process and postulated that antigen combines with some component of the antibody-forming cells to produce new specific catalysts which direct the formation of antibodies.<sup>1</sup>

Sevag (1945, 1951) also emphasized the catalytic role of antigen in antibody formation.<sup>57</sup> His hypothesis is based upon the assumption that introduced foreign protein resists enzymic digestion so that its intact existence, or that of its structurally specific part, is prolonged. Transferred by phagocytes to a center of protein metabolism, it influences the process of globulin synthesis, with the result that antibody is formed. The foreign protein or antigenic unit thus "exercises the role of specific catalytic modifier, or superimposes a new catalytic role of its own on the enzymes which synthesize globulin."

**Burnet's "Modified Enzyme" Hypothesis.** Burnet (1941)<sup>9</sup> and Burnet and Fenner (1949)<sup>10</sup> formulated an interesting hypothesis based upon the assumption that antigen causes adaptive alteration of cellular catalysts.

Synthesis of normal proteins was postulated to occur by a process of complete or partial replication of intracellular enzymes, considered to be "living" proteins whose nature is ultimately under genetic control. Complete replication of an intracellular enzyme yields another molecule of the enzyme; partial replication yields a "nonliving" protein such as serum globulin or albumin, or a digestive enzyme. Nonliving proteins lack the ability to reproduce themselves but possess certain distinctive structural features of the intracellular enzymes. Enzyme replication may occur within or closely associated with mitochondria.

Burnet believed that the normal function of certain intracellular enzymes is the disposal of damaged or effete body constituents such as blood cells. Each enzyme possesses a configuration which permits specific adsorption to a characteristic component ("self marker") of one of these body materials. None of these enzymes can immediately adsorb a foreign substance (i.e., an antigen), but gradual adaptation may occur until it can adsorb and destroy the antigen. Complete or partial replication of the modified enzyme yields a replica possessing the same modification.

The first antigenic stimulus causes adaptation of the enzyme to the most distinctive features of the antigen. Subsequent stimuli broaden the

modification and accelerate replication and liberation of partial replicas into the blood stream as antibody molecules. These cannot reproduce themselves but possess the induced specific complementary pattern and hence can combine with the antigen. The new character is carried into descendant cells which continue to produce antibody in the absence of antigen, but at a gradually diminishing rate as the modified enzyme reverts toward the normal form.

**Template Hypothesis versus Modified Enzyme Hypothesis.** Nature does not always use the same means to accomplish a given end. It is conceivable that both the template and modified enzyme mechanisms may participate in antibody formation under certain circumstances. Furthermore, these hypotheses may not be as far apart as they seem. Each is an attempt to visualize a process about which only indirect evidence is available.

Heidelberger recently suggested that antigens may serve as templates at the beginning of immunization while enzymes are undergoing adaptation.<sup>85</sup> An antigen which is subject to digestion within the tissues induces a rise in antibody during only the first two weeks, after which there is a rapid decrease as the template is removed. The enzyme mechanism then accounts for continuing formation of antibody at gradually lower levels. The cycle is rapidly renewed by another antigenic stimulus during this period. A more resistant antigen such as certain bacterial polysaccharides acts as a relatively permanent template, and a high level of antibody production is maintained for a long period. Antigen released from dead cells attaches to new globulin-synthesizing cells and continues to act as a template. Limited enzyme adaptation may occur but does not influence the rate of antibody formation appreciably. Rejection of such an antigen has little effect, as has been observed.

Heidelberger's proposal may help to explain some of the peculiarities of antibodies against different antigens. It may also account in part for the differing physical, chemical and biologic properties of antibodies formed during various periods in the course of immunization with a single antigen.

#### References

1. Alexander, 1931. *Protoplasma* 14, 296.
2. Barr and Glenvy, 1947. *Lancet* 2, 647.
3. Berger and Erlenmeyer, 1931. *Ztschr. Hyg. Infektkr.* 113, 79.
4. Bieling, 1919. *Ztschr. f. Immunitätsforsch.* 28, 246.
5. Bieling and Isaac, 1921. *Ztschr. ges exp. Med.* 25, 1; 1922, *ibid.*, 26, 251; 1922, *ibid.*, 28, 154.
6. Björneboe, 1941. *Ztschr. f. Immunitätsforsch.* 99, 245.
7. Breinl and Haurowitz, 1930. *Ztschr. physiol. Chem.* 192, 45.
8. Buchner, 1893. *Münch. med. Wschr.* 40, 449.
9. Burnet *et al.*, 1941. *The Production of Antibodies*, Macmillan & Co., Ltd., Melbourne.

10. Burnet and Fenner, 1949. *The Production of Antibodies*, 2nd ed., Macmillan & Co., Ltd., Melbourne.
11. Burnet and Freeman, 1938. Med. Jour. Aust. 2, 299.
12. Carrel and Ingebrigsten, 1912. Jour. Exp. Med. 15, 287.
13. Cohn and Pappenheimer, 1949. Jour. Immunol. 63, 291.
14. Coons and Kaplan, 1950. Jour. Exp. Med. 91, 1.
15. Dean and Webb, 1928. Jour. Path. Bact. 31, 89.
16. DeGara and Angevine, 1943. Jour. Exp. Med. 78, 135.
17. Deutsch, 1899. Ann. Inst. Pasteur 13, 689.
18. Dixon, Burkantz, Dammin and Talmage, 1953. In Pappenheimer, *The Nature and Significance of the Antibody Response*, Columbia University Press, New York.
19. Dixon, Maurer and Deichmiller, 1954. Jour. Immunol. 72, 179.
20. Dougherty and White, 1945. Amer. Jour. Anat. 77, 81.
21. Dreyer and Walker, 1909. Jour. Path. Bact. 14, 28.
22. Ehrlich, 1946. Ann. N. Y. Acad. Sc. 46, 823.
23. Ehrlich and Harris, 1945. Science 101, 28.
24. Ehrlich, 1897. Fortschr. Med. 15, 41.
25. Freund, 1947. Ann. Rev. Microbiol. 291.
26. Freund and Bonanto, 1944. Jour. Immunol. 48, 325.
27. Glenny, 1925. Jour. Path. Bact. 28, 241, 251.
28. Glenny and Pope, 1925. Jour. Path. Bact. 28, 273.
29. Harris, Grimm, Mertens and Ehrlich, 1945. Jour. Exp. Med. 81, 73.
30. Harris, Harris and Farber, 1952. Fed. Proc. 11, 470.
31. Haurowitz, 1952. Biol. Rev. 27, 247.
32. Haurowitz and Crampton, 1952. Jour. Immunol. 68, 73.
33. Haurowitz, Schwerin and Tunc, 1946. Arch. Biochem. 11, 515.
34. Haurowitz, Vardar and Schwerin, 1942. Jour. Immunol. 43, 327.
35. Heidelberger, 1953. In Pappenheimer, *The Nature and Significance of the Antibody Response*, Columbia University Press, New York.
36. Heidelberger, DiLapi, Siegel and Walter, 1950. Jour. Immunol. 65, 535.
37. Heidelberger and Kendall, 1935. Jour. Exp. Med. 62, 697.
38. Heidelberger, Kendall and Soo Hoo, 1933. Jour. Exp. Med. 58, 137.
39. Heidelberger, MacLeod, Kaiser and Robinson, 1946. Jour. Exp. Med. 83, 303.
40. Heidelberger, Treffers and Mayer, 1940. Jour. Exp. Med. 71, 271.
41. Hektoen, 1915. Jour. Infect. Dis. 17, 415; 1916, ibid., 19, 69; 1918, ibid., 22, 28.
42. Hektoen and Boor, 1931. Jour. Infect. Dis. 48, 588.
43. Hektoen and Carlson, 1910. Jour. Infect. Dis. 7, 319.
44. Hektoen and Curtis, 1915. Jour. Infect. Dis. 17, 409.
45. Herdegen, Halbert and Mudd, 1947. Jour. Immunol. 56, 357.
46. Hooker, 1946. Ann. Allergy 4, 425.
47. Hooker and Boyd, 1931. Jour. Immunol. 21, 113.
48. Hooker and Boyd, 1932. Jour. Immunol. 23, 465.
49. Jensen, 1933. Acta path. et microbiol Scand. 10, 137.
50. McMaster and Hudack, 1935. Jour. Exp. Med. 61, 783.
51. McMaster and Kruse, 1951. Jour. Exp. Med. 94, 323.
52. Meyer and Loewenthal, 1927. Ztschr. f. Immunitätsforsch. 54, 409.
53. Mudd, 1932. Jour. Immunol. 23, 423.
54. Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.
55. Pauling and Campbell, 1942. Jour. Exp. Med. 76, 211.
56. Sabin, 1939. Jour. Exp. Med. 70, 67.
57. Sevag, 1945. *Immunocatalysis*, Charles C Thomas, Springfield, Ill. Ibid., 2nd ed., 1951.
58. Simonds and Jones, 1915. Jour. Med. Res. 33, 183, 197.
59. Stavitsky, 1952. Fed. Proc. 11, 482.
60. Topley, 1930. Jour. Path. Bact. 33, 339.
61. Wager and Chase, 1952. Fed. Proc. 11, 485.
62. White and Dougherty, 1946. Ann. N. Y. Acad. Sc. 46, 859.
63. Wilson and Miles, 1946. *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., Edward Arnold & Co., London.

## Chapter 6

### THE ANTIGEN-ANTIBODY REACTION

THE REACTION between antigen and antibody may be observed by a variety of techniques. Precipitation, complement fixation or anaphylaxis can be demonstrated with soluble antigens. Particulate antigens may be used to illustrate agglutination, lysis or phagocytosis. The visible evidence of reaction is determined by the physical state of the antigenic substance and by accompanying conditions. Precipitation and agglutination have been used in most theoretic studies of antigen-antibody reactions.

*Table 23. Normal Agglutination and the Prozone Phenomenon*

	ANTISERUM DILUTIONS									Con-
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Normal reaction	++++	++++	++++	++++	++++	++++	+++	++	-	-
Prozone reaction	-	-	+	+++	++++	++++	+++	++	-	-

- = no agglutination

+ to +++ = varying degrees of agglutination

**Two-Stage Nature of Agglutination and Precipitation.** In the absence of a proper concentration of electrolyte a potent antityphoid serum does not agglutinate typhoid bacteria which have been adequately freed from electrolyte by washing with distilled water. However, addition of sodium chloride or various other electrolytes is followed by the customary agglutination. Specific agglutination by antiserum obviously requires the presence of three reagents: antiserum, antigen and electrolyte. The same requirement can also be demonstrated in precipitation. Antigen and antibody separately do not flocculate in the absence of electrolyte, so it appears that agglutination and precipitation are two-stage reactions. The

first stage is the union of antigen and antibody, and the second stage is the visible effect caused by electrolyte.

**The Zone Phenomenon.** The normal reaction when a bacterial antigen is incubated with homologous antiserum is illustrated in the first horizontal row of results of Table 23. Decreased agglutination in the higher dilutions of immune serum is caused by lack of sufficient antibody to clump all the antigen. Another not uncommon reaction is illustrated in the second row. Absence of agglutination with low dilutions of antiserum is known as the *prozone* or *prezone* phenomenon. Prozones are most commonly encountered in old antiseraums, although they are occasionally found in strictly fresh immune serums and on rare occasions in normal

Table 24. The Zone Phenomenon in Precipitation

Egg albumin (1 ml.)	1.500	1.1000	1:2000	1:4000	1:8000	1:16,000
Rabbit anti-egg albumin serum (undiluted)	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.	1 ml.
Precipitation	±	+++	++++	+++	++	+
Tests of supernates:						
* with antiserum	+	+	-	-	-	-
** with egg albumin	-	-	-	+	+	+

\* + in this row indicates excess antigen

\*\* + in this row indicates excess antibody

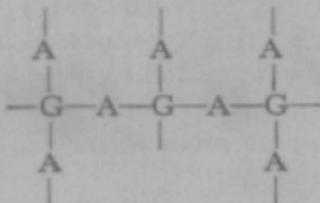
serums. In the old Widal reaction the patient's serum was diluted approximately 1:80 or 1:160 to avoid prozone effects. Older textbooks cite both heating and aging of serums as methods for experimentally producing prozones.<sup>36</sup> However, the artificial production of prozones is very erratic. Serums may be repeatedly evaporated to dryness at 56° C. and redissolved in distilled water without producing a prozone. On the other hand, heating serums for an hour or more at 56° C. may occasionally produce a clearly demonstrable prozone.

The prozone phenomenon was originally attributed by Ehrlich to the presence in certain antiseraums of two kinds of antibodies: (1) complete agglutinins which cause visible reaction and (2) a low concentration of incomplete but more highly reactive antibodies or "agglutinoids," which do not give a visible reaction. Coca and Kelley studied an antiserum which failed to agglutinate its homologous organism (*Hemophilus influenzae*) in any dilution and which inhibited agglutination of this organism by either of two other anti-*H. influenzae* serums.<sup>37</sup> They considered that the serum in question contained a specific inhibiting antibody because the effect was removed by adsorption with a small dose of *H. influenzae*.

Table 24 shows the zone phenomenon in precipitation. Egg albumin diluted 1:2000 yielded the maximum amount of precipitate when mixed

with one milliliter of undiluted antiserum. All the antigen combined with all the antibody in the mixture as shown by examination of the supernatant liquid after removal of the precipitate. Decreased precipitation with lower dilutions (higher concentrations) of antigens is called the "inhibition" or prozone phenomenon. Within the prozone there was insufficient antibody to precipitate all the antigen, although part of the antigen combined with the antibody. Some of the antigen-antibody complexes were evidently of such small size (low molecular weight) that they did not precipitate. The reaction mixture also contained uncombined antigen which was detected by addition of antiserum to the supernatant liquid. Antigen dilutions of 1:4000 and greater also yielded smaller precipitates than that obtained with 1:2000 egg albumin. All the available antigen was combined, so the decreased amounts of precipitate can be attributed to insufficient antigen to combine with the antibody present. Excess (uncombined) antibody was present in the supernatant fluid. Many antigen-antibody systems are not particularly sensitive to slight excesses of antigen, so there is often a range of antigen concentrations within which neither antigen nor antibody is present in the supernates. This is known as the "equivalence zone." Maximal precipitation occurs either within this zone or in mixtures containing a small excess of antigen.

Precipitates formed in the equivalence zone may disappear when more antigen is added. A point usually not made clear is that "dissolution" of the precipitate is merely conversion of a large aggregate into small, invisible complexes. An antigen-antibody precipitate is usually pictured as a chain or network of alternating antigen (G) and antibody (A) molecules:

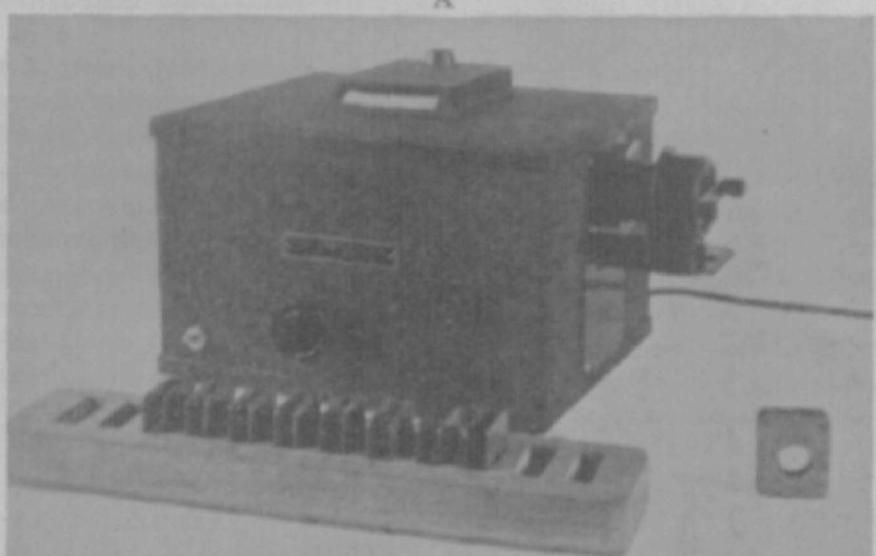


A large network yields a visible precipitate, but when the reaction mixture contains a high antigen-antibody ratio or when further antigen is added to a precipitate, small complexes are formed, such as



These are invisible and of such low molecular weight that the physical chemist speaks of them as "soluble complexes." Whether antigen-antibody complexes of this kind are truly soluble is debatable.

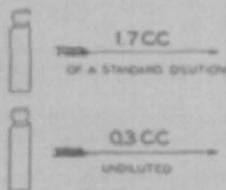
Precipitation is in some respects the simplest demonstrable expression of antigen-antibody reaction because it can be performed with purified antigens, thus eliminating some of the variables inherent in the use of



NEW PROCEDURE IN THE SEROLOGICAL STUDY OF ANIMAL RELATIONSHIP

THE REAGENTS

ANTIGEN SERUM



ANTISERUM

0.3 CC  
UNDILUTED

THE TEST

A - MIX  
B - INCUBATE  
C - READ

PHOTRONER CELL

AN ACTUAL EXPERIMENT

**A - HOMOLOGOUS TEST**

HUMAN SERUM (1:500)  
+  
ANTI-HUMAN SERUM



**B - HETEROLOGOUS TEST**

RHESUS SERUM (1:500)  
+  
ANTI-HUMAN SERUM



B

FIGURE 24. *A.* Photronreflectometer used to measure turbidity in precipitin tests. Turbidity in the cell causes diffraction of light onto sensitive areas of the photoelectric cell, which in turn deflects the galvanometer needle.

*B.* Use of the photronreflectometer in comparing two antigenic solutions. Galvanometer readings are proportional to the turbidity which develops in the cell containing the reagents. (From Boyden, 1942. *Physiol. Zool.* 15, 109.)

cells, which contain many antigenic components. Many features of the antigen-antibody reaction which have been discovered by study of precipitation apply with appropriate modification to other tests.

Early work suffered from ignorance of the zone phenomenon or lack of appreciation of its significance. Visible reaction sometimes does not occur if only a single or few dilutions of antigen are tested, depending upon the position of the dilutions in relation to the equivalence zone. Adequate study of a precipitating system requires tests with several dilutions of first one reagent and then the other.

Simple mixtures of antigen and antiserum, observed macroscopically for relative turbidity or amount of precipitate, yield only limited infor-

*Table 25. Effect of Antigen Dilution on the Flocculation Time of Antigen and Antibody*

<i>Antiserum: rabbit anti-horse serum (7·70)</i>	
<i>ANTIGEN (HORSE SERUM) DILUTION</i>	<i>FLOCCULATION TIME (min.)</i>
1:50	14 5
1:100	12.0
1:200	8.0
1:400	6 0*
1:800	8 5
1:1600	13 0
1:3200	28 0

(From Wilson and Miles,<sup>84</sup> by permission.)

mation. Precipitates are sometimes measured roughly by centrifugation in graduated tubes. Photoelectric devices have recently been adapted to determine the cloudiness or turbidity of precipitating mixtures (Figure 24).<sup>8</sup> Tests of this sort are usually read after a specified time interval because the amount of precipitate increases to some extent with continued incubation. Dean and Webb employed the reaction times of simple mixtures to determine the endpoint of a titration procedure which bears their name.<sup>11</sup> Antigen dilutions were mixed with a constant amount of antiserum and closely observed. The mixture in the series which flocculated most rapidly was noted. A typical set of results is shown in Table 25.<sup>84</sup> The first visible reaction occurred at a particular ratio of antiserum to antigen (40:1). Dean and Webb found that this "optimal ratio" was constant and characteristic of a given lot of antiserum. If the antiserum illustrated were used in a dilution of 1:5, it would flocculate most rapidly with antigen diluted 1:200, and so forth. Such a relationship makes it possible (1) to determine the amount of an antigen in an unknown solution by use of a standardized antiserum, or (2) to determine the relative amounts of antibody in two or more antisera.

The result obtained by the Dean and Webb method is called the constant antibody optimal ratio. When antigen is kept constant and antibody is varied, a mixture is often found which reacts most rapidly in a series of antiserum dilutions. This method is used in the Ramon flocculation test of diphtheria antitoxic serums. Both titration procedures employed with the same antigen and antibody yield results like those in Table 26.<sup>84</sup> Each horizontal row represents a constant antibody titration in which antigen concentration is varied and contains a mixture flocculating more rapidly than any other mixture in the same row. For example, 1:5 antibody reacted most rapidly with 1:200 antigen, and 1:20

*Table 26. Constant Antibody versus Constant Antigen Precipitation Reactions*

ANTIBODY DILUTIONS	ANTIGEN (HORSE SERUM) DILUTIONS						
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200
Flocculation time (minutes)							
1:2.5	2.75	<i>2.25*</i>	2.50	3.25	6.00	17.00	60.00
1:5	4.00	3.50	2.75	3.75	7.00	14.00	40.00
1:10	14.50	12.00	8.00	<i>6.00</i>	8.50	13.00†	<b>28.00</b>
1:20	134.00	39.00	30.00	19.00	13.50	18.50	<b>28.00</b>
1:40	—	—	83.00	65.00	50.00	38.00	45.00

\* Italicized figures are minimal flocculation times in constant antibody titration (horizontal rows).

† Bold face figures are minimal flocculation times in constant antigen titrations (vertical columns).

(From Wilson and Miles,<sup>84</sup> by permission.)

antibody most rapidly with 1:800 antigen. The constant antibody optimal ratio was 40:1. Each column of the table represents a constant antigen titration, antibody being varied. Minimum flocculation times in the last two columns indicate that the constant antigen optimal ratio was approximately 160:1.

Flocculation time is determined by several factors including the antigen:antibody ratio and the chemical compositions and molecular weights of the antigen and antibody. These factors and presumed differences in solubility of various antibodies and antigen-antibody complexes probably account, at least in part, for the observation that constant antibody and constant antigen optimal ratios rarely, if ever, coincide.

A more recent development is the application of quantitative chemical procedures to the analysis of precipitates formed in mixtures of antigens and antibodies. The quantitative approach does not represent a new serologic reaction but merely provides a method of determining the amounts of the reacting substances which appear in precipitates. Much of this work was done by Heidelberger and his associates.<sup>15</sup> It is unfor-

tunate in one sense that most of the precise studies were carried out with pneumococci and their antibodies because the student is likely to gain the impression that the phenomena described are applicable only to this and a few other systems. Actually, pneumococci and their purified polysaccharides provided unusually good material for this type of investigation and were excellent reagents for development of the procedures. The methods devised were later applied to other systems with almost as satisfactory results.

The quantitative method is based upon the fact that antibody is protein and hence contains nitrogen which can be determined with high accuracy

*Table 27. Analyses of Precipitates Formed by Adding Various Amounts of Egg Albumin to 1.0 ml. Rabbit Anti-egg Albumin Serum*

EGG ALBUMIN N ADDED	TOTAL N PRECIPITATED	EGG ALBUMIN N PRECIPITATED	ANTIBODY NITROGEN BY DIFFERENCE	RATIO IN PRECIPITATE		TESTS OF SUPERNATANT LIQUID
				Antibody N	Egg albumin N	
mg 0.0091	mg. 0.156	mg. Total	mg. 0.147	16.2		Excess Ab*
0.0155	0.236	Total	0.220	14.2		Excess Ab
0.050	0.632	Total	0.582	11.6		Excess Ab
0.065	0.740	Total	0.675	10.4		Excess Ab, tr. Ea†
0.074	0.794	Total	0.720	9.7		No Ab or Ea
0.082	0.830	Total	0.748	9.1		No Ab, < 0.001 Ea N
0.090	0.826	0.087	0.739	8.5		Excess Ea
0.124	0.730	0.087	0.643	7.4		Excess Ea
0.195	0.414	(0.048)	(0.366)	(7.6)		Excess Ea
0.307	0.106	(0.004)				Excess Ea

Values in parentheses uncertain.

\* Ab = antibody.

† Ea = egg albumin (antigen).

(From Heidelberger and Kendall,<sup>18</sup> by permission.)

by the micro-Kjeldahl or various other methods. Mixtures containing known amounts of antiserum and antigen are incubated under conditions of temperature and time favorable for maximal precipitation (one to seven days at 0° C.). The precipitates are washed and quantitatively analyzed for total nitrogen. The nitrogen content of the antigen alone is also determined. Subtraction of the amount of nitrogen in the antigen from the total in the precipitate yields the antibody nitrogen in the precipitate, when a test of the supernatant fluid indicates that all the antigen was precipitated. Excess (uncombined) antigen in the supernatant can be determined quantitatively by a second precipitation with additional antiserum. Certain antigens possess distinctive chemical characteristics by which their concentration in a precipitate can be ascertained. For example, hemocyanin contains copper, and synthetic azoprotein antigens often contain readily determinable elements, such as arsenic, iodine or

radioisotopes. Moreover, some polysaccharide antigens contain no nitrogen; hence, all the nitrogen in their specific precipitates represents antibody.

The variation in composition of an antigen-antibody precipitate is illustrated by quantitative data obtained with egg albumin and a rabbit anti-egg albumin serum (Table 27).<sup>18</sup> Addition of increasing amounts of egg albumin to a constant amount of anti-egg albumin yielded precipitates which increased to a maximum (0.830 mg. N), and throughout this range all the antigen was precipitated. The first four supernates contained excess antibody. Beyond the maximum, decreasing amounts of egg albumin precipitated, the excess remaining in the supernates, and the total quantity of precipitate diminished. The relative amounts of antibody

*Table 28. Molecular Composition of Precipitates with Rabbit Antisera*

ANTIGEN	INHIBITION ZONE (prozone)		EQUIVALENCE ZONE		EXTREME ANTIBODY EXCESS IN SUPERNATE
	Soluble com- pounds	Antigen excess	Slight antigen excess	Slight antibody excess	
Egg albumin (E)	(EA*)	EA <sub>2</sub>	E <sub>2</sub> A <sub>5</sub>	EA <sub>3</sub>	EA <sub>5</sub>
Pneumococcus 3 specific polysaccharide (S)	(S <sub>5</sub> A)	S <sub>4</sub> A HA <sub>38</sub>	S <sub>2</sub> A HA <sub>82</sub>	S <sub>3</sub> A <sub>2</sub> HA <sub>120</sub>	SA
Hemocyanin (H)		T <sub>2</sub> A	T <sub>2</sub> A <sub>3</sub>	TA <sub>4</sub>	
Diphtheria toxin (T)					TA <sub>8</sub>

\* A = antibody.

Composition of compounds in parentheses is uncertain.

(Reprinted by permission of the publishers from Karl Landsteiner's *The Specificity of Serological Reactions*, Revised Ed. Cambridge, Mass., Harvard University Press, 1947.)

and antigen in the precipitates varied markedly throughout the range of the experiment. The ratio of antibody nitrogen to antigen nitrogen was about 16 to 1 in the region where considerable antibody remained uncombined, whereas in the region of antigen excess the antibody:antigen nitrogen ratio was approximately 7.5 to 1. The corresponding ratio in the zone of equivalence was slightly more than 9 to 1. Assuming that the molecular weights of egg albumin and of rabbit antibody are 42,000 and 165,000, respectively, the molecular antibody:antigen ratio at equivalence was approximately 2.5 to 1. These and similar data with other systems permit calculation of the molecular compositions of precipitates or complexes formed when antigen and antibody in various proportions are allowed to react (Table 28).<sup>23</sup>

Obviously the mechanism of reactions between antigens and antibodies is complex. It should be emphasized that macroscopic inspection, observation of reaction time and quantitative analyses are merely three different ways of studying precipitation.

**Rate of Antigen-Antibody Reaction.** The first stage in the reaction between antigen and antibody is union of the two principal reagents. The second stage is the formation of a visible precipitate or other reaction product. There is evidence to indicate that the first stage is extremely rapid. Winkler and Westphal found that adsorption of typhoid antibodies was more than 70 per cent complete in five minutes.<sup>35</sup> Heidelberger and co-workers reported that egg albumin and its antibody combined in less than twenty seconds, even at 0° C., and that union between pneumococcal polysaccharides and antibodies was 90 per cent complete in less than three seconds at 0° C.<sup>20</sup> Further evidence is provided by determining the heat of serologic reactions. Boyd and his associates

*Table 29. Effect of Temperature on Combination of Sheep Hemolysin with Homologous Erythrocytes*

(Reaction time = 30 minutes)

TEMPERATURE (° C.)	PER CENT ADSORPTION OF HEMOLYSIN
0	90
15	94
25	94
37	90
40	88
45	82
50	78
55	60
60	38

(From Cromwell,<sup>10</sup> by permission)

employed calorimetric methods and detected considerable evolution of heat immediately upon mixing hemocyanin and its antibody and concluded that the reaction was complete within about three minutes.<sup>6</sup> Approximately 40,000 gram calories were evolved per mole of antibody.

Salts are not necessary for union of antigen and antibody, and in fact combination may be considerably reduced in hypertonic solutions. Heidelberger and co-workers found that agglutination of pneumococci and precipitation of pneumococcal polysaccharide were strongly inhibited by sodium chloride in concentrations up to 15 per cent.<sup>19</sup> It has been suggested that the inhibitory effect of strong electrolytes upon the combination of antigen and antibody may result from formation of a diffuse layer of electrolyte ions around the oppositely charged polar combining radicals of antigen and antibody, thus reducing their attraction for each other.<sup>24</sup>

Temperature variation does not markedly affect combination of certain antigens and antibodies, although it is difficult to generalize from the meager data available. Figures such as those of Table 29<sup>10</sup> indicate a

wide range of reactivity, from 0° to 60° C. Reaction is so nearly complete, even at 0° C., that increased temperature does not exert a very pronounced stimulating effect. Inhibition above 55° C. can be attributed in part to increased dissociation and in part to denaturation of the reagents. Dissociation of some antigen-antibody complexes occurs when the temperature is raised. Certain hemagglutinins react with erythrocytes at low temperatures but dissociate from them upon incubation at 25° to 37° C. Agglutinins have been separated from some bacteria by a similar procedure. Stuart and Carpenter observed flagellar agglutination of normal and intermediate Escherichia and paracolon strains after two hours at 37° C., but agglutination of approximately 65 per cent of the strains was partially or completely reversed when the tests were placed at 55° C. overnight.<sup>32</sup> Reincubation at 37° C. of tests in which dispersion had occurred at 55° C. resulted in complete reagglutination. Flagellar agglutination of Salmonella did not reverse under these conditions. The ease with which reversion took place varied with the degree of specialization of the organisms. Aerobacter redispersed at 48° C., Escherichia at 55° C. and Salmonella not uniformly at any temperature tried.

**Specificity and the Nature of the Forces Joining Antigen and Antibody.** The reaction between antigen and antibody derives much of its theoretic interest from its specificity. Antigens are chemically heterogeneous. Most proteins, some polysaccharides and possibly a few lipids are antigenic. It is therefore impossible to postulate a single type of attractive force or chemical reaction between antigen and antibody. The alternative appears to be a specific pattern or arrangement of one or more nonspecific forces.

Landsteiner and others clearly demonstrated the importance of highly polar radicals (with which strong fields of force are associated) as determinants of the specificity of synthetic antigens. Less strongly polar but larger radicals also exerted considerable influence upon specificity. It must be presumed that antibody, synthesized under the influence of an antigen determinant radical, contains sites capable of reacting with the determinant and having an affinity or attraction for it.

Marrack considered that specific attraction between antibody and antigen is the result of intermolecular forces whose specificity is attributable to atoms or radicals with suitable (and presumably opposite) fields of force so arranged that they can come into apposition.<sup>24</sup>

Pauling postulated that the attraction of antigen for antibody consists of several short-range forces, any or all of which may operate under appropriate conditions.<sup>20</sup> The least specific intermolecular force exerted between every pair of molecules is van der Waals attraction. This force attracts every atom in one molecule toward every atom in neighboring molecules. Its magnitude is inversely related to the distance between atoms. The mutual attraction between molecules is great if they possess sufficiently large areas of complementary configuration.

A second force between molecules is Coulomb attraction, which operates between positive and negative charges such as those associated with  $\text{--NH}_3^+$  and  $\text{--COO}^-$  ions. This force alone is too weak to yield a stable aggregate, but hydrogen bond formation may increase the strength of interaction sufficiently to provide a fairly strong union between the groups.

Hydrogen bonds may be formed between certain pairs of atoms, among which oxygen and nitrogen form the strongest bonds. It is believed that protein molecules are held in stable configurations by hydrogen bonds connecting amino and carboxyl radicals.

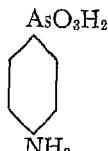


This linkage provides a moderately firm union.

Electric dipole or multipole forces may play some part in the attraction of one molecule to another but are less important than the preceding forces.

The various forces of attraction which have been described are not specific by themselves. However, two molecules with suitable surface structures may be mutually attracted by strong van der Waals forces as they approach each other under the influence of Brownian motion. An appropriate arrangement of hydrogen bond-forming groups and radicals of opposite electric charge strengthens the force between the two molecules. Pauling believes that the configuration of the specific combining sites of antigen and antibody correspond to within one or two Ångströms.<sup>80</sup> A lower degree of correlation in structure and arrangement provides less tendency for interaction. According to this concept, both specificity and mutual attraction are determined by the extent to which the molecules possess complementary surface configurations.

**Minimum Size and Number of Antigen Determinant Groups.** The minimum size of the reacting group or radical may be deduced from experiments with synthetic antigens by means of inhibition tests with simple haptenes. Compounds as small as arsanilic acid



are capable of combining specifically with homologous antibody and inhibiting precipitation of complete antigens containing the same determinant group. The minimum molecular weight of a determinant therefore appears to be about 250. Boyd considered that the active radicals in serology are at least as large as or slightly larger than those compounds

used by Landsteiner and others in azoantigens.<sup>5</sup> The active group might consist solely of arsanilic acid, for example, or it might also include some of the adjacent amino acid residues or other protein components to which the arsanilic acid is coupled. These neighboring areas might also be expected to influence the specificity of the complete antigen.

An antigen must also contain at least a minimum number of determinant groups to precipitate with homologous antiserum. The number of determinants can readily be found by chemical analysis in the case of artificial antigens containing distinctive elements such as arsenic. Hooker and Boyd reported that arsanilic azocasein with less than thirteen arsanilic radicals per molecule failed to precipitate with corresponding antiserum.<sup>21</sup>

*Table 30. Molecular Weights and Minimum Valences of Various Proteins*

PROTEIN	MOLECULAR WEIGHT	MINIMUM VALENCE
Ovalbumin	40,500	5
Serum albumin	70,200	6
Diphtheria toxin	70,000	8
Thyroglobulin	650,000	40
<i>Bufo</i> hemocyanin	6,760,000	74
<i>Viviparus</i> hemocyanin	6,630,000	231

(From Hooker and Boyd,<sup>22</sup> by permission.)

Haurowitz also concluded from similar observations that between ten and twenty determinant radicals were needed for precipitation, although compounds containing only one introduced group per molecule yielded specific antibodies in the animal body.<sup>14</sup>

The determinants in natural proteins are more difficult to estimate, and only minimum figures can be cited. The number of molecules of antibody entering into combination with a molecule of antigen can be calculated from quantitative precipitation data. If the figure is five, for example, the antigen must possess at least five combining sites or determinants and is said to have a "valence" of five. The larger the antigen molecule, the more determinant radicals it is likely to possess. A few minimal values are given in Table 30.<sup>22</sup>

Until the structures of naturally occurring proteins are known, it will be possible only to speculate upon the actual nature of their determinant radicals. It has been suggested that a protein consists of a peptide chain folded into a characteristic configuration and held in this form by hydrogen bonds. Proteins in solution exist in globular or ellipsoidal shape. Their behavior in serologic reactions indicates that only the surfaces are serologically active, and furthermore that particular surface areas determine their activity and specificity. These "active patches" presumably consist of certain amino acids or combinations of amino acids

which may recur at constant intervals throughout the length of the peptide chain (Bergmann's hypothesis).

**Factors Affecting Precipitation and Agglutination.** The rate of visible reaction varies widely, from a few seconds to several days. Certain precipitating systems and particularly strong antiseraums may produce opalescence, turbidity or even flocculation almost immediately. On the other hand, several hours may be required for opalescence with weak antiserum or with too little serum or antigen. Other factors which affect the rate of reaction include the concentration and kind of electrolyte present, pH, temperature, and agitation of the mixture.

Electrolytes are essential for precipitation and agglutination but their exact function is not clear. The salting-out of proteins and other colloidal solutions or suspensions by electrolytes is well known. Many kinds of proteins precipitate when treated with an electrolyte in sufficient concentration, this effect being more pronounced with polyvalent cations than with monovalent or bivalent cations.

Northrop and DeKruif demonstrated that under appropriate conditions electrolytes may also agglutinate bacterial suspensions in the absence of antiserum ("salt agglutination").<sup>25</sup> They maintained that the stability of suspensions is the resultant of two opposing forces: (1) the "cohesiveness" or "surface tension" of the cells, which tends to cause aggregation, and (2) the electric charges on the cells, which repel if alike. A suspension is stable as long as these two forces are properly balanced. Most proteins and bacteria in approximately neutral solutions possess a negative electric charge. Addition of electrolyte reduces the negative charges on the particles; this effect is greater with polyvalent cations than with monovalent. Aggregation takes place when the charge drops below a critical value, found to be about fifteen millivolts. However, electrolytes in concentrations greater than 0.01 to 0.1 normal decrease the cohesiveness of such particles or increase their attraction for water, thus counteracting the agglutinating effect of lowered electric charge. The surface tension reducing power of monovalent and bivalent cations is sufficiently great to prevent salt agglutination of such bacteria as *S. typhosa*. Trivalent cations like  $\text{Al}^{+++}$ , however, may cause the critical potential range of +15 to -15 millivolts to be attained at low electrolyte concentrations (less than 0.0001 normal).

Combination with antibody apparently protects antigenic particles against loss of cohesiveness in electrolyte solutions, so that aggregation occurs even with monovalent cations when the surface potential is below 15 millivolts. In the absence of antibody, bacteria behave as though they are hydrophilic, whereas after combination with antibody they become hydrophobic.

Electrolytes affect serologic precipitation in much the same manner as agglutination. Electrolyte in proper concentration is necessary for

precipitation. The composition of the precipitate may vary with the concentration of electrolyte. A precipitate of pneumococcal polysaccharide and homologous antibody partially dissociates when the sodium chloride is greater than the customary 0.15 molar, part of the antibody returning to solution. Heidelberger found that the precipitate of rabbit antibody formed in approximately 0.95 molar sodium chloride contained only one-third as much antibody nitrogen as that formed in 0.15 molar salt.<sup>19</sup> On the other hand, precipitation of egg albumin by homologous

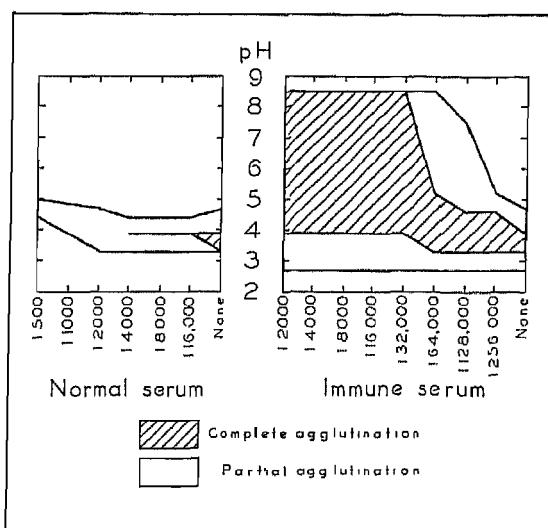


FIGURE 25. Effect of pH on agglutination of typhoid bacteria in the presence of (a) normal and (b) immune serum. (Adapted from Northrop and DeKruif, 1922. *Jour. Gen. Physiol.* 4, 639.)

antibody was relatively unaffected by high salt concentration. Schmidt reported that flocculation of diphtheria toxin-antitoxin mixtures did not take place in salt-free solution.<sup>31</sup> Addition of electrolyte permitted flocculation to occur, the optimal concentration of sodium chloride, bromide or iodide being between 0.05 and 0.25 molar.

The optimum hydrogen ion concentration for most serologic procedures is between pH 6.5 and 8.5, however, certain reactions proceed satisfactorily in more acid or more alkaline media. Precipitation occurs within the range from pH 4.5 to 9.5; the amount of precipitate formed is relatively constant between pH 6.6 and 8.0. Northrop and DeKruif found that a typhoid bacterial suspension agglutinated within a narrow range of acid pH values in the absence of serum or when mixed with normal serum (Figure 25), but that immune serum caused agglutination throughout a wide pH range.<sup>25</sup>

The visible stage of serologic reactions is usually accelerated as the temperature rises from 0° to 20° or 30° C. and in some cases to higher temperatures. The increased rate of precipitation or agglutination at higher temperatures results in part from accelerated Brownian movement, which may cause more frequent collisions between the reacting molecules or particles. Greater rates of molecular or particle contact are also obtained by incubating precipitation or agglutination tests with the water bath level at two-thirds the depth of the fluid within the test tubes. The cooler top surface of the liquid creates convection currents which promote faster visible reaction. Above 56° C. the rate of precipitation or agglutination usually decreases. The effect of temperature upon the amount of precipitate formed is variable. Some systems are little affected, but in other cases (e.g., pneumococcus 3 specific polysaccharide and horse antiserum) considerably less precipitate forms at 37° than at 0° C.,<sup>17</sup> and some precipitates produced at the lower temperature disappear at room temperature or 37° C. and reappear on cooling. However, egg albumin specific precipitates disappear when warmed to 55° C. but do not reappear when cooled.<sup>27</sup>

Some antigens and antibodies agglutinate only at very low temperatures. The cold isohemagglutinins or autohemagglutinins clump erythrocytes of the same species or individual at 0° to 35° C. but not at normal body temperature.<sup>18</sup> Moreover, certain animals such as the rabbit contain cold hemagglutinins for human erythrocytes of any or all blood groups as well as for erythrocytes of the rabbit, guinea pig, horse and sheep. Cells agglutinated at low temperatures redisperse when warmed and reagglutinate when again cooled. Antibody is eluted from the cells by warming but recombines upon cooling.

The optimum temperature for bacterial agglutination varies with the type of organism and antigen (somatic or flagellar). In fact, within a large group of related organisms a temperature gradient may exist through which the different organisms or antigens agglutinate optimally. Flagellar agglutination varies widely with the bacterial species. For certain organisms it is best at room temperature, for others at 37° C., and for some others best results are obtained at temperatures as high as 50° to 55° C. On the other hand, little if any somatic agglutination may occur after several hours at 37° C., whereas marked agglutination to high titers is obtained at 55° C. Some highly mucoid organisms agglutinate only after prolonged incubation (two or more days) of phenolized mixtures at 37° C. Osterman and Rettger obtained agglutination titers of only 20 to 40 in their study of the Friedländer-aerogenes group, but reactions appeared to be specific as shown by absence of cross-agglutination between types in dilutions as low as 1:5.<sup>28</sup> Adler and Humphries recently reported successful application of this method in another investigation of the

Friedländer organism.<sup>1</sup> This procedure is valuable for studying such organisms and has not received the attention it deserves.

Mechanical shaking accelerates the visible stage of antigen-antibody reactions, apparently by providing increased opportunity for contact between molecules or particles of the reagents. Agglutination, precipitation, complement fixation and cytology are hastened by frequent mixing, and gentle agitation of phagocytosis tests seems to permit more efficient ingestion.

#### MECHANISM OF PRECIPITATION AND AGGLUTINATION

Early investigators dealt with what appeared to be simple systems in their studies of the reactions between antigens and antibodies. Observations were carefully made and adequate in scope and were interpreted in a straightforward manner to yield the simplest hypothesis consistent with the known facts. That some of these hypotheses seem a little naive today is readily understood when it is appreciated that little was known of the chemical and physical nature of the reacting ingredients.

More recently masses of data, particularly of a quantitative nature, have accumulated. It appears at first glance that present information should provide the basis for an acceptable theory of the mechanism of antigen-antibody reactions. However, in step with increased knowledge of the conditions under which reaction occurs, has come a growing awareness of the chemical and physical complexity of the reacting substances. Consequently, the situation is about as confused today as ever.

**Ehrlich's Side-Chain Hypothesis.** Ehrlich postulated that antibodies are substances produced by body cells which are stimulated by antigen.<sup>12</sup> Antigens possess combining radicals or "side-chains" called *hapto**phore* groups. In addition, toxic antigens contain *toxophore* groups responsible for their poisonous properties. Antibodies also possess side-chains or hapto*phore* groups, and these are capable of combining specifically with those of antigens.

Three different kinds or "orders" of antibodies were postulated. "First order" antibodies neutralize toxins; each contains a hapto*phore* group which can combine with a specific toxin. Antibodies of the "second order" cause agglutination and precipitation. The visible reaction was attributed to *ergophore* or *zymophore* groups possessed by the antibodies. "Third order" antibodies participate in lysis and complement fixation, and contain two hapto*phore* groups, one of which combines specifically with antigen, the other with complement. These antibodies were called *amboceptors*. Complement possesses a zymophore group which causes cytology. Only when antigen and amboceptor have combined can complement exert its characteristic action.

Ehrlich postulated that the combination between antigen and antibody is a firm chemical union in constant proportions. This hypothesis encountered opposition when it was shown that constant proportions are not always found in the neutralization of toxin by antitoxin. It was then proposed that toxic bacterial filtrates contain substances with differing degrees of toxicity and/or affinity for antitoxin, and such names as toxone, prototoxoid, syntoxoid and epitoxoid were applied to them. It is of interest that the term *toxoid* is still in widespread use to designate toxin so modified that it does not produce symptoms in an experimental animal although it can combine with antitoxin; this is the same description as that given by Ehrlich.

The side-chain hypothesis emphasized the now well established chemical basis of specificity and the chemical nature of antigen-antibody reaction. It gradually became cumbersome as attempts were made to fit new discoveries to the old framework, but it persisted for a number of years. Recent observations, particularly concerning the serologic properties of viruses and erythrocytes, are currently described in terms reminiscent of Ehrlich.

**Arrhenius and Madsen's Mass Action Hypothesis.** Arrhenius was the first physical chemist to attempt an interpretation of the reaction between antigen and antibody. He and Madsen concluded from their studies of the neutralization of toxins by antitoxins that they were dealing with a reversible reaction like that between a weak acid and a weak base.<sup>2</sup> They proposed that antigen-antibody reactions follow the law of mass action and attain a state of equilibrium in which both free and combined antigen and antibody are present in concentrations which agree with the familiar mathematical relationship.

The most significant feature of this hypothesis is its emphasis upon reversibility. This was vigorously opposed by Ehrlich, to whom the reaction between toxin and antitoxin seemed to resemble that between a strong acid and a strong base. Subsequent investigations have made it very doubtful that true reversibility of antigen-antibody reactions occurs. This question was never adequately investigated by either Ehrlich or Arrhenius and Madsen because of the lack of technical methods at that time.

**Bordet's Adsorption Hypothesis.** Bordet attempted to account for the union of antigen and antibody in variable proportions and for the participation of electrolytes in agglutination and precipitation.<sup>3</sup> He postulated that antigen-antibody reactions are adsorption phenomena in which antigen is the adsorbing agent. The amount of antibody adsorbed is governed by the surface area of the antigen and the concentration of antibody according to the physical laws of adsorption. Antigen with its adsorbed antibody behaves essentially as a particle of globulin and is "salted out" by electrolyte in the visible reaction of agglutination or

precipitation. This "second stage" of antigen-antibody reaction was considered to be nonspecific. Eagle later proposed that combination with antigen produces a change in the structure of antibody protein similar to denaturation so that the solubility of antibody is decreased. This presumably requires considerable distortion of the antibody molecules when joined to antigen.

The most serious objection to Bordet's hypothesis was its failure to account adequately for specificity. Moreover, the laws of adsorption indicate that the amount of antibody adsorbed from solution should increase indefinitely with its concentration. Quantitative data reveal wide deviations from values predicted by this hypothesis, particularly at high concentrations of either reagent.

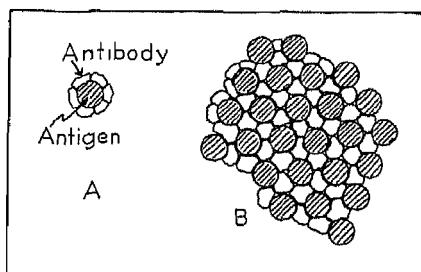


FIGURE 26. Two of Marrack's postulated arrangements of antigen and antibody molecules in the antigen-antibody complex. *A*, simple unit. *B*, complex structure at optimal proportions. (From Marrack, *The Chemistry of Antigens and Antibodies*, 1938, H. M. Stationery Office, London.)

The role of adsorption in antigen-antibody reactions is still not clear. It is now realized that adsorption is not a nonspecific, purely physical phenomenon, but is chemical as well. A number of examples of highly specific adsorption have been observed, such as the adsorption of particular dyes to crystals. Antigen-antibody reactions appear to involve a new kind of chemistry to which the laws of classical chemistry seemingly do not apply. Union of antigen and antibody may ultimately be considered a specific adsorption, promoted and directed by intermolecular forces.

**The Lattice Hypothesis.** The lattice or framework hypothesis utilizes portions of earlier hypotheses. The chemical basis of specificity, originally postulated by Ehrlich, is now universally accepted; likewise, union in variable proportions as proposed by Bordet and the participation of electrolytes in agglutination and precipitation.

The lattice hypothesis is the most widely favored today, particularly in the United States and England. It was described by Marrack in 1938,<sup>24</sup> and modifications were proposed by Heidelberger, Pauling, and various other workers. The basic concept is that an antigen-antibody aggregate

consists of a lattice or framework of alternating antibody molecules and antigen molecules or particles.

Marrack believed that the solubility of antibody globulin is attributable to its polar radicals, and that these are brought into close apposition and attract each other instead of water molecules when antibody combines with antigen (Figure 26 A).<sup>24</sup> This complex precipitates if the remaining free polar groups are insufficient to keep it in solution and if the surface potential is below a critical level. Assuming that both antigen and antibody are multivalent, large complexes (Figure 26 B) may build up through specific links provided by further antigen molecules and thus form a network or lattice.

Pauling supported the lattice hypothesis of Marrack, but saw no need for multivalent antibody except in certain situations involving poly-

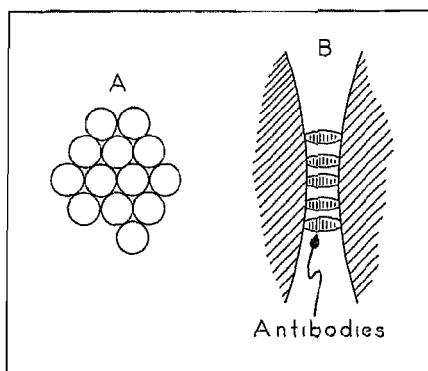


FIGURE 27. Pauling's pictorial representation of (A) agglutinated cells, (B) the region of contact of two cells, showing the postulated structure and mode of action of agglutinin molecules. (From Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.)

haptenic antigens.<sup>28</sup> He postulated that antibody is bivalent and antigen multivalent. Specificity was considered to require a definite bond between antigen and antibody.

The agglutination of cells was represented diagrammatically as in Figure 27,<sup>28</sup> the cells being held together at the regions of "contact" by antibody molecules. The relative sizes of antigen and antibody obviously limit the effective valence of antibody to two. Pauling likewise reasoned that the maximum possible valence of any antigen is determined by the ratio of its surface area to the area effectively occupied by one antibody molecule. It is likely that the actual valence of an antigen is never this high, because only a small proportion of its surface probably consists of determinant or combining sites.

Precipitation under optimal conditions was represented as in Figure

28 A.<sup>28</sup> A network formed with a greater than optimal proportion of antibody was indicated in Figure 28 B, and networks or complexes with less than optimal antibody in Figure 28 C, D and E.

Heidelberger's "quantitative" hypothesis was a modification of the lattice hypothesis based upon postulated chemical interaction of multi-valent antibody.<sup>16</sup> A series of successive bimolecular reactions was pro-

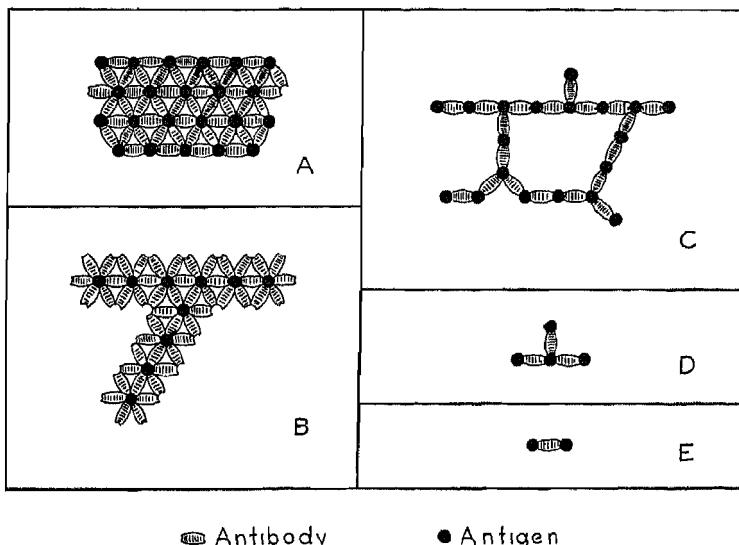


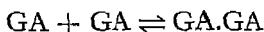
FIGURE 28. Complexes formed with soluble antigen and antibody postulated by Pauling. A, an ideal framework. B, a network formed with antibody excess. C, a network formed with antigen excess. D and E, soluble complexes formed with excess antigen. (Adapted from Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.)

posed, the rates of which are proportional to the concentrations of the reacting substances in accord with the mass law.

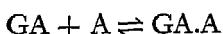
The first step is the reaction between single units of antigen (G) and antibody (A):



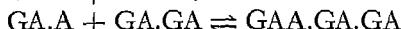
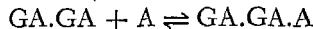
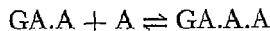
This is followed by a reaction similar to polymerization:



or, in the presence of more antibody, by combination of the primary product with another unit of A:



In the third step the products already formed react according to one or another of the following equations:



These various products continue to combine until particles are formed large enough to settle out as a precipitate. Electrolytes reduce electro-

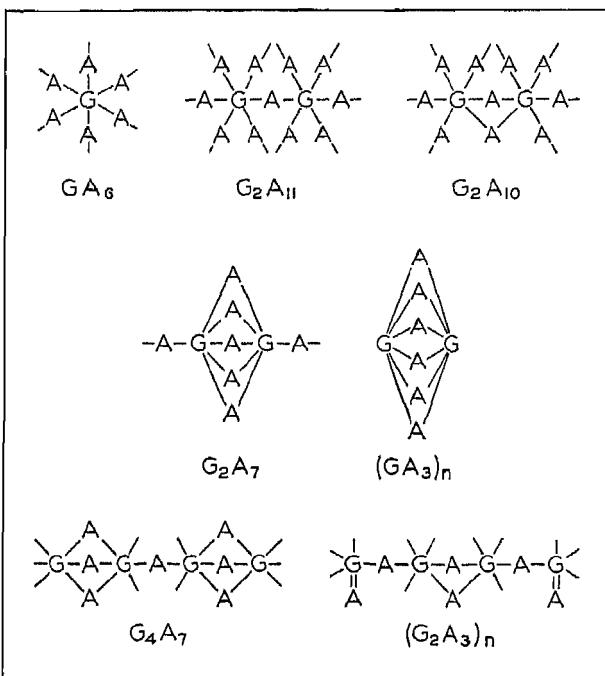


FIGURE 29. Structures postulated by Heidelberger for antigen-antibody complexes of various empirical formulas. (Redrawn from Heidelberger, 1939, Bact. Rev. 3, 49.)

static effects of ionized groups on the particles, which might otherwise interfere with formation of aggregates by chemical interaction.

The foregoing description of reactions was based upon observations with pneumococcal type 3 polysaccharide antigen. Precipitation of this substance by homologous antiserum was reversible in the sense that precipitates formed with excess antibody combined with added antigen and even "dissolved" in concentrated antigen solutions. However, it

was stated that over a large part of the reaction range there was no evidence of dissociation, so "the equilibria postulated evidently lie very far to the right."

Heidelberger diagrammatically represented precipitates by a three dimensional arrangement similar to Marrack's lattice (Figure 29).<sup>15</sup> He considered that specificity applied to the whole process of aggregation and not alone to the initial union between antigen and antibody. Heidelberger's hypothesis was admittedly founded upon several assumptions but provided a workable basis for further experimentation along quantitative lines.

Teorell also postulated a series of equilibrium reactions.<sup>33</sup> However, he assumed that antibody is *univalent* and did not attribute precipitation to lattice formation, but rather to insolubility of one or more of the various antigen-antibody compounds formed.

Boyd agreed that the lattice mechanism satisfactorily explains certain experimental observations. For example, Heidelberger and Kabat found that type 1 pneumococci treated with a large excess of antibody agglutinated in the form of small aggregates which could easily be resuspended.<sup>16</sup> These were washed repeatedly until the supernatant liquid contained no detectable antibody. The sediment was uniformly resuspended in saline and type 1 pneumococci or type 1 pneumococcal polysaccharide was added, whereupon larger clumps formed. Cells or polysaccharide of type 2 did not produce larger clumps. This experiment was interpreted to indicate that the added homologous antigen provided the specific links necessary for lattice formation with aggregates already maximally "coated" with antibody.

Boyd and Hooker, on the other hand, reported that erythrocytes after prolonged exposure to an excess of antibody agglutinated promptly when agitated, yielding a single large mass which could not readily be dispersed.<sup>7</sup> They concluded that the final visible reaction was a nonspecific aggregation of particles coated with antibody and that lattice formation had not occurred.

Boyd therefore proposed his "occlusion" hypothesis which attributed agglutination and precipitation to decreased solubility of the antigen-antibody compound as a consequence of mutual neutralization of antigen and antibody polar groups and "steric hindrance" of other polar groups, which thereby lost access to water.<sup>4</sup>

Acceptance of the lattice hypothesis, although quite general, is by no means universal. So far it has not been possible to devise experiments which unequivocally settle the question whether precipitation requires the formation of aggregates of a certain (large) size or whether precipitation may result simply from loss of affinity for water by small aggregates consisting of one or a few antigen and antibody molecules.

### Summary

The development of ideas regarding the mechanism of antigen-antibody reactions provides an interesting illustration of the increasing complexity that accompanies acquisition of factual knowledge. In fact, proper understanding of present hypotheses requires considerable familiarity with chemistry, physics and mathematics. The original explanation of Ehrlich, that agglutinin and precipitin molecules possess "zymophore" radicals which cause the visible reactions, left much to the imagination of the student but was perfectly intelligible if he did not seek to know what zymophore radicals actually are. Its emphasis upon chemical structure accorded well with present concepts of specificity, which have only been more clearly defined by Pauling and other investigators. The later concept, that particles of multivalent antigen coated with antibody agglutinate or precipitate because of some attraction or lack of repulsion, is also fairly understandable. Increasing knowledge of factors which affect antigen-antibody reactions seemed to necessitate the lattice and occlusion hypotheses with various modifications. It now appears that no single hypothesis may satisfactorily account for all instances of antigen-antibody reaction.

The present situation may be stated as follows:

(1) Agglutination and precipitation consist of rapid union between antigen and antibody, followed by a visible stage which is often slower and is modified by temperature, electrolytes and other factors.

(2) Antibody combines with antigen through surface structures possessing complementary physical configurations and patterns of force.

(3) It is generally agreed that the antigen-antibody complex consists of a network of alternate antigen and antibody molecules. This indicates bivalence or multivalence of one or both reagents, which makes possible union in variable proportions.

(4) The necessity for electrolyte implies that reduction in the number or effectiveness of polar radicals, which normally attract water, plays an important part in the visible reaction. This may consist of mechanical blocking of polar radicals, mutual neutralization, or reduction of charge by electrolytes.

### References

1. Adler and Humphries, 1954. *Jour. Bact.* *67*, 126.
2. Arrhenius, 1907. *Immunochemistry*, The Macmillan Co., New York.
3. Bordet, 1920. *Traité de l'Immunité*. Masson & Cie., Paris.
4. Boyd, 1942. *Jour. Exp. Med.* *75*, 407.
5. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
6. Boyd, Conn, Gregg, Kistiakowsky and Roberts, 1941. *Jour. Biol. Chem.* *139*, 787.
7. Boyd and Hooker, 1938. *Proc. Soc. Exp. Biol.* *39*, 491.
8. Boyden, 1942. *Physiol. Zool.* *15*, 109.

9. Coca and Kelley, 1921. Jour. Immunol. 6, 87.
10. Cromwell, 1923. Jour. Immunol. 7, 461.
11. Dean and Webb, 1926. Jour. Path. Bact. 29, 473.
12. Ehrlich, 1906. *Studies in Immunity*, John Wiley & Sons, New York.
13. Favour, 1944. Bull. N. E. Med. Ctr. 6, 157.
14. Haurowitz, 1936. Ztschr. physiol. Chem. 245, 23.
15. Heidelberger, 1939. Bact. Rev. 3, 49.
16. Heidelberger and Kabat, 1937. Jour. Exp. Med. 65, 885.
17. Heidelberger and Kendall, 1935. Jour. Exp. Med. 61, 563.
18. Heidelberger and Kendall, 1935. Jour. Exp. Med. 62, 697.
19. Heidelberger, Kendall and Teorell, 1936. Jour. Exp. Med. 63, 819.
20. Heidelberger, Treffers and Mayer, 1940. Jour. Exp. Med. 71, 271.
21. Hooker and Boyd, 1932. Jour. Immunol. 23, 465.
22. Hooker and Boyd, 1942. Jour. Immunol. 45, 127.
23. Landsteiner, 1945. *The Specificity of Serological Reactions*, rev. ed., Harvard University Press, Cambridge, Mass.
24. Marrack, 1938. *The Chemistry of Antigens and Antibodies*, H. M. Stationery Office, London.
25. Northrop and DeKruif, 1922. Jour. Gen. Physiol. 4, 639, 655.
26. Osterman and Rettger, 1941. Jour. Bact. 42, 741.
27. Ottensooser, 1923. Kolloidzschr. 33, 176.
28. Pauling, 1940. Jour. Amer. Chem. Soc. 62, 2643.
29. Pauling, 1945. In Landsteiner, *The Specificity of Serological Reactions*, rev. ed., Harvard University Press, Cambridge, Mass.
30. Pauling, 1948. Endeavour 7, No. 26.
31. Schmidt, 1930 Compt. rend. Soc. de Biol. 103, 101.
32. Stuart and Carpenter, 1949. Jour. Immunol. 61, 161.
33. Teorell, 1946. Jour. Hyg. 44, 227, 237.
34. Wilson and Miles, 1946. *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., Edward Arnold & Co., London.
35. Winkler and Westphal, 1944. Ztschr. f. Immunitätsforsch. 105, 154.
36. Zinsser, 1923. *Resistance to Infectious Diseases*, 3rd ed., The Macmillan Co., New York.

## Chapter 7

### PRECIPITATION

THE PRECIPITIN REACTION was first reported by Kraus in 1897.<sup>20</sup> Bacteria-free filtrates of *Vibrio cholerae* and *Salmonella typhosa* mixed with homologous antiserums became cloudy after a short period of incubation; by twenty-four hours finely flocculent precipitates settled to the bottoms of the tubes. The specificity of the reaction was shown by failure of anticholera serum to precipitate the typhoid culture filtrate and *vice versa*. It was not known at that time that the same antibody might be responsible for both precipitation and agglutination, so Kraus applied the name *precipitin* to the antibody reacting in precipitation and called the antigen which elicited its formation *precipitinogen*.

Two years later Tchistovitch, studying the inherent toxicity of eel serum, inoculated this material into rabbits, guinea pigs, dogs and goats.<sup>41</sup> Blood serum from animals surviving the toxic effects of the antigen yielded a copious precipitate when mixed with eel serum. About the same time Bordet reported that milk, injected into rabbits, stimulated the appearance of antibodies which caused flocculation when mixed with milk.<sup>2</sup> Likewise Myers, in 1900, obtained precipitins for egg albumin by intraperitoneal injection into rabbits.<sup>30</sup>

Within a few years precipitating antibodies were produced for a great variety of proteins derived from animals, plants and bacteria, and studies of theoretic, forensic and phylogenetic interest were undertaken.

#### PRODUCTION OF PRECIPITIN

**Precipitin Formation in Man.** Precipitating antibody is often formed in the normal course of an infection. The stimulus is provided by soluble antigenic bacterial constituents liberated by autolysis and other means of disintegration. Antibacterial precipitin tests are not often performed because suitable concentrations of antibody are difficult to obtain.

Precipitating antibody is sometimes found in the serums of individuals

who have had parenteral contact with foreign proteins. Such antibodies have been detected in the blood of patients with serum sickness. Precipitins have also been demonstrated by a highly sensitive technique in the serums of patients hypersensitive to egg, insulin and tuberculin.<sup>7</sup> The concentration of antibody is often low in such conditions, and negative results are not infrequent.

**Precipitin Production in the Laboratory.** Precipitins may be produced against most proteins, some carbohydrates and carbohydrate-lipid complexes. Certain substances like ragweed pollen extract which are usually considered poorly antigenic have been found to induce precipitin formation when injected with an adjuvant mixture containing killed tuberculosis bacteria, paraffin oil and a lanolin-like substance.<sup>21</sup> It has also been reported that nonantigenic carbohydrates such as glycogen, adsorbed onto aluminum hydroxide particles, produce antibodies and that the antigenicity of lipids is greatly enhanced by adsorption onto kaolin or other inert particles.<sup>18,43</sup>

Routine production of precipitins in rabbits requires a series of three or more injections. The dose intravenously is usually one to two milliliters of a 1 per cent protein solution, or two to five milliliters by the intraperitoneal route. Satisfactory precipitin titers against some substances such as egg albumin are obtained only after many injections, although small doses of many antigens suffice to yield detectable antibodies; positive results have been obtained with 0.35 milligram of a synthetic dye-egg albumin antigen. Very small amounts of bacterial extracts induce high titers of precipitating antibody in rabbits: 0.002 to 0.005 milligram per kilogram of body weight.

Precipitin formation parallels the ability of the various animal species to produce antibodies in general and is usually better in rabbits, horses, chickens and man than in guinea pigs, dogs and rats. Wide individual differences within any species are common. Some workers strongly advocate the use of paraffin oil and/or other adjuvants as a means of reducing the number of injections necessary to secure antiserums of high titer.

Precipitins are titrated in trial bleedings before and at intervals during the course of immunization. When the antibody concentration is sufficiently great, the animal may be bled out by cardiac puncture. Larger quantities of serum can be secured by bleeding the animal partially at two or three day intervals. After each bleeding an equal volume of sterile saline is injected intraperitoneally to replace the lost fluid.

#### PRECIPITATION TESTS

The reagents required for precipitation tests are antigen, antibody and saline. All solutions must be perfectly clear because traces of turbidity

or cloudiness obscure the results. Filtration or centrifugation of the reagents is sometimes necessary.

Precipitin tests, in contrast to agglutination tests, are usually set up with serial dilutions of antigen and a constant, high concentration of antibody (either undiluted or diluted approximately 1:5). Apart from the serodiagnosis of syphilis, precipitin tests are rarely employed in laboratory diagnosis. A single precipitin test ordinarily requires one milliliter of antiserum, an amount which suffices for eighty or more agglutination tests.

The amount of precipitate and titer rapidly decrease as antiserum is diluted and antigen is added in constant amount. Zinsser explained the necessity for concentrated antibody in precipitation as contrasted with agglutination tests by means of calculations based upon the particle size and surface area of the antigen, assuming that the antigen is equally "coated" with antibody whether it is of microscopic or of molecular dimensions.<sup>46</sup> He pointed out that the total available surface of a given mass of antigen increases inversely as the cube of the particle diameter when the antigenic material is subdivided into smaller particles. It is obvious therefore that a vastly greater amount of antibody is required for a given amount of antigen in molecular form than for the same amount of antigen in the size of bacterial cells.

The temperature at which precipitation tests are incubated varies according to the nature of the test and the particular antigen-antibody system employed. The rate of precipitation usually increases as the temperature rises to 40° or 45° C., so that qualitative tests are often incubated at 37° C. or slightly higher. More complete precipitation, however, is frequently obtained at 0° to 4° C., and quantitative precipitation tests practically always include an interval of one or more days of refrigeration before chemical analysis. The optimal temperature and time of incubation must be determined for each new antigen-antibody system investigated.

**Procedures. Simple Mixture.** Qualitative or semiquantitative precipitin tests can very easily be made by mixing undiluted or slightly diluted (e.g., 1:3 to 1:5) antiserum with antigen. Sufficient dilutions of antigen are employed to ensure a positive result which might otherwise be missed because of inhibition by too concentrated antigen. It should be reemphasized that diluting the antiserum only slightly causes a disproportional reduction in the titer observed by this procedure. It is therefore important in comparing antisera that the same concentration of antiserum be employed in all tests with the same antigen. Likewise, the same concentration of a given antiserum must be employed in tests against different antigens.

The following results may be obtained after a suitable period of incubation:

(1) Varying degrees of opalescence decreasing from strong to none. In most cases further incubation yields opalescence in only one or more additional dilutions.

(2) Distinct turbidity decreasing in density from the first tube to a negative tube. On continued incubation an amorphous precipitate settles to the bottom of the tube and can be observed in the supernatant fluid after shaking.

(3) Precipitates which cannot be distinguished from clumps of agglutinated bacteria. These are very easy to read (see Chapter 8).

It is difficult to grade the reactions as the dilution of antigen increases because, although the difference between the strongest and weakest positive tubes is extremely marked, gradations between successive tubes are often barely perceptible. Moreover, prozones may also occur. Only after the student has set up a considerable number of these tests can he work out a satisfactory system for recording results.

*Interfacial or Ring Test.* The interfacial or ring test was introduced by Ascoli in 1902 and is one of the most sensitive modifications of the precipitin reaction.<sup>1</sup> Very small test tubes or capillary tubes are usually employed to conserve antiserum, and the reagents are added in such a manner that mixing does not occur; antiserum usually constitutes the bottom layer and antigen the top layer. With large enough test tubes the antiserum may be allowed to run down the wall of the tube and flow under the antigen, or it may be added with a capillary pipette inserted to the bottom of the tube beneath the antigen. If small test tubes (e.g., 3 mm. inside diameter) are employed, the antigen may be added slowly with a capillary pipette as a layer above the antiserum. This method often gives sharper results but is more time consuming and tedious.

Tests are incubated either at room temperature or at 37° C. for periods up to four hours. Formation of a visible ring or layer of precipitate at the interface between antiserum and antigen may occur within a few minutes, depending upon the antibody content of the serum and the concentration of antigen. The endpoint or titer is represented by the highest antigen dilution giving a positive result. This method does not suffer greatly from inhibition by antigen excess because diffusion at the interface provides a zone of nearly optimal proportions in which precipitation occurs. A positive result may be obtained with antigens diluted one million times or more. This procedure is used to detect specific antigens but gives little information regarding the antibody content of an antiserum. It is commonly employed to identify proteins, as in the forensic determination of blood stains.

Additional information regarding the precipitating antibody content of an antiserum may be obtained by mixing the layers in a ring test after preliminary reading and continuing incubation for a period of

hours or days. Secondary incubation is carried out at refrigerator or room temperature or even higher. Overnight incubation at 55° C. has been found satisfactory with somatic bacterial antigens.

*Oudin's Agar Column Test.* Formation of a sharp interface in the ring test is facilitated by mixing the bottom reagent with gelatin or agar and allowing it to solidify before the upper layer is added. Oudin reported in 1946 that when antiserum is gelled with agar and overlaid with a single, pure antigen, the zone of precipitate which forms migrates downward into the serum-agar, the distance of migration being inversely related to the square of the time elapsed.<sup>38</sup> Two or more unrelated antigens tested in the same tube against a mixture of their homologous antibodies yielded a corresponding number of zones of precipitate, usually migrating at different rates.

Oudin's observation provided a method for determining the *minimum* number of antigenic substances in a mixture such as blood plasma or cell extract and for detecting the presence of antigenic impurities in protein solutions. Cooper employed this technique to study the proteins of frog embryos and adult frog serum.<sup>9,10</sup> She obtained evidence that there were at least six to eight antigens in saline extracts of frogs' eggs and in the neural plate and neural fold embryonic stages; adult frog serum contained a minimum of six or seven antigens. Cross-precipitation tests in agar columns indicated that at least five of the egg and neural plate antigens were also present in adult serum. This technique obviously offers considerable possibility for use in the serologic study of development and differentiation in higher forms of life, and in the investigation of phylogenetic relationships.

The Oudin technique has recently been used to study the homogeneity of diphtheria toxins, toxoids and antitoxins. Pope found that highly purified toxin produced several zones of precipitate with equine antitoxin, which indicated a corresponding number of antigen-antibody systems.<sup>37</sup> Bowen reported that an experimental lot of diphtheria toxoid purified by precipitation with cadmium chloride and ammonium sulfate contained one major antigenic fraction and at least six minor fractions.<sup>3</sup> Seventeen horse antitoxins were tested with six toxoids of various degrees of purity, and the presence of as many as fifteen different antigenic components was shown. Antitoxic serums all possessed a major antibody component, presumably antitoxin, and usually three or more minor antibody fractions. The number of minor antibodies was generally greater after long-term immunization than after short immunization. Despite the fact that toxins and toxoids previously purified by Eaton, Pappenheimer and Pillemser appeared to consist of pure protein according to their electrophoretic and solubility properties, the observations of Pope and Bowen suggest that immunologic purity of these materials has not yet been achieved.

*Dean and Webb Optimal Proportions Determination.* It was pointed out previously that the Dean and Webb titration can be used to determine the concentration of a specifically reacting antigen in an unknown solution. The antiserum is first titrated roughly against widely spaced dilutions of antigen as illustrated in Table 25 (page 100). The endpoint is represented by that tube in which the minimum flocculation time is noted. On the basis of this preliminary titration a "fine" test is performed in which the antigen dilutions are closely spaced about the preliminary endpoint. Taylor, Adair and Adair employed this procedure to determine the amounts of egg albumin in egg white and of globulin in horse serum.<sup>40</sup> Egg white contained 7.29 per cent egg albumin, which compared favorably with the average percentage of 7.25 previously determined by chemical means. They found 4.46 per cent globulin in horse serum, whereas chemical determinations indicated 4.32 per cent. It is obvious that the accuracy of such determinations by the Dean and Webb and other methods is dependent upon the purity of the antigen used to prepare the antiserum, because traces of contaminating antigen may induce formation of considerable quantities of other antibodies.

Culbertson suggested a modification of the Dean and Webb titration which has been called the "neutralization" method.<sup>11</sup> Mixtures containing constant amounts of antiserum and varying amounts of antigen are incubated until precipitates form. The supernatant liquid from each tube is divided into two aliquots, which are tested for uncombined antigen or antibody by adding antiserum or antigen. Absence of antigen and antibody in the supernatant liquid indicates complete precipitation of both substances, and occurs at the neutral point.

Martin proposed an antiserum dilution method of determining precipitin titers.<sup>26</sup> The principle is well illustrated by his experiment with rabbit pneumococcal type 1 antiserum and homologous polysaccharide (Table 31). The amount of antigen to employ in the final titration is determined with mixtures of antigen dilutions and any convenient constant dilution of antiserum such as 1:25. The smallest amount of antigen yielding a precipitate is selected for the subsequent titration of precipitin. This amount of antigen (0.25 microgram in Table 31) is then mixed with each of a series of dilutions of antiserum. The greatest antiserum dilution yielding a visible precipitate represents the precipitin titer. It is evident that the success of this method depends upon several factors, including time, temperature, volume, the nature of the antigen and the ability of the observer to detect small amounts of precipitate.

With the exception of the ring test, most of the foregoing procedures are not widely applied practically at present, although they are of considerable theoretic interest.

*Agglutination of Antigen-Coated Inert Particles.* Agglutination of inert particles to which soluble antigen has been adsorbed provides a

serum dilution method of titrating precipitin and also demonstrates the similarity between precipitation and agglutination. Collodion particles or cells unrelated to the antigen (e.g., bacteria or erythrocytes) are coated with the antigen and employed in constant amount in an agglutination test against serial dilutions of antiserum.

Cannon and Marshall prepared collodion particles by adding a water-acetone mixture in a fine stream to a constantly stirred solution of collodion in acetone.<sup>7</sup> The particles were freed from acetone by washing with distilled water. Differential centrifugation yielded particles between one and five microns in diameter. These were mixed with the antigen

*Table 31. Titration of a Rabbit Antipneumococcal Type I Antiserum with Homologous Polysaccharide Antigen*

ANTISERUM DILUTION	ANTIGEN CONCENTRATION ( $\mu$ G. PER ML.)										
	128	64	32	16	8	4	2	1	0.5	0.25	0.125
1:25	+	+	+	+	+	+	+	+	+	+	-
1:50	+	+	+	+	+	+	+	+	+	+	-
1:100	+	+	+	+	+	+	+	+	+	+	-
1:200	+	+	+	+	+	+	+	+	+	+	-
1:400	-	+	+	+	+	+	+	+	+	+	-
1:800	-	-	+	+	+	+	+	+	+	+	-
1:1600	-	-	-	-	+	+	+	+	+	+	-
1:3200	-	-	-	-	-	-	-	+	+	+	-
1:6400	-	-	-	-	-	-	-	-	-	+	-
1:12,800	-	-	-	-	-	-	-	-	-	-	-

+= precipitate

Boldface indicates tubes in which neither antigen nor antibody was detected in supernatant fluid.

(From Martin,<sup>26</sup> by permission.)

solution to permit adsorption and finally washed several times to remove unadsorbed material. Coated particles diluted to proper turbidity were then employed as test antigen in the agglutination reaction. Cannon and Marshall reported that this procedure was very sensitive. They obtained positive results with serums from some patients hypersensitive to egg, insulin and tuberculin by use of corresponding test antigens. Titers of 60 to 480 were obtained with rabbit anti-egg albumin serums which failed to precipitate by the antigen dilution method. Other investigators have successfully employed this technique in the study of antibodies against tissue and bacterial extracts.

Bozicevich recently used bentonite, a colloidal hydrated aluminum silicate, as an inert particulate carrier to adsorb a soluble antigen.<sup>8</sup> The particles were treated with an extract of trichina, a parasitic nematode worm, and with a dye (thionin) to facilitate reading flocculation. Tests were performed on large microscope slides. A drop of antigen was

added to 0.1 milliliter of serum dilution, the slide was rocked and tilted mechanically for fifteen minutes, and flocculation was read with the low power of the microscope. This test was used to detect antibody in the serums of patients with trichinosis. The flocculation reaction was as sensitive as the complement fixation test and apparently gave no falsely positive reactions.

Cells unrelated to the soluble antigen may be substituted for collodion or bentonite particles, the preparation of which requires an undue amount of time if only a few tests are to be performed. *Serratia marcescens* has been successfully used in this manner with tuberculin and various other antigens. The heat-killed bacterial cells are coated with the antigenic material by incubation at 37° C. and washed repeatedly to remove unadsorbed antigen. Constant amounts of the treated bacteria are mixed with serial dilutions of antiserum as in agglutination tests. Muether and MacDonald obtained titers of 64 to 1024 in tests of the serums of thirteen patients with active tuberculosis against *S. marcescens* coated with "old tuberculin."<sup>20</sup> Serums from eight other tuberculin positive individuals yielded titers of 16 to 64, whereas serums from seven out of eight tuberculin negative individuals failed to agglutinate the tuberculin-coated bacteria.

Erythrocytes coated with extracts of bacterial cells also provide a sensitive reagent for detecting and titrating the corresponding antibodies. Vi antibodies in the serums of normal and immunized humans have been studied by this means. Washed human group O or sheep erythrocytes are incubated at 37° C. with appropriate extracts of Vi-containing bacterial cells, washed thoroughly and employed as test antigen. Landy and Webster reported titers as high as 240 in humans two weeks after immunization with forty micrograms of Vi antigen.<sup>25</sup> A titer of 10,240 was obtained with antiserum against *S. ballerup*. It should be noted that the specificity of agglutination of coated bacterial cells or erythrocytes must be checked by appropriate controls lest the test serums contain antibody against the uncoated cells.

*Quantitative Precipitin Tests.* The quantitative precipitin test described by Heidelberger and his associates permits precise determination of the amount of antibody in an antiserum. A typical set of results was presented in Table 27 (page 102) and is illustrated graphically in Figure 30. Antibody nitrogen precipitated was calculated by subtracting the antigen nitrogen precipitated from the total nitrogen precipitated. It is apparent that the antiserum contained about 0.75 milligram of antibody nitrogen capable of reacting specifically with the homologous antigen. This figure represents the highest point of the antibody nitrogen curve.

The quantitative technique has been widely employed in recent studies of the nature of antigen-antibody reactions. Certain antisera produced in the horse, for example, yield a different type of reaction curve from

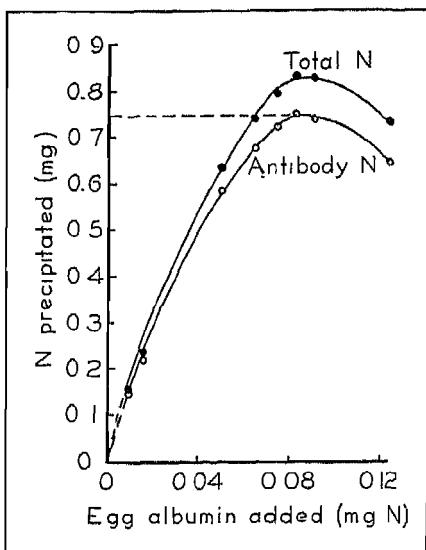


FIGURE 30. Total nitrogen and antibody nitrogen precipitated from 1.0 ml. of a rabbit anti-egg albumin serum by various amounts of egg albumin, illustrating the method of determining the quantity of homologous antibody in an antiserum. (Plotted from the data of Table 27.)

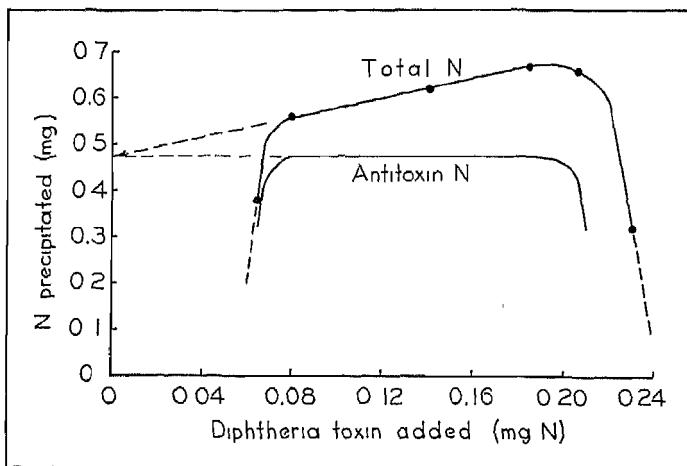


FIGURE 31. Total nitrogen and antitoxin nitrogen precipitated from 1.0 ml. of a diphtherial horse antitoxin, showing complete inhibition of flocculation outside the equivalence zone. (Plotted from data in Pappenheimer and Robinson, 1937. *Jour. Immunol.* 32, 291.)

that illustrated in Figure 30, which was obtained with rabbit antiserum. The reaction between diphtheria toxin and homologous horse antitoxin is shown in Figure 31.<sup>36</sup> Flocculation occurs only within a certain range of toxin concentrations and is completely inhibited both below and above this zone. This reaction is referred to by some authors as the "flocculation reaction" in contrast to the "precipitin reaction," typified by the behavior of rabbit antiserum against egg albumin. The precipitin reaction is characterized by a narrow equivalence zone and by gradually decreasing precipitates on either side of equivalence. The antibody content of flocculating antiserum is indicated by extrapolation of the straight portion of the curve to the vertical axis. Throughout most of the zone of flocculation all the antigen and antibody are precipitated, neither appearing in the supernatant liquid. Flocculating antibodies are observed only in antiserums derived from the horse, but not all horse antiserums are of this type. Flocculating antibodies have been described for diphtheria, tetanus, scarlet fever and botulinum toxins, chicken ovalbumin and conalbumin, human and rabbit serum albumins, and hemocyanin.

The quantitative precipitin technique is useful for the determination of specifically reacting substances for which no other analytic method is available. One advantage of this procedure is that the substance does not have to be in pure form but may be mixed with other components if the antiserum contains no antibodies capable of reacting with the heterologous substance. Globulin and albumin in normal and pathologic serums, lymph, edema and ascitic fluids have been measured quantitatively by means of homologous antiserums. Blood group polysaccharides in a variety of secretions have also been estimated by specific precipitation.

#### APPLICATIONS OF THE PRECIPITIN REACTION

Antibody can be detected in the serums of individuals with certain diseases by qualitative precipitin tests, and in some cases its amount can be determined by the quantitative test. The precipitin reaction is also used to detect specific proteins and to study the antigenic composition of bacteria and the phylogenetic relationships of plants and animals.

**Diagnostic Precipitin Tests.** The presence of plague or anthrax bacteria in animal tissue can be indicated by precipitin tests. Plague, for example, is primarily a disease of rats, and during epizootic periods considerable numbers of dead rats may be found. Isolation of the plague organism from carcasses is difficult, but the plague antigen may be detected, even in partly decomposed tissues, by the "thermoprecipitin" test. Organs or tissues are ground, the finely divided tissue boiled with five to ten volumes of distilled water and filtered. This constitutes the antigen solution. It is used in a ring test with known antiplague serum. Positive results demonstrate the presence of plague antigen.

Pure cultures of certain bacteria are sometimes identified or typed by precipitin tests. Diagnostic agglutination tests with hemolytic streptococci are of little value because of marked overlapping of antigens within the group and a strong tendency to spontaneous agglutination. Lancefield, however, found that strains isolated from the same source or disease condition could be grouped together by the precipitin test.<sup>22,23,24</sup> The organisms were extracted with dilute hydrochloric acid (pH 2.0 to 2.4) at 100° C., neutralized and centrifuged; the clear supernatant liquid constituted the test antigen. Eleven groups of hemolytic streptococci have been established; the majority of strains from human disease are in Group A. An unknown streptococcus is typed by testing its extract against antiseraums of the various groups. The ring test is usually employed, and tubes of narrow bore conserve serum.

The Neufeld "Quellungreaktion" may be considered a precipitin test in which the antigen is a specific polysaccharide in the bacterial capsule; the latter increases strikingly in size as a result of union with antibody.<sup>81</sup> This phenomenon occurs quickly and is observable with the microscope. It provides the easiest and most rapid method of determining the type of a capsulated pneumococcus, either in a clinical specimen such as sputum or in a young culture grown in suitable medium. A loopful of sputum or culture is mixed with an equal amount of antiserum on a slide, usually with the addition of methylene blue to facilitate observation of the capsules. In homologous antiserum the capsules become markedly swollen within a few moments. Capsules of type 3 organisms swell more than those of most others because this type produces polysaccharide in greatest abundance. Similar procedures are applicable in the identification or typing of certain streptococci, *Klebsiella pneumoniae*, *Hemophilus influenzae* and *Neisseria intracellularis*.

Several modifications of the precipitation test are employed in the laboratory diagnosis of syphilis. Blood serum from the patient is examined for the presence of an antibody-like substance or "reagin" which apparently is produced in response to the infection. Reagin appears within a few weeks after infection and persists in varying concentration throughout the course of the disease, usually disappearing when symptoms disappear.

The syphilis test antigen is a lipid extract of beef heart. Antigens first employed were aqueous or alcoholic extracts of syphilitic tissues, but it was subsequently found that similar extracts of normal tissues possessed equal or better reactivity. The antigen for the Kahn precipitation test is prepared from beef heart powder washed with ether and then extracted with alcohol. The lipid extract is treated with cholesterol, which enhances the sensitivity of the product, and diluted with saline for use in the test. The sensitivity of the antigen must be carefully

regulated because supersensitive preparations may give falsely positive reactions with the serums of normal individuals.

The Kahn test is performed with varying amounts of antigen and a constant amount of patient's serum which has been heated at 56° C. for thirty minutes.<sup>17</sup> The tubes are shaken vigorously for three minutes, after which saline is added to disperse the floccules and the degree of participation is read.

Other precipitation or flocculation tests for syphilis employ antigens prepared by different methods, together with different procedures for setting up, incubating, and reading the reactions. Both test tube and macroscopic and microscopic slide tests are in common use.

Serodiagnostic tests for syphilis are of interest because lipids are essential ingredients of the test antigens. The specifically reactive material was recently isolated by Pangborn and named *cardiolipin*.<sup>34</sup> This substance is now replacing previous lipid extracts in certain antigens for the diagnosis of syphilis. Cardiolipin is a complex phosphatidic acid containing linoleic and oleic acids and a polyester of glycerophosphoric acid and glycerol. The sodium salt appears to have a molecular weight of 2195.<sup>35</sup>

The fact that supersensitive antigens react with the serums of uninfected individuals indicates that reagin is not produced solely in response to syphilitic infection but may be present in normal persons. Moreover, in certain other diseases, such as malaria, leprosy and infectious mononucleosis, reagin is temporarily increased to such an extent that positive tests for syphilis are often obtained. In syphilis itself the reagin response is marked and remains at a high level until all symptoms of the disease have disappeared.

Kahn believes that reagin is an antibody against tissue lipids.<sup>18</sup> Lipids are presumed to be liberated from body tissue in the course of normal wear and tear. These, perhaps with the assistance of some "schlepper" agent in the individual serum, induce the formation of antibodies (reagins) within the same individual. These antibodies are normally low in concentration but may become increased in certain disease conditions as a result of increased tissue breakdown. In the final analysis the test for syphilis is quantitative rather than qualitative.

**Forensic Precipitin Tests.** One of the most dramatic applications of the precipitin test is the forensic identification of blood stains. It is often necessary, as in murder cases, to determine the species origin of blood dried on cloth, paper, a knife or some other material. The blood need not be fresh but must be thoroughly dried or otherwise well preserved. Positive results have been obtained with blood spots after many years, and Egyptian mummy tissue 5000 years old gave a detectable reaction.

Antiseraums should be highly potent, sterile and crystal clear. Anti-human serums of high titer can be prepared in rabbits by immunizing with human blood, plasma or serum. Schiff and Boyd recommended a series of intravenous one to two milliliter doses containing approximately 1 per cent protein, or three to six milliliter intraperitoneal doses.<sup>39</sup> If a rabbit is injected too frequently or with too great an amount of protein, or is bled too soon after the last injection, the antiserum will contain both human serum and anti-human precipitins and will be unsuitable for forensic work. Such a serum will react as an antigen against *properly* prepared antiserum and as an antibody against human serum. A forensic antiserum should give a positive ring test within twenty minutes at room temperature with a 1:20,000 dilution of homologous blood.

The stain is extracted with an amount of saline which varies with the size of the stain. The extract is clarified by filtration or centrifugation. If the stain is very small the solution will contain only a small amount of protein. Extracts representing a serum dilution of about 1:1000 develop a stable foam upon shaking. If only this amount of material is available, the test must be done in this dilution. A more concentrated extract is diluted serially and tested with constant antiserum as in the regular precipitin test. The presence of blood in the stain should be proven by the benzidine, hemin crystal or other suitable test, because stains of paint and other materials are sometimes indistinguishable from blood stains.

The ring test is always employed, antiserum constituting the lower layer and blood stain extract the upper layer. A positive result is apparent within five to twenty minutes at room temperature if the antiserum is of suitable potency.

In view of the importance of the forensic test, numerous controls are necessary to prove the specificity and reactivity of the antiserum. (1) Blood stain extract tested with normal rabbit serum must give a negative reaction. (2) Saline and antiserum must produce no precipitate. (3) An extract of an unstained portion of the material must not react with the antiserum. As an additional control, the extract from unstained material should not inhibit reaction between the antiserum and homologous blood. (4) Bloods from several species other than those involved in the test must be negative to confirm the specificity of the antiserum. (5) Several different specimens of known blood homologous to the antiserum must all react positively in the same test. For example, bloods from a number of humans must be shown to react with an anti-human test serum.

The forensic precipitin test is most widely known for its use in identification of human blood, but it has been employed for other purposes such as determination of the hosts of blood sucking insects. Suitably modified, the procedure is also used to identify the animal origin of semen, bones, milk, meat and other tissues or fluids.

"Cocto-antiseraums" are often used in the identification of meats and fish products. These antiseraums are prepared by immunizing rabbits with heated ( $70^{\circ}$  C. for one hour) serums of the animals in question or with heated tissue extracts. Cocto-antiseraums precipitate extracts of meat from the homologous animal sources, whether the meat is raw or cooked. Highly potent, specific antiseraums are required. They should give positive results with the homologous antigen diluted 1:10,000 to 1:100,000. Antiseraums which cross react with other meats are preferably discarded but are sometimes made specific by adsorption with small amounts of the cross reacting antigen and subsequent removal of the precipitate. The meat specimen to be examined is homogenized with saline in a Waring blender and filtered clear. Ring tests with the appropriate antiseraums and proper controls quickly indicate species sources of the meat or meats composing the specimen. This test is frequently used to detect horse meat in sausage, hamburger or other processed meat products.

**Phylogenetic Applications of the Precipitin Reaction.** Use of the precipitin test in the study of animal relationships was initiated shortly after discovery of the reaction itself. Bordet in 1899 reported that the serum of a rabbit injected with fowl serum precipitated fowl and pigeon serums.<sup>2</sup> The following year Myers found that antiserum against hen egg albumin reacted with duck egg albumin as well as with the homologous antigen.<sup>30</sup> He also found that antibodies against sheep globulin precipitated beef globulin less strongly than sheep globulin; conversely, antibeef globulin reacted less strongly with sheep globulin than with the homologous antigen. Several investigators promptly reported that anti-human rabbit serums reacted only with the bloods of primates. These observations provided the basis for the forensic precipitin test for identification of blood stains.

Early investigators employed simple mixtures of antiserum and antigen and reported the development of cloudiness or a definite precipitate after varying periods of incubation. Usually a constant amount of antiserum, such as 0.1 milliliter, was mixed with 0.5 milliliter of one or more dilutions of antigen. Nuttall included in some of his observations a record of the time required for visible reaction and also developed a "quantitative" procedure in which the volume of precipitate was measured in a capillary tube.<sup>32</sup> The ring test was subsequently employed in studies of animal and plant relationships.

Although the inhibition of precipitation in simple mixtures by excess antigen was known at that time, many precipitin tests were performed in which a single dilution of antigen was employed. It is therefore surprising that the results of early phylogenetic studies correlated as well as they did with the systematic position of the test materials when classified on morphologic and other grounds.

In 1901 Uhlenhuth reported that rabbit antiserums against human blood yielded precipitates only when mixed with human serum, not with the serums of eighteen lower animals.<sup>42</sup> Wassermann and Schütze reported similar experiments and noted that their anti-human serum precipitated

*Table 32. Precipitation of Primate Serums by Anti-human Rabbit Serum*

PRIMATE SERUMS	NUMBER TESTED	PER CENT POSITIVE TESTS
<i>Anthropoidea</i>		
Man	34	100
Simiidae (ourang-outang, chimpanzee, gorilla)	8	100
Cercopithecidae (Old World monkeys, 26 species)	36	92
Cebidae (New World monkeys, 9 species)	13	77
Hapalidae (Marmosets, 3 species)	4	50
<i>Lemuroidea</i>		
Lemuridae (Lemurs, 2 species)	2	0

(From Nuttall,<sup>32</sup> by permission.)

*Table 33. Relative Amounts of Precipitate Obtained with Anti-human Rabbit Serum and Equal Amounts of Serum of Various Anthropoids and Monkeys*

SERUM	PRECIPITATE
Human	100%
Chimpanzee	130%*
Gorilla	64%
Ourang-outang	42%
Cynocephalus mormon	42%
Cynocephalus sphinx	29%
Atcles (spider monkey)	29%

\* Loose precipitate

(From Nuttall,<sup>32</sup> by permission.)

with the serum of a baboon, although this reaction was slower and less intense than that with human serum. The same year Nuttall found that the serums of Old World monkeys reacted markedly with anti-human rabbit serum whereas serums of South American monkeys reacted only slightly.

Nuttall published a monograph in 1904 in which he tabulated and summarized the results of 16,000 precipitin tests.<sup>32</sup> He employed the

*Table 34. Precipitation of Animal Serums by Rabbit Antisera*  
*(Antisera prepared by immunizing with respective animal serums)*

ANTIGENS (serums)	NUMBERS OF ANTIGENS TESTED	RABBIT ANTISERUMS					
		Primate (man)	Insectivore (hedgehog)	Carnivore (dog)	Ungulate (ox)	Marsupial (wallaby)	Bird (fowl)
Primates	64-97	90	2	21	11	11	0
Insectivores	8-15	13	47	14	0	0	0
Carnivores	76-97	27	5	46	4	5	1
Ungulates	57-70	43	7	3	72	5	0
Marsupials	21-26	4	0	4	0	68	0
Birds	262-322	0.3		0.3	0.6	0	88
Reptiles	34-51	0		0	0	2	0
							18

*(From Nutall, 2, by permission.)*

blood serums of man and various animals as antigens and thirty rabbit antiserums against serum proteins of the various groups of animals. Results obtained in mixtures of primate serums and anti-human serum are presented in Table 32. Serums of ourang-outang, chimpanzee, gorilla and Old World monkeys were more closely related serologically to human serum than were those of New World monkeys and marmosets. Lemurs appeared to be quite unrelated to man. These results closely follow conclusions drawn from morphologic evidence. Precipitin tests by Nuttall's quantitative method gave similar results (Table 33). The relationships were confirmed by tests with anti-chimpanzee, anti-ourang-outang and anti-monkey serums.

Nuttall also studied mammalian, bird and reptile serums (Table 34). Antiserums against the various mammalian species gave the greatest percentage of positive precipitin reactions when tested with antigens derived from the same or other species of the same order. Thus, anti-ox serum yielded positive results with 72 per cent of the ungulate antigens, and anti-wallaby serum reacted positively with 68 per cent of the marsupial antigens. Birds constituted a distinct group with practically no evidence of relationship to the other animals tested. Reptiles were heterogeneous among themselves and cross reacted with only one other antiserum. It may be significant that the cross reaction occurred in the fowl group, of which reptiles were presumably the evolutionary ancestors.

Numerous interesting relationships have been shown by means of the precipitin test. As long ago as 1905 Friedenthal secured weak reactions between blood serum of the frozen Siberian mammoth and antiserum against Indian elephant.<sup>14</sup> Boyden and Noble in 1933 helped to clarify the relationships between certain amphibia.<sup>5</sup> They found that Siren, Necturus and Amphiuma are closely related to one another but are only distantly related to the primitive form *Cryptobranchus*. These relationships were represented graphically in a three dimensional model (Figure 32).<sup>4</sup> Boyden later employed a photoelectric method of measuring precipitation to demonstrate serologically that the mule, a hybrid of the horse and ass, possesses serum proteins found in the parent animals. Boyden has for many years been collecting blood and other protein specimens from all available sources. These are used for comparative serologic study and application to problems of physiology, systematics and evolution.

DeFalco showed that birds constitute a serologically separate group with respect to their blood, egg and lens proteins.<sup>15</sup> Cumley, Irwin and Cole studied serum antigens of *Streptopelia risoria* (Ring Dove) and *St. senegalensis* (Senegal) and their hybrids and backcross hybrids.<sup>12</sup> Senegal and Ring Dove shared major antigenic serum components and in addition Senegal possessed a small "species-specific" residue, probably consisting of at least four factors. These four species-specific factors may appear in

the hybrids but when the hybrids are backcrossed with Ring Dove, which lacks these factors, one or more of the specific Senegal antigens may be lost. Tests with appropriately adsorbed antiseraums indicated the genic control of these serum antigens. Irwin and his co-workers also demonstrated genic control of serum antigens in other birds.

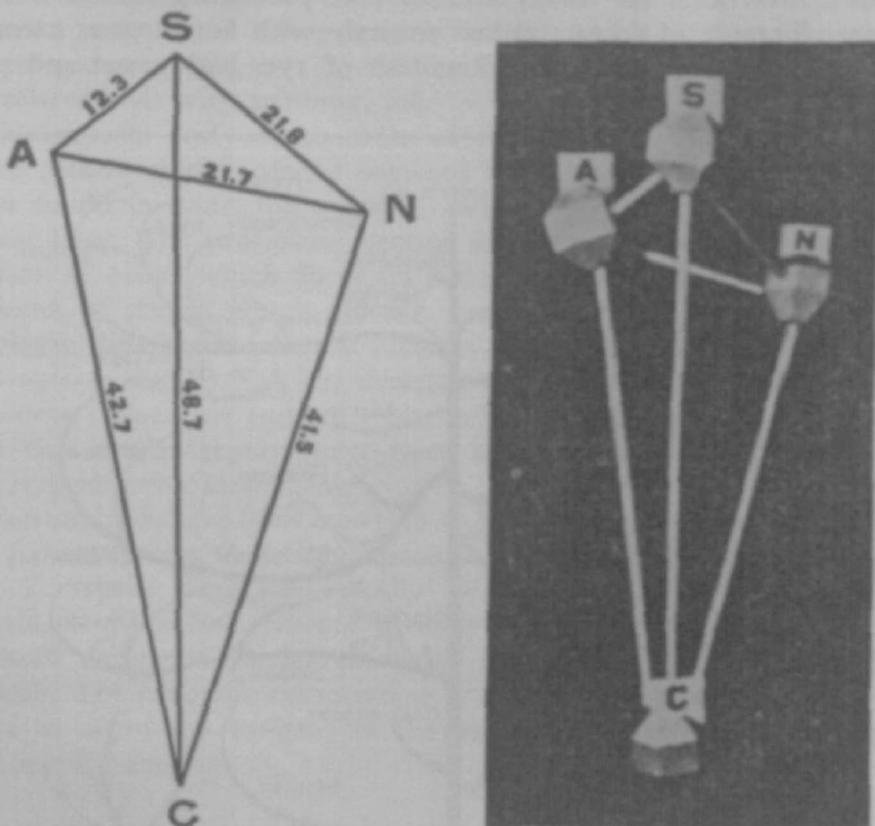


FIGURE 32. Diagram and three-dimensional model showing the interrelationships between *Cryptobranchus* (*C*), *Amphiuma* (*A*), *Siren* (*S*) and *Necturus* (*N*), based on ring tests with standard antigen dilutions. (From Boyden, 1942. *Physiol. Zool.* 15, 109.)

Moody and associates obtained evidence from the serologic behavior of rodent blood proteins which substantiated the morphologic classification of these animals.<sup>25</sup> They also found that the serum proteins of rabbits differ markedly from those of rodents, thus confirming the systematic position of rabbits in a separate order, Lagomorpha.

The precipitin reaction has also been applied to the study of plant materials.<sup>8</sup> Antiserums are prepared by immunizing rabbits with solutions of dried, macerated plant tissues in saline, water, buffer or weak alkali. Serologic work with plant materials is hampered by the presence of nonspecific precipitating substances such as organic acids, tannins, alkaloids and glucosides. Many of these substances can be removed by pre-

extraction of plant tissues with ether, alcohol, benzene, acetone or other solvents. Such treatment is, of course, subject to the objection that it may also remove specific antigenic materials. Nevertheless, valuable information has been secured by use of extracted tissues.

An early observation of the serologic nature of plant proteins was that of Kowarski.<sup>19</sup> He found in 1901 that precipitins against a heat resistant albumose of wheat reacted strongly with homologous extracts but weakly or not at all with albumoses of rye, barley, oat and pea.

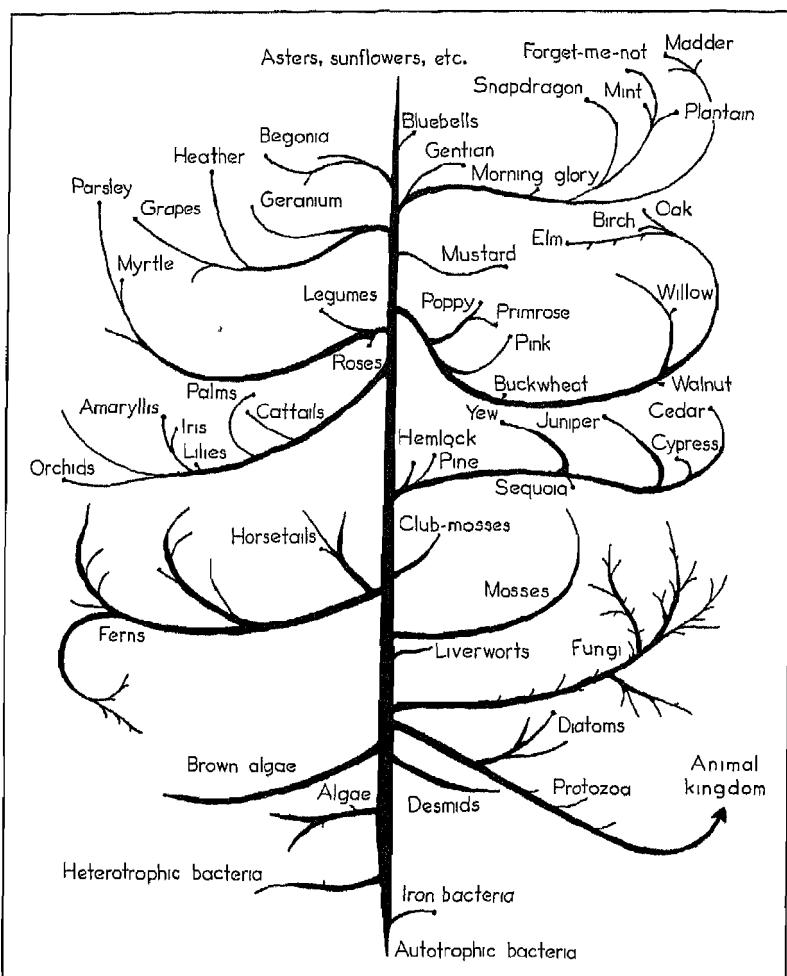


FIGURE 33. Excerpts from the Königsberg "Stammbaum" showing phylogenetic relations of plant species and genera as indicated by serologic reactions. (Modified and redrawn from Goertner, Outlines of Biochemistry, 1929, John Wiley & Sons, New York.)

In 1914 Zade employed the precipitin test to construct a genealogic scheme showing the relationships between species and varieties within the genera *Avena* (oats) and *Triticum* (wheat).<sup>44</sup> The relationships found agreed well with existing ideas of the classification of these plants based upon their morphology.

Mez and Ziegenspeck in 1926 summarized work of the preceding thirteen years of the Königsberg school in a serologic "Stammbaum" or genealogic tree (Figure 33).<sup>27</sup> The serologic relationships discovered correlated well with anatomy, morphology, cytology and paleontology.

Constituents and contaminants of flours, meals, fodders, drugs and other plant materials can be detected by the precipitin test.<sup>8</sup> Ergot has been found in flour, for example, and maize, rye, barley or potato in wheat flour. The precipitin reaction is also used in the serologic comparison of plants which do or do not graft readily. Rives reported that varieties of grapes which did not graft successfully possessed marked serologic differences whereas varieties which grafted successfully were serologically similar.<sup>38</sup> A like situation was found by Green in studies of Rosaceae, Solanaceae and other plants.<sup>16</sup> It is noteworthy in this connection that similar reports have been made regarding cross-fertilization and hybridization in animals.

Plant hybrids have been shown to be serologically intermediate between the parent species. Zade demonstrated in 1914 that *Trifolium pratense* and *T. repens* were serologically related but distinct and that their hybrid, *T. hybridum*, reacted strongly with antiseraums against extracts of both parents.<sup>44</sup> Similar situations have been found in other plant hybrids. The antigenic composition of a plant hybrid appears to include some or all of the antigens of the parents without the appearance of new specific antigens.

#### References

1. Ascoli, 1902. Münch. med. Wschr. 49, 1409.
2. Bordet, 1899. Ann. Inst. Pasteur 13, 225.
3. Bowen, 1952. Jour. Immunol. 68, 429.
4. Boyden, 1942. Physiol. Zoöl. 15, 109.
5. Boyden and Noble, 1933. Amer. Mus. Novitates 606, 1.
6. Bozicevich, Tobie, Thomas, Hoyem and Ward, 1951. Pub. Health Rep. 66, 806.
7. Cannon and Marshall, 1940. Jour. Immunol. 38, 365.
8. Chester, 1937. Quart. Rev. Biol. 12, 19, 165, 294.
9. Cooper, 1948. Jour. Exp. Zool. 107, 397.
10. Cooper, 1950. Jour. Exp. Zool. 114, 403.
11. Culbertson, 1932. Jour. Immunol. 23, 439.
12. Cumley, Irwin and Cole, 1943. Jour. Immunol. 47, 35.
13. DeFalco, 1942. Biol. Bull. 83, 205.
14. Friedenthal, 1905. Arch. Physiol. 1.
15. Gonzalez and Arnangué, 1931. Compt. rend. Soc. de Biol. 106, 1006.
16. Green, 1926. Genetics 11, 73
17. Kahn, 1928. *The Kahn Test, a Practical Guide*, Williams and Wilkins Co., Baltimore.

18. Kahn, 1949. Amer. Jour. Clin. Path. *19*, 347.
19. Kowarski, 1901. Dtsch. med. Wschr. *27*, 442.
20. Kraus, 1897. Wien. klin. Wschr. *10*, 736.
21. Kulkka and Hirsch, 1945. Jour. Immunol. *50*, 127.
22. Lancefield, 1925. Jour. Exp. Med. *42*, 377, 397.
23. Lancefield, 1928. Jour. Exp. Med. *47*, 91, 469, 481, 483, 857.
24. Lancefield, 1933. Jour. Exp. Med. *57*, 571.
25. Landy and Webster, 1952. Jour. Immunol. *69*, 143.
26. Martin, 1943. Jour. Lab. Clin. Med. *28*, 1477.
27. Mez and Ziegenspeck, 1926. Bot. Arch. *13*, 483.
28. Moody, Cochran and Drugg, 1949. Evolution *3*, 25.
29. Muehler and MacDonald, 1945. Jour. Lab. Clin. Med. *30*, 411.
30. Myers, 1900. Lancet *2*, 98; Zentralbl. f. Bakter. I *28*, 237.
31. Neufeld, 1902. Ztschr. Hyg. Infektkr. *40*, 54.
32. Nuttall, 1904. *Blood Immunity and Blood Relationship*, Cambridge University Press, Cambridge.
33. Oudin, 1946. Compt. rend. Acad. Sci. *222*, 115.
34. Pangborn, 1941. Proc. Soc. Exp. Biol. *48*, 484.
35. Pangborn, 1947. Jour. Biol. Chem. *168*, 351.
36. Pappenheimer and Robinson, 1937. Jour. Immunol. *32*, 291.
37. Pope, Stevens, Caspary and Fenton, 1951. Brit. Jour. Exp. Path. *32*, 246.
38. Rives, 1923. Rev. Viticolt. *58*, 300.
39. Schiff and Boyd, 1942. *Blood Grouping Technic*, Interscience Publishers, Inc., New York.
40. Taylor, Adair and Adair, 1932. Jour. Hyg. *32*, 340.
41. Tchistovitch, 1899. Ann. Inst. Pasteur *13*, 406.
42. Uhlenhuth, 1901. Dtsch. med. Wschr. *27*, 82, 260, 499.
43. Uhlenhuth and Remy, 1938. Ztschr. f. Immunitätsforsch. *92*, 171.
44. Zade, 1914. Ztschr. Pflanzenzücht *2*, 101.
45. Zinsser, 1930. Jour. Immunol. *18*, 483.

## Chapter 8

### AGGLUTINATION

BACTERIA, ERYTHROCYTES and other particulate antigens agglutinate when mixed with homologous antibodies under proper conditions. Antibodies presumably combine with specific antigenic substances on the particles to form a three dimensional lattice, as in the framework hypothesis of precipitation.

#### PROCEDURES

**Production of Agglutinin.** Rabbits are immunized by inoculation with living, killed or extracted bacteria or other cells. The details of preparation of immunizing suspensions vary from one laboratory to another and depend somewhat upon the nature of the antigen. After an appropriate course of injections, the animal is bled and the serum is removed and preserved.

A bacterial antigen should consist of a single species. The culture should be genetically pure, but variations such as smooth-rough, opaque-translucent, phase 1-phase 2, motile-nonmotile, and so forth, make it very difficult to know the exact serologic state of the antigen at any given moment. Organisms are usually grown on a suitable agar medium, although broth cultures are sometimes preferred. Traces of agar in immunizing antigens have been reported to induce antiagar antibodies which agglutinate completely unrelated bacteria grown on agar medium.<sup>2</sup>

The bacteria, suspended in saline, may be killed by moderate heat (e.g., 56° C.) or by chemicals such as formaldehyde, phenol or Merthiolate. The method of sterilization should be as mild as possible because drastic physical or chemical treatment sometimes alters the specificity of cell antigens. For this reason living bacterial vaccines are often preferred, although they may be more dangerous to the animal. Despite the fact that rabbits are not susceptible to infection by most of the organisms

with which they are immunized for experimental purposes, even small doses sometimes kill the animals for reasons not clearly understood. Very small initial doses can usually be tolerated, however, and larger amounts are withheld as immunity develops. An alternative procedure is to administer a few injections of killed bacteria followed by a series of doses of living organisms.

The vaccine is usually standardized to contain a specified number of bacteria such as 500,000,000 or 1,000,000,000 per milliliter. Suspensions are counted directly under the microscope using a counting chamber, or by the Wright method. In the Wright procedure equal volumes of bacterial suspension and blood are mixed, smeared and stained. Comparative numbers of erythrocytes and bacteria are determined in several fields; assuming a normal number of red cells (5,000,000,000 per milliliter of blood), the number of bacteria is easily calculated. Vaccines which are to be sterilized by heat must be standardized before sterilization because heat disrupts the cells. Vaccines are also standardized to a certain turbidity, either photoelectrically or by comparison with known suspensions. Some vaccines for commercial use are diluted to a specified nitrogen content (e.g., pertussis).

Rabbits are generally used for production of agglutinating serums in the laboratory, but commercial houses employ horses or other animals for large scale manufacture. The animals selected should be healthy. The age of the animal is a matter of debate. Young adult rabbits usually produce antibody most rapidly and yield highest titers. It is not difficult to secure antiserums against gram negative rod bacteria which have agglutination titers of 10,000 after a relatively short series of injections requiring less than three weeks. Older and larger animals often produce antibody more slowly but have the advantage of yielding more blood.

Rabbits usually are given a series of injections into the marginal ear vein. Intravenous inoculation provides most rapid distribution and absorption of antigen. The intraperitoneal route gives somewhat slower distribution and absorption, and subcutaneous inoculations are even slower. The later injections in a series, particularly those following several days of rest, are often given subcutaneously because slow dissipation of antigen from the site of inoculation is less likely to produce toxic reactions.

Inoculation schedules are highly variable, but it is usually considered desirable to secure the maximum attainable titer as quickly as possible. Injection routines are often dictated more by convenience than by the requisites of efficient antibody production. Daily injections yield anti-serums of high titer more rapidly than weekly injections. A common schedule consists of three injections at five to seven day intervals, followed by bleeding five to seven days after the last injection. Some investigators administer twelve or sixteen injections at the rate of three or four per week, followed by bleeding one week later. Excellent titers are

obtained against members of the Enterobacteriaceae by a series of six daily injections followed by one more injection after five days and bleeding five to seven days later.

The frequency, dosage and route of injections should be judged from daily observations of the animal's condition. The usual response of an animal to injection of foreign material is similar to that of a human: fever, malaise, loss of appetite. Fever is only transient unless a living vaccine produces actual infection, the temperature ordinarily rising several degrees within two or three hours but returning to normal by the next day. Loss of appetite and activity are the simplest indications of an unfavorable reaction. If an animal reacts unfavorably it is best to change the route of injection, decrease the dosage or omit an injection or two.

Periodic "trial" bleedings of a few milliliters during the course of immunization provide opportunity to determine the progress of antibody formation by agglutination tests and indicate when a final bleeding may be made to secure a large amount of serum. Antiserum preserved with 0.45 per cent phenol or 0.01 per cent Merthiolate keeps for years at refrigerator temperature with no loss of potency. Such serums occasionally develop a protein and/or lipid precipitate, but this does not affect the antibody titer appreciably. Perhaps the most satisfactory preservation is obtained by adding an equal volume of glycerine (highest purity), but this has the disadvantage of requiring double the storage space. Serum may be kept indefinitely in the frozen state without chemical preservative.

**Agglutination Tests. The Microscopic Slide Test.** The Widal agglutination reaction as originally performed was a microscopic slide test used in the laboratory diagnosis of typhoid fever. Drops of the patient's blood were collected on a glass slide, aluminum foil or glazed paper. The specimen, allowed to dry, might then be stored for considerable time if necessary. The dried blood was moistened with a small loopful of saline and gently emulsified. Sufficient of this solution to yield a delicate orange tint was mixed with a loopful of twenty-four hour broth culture of the typhoid organism on a cover glass, which was then inverted and sealed with Vaseline over the depression of a hollow ground slide. A control in which saline was substituted for serum was also prepared. After incubation for thirty to sixty minutes at room temperature or 37° C. both slides were observed with the high power dry objective. Positive results consisted of clumping of the organisms in the serum mixture but not in the control. The serum dilution represented by the "delicate orange tint" probably corresponded to about 1:80. The principal objection to this test was that it provided only one serum dilution, and occasionally a prozone gave a falsely negative result.

The process of agglutination may be followed continuously with the microscope in slide preparations. A hanging drop is prepared with a loopful of diluted serum and a loopful of a broth culture or saline sus-

pension of an agar slant culture. High concentrations of antibodies often cause almost immediate cessation of motility followed by clumping, but in greater dilution the rate of reaction is retarded and the gradual formation of large aggregates can be observed.

*The Macroscopic Slide Test.* The macroscopic slide agglutination technique is used in the diagnosis of many infectious diseases and in screening tests for the rapid identification of bacteria. It may be conducted on any flat glazed surface such as a microscope slide or glass plate ruled with a diamond point or marked with paraffin to keep tests from running together. Special hollow ground slides containing a dozen or more depressions are also available. A loopful or one small drop each of a low dilution of antiserum and a heavy bacterial suspension are used. The slide is rocked and tilted for one to three minutes by hand or mechanically, and flocculation or granulation is observed with the naked eye. A control containing saline instead of serum is provided for each organism under test. The agglutination of an unknown organism in any number of different antiseraums is rapidly determined, and often with single factor serums a complete identification can be made. The macroscopic slide technique is used particularly in identifying the gram negative intestinal bacteria.

*Tube Agglutination.* Bacteria are more definitely identified and antibodies in serum more accurately titrated by the tube agglutination test. Dilutions of serum are prepared in serologic tubes and a constant amount of bacterial suspension is added to each. The tubes are shaken thoroughly and incubated, preferably in a water bath, and the degree of agglutination in each is recorded. The titer of antibodies in the serum is expressed as the reciprocal of the highest dilution giving definite agglutination. Details of the procedure vary with individual preference. Usually the total volume of reagents is 0.5 to 1.0 milliliter. Serum dilutions are doubled in succeeding tubes (e.g., 1:10, 1:20, 1:40, etc.), and sufficient dilutions should be made to provide one or more tubes without agglutination. A control tube containing bacteria and saline is always included and must give a negative result. Agglutination in the control indicates that the antigen was at fault (perhaps in the "rough" state) or that there was some error in technique; in this case the whole test is meaningless and must be discarded.

Preparation of bacterial antigen for the agglutination test varies with the organism and with the information desired. Cultures may be taken from broth or from agar slants. Broth cultures are usually centrifuged and the packed cells resuspended in saline. Agar slants are washed off with saline and diluted. It is difficult to state a rule for diluting bacterial antigens; usually they should be as dilute as possible and still give readable results. Living antigens of many organisms are used, but it is safer to kill pathogens if the nature of the experiment permits; 0.3 per cent formalin is sometimes added to the saline used for this purpose.

Preparation of the test suspension is also determined by the particular antigenic fraction whose antibody is to be titrated. Motile bacteria contain at least two different kinds of antigenic substances: one associated with the cell bodies and known as somatic (O) antigens, the other presumably associated with the flagella and called flagellar (H) antigens. Somatic antigens are resistant to heat, whereas flagellar antigens are destroyed by heat. Fortunately, formalin used to kill bacteria does not affect either the somatic or flagellar antigens, and therefore animals immunized with living or formalin treated bacteria produce both somatic and flagellar antibodies. Pure somatic antibodies are obtained either by immunizing with a nonmotile variant or a boiled suspension, or by adsorbing flagellar antibodies from antiserum against a motile strain. Pure flagellar antibodies are secured by adsorbing somatic antibodies from antiserum produced with a motile organism.

Somatic test antigens are prepared by heating a suspension of the organisms. Such antigens, or suspensions of nonmotile bacteria, react with homologous antisera to form compact sediments which break up into sharply defined granules when shaken. Flagellar test antigens consist of living or formalin treated suspensions and produce a loose, flocculent sediment with homologous antiserum which breaks up on shaking into almost indistinguishable clumps. These characteristic types of agglutination are common, but gradients between the two extremes are encountered in which it is impossible to determine the type of agglutination by inspection.

The temperature and time of incubation of tube agglutination tests differ according to the nature of the organism and the antigen preparation. There is such marked variation between laboratories that the same reagents tested by different investigators may give widely divergent results. For example, recommendations for flagellar agglutination tests range from incubation at 37° C. for two hours followed by overnight refrigeration, to incubation at 55° C. for fifteen to twenty hours. However, recent work has shown that some motile bacteria display a flocculent, flagellar type of agglutination at 37° C., but not at the higher temperatures.<sup>41</sup> In fact, certain organisms which have been agglutinated at 37° C. may spontaneously disperse at 55° C. Some other motile organisms react somewhat better at 55° C. than at 37° C. The effect of incubation temperature upon specificity and titer needs thorough study before definite recommendations can be made.

It is generally conceded that somatic agglutination of bacteria requires a higher incubation temperature or a longer time or both than flagellar agglutination. Here again, however, recommendations range all the way from two hours at 37° C. followed by overnight refrigeration to twenty-four hours at 50° to 55° C.

Certain bacteria agglutinate only with difficulty. *Brucella* species are

often incubated for two days at temperatures from 37° to 55° C. Very light phenolized suspensions of highly mucoid bacteria may require one or two days at 37° C.<sup>31</sup> The optimum conditions for each organism or group of organisms must be worked out individually by trial and error.

When results are needed quickly, agglutination can be hastened by centrifuging the tubes lightly (e.g., 2000 r.p.m. for ten to fifteen minutes). All tubes *including the control* are then carefully shaken together in a rack before reading. Passage of the cells through the serum dilution provides opportunity for antigen-antibody contact and reaction, and packing speeds the process of aggregation. Final shaking redisperses cells which did not agglutinate and allows a reading to be made.

Bacteria possessing certain somatic or envelope antigens may fail to agglutinate in high titer homologous O antiserums. Such organisms are called O-inagglutinable. The K antigens of *E. coli* and the Vi antigen of *S. typhosa* are examples of antigens which confer O-inagglutinability.

Three kinds of K antigen are known, designated L, A and B.<sup>24</sup> (1) L antigens are thermolabile, and cultures containing them regain O-agglutinability after boiling for one hour. Antiserums containing L antibodies may be prepared by immunizing with appropriate living organisms; the O antibodies which are also formed can be removed by adsorption. Colonies of strains containing L antigens are somewhat more opaque than those lacking them. (2) The A antigens are thermostable capsular polysaccharides. They resist boiling for two and one-half hours but are destroyed in two hours at 120° C. Bacteria containing A antigen give a *Quellung* reaction in homologous antiserum. (3) B antigens, like L antigens, are thermolabile, being destroyed by boiling for one hour. They differ from L antigens in the ability of heated suspensions to adsorb homologous B antibody, although such heated suspensions do not agglutinate in B antiserum.

The Vi antigen is also said to be destroyed by heat. Peluffo, however, reported that it is relatively heat-stable in nonaqueous media like absolute alcohol, acetone or glycerine.<sup>32</sup> O agglutination of a culture containing Vi antigen may be obtained in anti-O serum by use of a suspension boiled thirty minutes. Antiserum against Vi antigen is prepared by immunizing rabbits with living organisms. Vi agglutination tests employ living or formalized (0.5 per cent formalin) test suspensions and are incubated at 37° C. for two hours followed by room temperature or refrigeration overnight. Vi agglutination also occurs to full titer of the serum if tests are kept at room temperature or in the refrigerator for twenty hours without preliminary 37° C. incubation.

*Quantitative Determination of Agglutinin.* The quantitative agglutination procedure of Heidelberger and Kabat is a modification of the quantitative precipitation test<sup>18</sup> and is laborious and time consuming. It provides a method for determination of agglutinating antibody in serum

on a weight basis and is valuable as a research tool. A small excess of washed bacteria or other antigen is allowed to react with antiserum and centrifuged. The packed cells are washed repeatedly to remove traces of nonantibody serum proteins, and their nitrogen content is then determined. An identical amount of bacteria is mixed with saline instead of antiserum and carried through the same procedure. Any increase in the nitrogen content of the antigen which has been in contact with antiserum is assumed to represent antibody capable of reacting specifically with that antigen. An excess of antigen is provided to ensure removal of all specific antibody, and the completeness of removal may be tested by treating the supernate with additional antigen and making further analyses.

This method was used by Heidelberger and Kabat to show that type 1 pneumococcal specific agglutinins in antiserum correspond in amount to the anticarbohydrate precipitins in the same antiserums.<sup>19</sup> Perlman and Goebel also obtained similar evidence of the probable identity of parady sentery type I-III agglutinins and precipitins.<sup>33</sup> Other investigations have demonstrated the percentage of antibody in certain antiserums capable of cross reacting with heterologous antigens; for example, in antipneumococcal and antidysentery serums. Quantitative determinations have also been made of antibodies against hemolytic streptococci, *Hemophilus influenzae*, *Neisseria gonorrhoeae*, *S. typhosa*, Brucella and other bacteria. Recently it was shown that convalescent human meningitis serums contained between 0.31 and 0.75 milligram of meningococcal antibody per milliliter.<sup>22</sup>

#### MECHANISM OF AGGLUTINATION

It will be recalled that Bordet considered agglutination to be a two-stage phenomenon in which antigen is first coated with antibody and then aggregated by electrolyte. Northrop and DeKruif showed that electrolytes in proper concentration produce conditions of cohesiveness and surface charge which cause agglutination of cells sensitized with antibody but do not agglutinate unsensitized cells. This hypothesis assumes that the second stage is a nonspecific salt effect. It would be expected, therefore, that a mixture of unrelated antigens, each coated with its homologous antibody, would join under the influence of electrolyte to produce a heterogeneous aggregate. Topley, Wilson and Duncan attempted to produce such a mixed aggregate by use of pneumococci and enteric bacteria and their antiserums but observed only homogeneous aggregates: cocci unmixed with rod forms or *vice versa*.<sup>42</sup> Other investigators, working with chicken and human erythrocytes, gram negative and gram positive bacteria, or human group O erythrocytes and type 1 pneumococci, reported mixed aggregates when the antibody concentration was great but homogeneous clumps with dilute antibody.<sup>1, 21, 46</sup> The rate of reaction

appeared to affect the results, rapid reactions yielding heterogeneous aggregates. Under such conditions mechanical entrainment of the heterologous antigen rather than a nonspecific salt effect might be expected to produce mixed clumps. The question is still not clearly settled.

The lattice hypothesis states that antigen and antibody react by means of radicals or groups which have a specific chemical affinity for each other and that the aggregate formed consists of antigen and antibody arranged alternately and combined by chemical bonds. Marrack considered that such union decreases the accessibility of polar groups to water, in consequence of which the solubility of the aggregates is reduced.<sup>30</sup> Heidelberger believed agglutination to be the same as precipi-

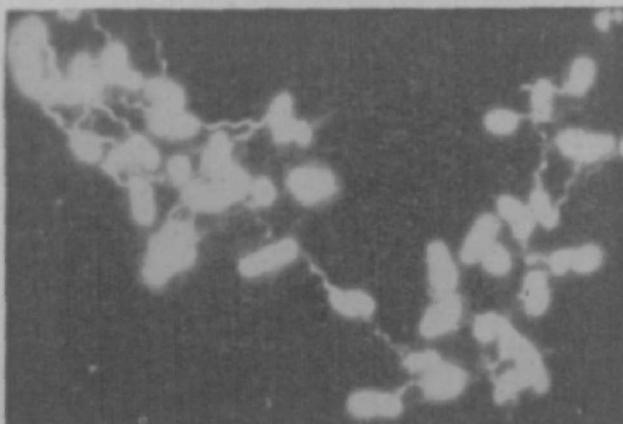


FIGURE 34. Flagellar agglutination of *Salmonella typhosa* by H antiserum: clumps of cells formed by intertwining of thickened flagellar structures, the bacterial bodies not touching one another. (From a photograph kindly supplied by Dr. Adrianus Pijper.)

tation within the spatial limitations imposed by location of the antigenic components on the surfaces of cells.<sup>17</sup> Bacterial agglutination was considered not merely nonspecific flocculation of sensitized antigen by electrolyte but a more dynamic process in which antigenic substances on bacterial surfaces react with antibodies in solution to form larger and larger aggregates, which eventually settle out. Electrolytes perform the secondary function of reducing electrostatic charges which might otherwise interfere with the chemical reactions leading to aggregation.

Observations of Pijper on the nature of flagellar, somatic and Vi agglutination are of interest in connection with the mechanism of aggregation. He employed very strong illumination (sunlight) and a darkfield microscope and made motion photomicrographs of the process of agglutination. Flagellar agglutination (Figure 34)<sup>34</sup> appeared to begin with deposition of antibody molecules (presumably radially) on the flagella, thereby causing thickening and immobilization of these structures, which later became mechanically entangled and caused aggregation of the cells.

In somatic agglutination (Figure 35),<sup>34</sup> antibody apparently combined with the bodies of the cells, which thereupon attracted instead of repelled each other. The behavior of the cells indicated to Pijper a possible alteration in their electric charges.



FIGURE 35. Somatic agglutination of *Salmonella typhosa* by O antiserum between slide and coverslip: polar attachment of cells gives a regular crystal-like structure in two dimensions. (From a photograph kindly supplied by Dr. Adrianus Pijper.)

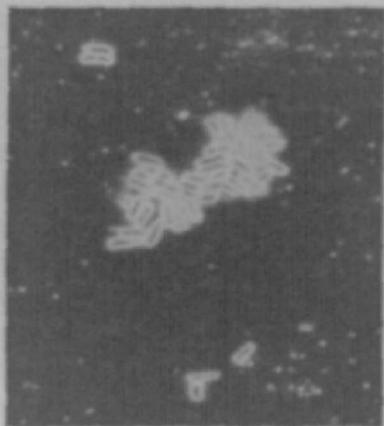


FIGURE 36. Vi agglutination of *Salmonella typhosa* by Vi antiserum; side-to-side attachment eventually yields a dense mass of agglutinated cells. This is a small clump. (From a photograph kindly supplied by Dr. Adrianus Pijper.)

Vi agglutination differed somewhat from flagellar and somatic agglutination (Figure 36).<sup>35</sup> Increasing paralysis led to highly erratic movements and gradual slowing of motility, but the cells appeared to exert continued normal mutual repulsion, as though their electric charges remained at customary levels. The cells, moving irregularly, occasionally collided by chance and stuck together if the initial contact involved sufficient area. Side-to-side contacts were effective but polar contacts were not.

Boyd suggested that somatic agglutination follows the Bordet mechanism, whereas Vi agglutination conforms to the more purely chemical

mechanism of the Marrack-Heidelberger hypothesis.<sup>5</sup> Landsteiner also agreed that both mechanisms may operate.<sup>28</sup>

One characteristic of certain agglutination tests which is difficult to explain by the Bordet hypothesis is the prozone phenomenon (see page 97), which occurs in the presence of excess antibody; all cells are presumed to be coated and hence should agglutinate if electrolyte is present. According to the lattice hypothesis, complete coating of all antigen cells with antibody leaves no connecting links of free antigen to permit framework formation.

#### APPLICATIONS OF AGGLUTINATION

**Detection of Antibodies.** Antibodies specifically directed against the causative agents of certain infectious diseases appear in the circulating blood a few days after infection. They increase in amount for several weeks, remain relatively constant for varying periods of time, then decrease slowly until little or no titer is detectable. The presence of such antibodies is therefore presumptive evidence for infection by the specific etiologic agent if the patient has not previously been infected or immunized with this organism. Unfortunately, from the diagnostic point of view, many individuals normally possess low or moderate amounts of certain antibodies. These "normal" antibodies, together with anamnestic reactions, complicate the interpretation of a positive agglutination test. In the absence of specific information regarding the history of the patient, two or more tests are performed at weekly intervals and a rising antibody titer is considered evidence of active infection.

Detection of agglutinins in patients' serum is of value in cases of typhoid and paratyphoid fevers and many other infections. The serum is diluted 1:10, 1:20, 1:40, 1:80, 1:160 or higher and tested with appropriate antigens. Enteric fever antigens include *S. typhosa*, *S. paratyphi A* and *S. paratyphi B*; usually both H (flagellar) and O (somatic) suspensions are employed. The results of tests for typhoid fever are interpreted as indicated in Table 35.<sup>10</sup> Tests for paratyphoid agglutinins have similar significance. A test for Vi agglutinins is also performed. These antibodies are not always found in active cases of typhoid fever but are present so frequently in typhoid carriers that the test for Vi antibody has been suggested to detect such individuals.

Diagnosis of undulant fever is sometimes aided by the agglutination test. This disease is notoriously difficult to diagnose by clinical methods; it runs a prolonged course, often subacute or chronic, and is easily confused with enteric or other infections. Agglutination tests with Brucella are often performed on the serums of suspected typhoid and paratyphoid patients. Before interpreting agglutination with Brucella antigen it is necessary to know whether the patient has constant opportunity for

infection by use of raw milk or by handling infected animals. Such a person is likely to have a relatively high agglutinin titer. A titer of 160 in individuals with infrequent opportunity for infection sometimes indicates active disease. This figure, however, may be within the normal range for persons continuously exposed to Brucella. In any case, a titer of 1000 is rare except in active brucellosis.<sup>47</sup>

Brucella antibodies can be detected also in the blood serum and milk of infected cows or other animals. Whole milk is tested by adding a heavy suspension of Brucella which has been treated with a suitable dye.<sup>15, 48</sup> The milk and antigen are thoroughly mixed and allowed to

*Table 35. Interpretation of Agglutination Test in Diagnosis of Typhoid Fever*

ANTIBODY TITER		SIGNIFICANCE
H (flagellar)	O (somatic)	
80 or higher	80 or higher	Almost always indicates active typhoid infection
80 or higher	Less than 80	Found in typhoid carriers or after previous infection or immunization; rare in active infection
Less than 80	80 or higher	Found in approx. 14% of active cases; usually in infections with related organisms

(From Coleman,<sup>10</sup> by permission.)

stand until the cream rises. The mass of agglutinated dyed bacteria is swept upward with the cream and is readily distinguished. This so-called "ring test" is sufficiently delicate to detect agglutinins from a single positive cow in the pooled milk from a large herd. Furthermore, the results with pasteurized milk are as satisfactory as with raw milk, which greatly increases its usefulness over methods of examination requiring isolation of the pathogenic agent.

Tularemia, like brucellosis, is prolonged, debilitating, subacute or chronic and rather difficult to diagnose. A titer of 160 or higher is considered indicative of infection, and titers of 2560 or 5120 are not uncommon.<sup>37</sup> Serums agglutinating *Pasteurella tularensis* may also agglutinate *Br. abortus*. Tularemia agglutinins often persist in low concentration for years.

Agglutination tests in bacillary dysentery and bubonic plague are of limited value, because detectable antibodies do not always develop. Titers of 20 or greater against *Shigella dysenteriae* and *Sh. sonnei* and of 160 or greater against *Sh. paradyENTERiae* are suggestive of active infection in areas where these organisms are not endemic. Any positive result is of value in bubonic plague, and the titers may be low (less than 40).

The Weil-Felix test for typhus fever is an example of a heterogenetic reaction of some theoretic as well as practical interest. In 1916, Weil and Felix isolated from the urine of a typhus fever patient a strain of *Proteus* which was agglutinated by the patient's serum and by serum from other typhus fever patients.<sup>46</sup> Serum from normal individuals did not agglutinate the organism. It was soon found that *Proteus* was not the cause of typhus fever, but the test is a highly useful diagnostic tool. The strain of *Proteus* was designated *X19*, and agglutinated to titers of 50 to 50,000 with typhus fever serums, but never over a titer of 25 with serum from patients without typhus fever. The reaction involves somatic antigens of the organism, so only nonmotile strains should be used. These are designated by the letter O (e.g., *Proteus OX19*). Two additional strains, *OX2* and

*Table 36. Agglutination Test for Rickettsial Disease*

DISEASE GROUP	PROTEUS TEST ANTIGEN		
	OX19	OX2	OX-K
Typhus fever	++++	0 or +	0
Spotted fever	0 or ++++	+++ or 0	0
Tsutsugamushi fever	0	0	+++
Q fever	0	0	0

(From Sabin et al.,<sup>38</sup> by permission. Copyright by The National Foundation for Infantile Paralysis, Inc.)

*OX-K*, are useful in the diagnosis of various other Rickettsial diseases (Table 36).<sup>36</sup> Normal agglutinins for *Proteus* occur with considerable frequency in man, so an increasing titer in second and third specimens taken at intervals of several days is considered more significant than the magnitude of the titer of a single specimen.

The chemical nature of the antigens which participate in the Weil-Felix reaction has not been completely determined. It is known that *Proteus OX19* and *Rickettsia prowazekii*, the cause of typhus fever, share a common glucolipid antigen.<sup>47</sup> The three *OX* strains possess similar glucolipid antigens. There is no direct evidence that *R. orientalis* (the cause of tsutsugamushi fever) shares the glucolipid of *OX-K*, although they apparently possess some antigen in common. It has been suggested that the *Proteus* strains represent variants of the Rickettsiae or that *Proteus* bacteria within the bodies of patients acquire new antigenic receptors which render them agglutinable by antibodies against the corresponding Rickettsiae. It has also been proposed that possession of common antigens by *Proteus X* strains and the respective Rickettsiae is coincidental, like the occurrence of common capsular antigens in certain gram positive cocci and gram negative rods. Conclusive evidence for any hypothesis is lacking.

An interesting diagnostic agglutination test is used in infectious mononucleosis or "glandular fever," a disease epidemic in children and sporadic in adults. Infectious mononucleosis is usually benign and is of uncertain etiology but thought to be caused by a virus. It is characterized by sudden onset with fever, usually a sore throat and pronounced enlargement of the cervical lymph nodes. There is moderate leukocytosis with marked increase in lymphocytes (50 to 90 per cent), which are somewhat atypical. The serologic test is performed by adding a suspension of sheep erythrocytes to dilutions of inactivated patient's serum and incubating at 37° C. for two hours. Agglutination titers of 320 or higher are considered significant and titers of 40,960 have been obtained. The laboratory diagnosis of infectious mononucleosis is complicated by the fact

*Table 37. Differentiation of Sheep Erythrocyte Agglutinins in Human Serum*

ANTIBODY	ANTIBODY REMOVED FROM SERUM BY	
	Guinea pig kidney	Boiled beef R.B.C.
Infectious mononucleosis	—	+
Serum sickness	+	+
Forssman	+	—

(From Stuart,<sup>40</sup> by permission.)

that injection of antitoxin or other horse serum preparations may induce a disease known as serum sickness, in which there is also an increase of sheep red cell agglutinins. Furthermore, normal individuals may possess a Forssman-like antibody which agglutinates sheep cells. These three sheep hemagglutinins can be distinguished from one another by adsorption of the serum with guinea pig kidney emulsion and boiled beef erythrocytes as indicated in Table 37.<sup>40</sup> It is usually sufficient to treat the patient's serum with the kidney preparation; after removal of the tissue by centrifugation, the agglutinin titer is compared with that of the unadsorbed serum. A diagnosis of infectious mononucleosis is confirmed if at least part of the hemagglutinin remains.

"Cold agglutinins" for human erythrocytes are occasionally important in laboratory diagnosis. These antibodies are sometimes found in certain disease conditions, such as atypical pneumonia, and may also be present in normal human serum and in some animal serums.<sup>14, 27, 45</sup> They agglutinate red blood cells at temperatures below that of the body, but the agglutinated cells redisperse when warmed to 37° C. Cold agglutination titers usually decrease as the test incubation temperature increases, but there is considerable variation between individuals and animals in the thermal range through which agglutination occurs, some serums reacting

only below 20° C., others as high as 35° C. The test is performed for diagnostic purposes at 2° to 5° C. Normal titers at this temperature are usually not more than 40. Titers of 160 or greater are not uncommon in atypical pneumonia, and there is some correlation between the severity of the infection and the titer observed. Washed human red cells, usually of blood group O, are employed as test antigen. Patient's serum is obtained from blood allowed to clot and centrifuged at room temperature or preferably 37° C., because cold agglutinins are adsorbed by erythrocytes of the same individual at refrigerator temperature. Serum dilutions plus antigen are incubated overnight in the refrigerator and agglutination is read immediately upon removing. Positive tests are warmed to 37° C. and reread after two hours to be sure that aggregation is caused by cold agglutinins, whose action is reversed under such conditions.

There is no satisfactory explanation of this reaction. It is not given by serums from all primary atypical pneumonia patients. Positive results have been obtained in more than 90 per cent of severe cases and in only about 20 per cent of mild cases.

The Hirst hemagglutination test is used in the diagnosis and investigation of viral infections. Hirst described the reaction in 1941 in a study of the influenza virus,<sup>20</sup> and it has since been employed with vaccinia, variola, mumps and several other viruses (see page 266). The test is performed by mixing dilutions of virus with erythrocytes, which agglutinate within an hour at room temperature. Chicken red cells have been most used, but human erythrocytes are satisfactory for a number of viral agents, and cells from most of the usual laboratory or domestic animals have been employed with one or more viruses.

This is clearly not an antigen-antibody reaction, but the mechanism is apparently similar to that described in the framework hypothesis. Virus particles are adsorbed to two erythrocytes simultaneously and act as "bridges" in the formation of a lattice.

The Hirst reaction is inhibited by convalescent or immune serum, which provides an *in vitro* serologic test for viral antibodies known as the *hemagglutination-inhibition* test. Serum dilutions are mixed with constant amounts of virus suspension and erythrocytes and incubated one hour at room temperature. Virus which reacts with antiviral antibody fails to agglutinate the red cells. The antibody titer is represented by the highest serum dilution which inhibits agglutination. Simultaneous tests with immune serum of known titer control the variation normally encountered in the red cells and virus. In the case of influenza, there is considerable correlation between the protective power of a serum and its titer in the inhibition test. This reaction affords a much more rapid and inexpensive method of testing serum than animal or egg inoculation, as was previously necessary.

**Identification and Classification of Bacteria.** Methods of identifying

and classifying bacteria have undergone considerable evolution since the early "morphologic" era. Morphology as a systematic tool was obviously limited in scope with such minute organisms as bacteria; its utility was hampered by the relatively small number of forms which bacteria might assume and by the visual acuity of the observers.

The discovery that certain bacteria produce characteristic changes in particular media, such as liquefaction of gelatin and fermentation of carbohydrates, led naturally to the "biochemical" period, in which major attention was focused on physiologic tests to distinguish species and even genera and families. The morphologists naturally resisted the taxonomic changes proposed by the "biochemists," maintaining the debatable position that the structure of an organism is more fundamental than its physiologic behavior. Practical considerations ultimately led to acceptance of biochemical properties as taxonomic criteria. The great variety of such reactions permitted much finer distinctions between organisms than were provided by morphology alone. In fact, subdivision compounded upon subdivision almost to the point of absurdity, so that controversy arose between the "splitters," who classified each new biochemical type as a separate species, and the "lumpers," who attempted to establish certain reactions as taxonomically important within particular groups and to ignore all other reactions.

Later, with recognition of the ability of serologic procedures to distinguish between organisms apparently identical morphologically and physiologically, serology became the "court of last appeal." This position was fostered by realization that serologic characteristics are basically chemical and hence presumably fundamental properties of an organism. In turn, the "biochemists" strenuously opposed the serologic approach, but presently the confusion engendered by the multiplicity of species, varieties and biotypes which they had fostered forced recognition of the value of antibodies as diagnostic reagents for identification and systematic study of bacteria. Now, however, it is evident that bacteria in widely different genera may possess antigens in common, and that serology is only one of a series of tools in determinative bacteriology. Morphology, physiology, habitat, pathogenicity and cultural behavior also contribute important information to the systematist and to the laboratory diagnostician.

So far, physics has not seriously turned its attention toward the problems of the systematic bacteriologist, but there are signs that this is the next step. Electrophoresis, ultracentrifugation and phase and electron microscopy are only beginnings. Recent reports hint at the role which infrared spectrophotometry may play in the identification of bacterial components and hence in classification of these organisms. The systematist may soon find himself faced with the necessity of learning physics as well as bacteriology, chemistry and serology.

The early serologist-bacteriologist picked colonies from, for example, a stool culture, tested their agglutination in known antityphoid serum and reported the presence or absence of typhoid bacteria without further examination. Later he found other bacteria in stool specimens which agglutinated in antityphoid serum but which differed in some biochemical properties and in the type of disease produced. Still others of these so-called paratyphoid bacteria were less clearly related to typhoid, but displayed relationships among themselves which could be demonstrated by appropriate antiseraums. Gradually a series of serologic "types" or varieties within the group of organisms became apparent, and the practice of "typing" fresh isolates was established. A similar situation arose within the pneumococci, hemolytic streptococci, meningococci and many other bacteria. Serologic typing is one of the steps in complete identification of an organism and is a necessary preliminary to specific antiserum therapy; it is also very useful in epidemiologic studies.

**Antigenic Analysis.** The serologic type of an organism is determined by its distinctive antigenic components or combination of components. Complete antigenic analysis includes detection of all such components possible. The various antigens of bacteria have not yet been chemically identified in detail, although they are principally proteins and polysaccharides. In the absence of more descriptive terminology they are usually designated by letters and numerals (e.g., A,B,C...I,II,III...1,2,3...). There is a definite system of antigenic nomenclature within each genus or other group of bacteria, but so far the system within one group usually bears no relationship to that within other groups. Thus, various species of *Salmonella* contain antigens customarily designated by Arabic numerals: 1,2,3...; certain *Escherichia* strains possess different antigens designated by the same numerals. It is necessary, therefore, to state the genus of an organism as well as its antigenic makeup. No doubt future investigators will devise a uniform system of nomenclature for all bacterial antigens.

Individual bacterial cells contain numerous antigenic components or determinants, each of which can induce the formation of a separate antibody. The antigenic structure of bacteria has been likened to a mosaic, each piece of which represents one antigen. Four organisms, for example, might possess the following antigenic structures and produce corresponding antiseraums:

ORGANISM	ANTIGENS	ANTISERUM	ANTIBODIES
I	A B C	1	a b c
II	B C D	2	b c d
III	C D E	3	c d e
IV	D E F	4	d e f

Each organism is related to those which immediately precede and follow it by common possession of one or two antigenic components. Organism

I, however, is completely unrelated in its antigenic structure to organism IV, although both are members in a series of related forms. There is growing evidence that the situation pictured here actually extends throughout the natural world, and that when all the "missing links" have been found each living form will appear as a member of a continuous intergrading series.

Antiserum 1 contains antibodies a, b and c, and can agglutinate organisms I, II and III, but cannot agglutinate IV because the latter possesses no antigen corresponding to an antibody in Antiserum 1. Antiseraums 2 and 3 can agglutinate all four organisms, and Antiserum 4 can agglutinate II, III and IV. Adsorption of Antiserum 1 with organism II removes all antibodies except a. This adsorbed serum can therefore be used to detect antigen A in any organism and is a so-called *monospecific* or *single factor serum*. It will agglutinate I but not II. Monospecific serums can be similarly prepared for any of the other antigenic components.

Table 38. Results of a Cross-Agglutination Experiment

ANTISERUM	AGGLUTINATION TITER WITH ORGANISM	
	A	B
A	5120	5120
B	10,240	10,240

The antigenic structure of an organism is ascertained by detecting its various serologically different components or antigenic determinants by use of appropriate unadsorbed and adsorbed antiseraums. The simplest case is that in which two organisms are compared. The first step is to prepare antiseraums against each organism. Cross-agglutination tests are conducted by preparing duplicate sets of dilutions of both serums and treating one series of dilutions of each serum with the homologous organism, the other with the heterologous organism. Agglutination results like those in Table 38 might be obtained. These two organisms are related, since each agglutinated in each antiserum.

Occasionally one organism agglutinates in both antiseraums, but the other reacts only in its homologous antiserum. The explanation of this nonreciprocal cross reaction is not clear. It can be supposed that a given antigenic component, common to both organisms, is so situated in one organism as to be readily accessible to antibody but in the other organism is either more deeply located or is masked by other surface substances. Agglutination appears to be a surface phenomenon in which only superficial antigens participate, because it is considered improbable that antibody molecules could penetrate far into the cell substance or could

cause agglutination if they did. Antibodies will be formed, however, against deeper or masked antigens as other bacterial components disintegrate within the antibody-producing cells. It is by no means universally accepted, it should be noted, that the somatic antigens of bacteria like the *Salmonellas* constitute a series of successive layers resembling an onion in structure.

An antigen will occasionally fail to remove from its homologous serum all antibodies capable of acting on heterologous organisms. It is generally assumed that the unadsorbed antibodies represent normal agglutinins, such as the alpha agglutinin,<sup>39</sup> which may be found in relatively high titer in some rabbits.

Cross-agglutination does not tell anything about the antigenic complexity of the two organisms. One or many antigenic determinants may

*Table 39. Results of a Reciprocal Adsorption Experiment*

ANTISERUM	ADSORBED WITH ORGANISM	ANTIBODIES REMAINING AFTER ADSORPTION	TESTED WITH ORGANISM	AGGLUTINATION TITER
A (x, y)*	B (Y, Z)†	x -	A (X, Y)† B (Y, Z)†	5120 0
B (y, z)*	A (X, Y)†	z	A (X, Y)† B (Y, Z)†	0 10,240

\* Antibodies present in the unadsorbed serum.

† Antigenic components.

be concerned. Furthermore, there is no indication whether each organism possesses all the determinants of the other. Organism A, for example, might contain antigens X and Y, whereas organism B might contain Y and Z.

The comparative antigenic composition of the two bacteria can be determined by reciprocal adsorptions or the "mirror test." Each antigen is adsorbed with the heterologous organism, and cross-agglutination tests with the adsorbed antiseraums indicate whether any antibodies remain uncombined. If, as supposed previously, organism A possesses antigens X and Y, and B possesses Y and Z, results might appear as in Table 39.

Occasionally each of two organisms may remove all antibodies from the heterologous antiserum in the usual reciprocal adsorption test, and yet differential adsorption reveals that the two organisms are not identical. Differential adsorption is performed by repeatedly treating antiserum with light doses of the adsorbing organism and titrating residual antibodies after each adsorption. Relatively few adsorptions with the homologous organism remove all antibodies, but many adsorptions with the heterologous organism may be required. This indicates that the two organisms are quantitatively different although qualitatively alike.

A problem in antigenic analysis becomes more complicated when additional organisms are compared, but much more information can be gained about each. Table 40 presents data from a study of three strains of *Sh. dispar*. Cross-agglutination (lines 1, 4 and 7) indicated that organism 171 was weakly related to 167 and 205, which in turn were quite strongly related to each other. The nature of these relationships was shown by reciprocal adsorption. Antiserum 171 adsorbed with strains 167 or 205 (lines 2 and 3) still agglutinated organism 171 to the original 5120 titer. This antibody and its corresponding antigenic component were designated by the letter A. Organisms 167 and 205 lacked this component. Adsorption of serum 167 with organism 171 (line 5) removed

*Table 40. Antigenic Analysis of Three Strains of Shigella dispar*

LINE	ANTISERUM	ADSORBED WITH ORGANISM	TITER WHEN TESTED WITH ORGANISM			ANTIBODIES IN ANTISERUMS (AND ANTIGENS IN CORRESPONDING ORGANISMS)
			171	167	205	
1	171	Unadsorbed	5120	640	1280	A, D
2		167	5120	0	0	
3		205	5120	0	0	
4	167	Unadsorbed	640	20,480	20,480	B, C, D
5		171	0	10,240	5120	
6		205	0	2560	0	
7	205	Unadsorbed	320	10,240	10,240	B, D
8		171	0	10,240	5120	
9		167	0	0	0	

all antibody for 171 as expected, but did not alter the titer for 167 significantly and reduced the titer for 205 slightly. It was not certain that the antibody agglutinating 167 was the same as that agglutinating 205, but at this point it was simplest to consider it so and to designate it B. Adsorption of antiserum 167 with organism 205 (line 6) removed all agglutinins except a portion capable of reacting with 167, which therefore contained an additional antigenic component, C. Adsorption of antiserum 205 with organism 171 (line 8) confirmed the previous finding that strains 167 and 205 shared an antigen (B) lacking in 171, and the final adsorption (line 9) showed that organism 167 had all the antigens possessed by 205. The major antigenic components of these organisms were therefore A, B and C. The weak cross reactions indicated by titers of 320, 640 and 1280 (lines 1, 4 and 7) were not accounted for, so a minor fraction, D, was postulated.

**Antigenic Structure of Bacteria.** Smith and Reagh showed in 1903 that motile organisms possess two different types of antigen, one associa-

ted with the flagella, the other with the cell bodies.<sup>38</sup> They encountered a nonmotile variant of *S. cholerae-suis* and prepared antiseraums against the motile form and the nonmotile variant. Positive cross-agglutination occurred in all combinations (Table 41). The motile organism agglutinated to high titer in homologous antiserum in the form of loose, flocculent clumps, whereas the nonmotile organism agglutinated in the same serum only to low titer and in a compact, granular mass. Both organisms were agglutinated in moderate dilutions of antiserum against

Table 41. Cross-Agglutination and Adsorption of Motile and Nonmotile *Salmonella cholerae-suis*<sup>38</sup>

ANTISERUM	TEST ANTIGEN	AGGLUTINATION	
		Titer	Type
Motile	Motile	20,000	Flocculent
	Nonmotile	200	Granular
Nonmotile	Motile	500	Granular
	Nonmotile	500	Granular
Motile antiserum adsorbed with non-motile antigen	Motile	20,000	Flocculent
	Nonmotile	0	
Nonmotile antiserum adsorbed with motile antigen	Motile	40*	Granular
	Nonmotile	40*	Granular

\* These low titers evidently resulted from inadequate adsorption

the nonmotile form, producing granular clumps. Each organism therefore possessed antigenic determinants also present in the other, but the titer and appearance of agglutination suggested a difference in the antigenic composition of the two bacteria.

The nature of this difference was made clear by adsorption. Antiserum against the motile form was adsorbed with the nonmotile organism. The adsorbed serum did not agglutinate the nonmotile strain but continued to agglutinate the motile form in undiminished titer. It is apparent that antiserum against the motile strain contained an antibody for which there was no corresponding antigen in the nonmotile organism. The motile organism therefore possessed an antigen lacking in the nonmotile. The reciprocal adsorption, in which antiserum against the nonmotile organism was adsorbed with the motile form, removed practically all antibodies. The low titers obtained with both the motile and nonmotile strains indicate that adsorption had not been thoroughly performed. The motile organism evidently contained all antigens present in the non-

motile culture and one or more in addition, presumably associated with the flagella.

The symbols H and O applied to flagellar and somatic antigens, respectively, were derived from terms used to describe the colonial growth of motile and nonmotile strains of *Proteus*.<sup>44</sup> Motile cultures of these organisms spread over the surface of moist agar as a thin film (German *Hauch*) and were called H forms. Nonmotile variants produced isolated colonies and were called "ohne Hauch" or O forms.

Somatic and flagellar antigens of bacteria like the *Salmonellas* behave as though they consist of several components. More than forty different somatic antigens have been identified among the various *Salmonella* species. In early serologic studies of *Salmonella* each serotype was characterized by several somatic antigens (e.g., *S. paratyphi* B: four O antigens designated 1, 4, 5, 12). As additional types were found and it became evident that many more might be encountered, the practice arose of distinguishing later isolates by a single somatic antigen (e.g., *S. champaign*, O antigen 39). The O antigens of *Salmonella* are indicated by Arabic numerals (1, 2, 3, etc.). Antigens 6 and 12 are known to be composed of more than one fraction; each fraction is distinguished by a subscript: 6<sub>1</sub>, 6<sub>2</sub>, 12<sub>1</sub>, 12<sub>2</sub>, 12<sub>3</sub>.

The various antigens of bacterial cells are not necessarily always separate chemical entities. Although *S. paratyphi* B appears to contain four O antigens, two (4 and 5) behave as though joined in a single unit or complex molecule having multiple antigenic determinants. Certain of the somatic antigens of paradsentery bacteria seem to be similarly bound together.

Some species which normally possess certain O antigens undergo a type of variation in which these antigens partially or completely disappear. This is known as "form variation." Antigen 1 is particularly susceptible to this change, and fraction 12<sub>2</sub> also undergoes similar variation. Form variation may be noted among colonies obtained from a single parent culture.

Variation in the Vi antigen of *Salmonella*, *Escherichia coli* and para-colon bacteria is called the V-W transformation. V forms contain the Vi antigen and for the most part are inagglutinable in O antiseraums. W forms lack Vi antigen and are O-agglutinable. Colonies possessing both V and W organisms are also found.

The S-R transformation affects bacterial O antigens. This phenomenon, sometimes called dissociation, has usually been described as a change from smooth to rough colony form with accompanying decreased virulence and increased spontaneous agglutinability in physiologic saline. Rough forms possess little or none of the original smooth somatic antigen. Antiserum for one R form of *Salmonella* may agglutinate the R forms

of other *Salmonellas* or even of *Shigellas* and more distantly related bacteria. The R antigen appears to be a polysaccharide but is different from those which determine the O antigenic properties of S cells. The S-R transformation brings about little if any alteration in the flagellar antigens of motile forms.

Qualitative changes in flagellar antigens are known as "phase variations." Andrewes discovered in 1922 that the flagella of certain *Salmonella* species (e.g., *S. paratyphi B*, *S. paratyphi C*, *S. typhimurium*, *S. newport*) existed in two alternative antigenic forms.<sup>8</sup> Flagellar antigens of one form appeared to be characteristic of the species and were called *specific antigens* (phase 1); those of the other form were found in several species

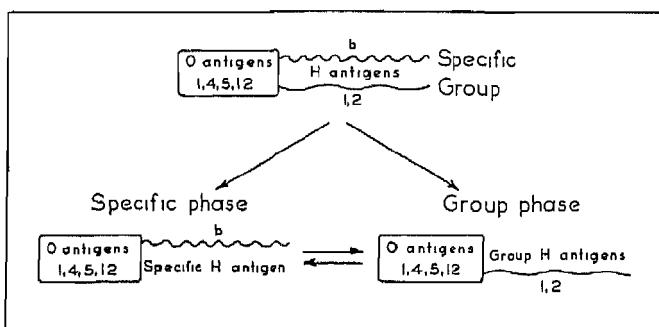


FIGURE 37. Phase variation, showing a culture in the diphasic form and the specific and group cultures which may be secured from it (diagrammatic).

of *Salmonella* and were designated *group antigens* (phase 2). Cultures possessed flagella in one phase or the other or both. Specific H antigens were labeled with lower case letters from a to z, and when the alphabet was exhausted subscript numerals were added to z (e.g.,  $z_2$ ,  $z_8$ ,  $z_{14}$ , etc.). Group H antigens were designated by Arabic numerals (1, 2, 3, etc.). It should be emphasized that the group or phase 2 antigens are just as specific as the phase 1 antigens and differ only in being more widely distributed.

Strains of *Salmonella* possessing flagellar antigens of both phases may be called *diphasic*, whereas those possessing flagellar antigens of only one phase may be termed *monophasic*. Diphasic organisms can be represented diagrammatically as in Figure 37. A culture in the specific phase (i.e., possessing specific H antigens) may spontaneously undergo variation to the group phase and *vice versa*.

Phase variation can be induced. To secure the specific phase from a group phase culture, the organism is grown in semisolid agar containing antiserum against the group phase. The spreading of group phase cells is inhibited by the antiserum, and motile cells in the specific phase can be isolated at a point distant from the original inoculation (Figure 38).

Repeated attempts are sometimes required to secure the second phase. This technique is often necessary to identify an organism. *S. paratyphi B* and *S. typhimurium* contain identical somatic and group flagellar antigens and can be differentiated only when in the specific phase.

Cultures in the specific phase can also sometimes be obtained from nutrient agar plates if a sufficient number of colonies is picked and tested.

The significant antigens of an organism are customarily listed in an expression known as an *antigenic formula*. The formula of *S. paratyphi B*,

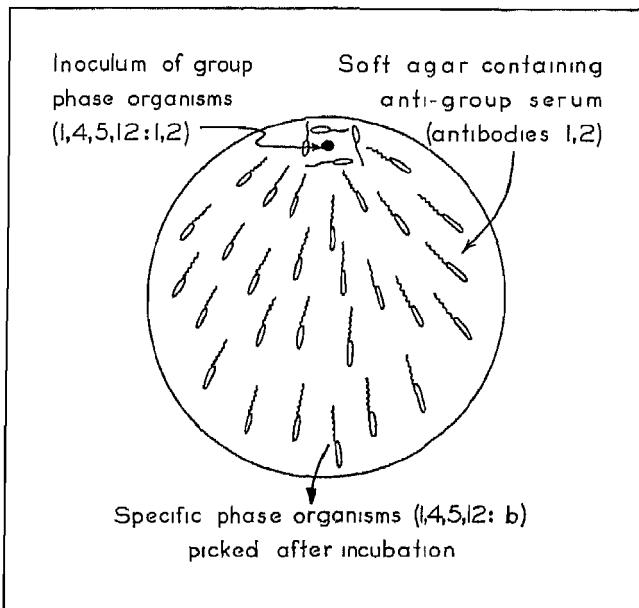


FIGURE 38. Isolation of a specific phase culture from a group phase culture by use of soft agar containing antiserum against the group antigens. Group phase organisms are prevented from spreading, but specific phase organisms will be motile and can be isolated at a point distant from the inoculation.

for example, is as follows: 1,4,5,12:b:1,2. The four somatic antigens are listed first, then the specific flagellar antigen, b, and finally the group flagellar antigens, 1,2. This formula means that *S. paratyphi B* is diphasic, and that cells in the specific phase possess antigens 1,4,5,12:b (see Figure 37), whereas those in the group phase contain 1,4,5,12:1,2. Some Salmonellas appear to be permanently monophasic with respect to either phase 1 or phase 2 antigens because the second phase has never been isolated. Until 1941, *S. paratyphi A* was considered to be in this group.

The distinction between specific and group phase antigens served a fairly definite purpose when the number of known Salmonellas was small. As the number of serotypes increased, the terms specific and group

antigens gradually lost significance. The confusion in phase variation increased when Kauffmann and Mitsui discovered a type of flagellar variation in which the second phase possessed e,n,x or other antigens previously considered to occur only in the specific phase.<sup>26</sup> This was called the  $\alpha$ - $\beta$  variation. Furthermore, Edwards and Bruner found that one phase of *S. worthington* possessed antigens l,w, and the other possessed z, both previously associated with phase 1.<sup>18</sup> As Kauffmann pointed out, however, both these phase variations are on the same level,<sup>25</sup> and there is

Table 42. Excerpts from Kauffmann-White *Salmonella* Diagnostic Antigenic Schema (1950)

TYPE	SOMATIC ANTIGENS	FLAGELLAR ANTIGENS	
		Phase 1	Phase 2
<i>S. paratyphi A</i>	1, 2, 12	a	---
<i>S. bispebjerg</i>	1, 4, 12	a	e, n, x
<i>S. paratyphi B</i>	1, 4, 5, 12	b	1, 2
<i>S. typhimurium</i>	1, 4, 5, 12	i	1, 2
<i>S. wien</i>	4, 12	b	l, w
<i>S. salinatis</i>	4, 12	d, e, h	d, e, n, z <sub>15</sub>
<i>S. montevideo</i>	6, 7	g, m, s	---
<i>S. potsdam</i>	6, 7	l, v	e, n, z <sub>15</sub>
<i>S. jerusalem</i>	6, 7	l, w	z <sub>10</sub>
<i>S. manchester</i>	6, 8	l, v	1, 7
<i>S. fayed</i>	6, 8	l, w	1, 2
<i>S. glostrup</i>	6, 8	z <sub>10</sub>	e, n, z <sub>15</sub>
<i>S. typhosa</i>	9, 12, Vi	d	---
<i>S. dar-es-salaam</i>	1, 9, 12	l, w	e, n
<i>S. meleagridis</i>	3, 10	c, h	l, w
<i>S. cambridge</i>	3, 15	c, h	l, w
<i>S. illinoi</i>	3, 15	z <sub>10</sub>	1, 5
<i>S. worthington</i>	1, 13, 23	l, w	z
<i>S. sundsvall</i>	1, 6, 14, 25	z	e, n, x

(From Kauffmann,<sup>25</sup> by permission.)

no fundamental difference whether antigens of phase 1 are alternative to phase 2 antigens or alternative to other phase 1 antigens. This is borne out by the work of Lederberg, who was able to replace the flagellar antigens of *S. miami* (9,12:a:1,5) with nine different phase 1 antigens and two different phase 2 antigens.<sup>29</sup> He also replaced the phase 1 antigen of *S. typhosa* (9,12:d-) with seven phase 1 antigens and one phase 2 antigen. The method of replacement (transduction) will be discussed on page 166.

The various *Salmonella* antigenic formulas are tabulated in the Kauffmann-White antigenic schema. This systematic listing is under constant revision as new serotypes are discovered. Excerpts from the 1950 schema are presented in Table 42 to illustrate different kinds of variation. Antigens such as n, x and z<sub>15</sub>, which are common among many strains and were formerly considered specific phase substances, are now listed solely in

phase 2. This shift to phase 2 will undoubtedly continue with other phase 1 antigens or antigen pairs such as e,h, and l,v and many more which are widely distributed. Certain other antigens like z, z<sub>10</sub>, and l,w are sometimes listed in phase 1 and sometimes in phase 2.

It should be noted that the *Salmonella* formulas in the diagnostic schema do not represent the complete antigenic structures of the organisms. Early investigators always employed the following form: 1,4,5,12 . . . : b . . . : 1,2 . . . , in which the series of dots after each group of symbols indicated that other antigens of the type designated could and probably did exist in the organism. It is unfortunate in one sense that this system is no longer used because the student is prone to accept what he sees in print as fixed and immutable. A formula written without express implication that other antigens are present leaves the impression that it indicates the entire antigenic makeup of the organism, which is far from the truth. Early investigators made some attempt to determine the complete antigenic composition of the various organisms, whereas present emphasis is principally upon diagnostic utility, for which only distinctive antigens suffice. It has already been mentioned (page 161) that only one somatic antigen is listed for most of the recently discovered *Salmonellas*, in contrast to three or four for each of those previously known. Furthermore, some flagellar antigens formerly listed<sup>1</sup> have been dropped from the schema in succeeding revisions.

**Induced Antigenic Variations.** Bruner and Edwards induced changes in the O antigens of *S. anatum* (3,10,26:e,h:1,6) by cultivation in semi-solid agar containing 3,10,26 antiserum which had been adsorbed with a type having antigens 3,15.<sup>9</sup> The form produced was indistinguishable from *S. newington* (3,15:e,h:1,6). The same results were obtained when 3,10,26 antiserum was adsorbed with *S. newington*, *S. cambridge* or *S. new brunswick*, all of which possess O antigens 3,15. The induced form resembling *S. newington* was then reverted to *S. anatum* by cultivation in adsorbed 3,15 antiserum. *S. meleagridis* (3,10,26:e,h:1,w) was similarly changed to a form identical with *S. cambridge* (3,15:e,h:1,w). It is not known whether these transformations depend upon residual antibodies in the adsorbed serums or upon bacterial components remaining in the adsorbed serums after removal of the adsorbing organisms by centrifugation. The new form in each case possessed the same somatic antigens as the adsorbing organism.

Numerous changes in flagellar antigens have been reported. Kauffmann cultivated a strain of the monophasic *S. typhosa* (9,12:d:---) in antiserum against antigen d and produced a form possessing another single flagellar antigen, i, never before found in *S. typhosa*.<sup>23</sup> The induced variant may be considered to contain an alternative single antigen in phase 1 (9,12:j:---).

Variants which are antigenically identical with other known types

have been produced by similar means. Two of the many transformations which have been accomplished are the conversion of *S. oranienburg* (6,7:m,t:---) into *S. montevideo* (6,7:g,m,s:---), and *S. thompson* (6,7:k:1,5...) into *S. cardiff* (6,7:k:1,10...).<sup>6,8</sup> Bruner and Edwards produced two alternate antigens, z<sub>5</sub> and z<sub>11</sub>, presumably phase 1, and a phase 2 form (1,5) of the normally monophasic *S. paratyphi A* (1,2,12:a:---).<sup>7</sup> Later a strain of *S. paratyphi A* identical with the induced phase 2 form was isolated in Egypt from the urine of a patient with enteric fever.<sup>12</sup>

It is sometimes possible to induce specifically directed changes in antigenic structure by repeated cultivation in antiserum-agar or by other methods. Altered serotypes of several Salmonellas were produced by Zinder and Lederberg through the process of *transduction*, defined as unilateral genetic exchange in which one character at a time is transferred from one bacterium to another.<sup>49</sup> Genetic material was transferred *via* a filterable agent in phage lysate of the donor organisms; selection of new or different serotypes was facilitated by the antiserum-agar technique. Suitable control tests indicated that the filterable agent played an active role in transduction. This method was used to produce eight new monophasic serotypes of *S. typhosa*. For example, the transduction from 9,12:d:--- to 9,12:i:--- was brought about by the filterable agent of *S. typhimurium* (1,4,5,12:i:1,2). Lederberg also reported (personal communication, 1952) use of *S. abony* (1,4,5,12:b:e,n,x) filterable agents in the following transductions:

1,9,12:a:1,5 <i>S. sendai</i>	→	1,9,12:a:e,n,x
1,4,5,12:i:1,2 <i>S. typhimurium</i>	→	1,4,5,12:b:1,2 <i>S. paratyphi B</i>
1,4,5,12:i:1,2 <i>S. typhimurium</i>	→	1,4,5,12:i:e,n,x

It is not known whether the changes induced by Bruner and Edwards in the O antigens of *S. anatum* and *S. meleagridis* (page 165) were caused by a similar filterable agent in the adsorbed serums which they employed.

In view of all these serologic transformations it is obvious that the original significance of specific-group, α-β, and other phase variations is rapidly being lost. The serology of flagellar variation is becoming a tool in the systematic study of groups of bacteria, and emphasis is properly being placed upon the distribution of flagellar antigens rather than upon their specificity.

One of the first examples of induced change in antigenic structure was the type transformation of pneumococci, demonstrated in 1928 by Griffith.<sup>18</sup> He injected mice subcutaneously with small amounts of non-encapsulated type 2 pneumococci in the rough (R) form, and simultaneously

introduced heat killed capsulated pneumococci of type 3. Some of the animals died and were found to be infected with capsulated type 3 pneumococci. Since the type 3 organisms injected had been killed by heat, it was obvious that the 2R organisms had been transformed into type 3 bacteria. The derived type 3 organisms maintained their type specificity through subsequent transfers in the laboratory. Several other similar pneumococcal type transformations were induced. The acquired specificity in each case was that of the dead, capsulated organism injected.

This transformation was carried out *in vitro* by Dawson and Sia in 1931.<sup>11</sup> They were successful in transforming a rough type 2 pneumococcus into a capsulated type 3 pneumococcus by cultivating the 2R organisms in broth containing anti-R serum and heat killed capsulated organisms of type 3 or a cell-free extract of them.

The substance derived from capsular material which induced this transformation was later shown by Avery and his colleagues to be a form of desoxyribonucleic acid.<sup>4</sup> It was effective in very minute amounts, 0.003 µg. being sufficient for two milliliters of broth inoculated with 0.000,005 milliliter of a culture of noncapsulated type 2 organisms. Serum appeared to be necessary. Effective serums usually contained some anti-R agglutinin which apparently clumped the growing pneumococci and helped to create and maintain local, perhaps reducing, conditions favorable for the transformation. Serum contributes in addition some form of phosphate and a protein component, both of which were found essential. These three constituents behaved as though they "conditioned" or sensitized R cells to the subsequent action of the transforming substance, desoxyribonucleic acid. Sensitization was only temporary, however, and was lost by delaying addition of desoxyribonucleic acid for three or four hours.

Other species, including *Hemophilus influenzae*, *E. coli* and *Sh. parasynteriae*, are also susceptible to serologic type transformation induced by specific desoxyribonucleic acids.

Induced changes in antigenic structure are of great evolutionary interest because they may indicate part of the natural mechanism and path by which the various serologic types of bacteria have arisen. They also emphasize the artificiality of species and even generic distinctions in such a continuous series of intergrading forms.

### References

1. Abramson, Boyd, Hooker, Porter and Purnell, 1945. Jour. Bact. 50, 15.
2. Alexander and Heidelberger, 1940. Jour. Exp. Med. 71, 1.
3. Andrewes, 1922. Jour. Path. Bact. 25, 505.
4. Avery, MacLeod and McCarty, 1944. Jour. Exp. Med. 79, 137.
5. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
6. Bruner, 1949. Jour. Bact. 57, 387.
7. Bruner and Edwards, 1941. Jour. Bact. 42, 467.

8. Bruner and Edwards, 1947. Jour. Bact. 53, 359.
9. Bruner and Edwards, 1948. Jour. Bact. 55, 449.
10. Coleman, 1950. In *Diagnostic Procedures and Reagents*, 3rd ed., American Public Health Association, New York.
11. Dawson and Sia, 1931. Jour. Exp. Med. 54, 681.
12. Edwards, Barnes and Babcock, 1950. Jour. Bact. 59, 135.
13. Edwards and Bruner, 1938. Jour. Hyg. 38, 716.
14. Favour, 1944. Bull. N. E. Med. Ctr. 6, 157.
15. Fleischhauer, 1937. Berl. tierärztl. Wschr. 53, 527.
16. Griffith, 1928. Jour. Hyg. 27, 113.
17. Heidelberger, 1939. Bact. Rev. 3, 49.
18. Heidelberger and Kabat, 1934. Jour. Exp. Med. 60, 643.
19. Heidelberger and Kabat, 1936. Jour. Exp. Med. 63, 737.
20. Hirst, 1941. Science 94, 22.
21. Hooker and Boyd, 1937. Jour. Immunol. 33, 337.
22. Kabat, Miller, Kaiser and Foster, 1945. Jour. Exp. Med. 81, 1.
23. Kauffman, 1936. Ztschr. Hyg. Infektkr. 119, 103.
24. Kauffman, 1947. Jour. Immunol. 57, 71.
25. Kauffman, 1950. *The Diagnosis of Salmonella Types*. Charles C Thomas, Springfield, Ill.
26. Kauffman and Mitsni, 1930. Ztschr. Hyg. Infektkr. 111, 749.
27. Kettel, 1928. Acta path. microbiol. Scand. 5, 306.
28. Landsteiner, 1945. *The Specificity of Serological Reactions*, 2nd ed., Harvard University Press, Cambridge, Mass.
29. Lederberg and Edwards, 1953. Jour. Immunol. 71, 232.
30. Marrack, 1938. *The Chemistry of Antigens and Antibodies*, H. M. Stationery Office, London.
31. Osterman and Rettger, 1941. Jour. Bact. 42, 721.
32. Peluffo, 1941. Proc. Soc. Exp. Biol. 48, 340.
33. Perlman and Goebel, 1946. Jour. Exp. Med. 84, 223, 235.
34. Pijper, 1938. Jour. Path. Bact. 47, 1.
35. Pijper, 1941. Jour. Path. Bact. 53, 431.
36. Sabin, Horsfall, Meyer, Scott and Snyder, 1952. In Rivers: *Viral and Rickettsial Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
37. Smith and Conant, 1952. *Zinsser's Textbook of Bacteriology*. 10th ed., Appleton-Century-Crofts, New York.
38. Smith and Reagh, 1903. Jour. Med. Res. 10, 89.
39. Stamp and Stone, 1944. Jour. Hyg. 43, 266.
40. Stuart, 1950. In *Diagnostic Procedures and Reagents*, 3rd ed., American Public Health Association, New York.
41. Stuart and Carpenter, 1949. Jour. Immunol. 61, 161.
42. Topley, Wilson and Duncan, 1935. Brit. Jour. Exp. Path. 16, 116.
43. Weil and Felix, 1916. Wien. klin. Wschr. 29, 33, 974.
44. Weil and Felix, 1917. Wien. klin. Wschr. 30, 1509.
45. Wheeler, 1938. Jour. Immunol. 34, 409.
46. Wiener and Herman, 1939. Jour. Immunol. 36, 255.
47. Wilson and Miles, 1946. *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., Edward Arnold & Co., London.
48. Wood, 1949 Dissertation, Johns Hopkins University.
49. Zinder and Lederberg, 1952. Jour. Bact. 64, 679.

## Chapter 9

### ISOHEMAGGLUTINATION

THE ERYTHROCYTES of one individual may be strongly agglutinated when mixed with the normal serum of another individual and incubated at body temperature. This phenomenon is known as isoagglutination. It first attracted great attention as an explanation of transfusion accidents in which hemoglobinuria, jaundice and occasionally death resulted. Normal antibodies in the serum of the recipient agglutinate the injected erythrocytes within the vascular system, producing embolism, or sensitize them to the hemolytic action of complement.

#### THE BLOOD GROUPS

Isohemagglutination was clearly described by Landsteiner in 1900.<sup>8</sup> Within the next two years the existence of four human blood groups had been reported.<sup>23</sup> These were differentiated by the presence or absence of one or both of agglutinogens A and B in the erythrocytes, and are usually designated by the letters, A, B, AB and O.

The normal antibodies corresponding to these agglutinogens are anti-A and anti-B, respectively. In no case did Landsteiner find anti-A in the serum of an individual who possessed antigen A in his cells, nor did anti-B occur in the serum of a person with antigen B. Furthermore, he observed that human serum always contained antibodies corresponding to the A and B agglutinogens which were missing from the red cells of the same individual. Thus, serum from a person of group O possessed agglutinins anti-A and anti-B, and so forth. Anti-A clumps cells containing agglutinogen A, and anti-B reacts similarly with cells containing the B antigen. Serums and cells of the four blood groups yield agglutination when mixed in the various combinations indicated by the + signs in Table 43.

The terminology of the blood groups was in a state of confusion for some time. Moss and Jansky independently suggested numerical designa-

tions but employed different numerals for two of the groups. Ultimately the "International" or Landsteiner classification (A, B, AB, O) was widely accepted, although occasional use is still made of the numerical systems.

Human group O cells, as well as A, B, and AB cells, stimulate the production in other animals of antibodies which react with all human red blood cells because they possess common human species antigens. Antisera produced in other animals against A, B, and AB cells contain in addition anti-A and/or anti-B agglutinins.

Blood grouping formerly consisted of testing the erythrocytes for agglutination with normal human serums containing anti-A and anti-B antibodies. Today, however, immune serums are available and are much more satisfactory if they have been rendered specific by suitable adsorption. The diagnostic reactions are readily determined from Table 43.

*Table 43. Agglutination in Mixtures of Serum and Red Blood Cells of the Four Principal Human Blood Groups*

BLOOD GROUP DESIGNATIONS			AGGLU-TINOGENS IN CELLS	HUMAN SERUMS CONTAINING AGGLUTININS				PER CENT IN U.S.A.
Inter-national	Jansky	Moss		Anti-A	Anti-B	Anti-A	None	
Agglutination								
O	I	IV	O	-	-	-	-	45
A	II	II	A	+	-	+	-	41
B	III	III	B	+	+	-	-	10
AB	IV	I	AB	+	+	+	-	4

Two techniques for determining blood groups are in common use. A test tube method is preferred for research, although most diagnostic laboratories employ slide agglutination. The test tube procedure requires 0.1 milliliter of test serum and 0.2 milliliter of erythrocyte suspension in small serologic tubes. The cells are secured from a vein or from an earlobe or finger and diluted in saline or saline-citrate solution in the proportion of one drop of blood to about one milliliter of diluting fluid. Tubes containing serum and cells are shaken and observed for agglutination after thirty minutes to one hour at 37° C.

The slide test is performed by placing drops of the test serums in opposite halves of a microscope slide and adding a drop of cell suspension to each. The reagents are mixed with a platinum loop or applicator stick and the slide is rocked to hasten agglutination and observed against a white background. Agglutination often occurs almost immediately and should take place within five or ten minutes.

The agglutinogens A and B are stable polysaccharide-amino acid complexes containing galactose and an amino sugar, N-acetylglucosamine, and

are closely related chemically. They are found not only in the erythrocytes but also in other tissue cells such as sperm, liver, muscle, spleen, kidney and lung cells. The blood group substances are also present in various body fluids of the majority of individuals, including saliva, seminal fluid, gastric juice and sweat. Those persons are known as "secretors." Blood group substances in such fluids can be detected by a precipitin test with anti-A and anti-B serums or by an inhibition test. The blood group substance reacts with the corresponding antibody and inhibits agglutination of homologous erythrocytes subsequently added. Saliva, for example, is mixed with anti-A and anti-B serums; A and B erythrocytes are then added to the respective serum-saliva mixtures. If agglutininogen A is present in the saliva it combines with anti-A and prevents agglutination of A red cells; likewise, agglutininogen B in the saliva prevents agglutination of B red cells. The capacity to secrete blood group substance is inherited.

The specific substances may be present in aqueous extracts of the organs of secretors, and similar blood group substances are detectable in alcoholic extracts of the organs of nonsecretors as well as secretors. It is therefore possible to determine the blood group of an individual by examination of almost any tissue.

The blood group A substance is chemically related to the Forssman haptene. Moreover, some anti-A rabbit serums lyse sheep erythrocytes, and certain rabbit antiserums against sheep erythrocytes agglutinate human cells containing A. The same or a similar substance is also present in various bacteria, the gastric mucosa of swine and horses, and in other widely distributed members of the animal and plant kingdoms. A substance related to B is also present in the gastric mucosa of individual horses.

Two major subdivisions of blood factor A were discovered by Landsteiner and Levine.<sup>11</sup> This agglutininogen is apparently composed of two separate factors, A<sub>1</sub> and A<sub>2</sub>, which occur either alone or associated with the B substance, so four subgroups are possible: A<sub>1</sub>, A<sub>2</sub>, A<sub>1</sub>B, A<sub>2</sub>B. Normal serums of groups B or O individuals may contain two qualitatively different antibodies, one of which reacts with both A<sub>1</sub> and A<sub>2</sub>, the other with only A<sub>1</sub>. Anti-A serum adsorbed with A<sub>2</sub> cells until the latter are no longer agglutinated still contains agglutinin for A<sub>1</sub> cells. A<sub>1</sub> cells are more sensitive than A<sub>2</sub> cells to serum hemolysis when freshly drawn anti-A (group B or O) serums are used. Cells of subgroups A<sub>1</sub> or A<sub>1</sub>B can also be differentiated by their stronger agglutination in inactivated (56° C. for fifteen minutes) anti-A test serums. The A<sub>1</sub> factor is five to six times as frequent as A<sub>2</sub>. Their occurrence is determined by inheritance, A<sub>1</sub> being dominant over A<sub>2</sub>.

The lifelong permanence of the blood groups is well confirmed. Agglutinogens A and B are already established in newborn infants and even in

fetuses. The normal isohemagglutinins in the serum, however, are not demonstrable at birth, but become evident within a few months, or at least by the age of two years.

The A and B characteristics of the erythrocytes are inherited according to Mendelian law, behaving as strict dominants. Neglecting the subgroups of A, there are six genotypes and the four phenotypes already described:

<i>Genotypes</i>	<i>Phenotypes</i>
AB	AB
AA}	A
AO}	
BB}	B
BO}	
OO	O

It is therefore possible to calculate the average frequency with which individuals of the various groups will result from any mating. A few examples are given in Table 44. Twenty-one different types of mating

*Table 44. Illustrations of Blood Group Inheritance*

GENOTYPES OF PARENTS	AA × AA	AA × BB	AO × OO	BO × AB	AO × BO
Genetic combinations	A A	A A	A O	B O	A O
	A   AA AA	B   AB AB	O   AO OO	A   AB AO	B   AB BO
	A   AA AA	B   AB AB	O   AO OO	B   BB BO	O   AO OO
Genotypes of children	AA	AB	AO OO	AO BO BB AB	OO AO BO AB
Phenotypes of children	A	AB	A O	A B AB	O A B AB
Expected percentage	100	100	50 50	25 50 25	25 25 25 25

are possible. From charts such as those illustrated, it may be ascertained whether a given child could or could not be the offspring of certain parents. These facts are useful in cases of disputed parentage and questioned paternity, as will be discussed later.

#### THE MNS AND P SYSTEMS

In 1927 Landsteiner and Levine immunized rabbits with the cells of a number of different group O individuals. Adsorption of the antiserums with some group O erythrocytes removed all agglutinins; adsorption with

other group O cells, however, left agglutinins which reacted with cells of certain individuals.<sup>10, 12</sup> The rabbit immune serums were used to describe two agglutinogens, M and N, independent of the A and B factors. All human red blood cells contain either M or N or both. They are inherited according to Mendelian principles. Consequently, there are three genotypes, MM, NN, MN, and corresponding phenotypes, M, N, MN. Blood specimens are classified as AM, BM, ON, ABN, ABMN, . . . , depending on the presence in the cells of the A, B, M and N factors.

The M and N agglutinogens are detected with rabbit immune serums because normal isoagglutinins for M and N do not occur in humans. Rabbits are immunized with OM and ON cells and the antiserums are adsorbed with cells containing N and M (e.g., AN and AM), respectively, to remove human species agglutinins. A single adsorption suffices to remove the species antibodies. Despite the fact that M cells are used to adsorb N serum and *vice versa*, repeated adsorption markedly reduces or eliminates the type specific agglutinins. Why this is so is not clear.

Like the A and B antigens, M and N are well established in the newborn and are stable throughout life. Type MN is found in about 50 per cent of individuals, M in 30 per cent and N in about 20 per cent.

Walsh and Montgomery in 1947 discovered an antigenic factor, S,<sup>24</sup> and Levine *et al.* in 1951 reported a related antigen, s.<sup>17</sup> Erythrocytes were found containing either S, s or Ss, but none lacking both factors. S was present in about 54 per cent of individuals and s in 88 per cent. The genetic relationship of S to M and N was indicated by observations of Sanger and Race that different percentages of positive reactions were obtained with anti-S serum according to the MN type of blood tested.<sup>21</sup> S was present in 73.4 per cent of type M individuals, 54.1 per cent of type MN and 32.3 per cent of type N persons. Antigen S is inherited as a simple Mendelian dominant.

Landsteiner and Levine encountered serums which reacted with another agglutinogen of human erythrocytes, P.<sup>10, 12</sup> This factor is also inherited as a Mendelian dominant. Blood cells possessing it are designated P+, those lacking it P-. Antigen P is present in about three-quarters of white individuals and nearly all Negroes. Anti-P agglutinin has been reported in some human blood serums, but typing is usually accomplished by means of various animal bloods which normally contain anti-P. Serums of horses and hogs are especially useful.

#### THE Rh FACTOR

A recent development of great interest is the discovery of the Rh factor and its role in certain disease conditions. Previous to 1940 there were numerous reports of newborn infants with general edema and more or less marked anemia, a condition called *erythroblastosis fetalis* or

*hemolytic disease of the newborn.* In 1939, Levine and Stetson observed a transfusion reaction in a pregnant woman following administration of blood from her husband.<sup>18</sup> Serum from this woman contained an agglutinin which reacted with about 80 per cent of group O bloods examined.

The following year, Landsteiner and Wiener, investigating the M factor in monkeys, found that serums produced in rabbits inoculated with rhesus monkey blood possessed antibodies which agglutinated the erythrocytes of about 85 per cent of humans tested.<sup>14</sup> They named the red cell agglutinogen the *Rh antigen*, because it also occurred in rhesus monkey blood cells, and designated as Rh positive those individuals who possessed it. Wiener and Peters observed that after several transfusions of Rh positive blood into an Rh negative individual, the recipient might show increasing signs of transfusion reaction and that his serum contained agglutinins for the Rh factor.<sup>26</sup>

Levine then found that the patient who had had a transfusion reaction in 1939 possessed Rh antibody in her serum and that her husband's red cells were Rh positive.<sup>15, 16</sup> He suggested that this woman, who was Rh negative, had been immunized ("isoimmunized") by the Rh factor of the fetus which was inherited from the Rh positive father. Although only about 15 per cent of the normal population is Rh negative, nearly all mothers of erythroblastotic infants are Rh negative, but the fathers and babies are Rh positive.

The role of Rh in transfusion reactions appears fairly clear. An Rh negative recipient of Rh positive blood produces antibody against the Rh antigen. If sufficient Rh antibody is formed, subsequent transfusions with Rh positive blood provide opportunity for intravascular reaction with the introduced erythrocytes. The Rh factor may be poorly antigenic in some individuals, and consequently several transfusions can often be given before the recipient develops enough antibody to cause complications.

Present explanations of erythroblastosis fetalis closely follow the original suggestion of Levine and Stetson. The child of an Rh positive father and Rh negative mother may possess Rh positive red blood cells. Fetal erythrocytes or the slightly soluble Rh substance derived from them pass through the single layer of cells separating fetal and maternal circulations and stimulate formation of Rh antibodies by the mother. Some of these antibodies then recross the placenta into the fetus, where they react with the Rh positive red cells, producing anemia and jaundice. The anemic condition causes the fetus to produce immature red blood cells (erythroblasts) at a high rate.

Erythroblastosis fetalis does not occur in all cases in which it appears theoretically possible. The percentage distribution of Rh positive and Rh negative individuals in the general population (85 and 15 per cent, respectively) indicates that approximately 13 per cent of matings involve an Rh positive father and an Rh negative mother. Actually, erythro-

blastosis occurs in only one of every 200 to 400 births, or less than 5 per cent of cases in which it might be expected. Many factors probably contribute to this low percentage. Only about 50 per cent of Rh positive persons are homozygous for Rh antigen. Half the children of heterozygous fathers and Rh negative mothers are therefore Rh negative. Furthermore, erythroblastosis fetalis is relatively rare in a first pregnancy but becomes more common with succeeding pregnancies.

Six major Rh factors are now known. They are designated in the Fisher-Race system by the letters, C, D, E, c, d, e, and in the terminology of Wiener by the corresponding symbols rh', Rh<sub>o</sub>, rh'', hr', Hr<sub>o</sub>, hr''. The antigens are paired as follows: C and c, D and d, E and e; every erythrocyte contains one or both antigens of each pair. Red cells from one individual, for example, may contain CDE; from another individual, CcDdEe. The two systems of nomenclature and the incidence of the various antigens in human erythrocytes are listed in Table 45.<sup>10</sup>

*Table 45. Nomenclature and Incidence in Caucasoids of Rh-Hr Antigens and Antibodies*

FISHER-RACE SYSTEM	WIENER SYSTEM	INCIDENCE OF	
		Antigens	Antibodies
D	Rh <sub>o</sub>	85%	Very frequent
d	Hr <sub>o</sub>	63%	Extremely rare
C	rh'	73%	Very rare
c	hr'	80%	Occasional
E	rh''	30%	Occasional
e	hr''	97%	Very rare

(From Levine,<sup>15a</sup> by permission. Copyright by the National Foundation for Infantile Paralysis, Inc.)

The Rh antigen first discovered was that now designated D (Rh<sub>o</sub>) and occurs in about 85 per cent of individuals. There are theoretically twenty-seven possible combinations of the six Rh subtypes, nearly all of which have been found, although many in only a small percentage of persons. Antigen D is most important clinically, and d and c are least important. C and E occur only infrequently in the absence of D (about 1 per cent of individuals each). Partly for these reasons and partly because of difficulty in securing test serums for c, d and e, routine Rh typing usually employs only serums containing anti-C, anti-D and anti-E. Eight subtypes are differentiated by use of these three antibodies (Table 46).<sup>10</sup> In the absence of a positive reaction with one of the three test serums, the corresponding paired antigen of the cde series is assumed to be present and is often included in the phenotypic formula. For practical purposes individuals possessing antigen D are considered Rh positive, those lacking D (and therefore possessing d) are Rh negative.

Table 46. Rh Subtypes Differentiated by the Three Principal Anti-Rh Serums

	REACTION WITH ANTI-D	ANTIGENS PRESENT	INCIDENCE	REACTION WITH ANTI-C	ANTIGENS PRESENT	INCIDENCE	REACTION WITH ANTI-E	ANTIGENS PRESENT (PHENOTYPE)	INCIDENCE
Rh+	+	D	85%	+	DC	66%	+	DCE	15%
				-	Dc	19%	+	DCe	51%
Rh-	-	d	15%	+	dC	1%	-	Dce	16.5%
				-	dc	14%	+	dCE	2.5%
							+	Rare	
							-	dCe	1%
							+	dcE	0.5%
							-	dce	13.5%

(From Levine and Wigod,<sup>19</sup> revised by Dr. Levine, 1955. By permission.)

Several different kinds of antibody may be present in the serums of individuals isoimmunized against Rh antigens (Table 47). Classic agglutinating antibodies aggregate homologous red blood cells suspended in saline. These are known as "complete antibodies" or "saline agglutinins." So-called "incomplete antibodies" fail to agglutinate saline suspensions of homologous erythrocytes, but their specificity can be shown by one or more of several other methods.

*Table 47. Types and Methods of Detecting Rh Antibodies*

SYNONYMS	1	2	3	4
	COMPLETE ANTIBODY	INCOMPLETE ANTIBODIES		
	Saline agglutinin	Albumin agglutinins		
Agglutination of R.B.C. in saline	+	—	—	—
Agglutination of R.B.C. in protein, etc	+*	+	+	—
Blocking test	—*	+	—	—
Coombs test	+*	+	+	+
Agglutination of trypsinized R.B.C. in saline	+*	+	+	—

\* Not ordinarily tested because these antibodies are readily detected by agglutination of saline suspensions of R.B.C.

Some incomplete antibodies agglutinate red cells suspended in human serum, bovine serum albumin or other protein solutions, and have been designated "albumin agglutinins" because albumin appears to be most effective in providing the nonspecific linkage or condition necessary for clumping. Protein *per se* does not appear to be essential, inasmuch as erythrocytes suspended in polyvinyl alcohol, gum acacia or certain other menstruums can also be aggregated by albumin agglutinins. One kind of incomplete antibody can also be demonstrated by the "blocking test." Saline suspensions of erythrocytes mixed with certain Rh antiserums fail to agglutinate when subsequently mixed with homologous saline agglutinin. "Blocking antibodies" apparently combine with the corresponding agglutinogens and specifically prevent later reaction with saline agglutinins.

The Coombs test is a very sensitive method of detecting both complete

and incomplete antibodies.<sup>4</sup> Saline suspensions of Rh positive red cells are incubated with Rh antiserum, washed with saline, and mixed with anti-human globulin serum. Rh antibody first combines specifically with its homologous agglutinogen and then, as a globulin, reacts with the antiglobulin to cause agglutination of the erythrocytes.

Treatment of erythrocytes with trypsin, pepsin or other proteolytic enzymes provides another delicate reagent for detecting and identifying certain incomplete Rh antibodies. Trypsinized red cells in saline suspension agglutinate when mixed with homologous albumin agglutinins. The enzyme apparently digests some surface protein of the red cells and decreases resistance to clumping.

There is growing evidence that the expression "incomplete antibody" may be inaccurate. It was originally assumed that such antibodies were monovalent instead of bivalent or polyvalent and hence lacked sufficient antibody combining sites to participate in lattice formation. They could combine with homologous antigenic determinants of erythrocytes as in the blocking test or the Coombs reaction, but were unable to cause aggregation.

There still appears to be no satisfactory explanation of the role of protein, gum acacia and other colloidal substances in "albumin agglutination." However, the increased sensitivity of trypsinized red cells to agglutination by albumin antibodies has raised the question whether such antibodies are really monovalent or whether the enzyme merely removes a protein inhibitor of agglutination from the erythrocytes. Zwicker *et al.* assumed the antibody to be potentially bivalent.<sup>27</sup> They noted that a carboxypeptidase, which attacks protein at certain terminal amino acid residues instead of within the molecule, is also effective in rendering saline suspensions of red cells agglutinable by "incomplete antibodies," and concluded that only terminal groups of surface proteins are degraded. Raffel postulated that the combining sites of saline agglutinins are situated far apart, as at opposite ends of the molecule, whereas "incomplete antibody" possesses two combining sites located close together.<sup>20</sup> He also suggested that the Rh agglutinogens are relatively deep within the erythrocyte stromata and that when one combining site of incomplete antibody reacts with its Rh antigen, the other combining site is masked or occluded and cannot attach to another cell. Enzymatic digestion removes a surface protein layer and permits union of the second antibody site and another cell.

Rh typing serums are secured from humans immunized against the various Rh antigens. Principal sources of potent antisera are Rh negative mothers of erythroblastotic infants and volunteer Rh negative donors who have received injections of Rh positive blood cells. Typing serums as purchased at present are labeled with both the Wiener and Fisher-Race designations; for example, Anti-Rh<sub>o</sub> (Anti-D). Routine typing of blood

cells includes tests with one to four monovalent or polyvalent antiserums (e.g., anti-D, anti-CD, anti-DE, anti-c).

Most typing serums in use today contain blocking antibodies, so saline must not be used as a diluent. Fresh whole blood or blood taken into a tube containing dry oxalate as anticoagulant is employed in the slide test. The test serum and blood are mixed on a microscope slide or glass plate and rocked slowly over a lamp or other source of heat which maintains a temperature of about 40° C. Agglutination should be seen within thirty to sixty seconds, and at the end of two minutes nearly all red cells should be clumped into large masses. These tests are observed without the aid of the microscope and are discarded after two or three minutes to avoid errors in reading caused by drying or rouleaux formation.

#### INDIVIDUALITY OF HUMAN BLOOD

It is evident at this point that human blood displays considerable individuality. In addition to the six combinations in the AB system, including the subgroups of A, there are three combinations of MN, three of S and s, two involving P, and two concerned with secreting ability. This makes a total of 216 different combinations, neglecting the Rh factor. The subgroups of this antigen system raise the total to many hundred. In addition to the groups or types already discussed, at least seventeen others have been described, some of which have been studied only in isolated cases or relatively small populations. Certain of these factors participate in individual transfusion reactions and in hemolytic disease, but their general importance does not seem to be great. It can readily be calculated that the blood types now known permit the existence of many million different kinds of human red cells, and it has been postulated that each person has his own peculiar blood type.

Human individuality is further indicated by attempts to transplant tissues such as skin.<sup>3</sup> These attempts are almost universally unsuccessful; the transplanted tissue incites an antigen-antibody reaction which leads to its eventual death. There are two situations, however, in which transplants grow and establish themselves in the recipient. Tissues may be transferred from one individual to his identical twin, and corneal grafts are often successful in any recipient. The first instance involves genetically (and therefore presumably antigenically) identical individuals, and the second involves a tissue which has a low blood supply and hence in its normal situation does not incite antibody formation or encounter antibodies.

#### APPLICATIONS OF BLOOD GROUPING

**Preparation for Transfusion.** One of the most important applications of blood grouping is that concerned in preparation for transfusion. It is

advisable always to employ blood of the same AB group as that of the recipient. This means that the bloods of the recipient and of the prospective donors must be tested. Ideally, not only are the agglutinogens in the respective cells determined but also the agglutinins in the serums, although the latter tests are often omitted. The importance of Rh isoimmunization also makes it necessary to determine the Rh type of recipient and donor, lest Rh positive blood be introduced into an Rh negative recipient and induce sensitivity. Presence of the M, N, S and P factors is not usually determined, because they are not strongly antigenic in man. However, complications may arise in repeated transfusions as a result of immunization against any of the blood cell agglutinogens. Natural and immune agglutinins can be detected by "cross match" tests, which are always performed. Serum from the patient is tested for its power to agglutinate cells of the donor. The reverse cross match is also recommended if time permits.

The chief danger from incompatible transfusions lies in the fate of the injected cells. The patient may suffer a transfusion reaction if there is sufficient antibody in his circulation to cause agglutination or hemolysis of the donor's erythrocytes. Despite the presence of anti-A and anti-B agglutinins in the serums of group O individuals, their blood can usually be given to persons of any group. The titer of normal isohemagglutinins is ordinarily low, and they are diluted by the blood of the recipient to such an extent that they do not agglutinate or hemolyze the patient's cells. Members of group O are called "universal donors." Group AB individuals, or "universal recipients," can accept blood of any group because their serum does not contain anti-A or anti-B, and any introduced agglutinins are diluted within the vascular system. Heterologous transfusions of these kinds are not recommended when homologous blood can be obtained without too much difficulty.

The hemagglutination reaction is used to determine the survival time of transfused blood. For this purpose the donor and recipient must be of different types. Group O blood, for example, is transfused into a group A recipient. The group O cells mingle with those of the patient and persist in the blood for some time. Samples of the recipient's blood are mixed with anti-A serum, and only his own cells are agglutinated; the type O cells from the donor remain dispersed. The test is observed microscopically and the agglutinated and unagglutinated cells are counted. Other blood types may be used in the same manner. Tests of this sort indicate that the survival time of introduced cells is as great as 80 to 120 days.<sup>1, 22</sup> Blood cells stored for twenty-one days before transfusion survive only about twenty-four hours in the circulation of the recipient.

**Medico-Legal Applications.** Problems of relationship including disputed paternity may sometimes be solved by isohemagglutination since blood group characteristics are inherited in a known manner (Table 44).

This method has been used in the past to identify babies born at the same time in a hospital and accidentally given to the wrong parents, although present practices almost certainly obviate this chance of confusion. One of the best known cases of this kind occurred when Mrs. B., on returning home from the hospital, found that her baby had a piece of adhesive tape on its back with the name W.<sup>22</sup> Mrs. W. likewise found her baby to be labeled B. Blood tests of the two babies and four parents showed the following results:

<i>Group</i>	<i>Group</i>
Mr. W. .... O	Mr. B. ... AB
Mrs. W. .... O	Mrs. B. .... O
Baby B. .... A	Baby W. .... O

Obviously Baby B. could not have been the child of Mr. and Mrs. W. nor could Mr. and Mrs. B. have been the parents of Baby W. The children had been accidentally switched before the mothers took them home, and the labels they bore were correct.

The known information in cases of disputed paternity consists of the blood characteristics of the mother and of the child. The A, B, M, N, Rh and other factors are used. The rules of genetics sometimes permit exclusion of certain men as fathers of the child in question. It is never possible to state that the putative father is in fact the real father. It can only be stated that he may be or cannot be the father. Table 48 shows the AB blood groups possible or impossible from any mating combination.<sup>22</sup> Similar tables can readily be prepared for the MN, Rh, and other systems. The use of such tables is self apparent. Taking into account the frequency distribution of the various blood groups in this country, an innocent man has, on the average, one chance in seven of proving non-paternity by the AB groups alone. Addition of the M and N factors increases his chance of establishing nonpaternity to about one in three.

There are many other forensic applications of hemagglutination in the solution of violent crimes such as murder and rape, and in the identification of individuals concerned in inheritance suits and other legal proceedings. Under suitable conditions and by use of extremely careful technique it is possible to determine the blood type of an individual from almost any kind of specimen: blood stain, tissue fragment, saliva (e.g., a cigarette stub), semen and even from a corpse.

Stains should first be proven to be blood by chemical tests and to be human blood by the precipitin test. There are two chief methods for typing a blood stain. The first is by detection of isoagglutinins in relatively fresh specimens. A small flake of the dried smear or some of the powdered material is placed on a slide and near it a suspension of group A erythrocytes. A similar preparation is made with group B cells. Cover glasses are put in position and adjusted so that the specimen and cells

make contact, and the preparations are observed for agglutination of the known erythrocytes near the region of contact. A second method, suitable for older stains, is to detect agglutinogens in the specimen by the inhibition test previously described (page 171).

It is imperative that numerous controls be included in these tests, using all blood types and including an extract of the substrate (cloth, etc.) from which the blood stain was taken. Fresh blood stains are most satisfactory, but fairly reliable results have been obtained with stains as old as forty years.

*Table 48. Blood Groups of Offspring Possible or Impossible from Any Combination of Parents*

ALLEGED FATHER	KNOWN MOTHER	POSSIBLE CHILDREN	CHILDREN NOT POSSIBLE (decisive for nonpaternity)
O	O	O	A, B, (AB)
O	A	O, A	B, AB
O	B	O, B	A, AB
O	AB	A, B	AB, (O)
A	O	O, A	B, (AB)
A	A	O, A	B, AB
A	B	O, A, B, AB	
A	AB	A, B, AB	(O)
B	O	O, B	A, (AB)
B	A	O, A, B, AB	
B	B	O, B	A, AB
B	AB	A, B, AB	(O)
AB	O	A, B	O, (AB)
AB	A	A, B, AB	O
AB	B	A, B, AB	O
AB	AB	A, B, AB	(O)

Blood groups in parentheses represent individuals who could not be children of the corresponding mothers.

(From F. Schiff and W. C. Boyd. *Blood Grouping Technic*. Copyright 1942, Interscience Publishers, Inc., New York-London.<sup>22</sup>)

The blood type of a corpse can be determined quite readily, if it is not too old, from examination of the clotted blood. After partial decomposition, it may still be possible to secure pericardial fluid which is satisfactory for typing. Muscles, various organs and even bones may also be employed. Agglutinogens have been detected in corpses of considerable age. Several Egyptian mummies have been claimed to possess group A or B substances after about 5,000 years of preservation.<sup>2</sup>

Other applications of the blood grouping reactions are mainly of scientific interest, although some have practical significance. The blood

group A and B substances in purified form are now often added to blood (e.g., type O) used in transfusions to combine with and inactivate anti-A and anti-B antibodies derived from the donor and thus reduce a possible cause of transfusion reaction.

**Phylogeny of Primates.** The occurrence of human blood group substances in apes and monkeys is of interest to students of evolution. As might be anticipated, the higher an animal is in the primate scale, the more closely its blood characteristics resemble those of man. Substances indistinguishable from A and B are widely distributed among the higher primates, and in some of the lower members of the group there are substances which resemble A and B closely but are distinguishable from

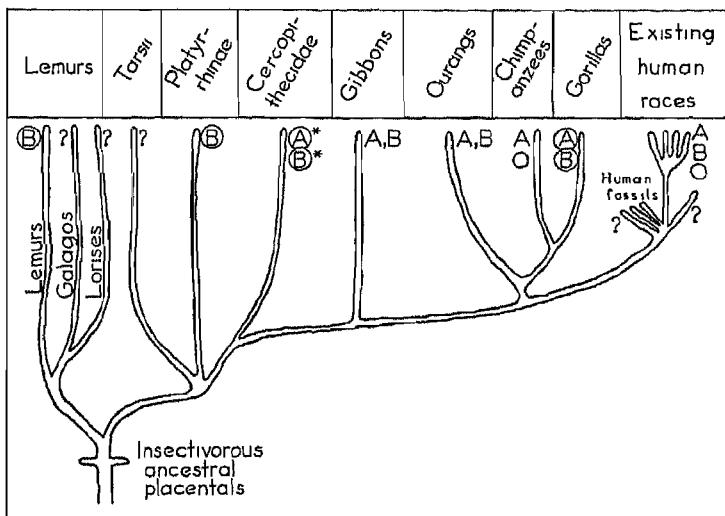


FIGURE 39. Distribution of blood factors A, B and O in man and other primates. Antigens within circles are similar to A and B respectively. Asterisks designate antigens present in tissues and secretions. (Adapted from Landsteiner: *The Specificity of Serological Reactions*, 2nd ed., 1945, Harvard University Press, Cambridge, Mass.)

them. Likewise, a substance capable of reacting with anti-M serums has been found in certain primates. Much of the work with monkeys and anthropoids is summarized in Figure 39.<sup>9</sup>

Blood group determinations have provided anthropologists with an objective tool for the study of various racial and other groups. It is noted, for example, that the factor B occurs with unusual frequency in central Asia, whereas A is especially prominent in the Spanish peninsula, Scandinavia and certain isolated areas of North America. A few figures illustrating the distribution of the various groups are presented in Table 49.<sup>25</sup>

**Hemagglutination in Studies of Animal Genetics.** Landsteiner, in

1924, was apparently the first to perform comparative experiments on the antigenic composition of animal red blood cells.<sup>18</sup> He employed anti-serums against horse, donkey and mule cells and showed that these three kinds of erythrocytes are related but demonstrated by adsorption that they are not identical. It will be recalled that the mule is the hybrid resulting from mating a female horse with a male donkey. The results of Landsteiner's experiments indicated that red cells of all three animals

*Table 49. Distribution of the AB Blood Groups among Selected Human Populations*

	PER CENT OF BLOOD GROUP			
	O	A	B	AB
American Indians				
Blackfoot and Blood (pure)	22.8	76.7	0.0	1.0
Navajo	69.1	30.6	0.0	0.0
Peru (pure)	100.0	0.0	0.0	0.0
Yucatan (pure)	97.7	1.3	0.5	0.5
Arabs	34.1	30.8	28.9	6.2
Australian aborigines	53.1	44.7	2.1	0.0
Bushmen	56.1	29.6	7.5	6.8
Chinese (Peking)	30.0	25.0	35.0	10.0
Danes	42.0	43.6	10.4	3.9
Egypt (Moslems, Cairo)	26.9	36.9	26.4	9.8
English (London)	40.4	46.8	9.6	3.2
Eskimos (Baffin Land)	55.4	43.6	0.6	0.6
French	43.2	42.6	11.2	3.0
Germans	39.1	43.5	12.5	4.9
Hindus	30.2	24.5	37.2	8.1
Japanese	31.1	36.7	22.7	9.5
Negroes (Congo)	45.6	22.2	24.2	8.0
(North America)	47.0	28.0	20.0	5.0
North Africans (Algiers)	39.0	37.6	18.6	4.6
Norwegians	39.2	48.7	8.5	3.5
Russians	32.9	35.6	23.2	8.1
Spanish	43.6	51.2	3.9	1.1
United States	45.0	41.0	10.0	4.0

(From Wiener,<sup>25</sup> by permission.)

share a common antigen but that horse and donkey cells each possess specific components and that some of these antigens from both horse and donkey appear in the mule.

A slightly different situation in hybrids was shown by Irwin with two species of birds, pearlneck (*Streptopelia chinensis*) and ring dove (*S. risoria*).<sup>7</sup> Cross-agglutination and reciprocal adsorption revealed that not only did the F<sub>1</sub> hybrid of these two birds possess the blood cell antigens of each parent, but in addition an antigen of its own. Similar results were obtained with certain species of ducks.

Ferguson and co-workers reported the presence of forty distinct antigenic factors in the erythrocytes of cattle.<sup>5, 6</sup> Many of these were found to be inherited singly. The number of different combinations which might occur,  $2^{40}$ , or over a trillion, was considered to indicate that biochemical individuality within the bovine species is a definite possibility.

The use of serologic techniques in genetics is expanding. It is generally agreed that there is a one-to-one relationship between genes and antigens and that detection of antigens provides an objective and useful tool for study of genes. The term *immunogenetics* is applied to this field of research, which is fruitful from the theoretic standpoint and offers unrealized possibilities for practical application in breeding.

### References

1. Ashby, 1919. *Jour. Exp. Med.* **29**, 267.
2. Boyd and Boyd, 1934. *Proc. Soc. Exp. Biol.* **31**, 671.
3. Burnet and Fenner, 1949. *The Production of Antibodies*, The Macmillan Co., Ltd., Melbourne.
4. Coombs, Mourant and Race, 1945. *Brit. Jour. Exp. Path.* **26**, 255.
5. Ferguson, Stormont and Irwin, 1942. *Jour. Immunol.* **44**, 147.
6. Irwin, 1947. In Demerec, M. (editor), *Advances in Genetics I*, 133. Academic Press, New York.
7. Irwin and Cole, 1936. *Jour. Exp. Zool.* **73**, 85.
8. Landsteiner, 1900. *Zentralbl. f. Bakter. I.* **27**, 357.
9. Landsteiner, 1945. *The Specificity of Serological Reactions*, 2nd ed., Harvard University Press, Cambridge, Mass.
10. Landsteiner and Levine, 1927. *Jour. Exp. Med.* **47**, 757.
11. Landsteiner and Levine, 1927. *Proc. Soc. Exp. Biol.* **24**, 600, 941.
12. Landsteiner and Levine, 1928. *Jour. Exp. Med.* **48**, 731.
13. Landsteiner and Van der Scheer, 1924. *Jour. Immunol.* **9**, 213.
14. Landsteiner and Wiener, 1940. *Proc. Soc. Exp. Biol.* **43**, 223.
15. Levine, 1944. *Arch. Path.* **37**, 83.
- 15a. Levine, 1952. In Dubos, *Bacterial and Mycotic Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
16. Levine and Katzin, 1940. *Proc. Soc. Exp. Biol.* **45**, 343.
17. Levine, Kuhmichel, Wigod and Koch, 1951. *Proc. Soc. Exp. Biol.* **78**, 218.
18. Levine and Stetson, 1939. *Jour. Amer. Med. Assoc.* **113**, 126.
19. Levine and Wigod, 1950. In *Diagnostic Procedures and Reagents*, 3rd ed., American Public Health Association, New York.
20. Raffel, 1953. *Immunity, Hypersensitivity, Serology*, Appleton-Century-Crofts, New York.
21. Sanger, Race, Walsh and Montgomery, 1948. *Heredity* **2**, 131.
22. Schiff and Boyd, 1942. *Blood Grouping Technic*, Interscience Publishers, Inc., New York.
23. Von Decastello and Sturli, 1902. *Münch. med. Wschr.* **49**, 1090.
24. Walsh and Montgomery, 1947. *Nature* **160**, 504.
25. Wiener, 1943. *Blood Groups and Transfusion*, 3rd ed., Charles C Thomas, Springfield, Ill.
26. Wiener and Peters, 1940. *Ann. Int. Med.* **13**, 2306.
27. Zwicker, Giordano and Hoyt, 1952. *Jour. Immunol.* **69**, 415.

## Chapter 10

### TOXINS AND ANTITOXINS

#### TOXINS<sup>97</sup>

**Exotoxins versus Endotoxins.** Bacterial exotoxins are released or secreted into the surrounding medium during the life of the cells, whereas endotoxins are held within the cells until death and autolysis have occurred. Some of the properties of these substances are compared in Table 50.

*Table 50. Properties of Exotoxins and Endotoxins*

	EXOTOXINS	ENDOTOXINS
Source	Excreted by living cells	Liberated after autolysis
Thermostability	Most are inactivated at 60°–80° C	May resist 120° C. for 1 hour
M.L.D.	Small	Large
Pharmacologic action	Usually specific	Relatively nonspecific
Antigenicity	High	Low to moderate
Neutralizing power of antitoxin	High	Low

Exotoxins cannot be distinguished from endotoxins as clearly as was at first thought or as the tabulation appears to indicate. Some microbial products possess properties of both exotoxins and endotoxins. Filtrates of the meningococcus, for example, are quite toxic, but it is not clear whether such toxicity is attributable to an exotoxin or an endotoxin. This organism dies and autolyzes so readily and rapidly that it is practically impossible to determine by the customary method of filtration whether a soluble poison is excreted from the living cells. Likewise, some substances such as staphylococcus enterotoxin appear to be exotoxins but possess a high degree of thermostability. Furthermore, the differences in minimal lethal dose between exotoxins and endotoxins are not as sharp as once believed,

and there is a considerable range of variation among substances otherwise behaving as exotoxins. *Clostridium perfringens* toxin kills guinea pigs in doses of 0.2 to 0.3 milliliter, whereas the same animal species is killed by 0.0001 to 0.0005 milliliter of crude *Cl. tetani* or *Cl. botulinum* toxins, or by 0.0004 to 0.002 milliliter of crude diphtheria toxin. The minimal lethal dose for mice of purified endotoxic glucolipids from gram negative bacteria is 0.1 to 1.0 milligram; for the same animals the minimal lethal dose of diphtheria toxin is approximately 0.006 milligram, and of tetanus toxin about 0.000,000,1 milligram.

Specificity of site of action is characteristic of most exotoxins. Tetanus and botulinum exotoxins affect only nerve tissues. Tetanus exotoxin acts upon the neuromuscular junctions of voluntary muscles and may also damage the anterior horn cells of the central nervous system, and botulinum exotoxin is specifically toxic to certain peripheral motor nerves. Hemolytic streptococcus filtrates attack vascular endothelium, and *Shigella dysenteriae* produces a neurotoxin. Diphtheria exotoxin damages a wider variety of cells than many other exotoxins, including the skin, heart muscle, adrenals, liver and nerves.

The response to endotoxin is relatively uniform, no matter from what organism derived. Typical effects consist of weakness, diarrhea and increase in blood sugar followed by decrease to subnormal.

Difference in antigenic capacity is the best single criterion distinguishing exotoxins from endotoxins. Zinsser, Enders and Fothergill stated, "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."<sup>140</sup> Bacterial exotoxins may therefore be defined as toxic, antigenic substances secreted by living microorganisms. Some plants and animals also produce highly toxic, antigenic substances, such as ricin from the castor bean, abrin from the seed of Indian licorice (*Abrus praecatorius*) and venoms from various snakes, spiders and scorpions.

**Chemical Properties of Exotoxins.** Most toxins lose their toxicity when heated to 60° C. for a few minutes but do not necessarily lose their ability to induce antitoxin formation. Detoxified but still antigenic toxin is called *toxoid*.

Toxoid formation results from simple aging of most toxin solutions in the refrigerator<sup>141</sup> but occurs more rapidly at room temperature. Formaldehyde also converts exotoxins into toxoids. Only toxic groups are affected by this conversion; the ability to produce antibodies and to unite with antitoxin remains unaltered. This phenomenon recalls the combination of formaldehyde and amino radicals in the Sörensen formol titration<sup>142</sup>; but the formation of toxoid is different because it is slow and irreversible, whereas the reaction between proteins or their breakdown products and formaldehyde is rapid and reversible.

Diphtheria toxin can be detoxified by ketene, an acetylating agent which combines with free amino groups. Thirty to 50 per cent of the radicals are affected, with an accompanying reduction in toxicity of 83.3 to 99.7 per cent. Iodine, diazo compounds, ascorbic acid and various other compounds also accelerate toxoid formation.

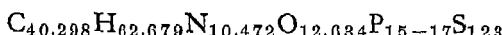
The chemical and physical changes in toxoid formation are not known with certainty. Purified toxin and toxoid may have the same optical rotation, which indicates that the transformation does not consist of a drastic physical rearrangement.<sup>5</sup> Pillemer recently observed that tetanus toxoid possesses a sedimentation constant nearly twice that of tetanus toxin and suggested that formation of this toxoid consists of condensation of two molecules of toxin through their toxic groups.<sup>27</sup>

*Table 51. Properties of Crystalline Botulinum (Type A) and Purified Diphtheria Toxins*

	EXOTOXINS		HORSE SERUM ALBUMIN
	Botulinum	Diphtheria	
Nitrogen	16.3%	16.0%	15.9%
Tyrosine	13.5%	9.5%	4.8%
Tryptophane	1.86%	1.4%	0.53%
Arginine	4.62%	3.8%	4.9%
Histidine	1.03%	2.4%	3.4%
Lysine	7.74%	5.3%	13.2%
Molecular weight	900,000	72,000	70,000

(From MacLeod and Pappenheimer,<sup>18</sup> by permission. Copyright by The National Foundation for Infantile Paralysis, Inc.)

Some toxins are destroyed or inactivated by proteolytic enzymes, oxidizing agents and ultraviolet light, and are precipitated by salts of heavy metals and by weak acid. These are properties typical of proteins. Qualitative and quantitative analyses also indicate that all exotoxins which have been purified are proteins. Relatively pure diphtheria, botulinum and tetanus toxins have been obtained, the two latter in crystalline form. The partial analyses in Table 51<sup>18</sup> indicate nitrogen percentages within the range expected of proteins. Buehler, Schantz and Lamianna performed a complete amino acid analysis of type A botulinum toxin and found nineteen amino acids.<sup>2</sup> They calculated that the toxin has an elementary formula of



It is a heat coagulable protein having the solubility characteristics of a globulin. It is the most potent poison known.

No unusual chemical groupings which might account for the extreme

toxicity of botulinum or of any other toxin have been found. It has been suggested that some peculiar stereochemical arrangement of amino acids is responsible for toxicity and that disarrangement of the amino acids reduces toxicity.<sup>18</sup>

**Mode of Action of Toxins.** It was formerly believed, probably because investigations were carried out with crude impure materials, that injections of exotoxins produced toxic symptoms only after several hours, no matter how large the dose of toxin. The incubation period following injection of tetanus toxin into mice was stated to be at least eight hours, during which toxin was bound by susceptible cells. A recent report by Pillemeyer *et al.* indicated that with a highly purified crystalline tetanus toxin the incubation period could be varied almost at will by regulating the size of the dose (Table 52).<sup>26</sup> Other observations suggested that the

*Table 52. Relationship between the Dose of Crystalline Tetanus Toxin and the Time Required for Onset of Symptoms and Death of White Swiss Mice*

M.I.D.	ONSET OF SYMPTOMS	DEATH
500,000	35 min.	1 hr.
100,000	1 hr.	2 hrs
10,000	4 hrs.	10-15 hrs.
10	15 hrs.	30-48 hrs.
1	30 hrs.	90-96 hrs.

(From Pillemeyer and Wartman,<sup>26</sup> by permission.)

toxin itself is not the actual agent responsible for production of symptoms but represents an intermediate agent which stimulates formation within the host of the actual toxic substance. Therefore, the only incubation period is that required for formation of this toxic agent.

An excellent example of the marked affinity of toxin for body cells is afforded by mixtures of tetanus toxin with brain and spinal cord tissue. If such a mixture is prepared and injected almost immediately into a susceptible animal, it is nontoxic. The high avidity of tetanus toxin for nerve tissue is one of the important factors in the pathogenesis of the disease. Lipid substances play an important role in tissue susceptibility to diphtheria and other exotoxins, since lipid extracts neutralize some toxins.

A lethal dose of diphtheria toxin is neutralized by a small amount of antitoxin if the two are mixed before injection. If the toxin is injected first and the antitoxin a few minutes later, many thousand times as much antitoxin may be insufficient to prevent death.<sup>24</sup> This shows that toxin is neutralized *in vivo* in the blood or body fluids and not in the cells, and it demonstrates the affinity of diphtheria toxin for susceptible tissue.

There is probably no single mechanism by which exotoxins exert their

destructive effects. Botulinum toxin appears to affect peripheral motor nerve endings by interfering with the release of acetylcholine; death is attributed to respiratory paralysis.<sup>37</sup> The peripheral action of tetanus toxin seems to be twofold: (a) spasm caused by stimulation of the neuromuscular junctions in voluntary muscles and (b) paralysis similar to that caused by botulinum toxin.<sup>37</sup> Brain and spinal cord tissue of animals injected with tetanus toxin display no significant histologic changes. Diphtheria exotoxin causes local necrosis at the site where it is produced (in disease) or injected, congestion and acute inflammation of heart muscle fibers, inflammation of the adrenals, and degeneration of certain nerves with resulting paralysis. It also produces changes in the carbohydrate metabolism of rabbits resembling those induced by abnormally high secretion of adrenalin. Isolated liver tissue from toxemic rabbits exhibits diminished respiration and impaired capacity to store glycogen and to synthesize carbohydrates. Pappenheimer and Hendee suggested that diphtheria exotoxin damages tissues by interfering with their synthesis of cytochrome *b* or related cytochrome components, which are important in cellular oxidations.<sup>22</sup>

*Clostridium perfringens* exotoxin acts directly on the central nervous system and on peripheral arterioles and liberates histamine from tissues, together with adenyl compounds and a substance which causes slow contraction of involuntary muscle. The  $\alpha$ -toxin of this organism was the first exotoxin shown to be an enzyme. It is a lecithinase and is more thermostable than most exotoxins. Its hemolytic, necrotizing and lethal properties result from its power of splitting lecithin and lecithoprotein complexes, which are important constituents of nearly all animal and vegetable tissues. *Clostridium perfringens* lecithinase hydrolyzes lecithin to phosphorylcholine and a diglyceride. It acts more rapidly than most other exotoxins and is neutralized by specific antitoxin. Certain snake venoms, such as cobra venom, contain a different lecithinase whose products of hydrolysis include an unsaturated fatty acid and lysolecithin.<sup>37</sup>

One milligram of type A botulinum toxin contains about 31,000,000 minimal lethal doses for the mouse. It can readily be calculated that one lethal dose consists of 20,000,000 molecules. The mouse possesses about 2,500,000 nerve cells (van Heyningen), which are believed to constitute the susceptible cells. Evidently, therefore, a fatal result is obtained at a concentration of about eight exotoxin molecules per nerve cell. Similar calculations indicate that the fatal dose for the guinea pig is about four molecules per nerve cell.

**Determination of Potency of Exotoxins.** The importance of the species of animal in which an exotoxin is assayed is indicated in Table 53.<sup>18</sup> The mouse and guinea pig differ in their susceptibility to diphtheria, tetanus and botulinum toxins, even when the results are expressed on the basis of equal weights of the animals. Some animals, such as dogs and chickens.

are extremely resistant to tetanus toxin, whereas humans, guinea pigs and horses are highly susceptible. Within a species, larger individuals usually tolerate greater amounts of exotoxin. In consequence of the variation between breeds and even individual animals, it is often necessary to

*Table 53. Number of Minimal Lethal Doses per Kilogram Body Weight Contained in 1 mg. Toxin*

TEST ANIMAL	DIPHTHERIA TOXIN	TETANUS TOXIN	BOTULINUM TOXIN
Guinea pig	3500	1,200,000	1,200,000
Mouse	3.5	200,000	620,000

(From MacLeod and Pappenheimer,<sup>18</sup> by permission Copyright by The National Foundation for Infantile Paralysis, Inc.)

inoculate several animals with each dose of toxin. The average outcome may be analyzed and the degree of statistical confidence to be placed in the results can be ascertained.

The route of administration of toxins is often important. Unlike most other exotoxins, botulinum toxin is highly toxic by mouth, partly because it is not destroyed by proteolytic enzymes.<sup>37</sup> Tetanus toxin is often introduced intramuscularly; diphtheria toxin is usually administered by subcutaneous injection, and certain exotoxins may be given intravenously.

The reactions which follow administration of exotoxin depend upon the kind of toxin, dosage and route of inoculation. The time at which

*Table 54. Death Times of Mice Injected with Small Amounts of Tetanous Toxin<sup>35</sup>*

(Three mice injected subcutaneously with each dose of toxin)

TOXIN PER 20 GM. MOUSE	DEATHS (DAYS)
0.000400 ml.	3 3 3
0.000200 ml.	4 4 4
0.000176 ml.	4 5 5
0.000140 ml.	5 5½ 6½
0.000114 ml.	6 S S
0.000100 ml.	S* S S

\* S = survived

symptoms appear usually varies inversely with the dosage. The relationship between dosage of tetanus toxin and the time of death of mice is illustrated in Table 54.<sup>35</sup> Very small doses may produce only local symptoms or no clinical signs whatever. Ipsen derived an equation and curve which relate the death times of mice with the dosage of tetanus toxin injected (Figure 40).<sup>14</sup>

The results presented in Table 54 and Figure 40 illustrate a "graded response" to a toxic agent. Graded response may also be expressed in terms of areas of necrosis, degrees of paralysis or other measurable reactions. Evaluation of a graded response requires the establishment of some arbitrary limit or endpoint. Most mice injected with fatal doses of tetanus toxin die within five days. This is a convenient time interval, feasible in ordinary laboratory practice and it has been widely adopted as the period of observation. Shorter or longer periods of observation are used with other animals and other toxins. The most common unit determined at such an endpoint is the *minimal lethal dose*, defined as the

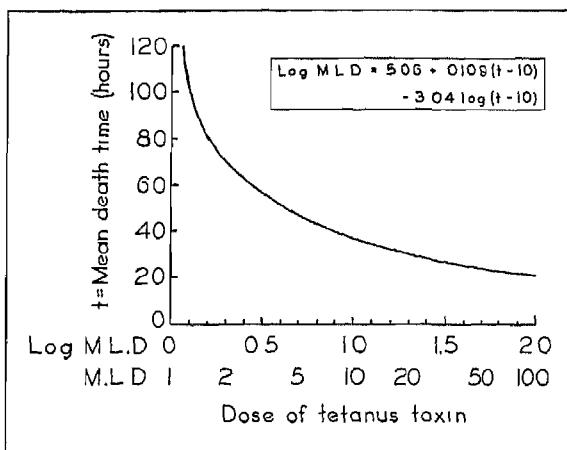


FIGURE 40. Correlation between dose of tetanus toxin and mean death times of mice.  
(Adapted from Ipsen, 1951. Jour. Immunol. 66, 691.)

smallest amount of toxin which will kill a given animal within a certain period after inoculation. The route of injection and weight of the animal are usually specified.

The tetanus toxin tested in the experiment shown in Table 54 had a minimal lethal dose at the five-day observation of slightly less than 0.000176 ml. The value of injecting several animals instead of one with each dose is obvious, because deaths occurred on the fifth day among animals which received two different dosages. In this case the minimal lethal dose can be estimated by interpolation.

Some investigators prefer to express toxicity in  $LD_{50}$  units. The  $LD_{50}$  is the dose of toxin required to kill 50 per cent of a group of animals within a specified period. The experimental results often do not contain a group in which exactly half the animals died, but the dosage which would be expected to produce this result can be calculated. The method of Reed and Muench<sup>80</sup> gives a fairly accurate figure without complicated mathematics and is illustrated with the data just discussed (see Table 55).

The results are first summarized to show the number of animals in each dosage group which survived the specified observation period and the number which died. Cumulative survivor totals are calculated, beginning with the largest dose, and also cumulative death totals, beginning with the smallest dose. From these cumulative totals the per cent mortality at each dosage level is determined. It appears that the 50 per cent mortality dose is between 0.000176 ml. and 0.000140 ml. of toxin. A simple proportion indicates that the LD<sub>50</sub> is 0.000149 ml.

*Table 55. Calculation of LD<sub>50</sub> of Tetanal Toxin  
(Data from Table 54)*

DOSE (ml.)	RESULTS IN 5 DAYS		$\Sigma$		PER CENT MORTALITY
	Lived	Died	Lived	Died	
0.000400	0	3	0	10	100
0.000200	0	3	0	7	100
0.000176	0	3	0	4	100
0.000140	2	1	2	1	33
0.000114	3	0	5	0	0
0.000100	3	0	8	0	0

$$\text{LD}_{50} = 0.000176 - \left( \frac{100 - 50}{100 - 33} \right) (0.000176 - 0.000140)$$

$$= 0.000176 - (0.75)(0.000036) = 0.000176 - 0.000027$$

$$= 0.000149 \text{ ml.}$$

The relative values of the M.L.D. and LD<sub>50</sub> depend upon the steepness of the dose-response curve, that is, the range of dosages through which only a fraction of the animals in a group die. The LD<sub>50</sub> is favored by theoretic investigators because it is likely to give a sharper endpoint. It is expensive, however, because many more animals are required. Practical workers, particularly those involved in preparation of biologic products for human use, find that the M.L.D. or 100 per cent endpoint is as satisfactory for their purposes and in some applications provides an additional slight margin of safety. It is usually recommended that critical tests be made with at least two animals per dosage to reduce the chance of misleading results caused by faulty injections or animal variation.

#### ANTITOXINS

**Preparation and Purification of Antitoxins.** Antitoxin is produced by immunization of animals, usually the horse, with toxin or toxoid. The procedure for preparing toxin is in part determined by the nature of the organism; that is, its nutrient, temperature and oxygen requirements. Synthetic media which yield toxin of high titer offer the important

advantage that less purification of the toxin is necessary to minimize undesirable side reactions.

After maximum development of toxin, the bacterial cells are removed by centrifugation or filtration. The toxin may be concentrated by precipitation and is titrated so that sublethal doses may be given during the early injections. Toxin may also be converted into toxoid by incubation with 0.2 to 0.5 per cent formaldehyde for several days or weeks at 35° to 37° C.

The kind of animal used for production of antitoxin depends almost wholly upon economic considerations. Larger animals are more satisfactory, because greater amounts of antitoxic serum are secured with less labor. Horses can be immunized readily and yield several liters of blood at each bleeding.

Any antitoxic preparation possesses the specific serum proteins of the animal from which it is derived. An individual receiving antitoxin prepared in horses may become sensitized to horse serum proteins, so that subsequent injection of horse antitoxin induces anaphylactic shock. Methods have been developed for purifying antitoxin which greatly reduce the possibility of such untoward reactions. Antiseraums from animals other than the horse are sometimes given to individuals sensitive to horse proteins. Cattle and goat serums have been employed, and even rabbit serum, although the latter is very expensive to manufacture.

Among individuals of any given species there is wide variation in antibody producing power. Some laboratories select animals by preliminary trial. Each of a considerable group of animals is given a single injection of toxin or toxoid or a short course of injections. Several weeks or months later an additional injection is given, and blood samples are taken after another ten to fourteen days. The antitoxic potency of these samples is determined, and only those animals are selected for further injection whose serums possess the highest titers.

Diphtheria antitoxin is usually produced by injections of diphtheria toxoid; tetanus and botulinum antitoxins are produced by injections of the corresponding toxins and/or toxoids.<sup>39</sup> Toxoids are preferred if they yield satisfactory antitoxin because larger doses of antigenic material may be administered with relative safety. Early injections with certain toxins, such as type B botulinum, produce severe reactions. In such cases immunization begins with a series of toxoid injections, followed by toxin when the animal has developed some antitoxin.

Wadsworth recommended that tetanus antitoxin be produced in relatively young horses.<sup>39</sup> These are given at least two immunizing subcutaneous injections of tetanus toxoid, allowed to rest six months or more, and then given twenty milliliters of tetanus toxoid containing aluminum potassium sulfate. One month later injections of toxin at three-day intervals are commenced. An animal which has produced sufficient antitoxin

in response to the toxoid may receive a first dose consisting of 5,000 guinea pig M.L.D. Subsequent injections increase in amount until the eighth injection, which contains 100,000 M.L.D. The animal is allowed to rest one month, after which toxin containing aluminum potassium sulfate is administered, beginning with 5,000 M.L.D. again and increasing rapidly at four-day intervals. Trial titrations are made and bleedings for plasma are taken when the serum contains 700 to 800 antitoxic units per milliliter. Blood is drawn seven days after the last injection, and nine liters may be secured at a time. Four or five additional injections of toxin at four-day intervals are followed by further bleeding. This process is repeated until the titer drops below 500 units per milliliter. Horses have been known to continue producing potent antitoxin for years.

Blood is collected in citrate, stored in a cold room and the supernatant plasma removed after the cells have settled. This crude antitoxin contains nonantitoxic proteins such as fibrinogen and albumins, together with salts, lipids and other materials. The antitoxin is principally pseudoglobulin. Fibrinogen and euglobulin are precipitated by 29 per cent saturation with ammonium sulfate and heat. The precipitate is removed and the solution is treated with additional ammonium sulfate to 48 per cent saturation, whereupon pseudoglobulins precipitate, leaving albumins and other materials in solution. The antitoxin-containing precipitate is dialyzed in cellophane bags against running water to remove ammonium sulfate, made isotonic with sodium chloride, treated with a preservative and stored. During the first few months in the refrigerator some loss of potency occurs, but the final product is relatively stable. It is then filtered to remove any bacteria, tested for sterility and its antitoxic activity is titrated.

Pepsin digestion of plasma proteins sometimes precedes ammonium sulfate fractionation. This treatment does not affect the antitoxic power of the plasma but greatly reduces its ability to produce undesirable side reactions.

Observations of theoretic interest have been made with enzymatically digested antitoxin. Whereas purified diphtheria antitoxin contains 2.6 per cent carbohydrate, Petermann and Pappenheimer found that partial digestion with pepsin yielded a fraction possessing virtually all the antitoxic activity of the untreated antitoxin but containing 3.8 per cent carbohydrate.<sup>25</sup> The increased percentage of carbohydrate in digested antitoxin seemed to indicate that carbohydrate is directly associated with the immunizing properties of this antibody. Furthermore, half molecules of antitoxin having a molecular weight of 90,000 have been obtained by tryptic digestion and found to contain nearly all the antitoxic activity of the original material. This observation implies that the antitoxic groups are distributed asymmetrically about the molecule.

**Standardization of Antitoxins. *In vivo Methods.*** The instability of

exotoxins is so marked that they cannot be used as permanent standards of toxicity for the assay of antitoxins. Antitoxins, however, are extremely stable under proper conditions of storage (see Table 56).<sup>12</sup> A standard diphtheria antitoxin has been maintained in the National Institutes of Health since 1905, and a preparation of tetanus antitoxin since 1907. Standardized antitoxins are supplied to qualified laboratories for use as reference in determining the potency of new lots of antitoxin and for control purposes by governmental agencies.

*Table 56. Effect of Storage Temperature on Potency of Antitoxin*

(Antitoxin preserved with 0.3% cresol)

TEMPERATURE	LOSS OF POTENCY IN 12 MONTHS
0-5° C.	0%
15° C	9%
22° C.	11%
30° C.	20%
37° C.	60%

(From Glenny,<sup>12</sup> by permission of the Controller of Her Britannic Majesty's Stationery Office.)

The strength of antitoxins is expressed in arbitrary units whose value differs from one kind of antitoxin to another. The official unit of diphtheria antitoxin in the United States is the amount contained in 1/6000 gram of a certain dried unconcentrated horse serum antitoxin. The official standard unit of tetanus antitoxin in this country is the amount contained in 0.00015 gram of a particular horse serum antitoxin. The international unit of tetanus antitoxin is one-half the amount of the American unit.

The response of animals to the injection of mixtures of toxin and antitoxin depends upon the proportions in which the two reagents are mixed. Excess toxin causes characteristic symptoms and death, whereas excess antitoxin completely protects against visible reaction. The most convenient endpoint is the same as that employed in titrating the corresponding exotoxins: for example, death of all or a certain percentage of the animals within a specified period. Parallel tests employing a constant amount of a given exotoxin preparation and varying amounts of standard and unknown antitoxins permit determination of the antitoxic potency of the unknown.

Titration of a tetanus antitoxin in mice is illustrated in Table 57.<sup>81</sup> The observation period in this experiment was limited to four days and dosages of antitoxin which protected against death until the fourth day were compared in order to calculate the number of units per milliliter contained in the unknown antitoxin No. 3903. The standard antitoxin diluted 1:10 contained 1 unit per milliliter, and 0.10 unit protected against 0.14

milligram of toxin. Equal protection was afforded by 0.09 milliliter of antitoxin 3903 diluted 1:400. Undiluted antitoxin 3903 therefore contained 444 units per milliliter.

The least amount of tetanus toxin which when combined with 0.1 unit of antitoxin kills a test animal within the specified period is known as the

*Table 57. Comparison of Standard and Unknown Tetanal Antitoxins in Mice<sup>31</sup>*

(Toxin-antitoxin mixtures were diluted with buffer to a total volume of 0.1 ml. After one-half hour, mice were injected subcutaneously. The endpoint was death on the fourth day.)

	ANTITOXIN (1:10)	TOXIN	RESULTS			
			1 day	2 days	3 days	4 days
Standard anti-toxin (10 units/ml.)	0.12 ml.	0.14 mg.	—	—	?	±*
	0.11 ml.	0.14 mg.	—	±	+	++
	0.10 ml.	0.14 mg.	±	+	++	D
	0.09 ml.	0.14 mg.	+	++	D	
	0.08 ml.	0.14 mg.	++	D		
Antitoxin 3903 (x units/ml.)	0.12 ml.	0.14 mg.	—	—	—	—
	0.11 ml.	0.14 mg.	—	—	—	±
	0.10 ml.	0.14 mg.	—	±	+	++
	0.09 ml.	0.14 mg.	±	++	++	D
	0.08 ml.	0.14 mg.	+	+++	D	

Calculation:

Standard antitoxin diluted 1:10 contains 1 unit/ml.

0.09 ml. antitoxin 3903 diluted 1:400 ≡ 0.10 unit

0.09 ml. antitoxin 3903 undiluted ≡ 400 × 0.10 = 40 units

1.0 ml. antitoxin 3903 undiluted ≡ 40/0.09 = 444 units

\* + signs indicate severity of symptoms; D indicates death.

*test dose.* It may be a few hundred to a few thousand times greater than the minimal lethal dose.<sup>17</sup> An amount of diphtheria toxin, called the L+ dose, employed for a similar purpose, is defined as the smallest amount of toxin which when combined with one unit of antitoxin kills a test animal within a specified period.

The official test animal for titration of both tetanus and diphtheria antitoxins in the United States is the guinea pig. Animals of 350 grams weight are specified for tetanus titrations and animals of 250 grams for diphtheria. In both cases the period of observation is four days. Mouse titrations of tetanus toxin and antitoxin are being increasingly used, particularly abroad, because they appear to give equivalent results and are somewhat less expensive.

Römer observed in 1909 that intracutaneous inoculation into guinea

pigs of very small doses (1/500 to 1/250 M.L.D.) of diphtheria toxin produced a mild local reaction consisting of swelling and erythema.<sup>32</sup> Slightly larger doses induced definite necrosis within forty-eight hours. Mixture of the toxin with adequate amounts of antitoxin before injection prevented any sign of local reaction. When insufficient antitoxin was employed, the area of erythematous swelling and necrosis was directly proportional to the amount of free toxin in the mixture. These principles form the basis of the Römer titration of diphtheria antitoxin. A dose of toxin is employed which when mixed with 1/20 or 1/500 unit of antitoxin and injected intracutaneously into a guinea pig or rabbit produces a minimal skin reaction. Standard and unknown antitoxic serums are compared in the same animal, a procedure which reduces the variation inherent in the use of several animals for a biologic titration. The test is highly sensitive and has been used to detect minute amounts of either antitoxin or toxin in blood, milk and other secretions. As little as 1/50,000 unit of diphtheria antitoxin can be detected in 0.1 milliliter of serum.

*Toxin-Antitoxin Flocculation.* Danysz in 1902 observed that flocculation occurred in nearly neutral mixtures of ricin and antiricin.<sup>4</sup> The precipitates in such mixtures contained all the ricin and antiricin originally present. Later Calmette and Massol measured the protective value of antivenins by flocculation,<sup>8</sup> and Ramon developed *in vitro* titration methods for diphtheria and tetanus.<sup>28</sup> Flocculation has been employed with several other systems, including toxin-antitoxin mixtures for *Cl. botulinum*, *Cl. perfringens*, *Str. scarlatinae* and *Sh. dysenteriae*.

The Ramon procedure consists of mixing varying amounts of antitoxin and a constant amount of toxin and incubating in a waterbath at 44° to 46° C., with frequent observation. The mixture which flocculates first is approximately neutral by animal inoculation. Mixtures containing less antitoxin are toxic to animals, and those containing more antitoxin produce no symptoms.

The antitoxin titer of an unknown serum is determined with reference to a known antitoxin. Homologous toxin is standardized against the known antitoxin by flocculation, and this standardized toxin is then tested with the unknown serum. A sample protocol illustrating the flocculation of tetanus toxin and antitoxin is shown in Table 58. Flocculation first appeared in the tube containing 0.15 milliliter of antitoxin, the strength of which was 500 units per milliliter. The unit of toxin measured by this procedure is called the *Lf dose*, which is the amount of toxin flocculating most rapidly with one unit of antitoxin. The Lf content of a toxin is calculated as follows:

$$\text{Lf/ml. toxin} = \frac{\text{Antitoxin units/ml.} \times \text{ml. of antitoxin}}{\text{ml. of toxin}}$$

Flocculation of toxin and antitoxin is analogous to precipitation of a

protein antigen by its antibody. It is a test of combining power and is independent of toxicity. The Lf value of a toxin solution does not decrease as its toxicity diminishes with age or with chemical conversion to the toxoid state. The Lf value is therefore more directly related to antigenicity than is the M.L.D. and is useful as an *in vitro* indication of the potency of toxoids employed in immunization.

An interesting application of optimal proportions in the precipitation of toxin by antitoxin is provided by the *in vitro* virulence test for diph-

*Table 58. Flocculation of Tetanum Toxin and Antitoxin  
(Incubation at 46° C.)*

ANTITOXIN (500 u /ml.)	0.13 ml.	0.14 ml.	0.15 ml.	0.16 ml.	0.17 ml.
TOXIN	2.0 ml.				
4 min.	Op	Op	Cl	Cl	Op
8 min.	Cl	Cl	Cl	Cl	Cl
12 min.	Cl	Cl	Fl	Cl	Cl
14 min.	Fl	Fl	Fl	Fl	Fl

Calculation.

$$500 \times 0.15 \div 2.0 = 37.5 \text{ Lf/ml. toxin}$$

$$1 \text{ Lf toxin} = 1/37.5 = 0.0267 \text{ ml.}$$

Op = opalescent

Cl = cloudy

Fl = flocculent

theria bacteria, described by Elek in 1948<sup>8</sup> and subsequently improved by Frobisher.<sup>10</sup> A narrow strip of sterile filter paper soaked in diphtheria antitoxin is covered with agar medium in a Petri dish. The hardened agar is streaked at a ninety degree angle to the length of the paper strip with the cultures under test. Antitoxin diffusing from the paper encounters toxin diffusing at a right angle to it from the streak of growth. A narrow line of precipitate forms where the optimal ratio of antitoxin to toxin occurs (Figure 41).<sup>9</sup>

#### MECHANISM OF REACTION BETWEEN TOXIN AND ANTITOXIN

**The Danysz Phenomenon.** Danysz discovered that the toxicity for animals of a mixture of toxin and antitoxin depends upon the manner in which the reagents are mixed as well as upon their relative final concentrations.<sup>4</sup> An amount of toxin can be determined which is just completely neutralized by a given quantity of antitoxin if the toxin is added in a single dose. This mixture produces no symptoms in an experimental animal. A mixture composed of the same amount of toxin added to the antitoxin in two or more portions at intervals of fifteen minutes is highly toxic when injected. This toxic mixture, when allowed to stand for several

days, gradually reverts to a nontoxic condition, evidently by dissociation and recombination. Toxin and antitoxin apparently combine in varying proportions depending upon the ratio in which they are mixed. The resulting complexes slowly dissociate. The first portion of toxin added to antitoxin in the Danysz experiment combines with more antitoxin than would be expected from its proportion in a neutral mixture. Toxin added a short time later is unable to combine completely with antitoxin and the mixture is therefore toxic.

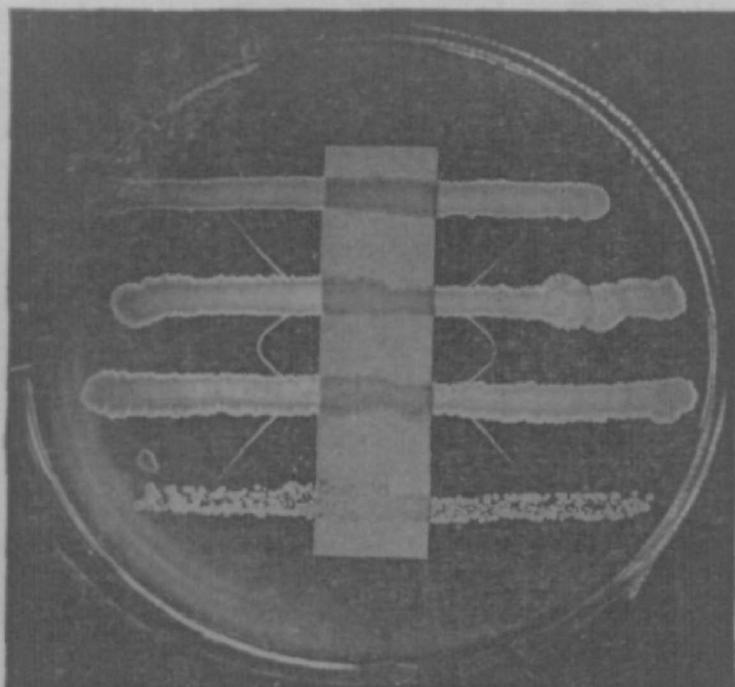


FIGURE 41. *In vitro* test for virulence. Serum agar covers a strip of filter paper saturated with diphtheria antitoxin. The agar was then inoculated at a right angle to the paper with strains of *Corynebacterium*. A thin line of precipitate formed when antitoxin diffusing from the paper met toxin diffusing from the growth in proper concentration. (From specimens prepared by Miss Elizabeth O. King. Photo courtesy U. S. Public Health Service, Communicable Disease Center, Atlanta, Ga.)

Healey and Pinfield found that a neutral mixture containing equivalent amounts of toxin and antitoxin (TA) can combine with another equivalent of antitoxin during incubation for ten hours at 40° C. followed by two days at room temperature.<sup>18</sup> The resulting complex is designated TA<sub>2</sub>.

Quantitative data clearly demonstrating combination in variable proportions are presented in Table 59.<sup>23</sup> A constant amount of antitoxin was mixed with varying amounts of toxin and the precipitates were analyzed for nitrogen. Tests of the supernates indicated whether all of each reagent had combined and which was in excess. The precipitated antitoxin nitro-

gen was calculated by difference, and the ratios of antitoxin nitrogen to toxin nitrogen determined. No precipitate formed in the region of great antitoxin excess. Partial combination was indicated by flocculation tests of the supernates, which showed the presence of 32 to 63 per cent of the antitoxin originally provided. Mixtures in the region of great toxin excess likewise failed to precipitate completely and contained uncombined toxin amounting to 20 to 40 per cent of the quantity employed.

*Table 59. Quantitative Analyses of Diphtheria Toxin-Antitoxin Precipitates*

(Antitoxin: 1 ml. containing 300 units)

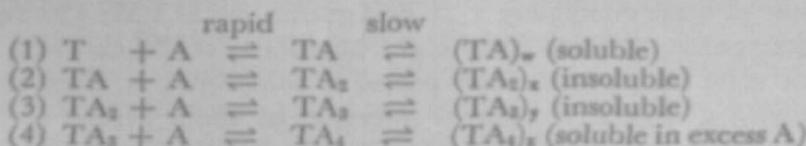
TOXIN		NITROGEN PPTED.		RATIO: Antitoxin N Toxin N	EXCESS IN SUPERNATE
Lf units	Nitrogen (mg.)	Total (mg.)	Antitoxin (mg.)		
50	0.023	0.000			Antitoxin (63%)
100	0.046	0.000			Antitoxin (32%)
150	0.069	0.386			Antitoxin
175	0.081	0.554	0.473	5.8	Antitoxin*
200	0.092	0.564	0.472	5.1	Neither
225	0.103	0.579	0.476	4.6	Neither
300	0.138	0.612	0.474	3.4	Neither
400	0.184	0.661	0.477	2.6	Toxin*
450	0.207	0.652			Toxin*
500	0.230	0.359			Toxin (20%)
600	0.276	0.000			Toxin (40%)

\* Traces, detectable by the rabbit intracutaneous test.

(From Pappenheimer and Robinson,<sup>20</sup> by permission.)

The precipitated antitoxin nitrogen was constant at about 0.474 milligrams throughout most of the zone of complete precipitation, although the toxin in the precipitates varied from 175 to nearly 400 Lf units. The ratios of antitoxin nitrogen to toxin nitrogen in the precipitates therefore varied from about 6.0 to 2.5.

Pappenheimer proposed the following equations for the reaction between diphtheria toxin (T) and antitoxin (A):<sup>21</sup>



Neutral mixtures probably have the composition  $\text{TA}_2$ , these molecules gradually aggregating to form floccules of  $(\text{TA}_2)_s$ . When only part of the neutralizing dose of toxin is added to the total amount of antitoxin, the composition is represented by  $\text{TA}_4$ , which polymerizes to  $(\text{TA}_4)_s$ . This complex is assumed to be only slowly dissociable. Addition of the remainder of the neutralizing dose of toxin produces no further immediate

change in the composition of the complexes and the mixture is toxic because all the available antitoxin is combined as  $(TA_4)_z$ .

**Relation between  $L_+$  and  $L_0$  Doses of Toxin.** Ehrlich designated as the  $L_0$  dose of toxin the largest amount which when combined with one unit of antitoxin fails to produce a toxic reaction in the test animal. The  $L_+$  dose of toxin is the smallest amount which when mixed with one unit of antitoxin kills the test animal in a specified time interval. It might be anticipated that the difference between the  $L_+$  and  $L_0$  doses is one M.L.D. Actually, however, it is usually between ten and 100 M.L.D. Ehrlich suggested that this phenomenon results from differing "avidity" of toxin and toxoids for antitoxin. He postulated a number of toxoids (epitoxoid, toxon, prototoxoid) possessing different affinities for antitoxin. This explanation did not meet with universal acceptance, particularly when quantitative data became available. It is now obvious that complexes of toxin and antitoxin may be formed containing a higher proportion of toxin than at the neutral point. Although these complexes dissociate slowly to yield traces of free toxin, considerable excess of toxin must be added before there will be sufficient free toxin to kill the test animal within the usual four or five day observation period.

**Neutralization of Toxins by Antitoxins.** The fact that antitoxins neutralize homologous toxins is undisputed. The mechanism by which they do so is not known. Combination with antitoxin apparently does not permanently change toxin. Diphtheria toxin has been recovered from toxin-antitoxin floccules in a high state of purity and with high toxicity and combining power. Boyd suggested, "Perhaps the action of antibody is simply to cover the toxin . . . and thus prevent it from coming into contact with the susceptible tissue."<sup>1</sup>

#### IN VIVO USES OF TOXINS, TOXOIDS AND ANTITOXINS

**Tests of Antitoxic Immunity.** Schick discovered that a minute amount of diphtheria toxin injected intracutaneously into nonimmune persons caused erythema and edema about the site of inoculation, whereas immune individuals usually gave no reaction.<sup>38</sup> The Schick test employs 0.1 milliliter of toxin containing 1/50 of a guinea pig M.L.D. The reaction is read after twenty-four to forty-eight hours and at the end of a week. A positive zone of erythema and edema is sometimes as large as three centimeters in diameter. A control injection of toxin heated at 60° C. for fifteen minutes is used to detect allergic sensitization to diphtheria cellular proteins. Slight inflammation, no more than three millimeters in diameter, may appear at the control site. No reaction at either site constitutes a negative test. Diluted toxoid substituted for the heated toxin control (Moloney test) detects sensitivity to toxoid which might cause severe reactions in routine immunization.<sup>20</sup>

A positive Schick test indicates lack of immunity to diphtheria. A negative test shows the presence of antitoxin which combines with the injected toxin. The "Schick level" of immunity is considered to represent between 1/100 and 1/30 unit of antitoxin per milliliter of blood and appears empirically to be sufficient to protect the great majority of individuals. The disease usually runs a milder course in the small percentage of Schick negatives who do contract diphtheria than in Schick positives.

Early surveys of urban populations indicated that about 85 per cent of newborn babies were immune to diphtheria but that by the age of one year at least 90 per cent were nonimmune. Thereafter the general level of immunity increased with age until the seventeenth year, when 85 per cent were immune. This level continued throughout older groups. Immunity of the newborn was believed to result from placental transfer of antitoxin from the maternal circulation and hence to be of short duration. The increasing immunity of children over one year of age undoubtedly reflected active immunization by actual cases of the disease or by unrecognized infection with relatively avirulent strains of *Corynebacterium diphtheriae*. In rural areas the percentage of immunes remained low later in life than in urban populations, and fewer children in private schools were immune than in public schools. These facts point to the role of personal contacts in distributing the organisms which induce active immunity.

The incidence of diphtheria has decreased to such an extent within the past generation that opportunities for subclinical infection and active immunization are relatively rare. As a consequence, the adult population in many areas is composed principally of nonimmune individuals. Between 13 and 76 per cent immunity has been reported in groups tested in the United States, Canada, Europe and Australia. It is not particularly surprising that the incidence of diphtheria increased greatly during the decade from 1940 to 1950.

The Dick test of immunity to scarlet fever is similar to the Schick test. One-tenth milliliter of scarlet fever toxin containing one skin test dose is injected intracutaneously. A positive reaction, indicating lack of immunity, consists of a circumscribed area of erythema at least one centimeter in diameter, developing within sixteen to twenty-four hours and fading rather rapidly. Heated toxin is injected as a control. The skin test dose is the smallest amount of toxin which causes a positive reaction in a majority of nonimmune individuals. Preliminary titration of toxin is performed by flocculation or by injection into rabbits, but the final titration is usually conducted in humans.

The results of the Dick test applied to large populations are similar to those of the Schick test and confirm the importance of personal contacts in the acquisition of immunity against scarlet fever.

A positive Dick reaction does not develop in nonimmune persons in-

jected with a mixture of Dick toxin and serum from convalescent scarlet fever patients. Moreover, Schultz and Charlton found that convalescent serum injected intradermally caused local blanching of the typical red rash of the disease.<sup>34</sup> This became the basis of the Schultz-Charlton test, which is sometimes used to distinguish scarlet fever from German measles and other rash-producing infections with which it may be confused. Convalescent serum or horse antitoxin is injected into an area with a bright red rash, and blanching occurs in positive cases between six and fourteen hours later over an area from one-half inch to several inches in diameter. The reaction usually persists throughout the duration of the rash. This test is not completely indicative of the presence or absence of scarlet fever but is often useful. It is considered to be a true toxin-antitoxin reaction *in vivo*.

**Active Immunization.** Toxoid is the most widely employed immunizing agent against diphtheria. It is especially useful in young children, in whom it usually produces no severe reactions. Individuals beyond the age of ten or twelve years may possess sensitivity to other diphtheria bacterial constituents or to toxoid itself. Immunization of such persons poses a considerable problem which is met only in part by the use of highly purified materials. Toxoid has the advantages that it is nontoxic and cannot revert to a toxic condition, is unaffected by aging or by heat to a temperature of 65° C., and can be used in relatively high concentrations. Three injections are desirable, consisting of 0.5, 1.0, and 1.0 milliliter amounts at intervals of two to three weeks. About 95 per cent of individuals become Schick negative after the usual course of injections.

Precipitation of toxin by alum was used by Roux and Yersin as early as 1889 in an attempt to purify the material, but apparently they did not determine the antigenicity of the precipitates. Within the last twenty or twenty-five years alum-precipitated toxoid (A.P.T.) has been found to be more effective as an immunizing agent than fluid toxoid. Toxoid is precipitated by 1 to 2 per cent alum and the precipitate, washed and resuspended in saline, is administered subcutaneously. Two doses a month apart usually give an adequate response.

Alum-precipitated toxoid is retained in the tissues much longer than fluid toxoid, the antigenic component being released slowly over a period of weeks. It has been detected locally in guinea pigs as long as seven weeks after injection. The alum incites an inflammatory response at the site of injection, which is believed to induce more effective antitoxin formation. Alum-precipitated toxoid, either alone or combined with pertussis vaccine and tetanus toxoid, is employed in infants and young children, in whom the advantage of reducing the number of injections is obvious.

Toxin-antitoxin mixtures are sometimes used for active immunization and may cause less severe reactions than toxoid. Three injections of one

milliliter are given at intervals of one to two weeks. Antitoxin develops gradually, and the Schick reaction may remain positive for six months or longer. Approximately 85 per cent of persons treated eventually develop immunity. Preparations currently employed consist of 0.1 L+ dose of toxin nearly neutralized by antitoxin. Slow dissociation within the body releases toxin which stimulates active immunization. An important disadvantage of toxin-antitoxin is the possible development of hypersensitivity to the horse serum components of the antitoxin.

The duration of immunity against diphtheria following artificial active immunization is considered to be less than five years. Although circulating antitoxin may disappear, the capacity of such an individual to respond to further antigenic stimulus may remain high for years or for life. Even the small amount of toxin used in the Schick test often constitutes an effective "booster" dose for previously immunized persons. Twenty-five to 40 per cent of Schick positive young adults become Schick negative as a consequence of the antigenic stimulus afforded by the test.<sup>7</sup> Inapparent infections in previously immunized persons also constitute important secondary stimuli to antitoxin production and assist in maintenance of a satisfactory state of resistance.

It is to be expected that some cases will occur in artificially immunized individuals who, according to the results of all known tests, should be immune. The size of the infecting dose, the virulence and serologic properties of the organisms and factors associated with the host may outweigh the results of the immunization procedure. On the whole, however, fewer cases occur in immunized persons, and those that do occur are usually less severe.

Frobisher considered that antitoxic immunity may be somewhat overrated as protection against diphtheria.<sup>11</sup> Numerous cases and outbreaks of all degrees of severity have been reported in individuals and groups supposedly immunized with toxoid. He described experiments which indicated that antiendotoxic immunity was important in prevention of diphtheria and suggested that highly purified toxoids actually produce less protection than commonly believed. He feels that antiendotoxic or antibacterial immunity localizes the initial infection and that antiexotoxic immunity constitutes a second line of defense. Endotoxin and exotoxin are related immunologically but are not identical, as is shown by the failure of antiexotoxin to neutralize endotoxin. Animals immunized against endotoxins withstood intradermally injected living, virulent diphtheria bacteria and exotoxin, although they contained no circulating antitoxin. Tissues near the sites of injection were damaged, but the challenge organisms were effectively localized.

Immunity against tetanus appears to be almost entirely associated with circulating antitoxin and with the heightened tissue reactivity resulting from active immunization. Individuals are not born with immunity to

tetanus, nor do they acquire it naturally.<sup>15, 16</sup> Subclinical immunizing infections do not occur, as in diphtheria. An active case of the disease from which the patient recovers does not necessarily lead to immunity; second and even third attacks have been reported.<sup>38</sup> Spontaneous recovery appears to be caused by light infection and fortuitous circumstances rather than by active immunization.

The only recommended immunizing agent against tetanus is toxoid. Tetanus toxoid is even less irritating than diphtheria toxoid and can be used in relatively enormous amounts equivalent to several thousand guinea pig minimal lethal doses of toxin. The practice of the United States Army during World War II was to administer three doses of one milliliter each at intervals of three to four weeks, followed by a single stimulating dose of one milliliter after one year and an emergency booster dose upon the occurrence of a wound or other injury that might result in tetanus. The effectiveness of this procedure is indicated by the report that only fifteen cases of tetanus occurred among over 2,500,000 wounded men in the U. S. armed forces—and nine of these had not received the scheduled full immunization.<sup>7</sup> The case rate during World War I was 13.4 per 100,000 wounded men.<sup>6</sup>

Two to three weeks after the first three injections of tetanus toxoid, the majority of individuals possess a protective level of circulating antitoxin. A stimulating dose some months later increases the titer to far higher levels which are longer maintained, and repeated booster injections induce more striking responses. Periodic injections of toxoid should be given at intervals of three to four years thereafter to maintain a high antitoxic level.<sup>8</sup>

Two injections of alum-precipitated tetanus toxoid four to six weeks apart have been found fully as effective as three injections of fluid toxoid for primary immunization, but booster injections of fluid toxoid give more rapid response than A.P.T. (in most subjects by the fourth or fifth day). The speed of antitoxin formation following booster injection depends upon how recently the last previous injection was given. After four years the response is somewhat delayed. It has been questioned whether emergency injection of toxoid following injury stimulates antitoxin formation quickly enough to prevent death, because fulminating, rapidly fatal cases occasionally occur. In such instances it is recommended that prophylactic administration of antitoxin be considered, particularly if the patient has not received toxoid recently and the injury occurred within the central nervous system.<sup>6</sup>

**Passive Immunization.** Passive immunization with antitoxin formed in another individual or species is usually employed prophylactically following exposure to infection. It may also be used as a therapeutic measure after signs of disease are apparent. Prophylactic doses are relatively small, but therapeutic doses must usually be very large. Antitoxin

is more or less effective against diphtheria, tetanus, botulism, gas gangrene and certain other diseases.

It is important in treating diagnosed cases of disease to administer adequate amounts of antitoxin as quickly as possible. The union between toxins and body cells is firm and undissociable, and tissue damage may be rapidly initiated and irreversible. Nerve destruction is practically irreparable because nerves have much less regenerating capacity than most other tissues. The speed with which diphtheria toxin combines with tissues is indicated by Table 60,<sup>24</sup> in which it is shown that a delay of

*Table 60. Diphtheria Antitoxin Necessary to Save Rabbits Given Ten Fatal Doses of Toxin Intravenously*

INTERVAL BETWEEN ADMINISTRATION OF TOXIN AND ANTITOXIN	THERAPEUTIC DOSE OF ANTITOXIN
10 min.	5 A. U.
20 min.	200 A. U.
30 min.	2000 A. U.
45 min.	4000 A. U.
60 min.	5000 A. U.
90 min.	No amount

(From Park and Schrader,<sup>24</sup> by permission.)

only fifty minutes increased a thousandfold the amount of antitoxin required to save the life of an experimentally injected rabbit, and that after another half hour no amount of antitoxin sufficed. Delay of only a few hours in treating human cases of diphtheria, tetanus or botulism may make the difference between life and death.

The route of injection determines how quickly the antitoxin can neutralize toxin. The intravenous route ensures thorough distribution about the body in the minimum time. Several hours are required for intramuscularly administered antitoxin to attain maximum concentration in the blood, and two to three days may elapse after subcutaneous injection before the circulating antitoxin is at a peak. Introduction of antitoxin directly into infected tissues is sometimes desirable.

Tetanus antitoxin prophylaxis of injured, unimmunized individuals is recommended. The dose is usually between 500 and 5000 units, and it may be repeated at five to seven day intervals.<sup>30</sup> Some authorities advise simultaneous initiation of active immunization with toxoid.<sup>19</sup> Antitoxin, if given soon enough, usually prevents appearance of symptoms. Treatment of established cases by means of antitoxin is less certainly effective. Larger doses are required, repeated at short intervals, but there seems little advantage in giving a total of much more than 100,000 units.<sup>40</sup>

Five types of *Clostridium botulinum* are known whose antitoxins are

type specific. Types A and B are most commonly encountered in human botulism, and a polyvalent antitoxin against these is often employed because there is usually insufficient time for identification of the cause of a given case or outbreak. Antitoxin therapy of botulism has not given as good results as laboratory experiments indicate,<sup>29</sup> doubtless because there is often too great delay before treatment is begun. Protective administration to persons suspected of ingesting the toxin is probably most effective.

Prevention of diphtheria in exposed persons is usually accomplished by administration of 500 (infants) to 2000 (adults) units of antitoxin. Protection is of short duration and should be augmented by active immunization. Specific treatment of cases of diphtheria consists of larger doses of antitoxin, the amount being determined by the severity of symptoms and the duration of disease. Five thousand units on the first day is more effective than 100,000 units on the fourth or fifth day.<sup>30</sup> Mild cases in children respond satisfactorily to 3000 to 5000 units, whereas severe cases in adults require 20,000 units or more.

Any immunization procedure, whether passive or active, entails certain elements of risk. Passive immunization with foreign serum subjects the patient to the possibility of allergic reactions, particularly if he has previously received injections of serum from the same species. An intradermal test with diluted serum will indicate whether the antiserum can be safely administered.

Active immunization with bacteria or bacterial products may be accompanied by unpleasant or even incapacitating side reactions. Some immunization procedures are occasionally followed by disease conditions which are at present unexplained: for example, postvaccinal encephalitis shortly after smallpox vaccination.

The vast majority of physicians and investigators believe that the benefits derived from active and passive immunization far outweigh the possible dangers. Public health statistics for the past sixty years generally confirm this belief.

### References

1. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
2. Buehler, Schantz and Lamanna, 1947. Jour. Biol. Chem. 169, 295.
3. Calmette and Massol, 1909. Ann. Inst. Pasteur 23, 155.
4. Danysz, 1902. Ann. Inst. Pasteur 16, 331.
5. Eaton, 1938. Bact. Rev. 2, 3.
6. Edsall, 1949. N. E. Jour. Med. 241, 18, 60, 99.
7. Edsall, 1952. Amer. Jour. Pub. Health 42, 393.
8. Elek, 1948. Jour. Clin. Path. 2, 250.
9. Frobisher, 1953. *Fundamentals of Microbiology*, 5th ed., W. B. Saunders Co., Philadelphia.
10. Frobisher, King and Parsons, 1951. Amer. Jour. Clin. Path. 21, 282.

11. Frobisher and Parsons, 1950. Amer. Jour. Hyg. 52, 239.
12. Glenny, 1931. In *A System of Bacteriology in Relation to Medicine*, 6, H. M. Stationery Office, London.
13. Healey and Pinfield, 1935. Brit. Jour. Exp. Path. 16, 535.
14. Ipsen, 1951. Jour. Immunol. 66, 691.
15. Ipsen, 1952. Personal communication.
16. Kolmer and Tuft, 1941. *Clinical Immunology, Biotherapy and Chemotherapy*, W. B. Saunders Co., Philadelphia.
17. League of Nations, 1940-41. Bull. Health Org. 9, 470.
18. MacLeod and Pappenheimer, 1952. In Dubos, *Bacterial and Mycotic Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
19. Miller and Ryan, 1950. Jour. Immunol. 65, 143.
20. Moloney and Fraser, 1927. Amer. Jour. Pub. Health 17, 1027.
21. Pappenheimer, 1942. Jour. Bact. 43, 273.
22. Pappenheimer and Hendee, 1949. Jour. Biol. Chem. 180, 597.
23. Pappenheimer and Robinson, 1937. Jour. Immunol. 32, 291.
24. Park and Schroder, 1932. Amer. Jour. Pub. Health 22, 7.
25. Petermann and Pappenheimer, 1941. Jour. Phys. Chem. 45, 1.
26. Pillement and Wartman, 1947. Jour. Immunol. 55, 277.
27. Pillement, Wittler, Burrell and Grossberg, 1948. Jour. Exp. Med. 88, 205.
28. Ramon, 1922. Compt. rend. Soc. de Biol. 86, 661, 711, 813.
29. Reed, 1952. In Dubos, *Bacterial and Mycotic Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
30. Reed and Muench, 1938. Amer. Jour. Hyg. 27, 493.
31. Regamey, 1947. Bull. Serv. féd. hyg. pub. 17.
32. Römer, 1909. Ztschr. f. Immunitätsforsch. 3, 208.
33. Schick, 1908. Münch. med. Wschr. 55, 504.
34. Schultz and Charlton, 1918. Ztschr. f. Kinderh. 17, 328.
35. Smith, 1942-43. Bull. Health Org., League of Nations 10, 104.
36. Smith and Conant, 1952. *Zinsser's Textbook of Bacteriology*, 10th ed., Appleton-Century-Crofts, New York.
37. Van Heyningen, 1950. *Bacterial Toxins*. Basil Blackwell & Mott, Ltd., Oxford.
38. Veneer and Bower, 1940. Jour. Amer. Med. Assoc. 114, 2198.
39. Wadsworth, 1947. *Standard Methods*, 3rd ed., Williams and Wilkins Co., Baltimore.
40. Zinsser, Enders and Fothergill, 1940. *Immunity, Principles and Applications in Medicine and Public Health*, The Macmillan Co., New York.

## Chapter 11

### PHAGOCYTOSIS

BORDET AND MANY OTHER INVESTIGATORS maintained that the resistance of an animal to a parasite is closely paralleled by the phagocytic vigor of the animal's cells. Injected bacteria are usually removed from the blood within a few minutes. The bactericidal power of the body fluids is limited, so apparently a cellular mechanism is also involved. This is confirmed by microscopic examination of tissues from recently injected animals. The organisms are often found in the liver, spleen, lung and other organs. Furthermore, pathogenic bacteria are less likely to produce fatal disease when introduced into an area rich in phagocytic cells, and a favorable turn in an infection is often accompanied by an increase in the number of circulating phagocytes.

The virulence of microorganisms is frequently associated with an antiphagocytic substance such as the capsular material of pneumococci. Noncapsulated variants of the pneumococcus are readily ingested by phagocytes, whereas the normal capsulated form is not.

**Kinds of Phagocytic Cells.** There are two chief varieties of phagocytic cells in the mammalian body: macrophages and microphages. Macrophages comprise the cells of the R-E system and are either "fixed" or "wandering." Fixed macrophages line the endothelium of capillaries and of the sinuses of organs such as the spleen, bone marrow and lymph nodes. Reticular or supporting cells of various organs, especially the liver, also possess phagocytic properties. Fixed macrophages normally appear to participate in the disposal of worn out and fragmented red blood cells. Wandering macrophages migrate through tissues and assist in the repair of damage by destroying and absorbing dead materials and by aiding in the disposal of erythrocytes which have passed out of blood vessels. Microphages include the polymorphonuclear leukocytes of the blood. Of these, neutrophiles possess the greatest phagocytic activity; eosinophiles and basophiles possess much less activity, if any.

**Opsonic Theory.** The presence of bacteria within leukocytes was

noted as early as 1870, but the observation was not associated with resistance to disease. As an outgrowth of his studies of intracellular digestion in the waterflea, daphnia, Metchnikoff was apparently among the first to suggest that leukocytic ingestion plays a role in resistance. Daphnia, being transparent, provided an excellent subject for the microscopic study of digestion. Metchnikoff observed ameboid cells within daphnia which could ingest small numbers of certain yeasts. Large numbers of the yeast cells, however, injured the ameboid cells, and the waterflea itself might be killed. Metchnikoff proposed that these phagocytic cells, as he called them, constituted a defense of the animal against fatal infection.

The importance of noncellular blood components in phagocytic ingestion was later shown. Denys and Lecler in 1895 and 1898 reported that immune serum promoted active phagocytosis much more effectively than normal serum. Metchnikoff maintained that this indicated the presence in immune serum of a substance, *stimulin*, which increased the phagocytic activity of leukocytes. The stimulin hypothesis was shown to be incorrect when Wright and Douglas demonstrated that the enhancement of phagocytosis by serum results from the action of a serum component on the bacteria rather than on the leukocyte.<sup>37</sup> Wright named this component *opsonin*, a term which is still used.

#### METHODS OF STUDYING PHAGOCYTOSIS

The most precise investigations of phagocytosis have been conducted *in vitro*, usually with neutrophiles as the phagocytic cells. Neutrophiles are the most abundant leukocytes in whole blood and can be secured with relative ease. Blood from humans or other animals is either defibrinated or treated with sodium citrate or heparin to prevent clotting. The leukocytes may be concentrated by centrifuging the blood at moderate speed and carefully removing the top layer of sedimented cells with a capillary pipette. Sterile exudates containing large numbers of neutrophiles can be obtained by injecting rabbits of 2.5 to 4 kilograms intraperitoneally with 300 milliliters of 1 per cent sodium chloride or Ringer's solution and removing the peritoneal fluid eighteen hours later.<sup>37</sup> Injection of sterile olive oil yields exudates containing principally mononuclear cells after five days.

The composition of the diluting fluid is an important factor in the success of phagocytosis experiments. Normal physiologic saline has been widely used, but a balanced salt solution maintains the cells in better condition. Small amounts of calcium chloride favor phagocytosis. Victor and co-workers recently used a modified Krebs solution in studies with Brucella.<sup>44</sup> This solution contained gelatin, potassium chloride, calcium chloride, magnesium sulfate, potassium phosphate and sodium bicarbonate.

Several methods of measuring the extent of phagocytosis have been devised. The procedure of Leishman,<sup>20</sup> further developed by Wright and Douglas, determined the average number of particles ingested per leukocyte in a given time. Equal parts of whole blood or leukocyte suspension and bacterial suspension were mixed and incubated at body temperature. Smears were stained and the bacteria engulfed by fifty or 100 neutrophiles were counted. The average number of bacteria per leukocyte was called the *phagocytic index*.

Hamburger modified the Wright method by determining the percentage of leukocytes which engulfed particles in a given time.<sup>22</sup> This procedure gave a measure of the number of cells at work but not of the amount of work done. McKendrick showed, however, that the average number of bacteria per leukocyte could be estimated from the percentage of empty leukocytes, assuming a statistically normal distribution of bacteria ingested.<sup>27</sup>

Fenn employed known numbers of cells and particles and counted the uningested particles at various intervals of time.<sup>16</sup> This method also measured the amount of work done. He employed gentle but constant agitation to secure more consistent results in duplicate experiments. Test mixtures in sealed tubes were mounted horizontally on a drum which revolved slowly about its horizontal axis.

A somewhat similar technique was applied by Maaløe to the phagocytosis of *Salmonella breslau*.<sup>28</sup> Approximately 30,000 bacteria were mixed with a saline suspension of washed phagocytes and incubated for thirty minutes. The reaction was then stopped by diluting with ten volumes of cold saline and the blood cells with ingested bacteria were thrown down by moderate centrifugation. Unphagocyted bacteria in the supernatant liquid were counted by plating. Similar counts were made in control tubes lacking phagocytes, and the number and percentage of ingested bacteria were calculated.

Phagocytosis may be studied qualitatively *in vivo* by histologic methods. A somewhat quantitative approach consists of counting viable bacteria in the blood of animals at intervals after intravenous injection. Best results are secured with organisms such as pneumococci, streptococci and staphylococci, which are resistant to lysis by complement in the presence of antibody. Microscopic examination and plate counts of emulsions of various organs like the spleen, liver, lungs and bone marrow indicate the location and relative effectiveness of the phagocytic cells of the body. The important role of reticuloendothelial cells is shown by such experiments. Bull, Wright and others injected animals intravenously with large numbers of pneumococci or streptococci and found that the bacteria in the blood decreased rapidly until about the fifth hour after injection.<sup>6, 7, 8, 58</sup> In some experiments with organisms of high virulence, a secondary rise in the number of circulating bacteria occurred.

## ROLE OF SERUM CONSTITUENTS IN PHAGOCYTOSIS

The importance of humoral blood components in phagocytosis was indicated by early experiments. "Spontaneous phagocytosis" of bacteria by leukocytes which have been washed with saline results in the ingestion of very few organisms per leukocyte. The phagocytic index in saline by the Wright technique is usually 1.0 or less. Addition of normal serum increases the phagocytic index to 5 or 10 or occasionally to 20. Immune serum may promote such extensive phagocytosis that the leukocytes are crowded with bacteria; phagocytic indexes of 80 or more have been noted.

By experiments such as the following, Wright showed that the phagocytosis-promoting substance of serum acted on the bacteria and not on the leukocytes:

MIXTURES	AVERAGE NUMBER OF BACTERIA PER LEUKOCYTE
(1) Washed leukocytes + saline + staphylococci	1.2
(2) Leukocytes + serum + staphylococci	20.0
(3) Leukocytes + serum, washed, + staphylococci	2.4
(4) Staphylococci + serum, washed, + leukocytes	18.4

The phagocytic activity of leukocytes exposed to serum and washed with saline (3) was little better than that of untreated leukocytes suspended in saline (1). The bacteria were sensitized by serum, however, whether they remained in contact with it (2) or were washed to remove uncombined serum constituents (4). Heating the serum to 60° C. for fifteen minutes markedly reduced phagocytosis: only 4.2 bacteria were ingested per leukocyte instead of 20. The thermolabile phagocytosis-promoting component of serum was *opsonin*.

The possible identity of normal serum opsonin and complement was suggested by their thermolability. Moreover, both normal opsonin and complement may be adsorbed from serum by red blood cells and other antigens sensitized with homologous antibodies. Zinsser, Enders and Fothergill pointed out, however, that this does not necessarily mean that normal opsonin is the same as complement.<sup>58</sup> Normal opsonic action may, instead, require the participation of two serum constituents. Phagocytosis in normal serum is therefore attributed either to the action of the thermolabile opsonin or to normal antibody with or without the cooperation of a thermolabile substance resembling complement. Normal antibody appears to be thermostable, according to observations of Cowie and Chapin,<sup>10</sup> Ward and Enders,<sup>45</sup> and others.

Neufeld and Rimpau demonstrated that the phagocytosis-promoting substance in immune serum was thermostable and specific and called it *bacteriotropin*.<sup>35, 36</sup> Ward and Enders found pneumococcal bacteriotropin to be identical with antibody which causes precipitation and bactericidal

action. Phagocytosis of type 2 pneumococci in the presence of homologous antiserum was almost completely inhibited by type 2 specific polysaccharide but was practically unaffected by type 1 or type 3 polysaccharides. Type 2 polysaccharide combined with homologous antibody and thus prevented sensitization of the pneumococci.

Ward and Enders found that complement accelerated phagocytosis in the presence of diluted immune serum.<sup>48</sup> Pneumococci were employed with 1:128 rabbit antiserum. In the presence of human complement each leukocyte ingested an average of fourteen bacteria in two hours, whereas in saline controls lacking complement eight hours were required for the same amount of phagocytosis.

Zinsser, Enders and Fothergill concluded that specific serum factors or antibodies may participate in both normal and immune opsonic action.<sup>68</sup> The thermolabile substance resembling complement accelerates phagocytosis, particularly when antibody is present in relatively low titer. The action of this component is less apparent if large amounts of antibody are employed.

It is difficult to evaluate much of the work on opsonization because many investigators did not record whether they employed normal or immune serums, native or inactivated by heat, washed leukocytes or whole blood.

Many authors agree that, in the light of present knowledge, the term bacteriotropin should be discarded because it refers to the same antibody as that which causes agglutination, precipitation and bactericidal action. Ward and Enders and others prefer the word opsonin, even though it was originally employed to designate the heat-labile phagocytosis-promoting substance of normal serum. Maaløe, on the other hand, refers to the thermostable component or bacteriotropin of immune serum simply as antibody. He discards the word opsonin entirely and believes complement to be the sole thermolabile serum constituent active in phagocytosis.

#### FACTORS AFFECTING PHAGOCYTOSIS

Phagocytosis of bacterial cells or other particles is a two-stage process. In the first stage contact between the phagocyte and the cell is established; the second stage consists of ingestion of the cell. Factors which affect these steps *in vivo* can be deduced only in part from *in vitro* studies. Furthermore, phagocytosis by leukocytes freely suspended in fluid differs from phagocytosis by leukocytes attached to a surface. White blood cells are generally believed to be incapable of swimming in a liquid and possess independent locomotion only upon solid substrates.

**Phagocyte-Particle Contact.** Fenn found that the rate of phagocytosis of quartz and carbon particles followed the equation for a monomolecular reaction; that is, the percentage of particles ingested per unit of time was

constant, whether few or many particles were present.<sup>16</sup> He concluded that the rate of phagocytosis is determined under ideal conditions by the chance of collision between phagocytes and particles. The chance of collision in liquid suspensions depends upon the size and density of the particles in relation to the size and density of the phagocytic cells. Dense particles settle more rapidly in a gravitational field and overtake the phagocytes more quickly. Moreover, large particles and clumps of cells have a greater opportunity of colliding with phagocytes than small particles and dispersed cells. He also found that the rate of phagocytosis varies directly with the speed of mixing during incubation.

Hanks investigated the effect of the relative number of bacteria and leukocytes and the total number of each per unit of volume.<sup>23</sup> A greater percentage of leukocytes ingested bacteria and a greater number of cells was ingested per leukocyte when increasing numbers of bacteria were mixed with a constant amount of leukocytes. The effectiveness of the system as a whole increased when more leukocytes were mixed with a constant number of bacteria, but fewer bacteria were ingested per leukocyte and a smaller percentage of leukocytes ingested bacteria. Greater phagocytic action also resulted from increasing the absolute numbers of bacteria and leukocytes in a constant ratio.

Fenn found that clumping of phagocytes or particles increased the rate of phagocytosis.<sup>17</sup> Carbon particles had a tendency to cause aggregation of leukocytes, and a greater percentage of aggregated than of unaggregated leukocytes contained carbon particles. Serum was essential for phagocytosis of carbon. The active serum constituent was obviously not antibody, but was thermolabile like complement, as shown by almost complete lack of phagocytosis in heated serum.<sup>20</sup>

Carbon particles were ingested by leukocytes in liquid suspension about four times as readily as quartz when both kinds of particles were present. Fenn repeated the tests by a cover slip method in which the particles and cells settled out and the leukocytes moved about on the slide. Carbon was again ingested more rapidly than quartz. The rate of phagocytosis decreased after the first hour but the ratio of carbon to quartz taken up generally increased with time. Carbon suspensions were less stable than quartz suspensions and it was suggested that more rapid ingestion of carbon might be caused by the factor responsible for greater instability. Fenn tentatively suggested that this factor might be surface tension. Mudd proposed that carbon and quartz might differ in their ability to adsorb phagocytosis-promoting substances from serum.<sup>22</sup> In the carbon and quartz experiments, such substances are evidently nonspecific proteins.

Collision between phagocytes and quartz or carbon particles in either the suspension or cover glass method seemed to be controlled by chance. Direct observation of one microscope field revealed approximately equal numbers of contacts between leukocytes and quartz and carbon particles

respectively, and yet twelve times as many carbon particles were ingested as quartz. Similar experiments with  $MnO_2$  and  $MnSiO_3$  particles showed that collision of leukocytes with  $MnO_2$  was not entirely fortuitous.<sup>18</sup> Equal numbers of the two kinds of particles were present, but 2.4 times as many contacts occurred with  $MnO_2$  as with  $MnSiO_3$ . The phagocytes were evidently attracted chemically by  $MnO_2$  particles and frequently advanced toward them instead of wandering in random fashion.

Ameboid migration of phagocytic cells toward bacteria and certain other particulate materials is probably the result of *chemotaxis* (response to chemical stimuli). Positive chemotaxis (i.e., attraction) is exerted on leukocytes by living or dead bacteria, by bacterial extracts and by proteoses, peptones and dextrans arising from partial decomposition of proteins and polysaccharides of bacterial and tissue origin.<sup>12</sup> Specific polysaccharides of staphylococci and certain other gram positive bacteria are positively chemotactic in the animal body as are polysaccharide haptenes of typhoid and paratyphoid B, Shiga and Flexner dysentery and coliform bacteria.<sup>4</sup> Nonbacterial polysaccharides such as soluble starch, gum arabic and inulin, and the glucoproteins of gastric mucin and vitreous humor are also distinctly chemotactic. Leukotaxine, isolated by Menkin from sterile inflammatory exudates, increases the permeability of capillary walls and permits leukocytes to approach the site of liberation of leukotaxine.<sup>20</sup> This process is known as *diapedesis*. Leukotaxine appears to be a relatively simple polypeptide containing an indol nucleus, an alpha-amino group and possibly a carbohydrate or lipid prosthetic radical.

Endotoxins of the gram negative bacteria strongly inhibit diapedesis of leukocytes *in vivo* and exert a "negatactic" effect. Boivin and Delaunay suggested that in some manner these agents modify the vascular endothelium through which leukocytes migrate.<sup>4</sup> They also pointed out that, when liberated from combination with the lipid-protein complex by heating in a slightly acid medium, the specific polysaccharide component of the endotoxin of gram negative bacteria behaves exactly like the polysaccharide of gram positive organisms: not only does it fail to inhibit diapedesis but it energetically attracts leukocytes.

The mechanism of chemotactic migration is not clear. Osmotic effects and surface potential differences between the leukocyte and the bacterial cell or particle have been suggested as causes of migration. Local changes in leukocyte surface tension may play a major role. Positively chemotactic substances diffusing from the particle are assumed to lower the surface tension of the proximal side of the leukocyte, which thereupon migrates toward the source of the substance.

**Ingestion.** Hypotheses of the ingestion of particles by leukocytes are derived in part from studies of the ingestion of food by protozoa. Four methods of food intake have been recognized in amebae: (1) a food

particle in contact with the surface of the ameba sinks into the protoplasm without any unusual movement by the cell; (2) the protoplasm of the ameba flows around the particle in immediate contact with its surface; (3) protoplasmic pseudopods surround the food particle without coming into direct contact with it; (4) the food particle adheres to the sticky surface layer of the ameba and is invaginated into the interior of the cell. Phagocytic ingestion is believed to occur by the first and second processes: that is, either the particle sinks into the phagocyte protoplasm, or the latter flows around it.<sup>33</sup>

Physical considerations indicate that whether or not ingestion takes place after contact has been established is determined by the surface energy forces between the particle and the fluid medium, the phagocyte and the fluid, and the phagocyte and the particle. Ingestion is favored by: (1) increased surface energy between the particle and the suspending fluid, (2) decreased surface energy between phagocyte and suspending medium and (3) decreased interfacial energy between particle and phagocyte.

Mudd and co-workers demonstrated that homologous antiserum altered the surface properties of acid-fast bacteria, erythrocytes and collodion particles coated with protein as measured by the agglutinability, electrophoretic charge and wetting characteristics in an oil-water interface.<sup>32</sup> These effects decreased as the concentration of sensitizing antiserum decreased. Phagocytic ingestion of the particles closely paralleled their surface properties. Complement was apparently not an essential reagent, because heating the serums at 56° C. for thirty minutes had little effect upon either the surface properties or phagocytic behavior of sensitized bacteria. Fractionation of the serums revealed that all these effects were attributable to the euglobulin and pseudoglobulin components. The iso-electric points of a variety of bacteria were all brought to approximately pH 5.2 by sensitization with adequate amounts of homologous antisera; the iso-electric points of unsensitized bacteria varied from pH 2.7 to pH 3.8. Specific immune serum evidently combined with the antigen surface and favored phagocytosis by altering the surface energy of the organism.

Berry confirmed the role of surface tension in phagocytosis by treating phagocytic cells with surface tension depressants.<sup>2</sup> Three anionic and two nonionic detergents approximately doubled the average number of bacteria ingested by neutrophiles. Incubation of bacterial cells with detergent before mixing them with leukocytes depressed phagocytosis. It was concluded that phagocytosis increases when the surface tension or surface energy of the leukocyte is lowered, but phagocytosis is decreased by lowering the surface tension of the bacteria.<sup>1</sup>

Phagocytosis may be affected by various ions. Iodides and the fluoride, sulfite and citrate of sodium decrease phagocytosis.<sup>21</sup> Calcium chloride markedly increases the rate of phagocytosis, whether tests are performed

in saline or in serum, but does not alter the final percentage of cells which ingest particles. The optimum concentration of calcium chloride is 0.02 per cent. This concentration in the presence of serum promotes most complete coating of the particles with protein and thus prepares them for phagocytosis. Calcium chloride in the absence of serum induces clumping of the leukocytes and therefore increases their chances of collision with particles.

Hektoen and Ruediger observed that all salts tested other than sodium chloride inhibited phagocytosis when added to mixtures of defibrinated blood and bacteria.<sup>24</sup> Previous treatment of the bacteria or the leukocytes with the same salts did not reduce phagocytosis. They concluded that those salts which inhibit phagocytosis interfere with opsonization of bacteria. Mudd believed that such experiments indicate that ions affect the adsorption of serum components to the test particles and also influence the surface properties of serum-sensitized particles.<sup>23</sup>

Homologous bacteriophage specifically increases phagocytosis of phage-susceptible organisms but not of strains resistant to the phage.<sup>24</sup> The action of bacteriophage appears to be principally upon the bacteria, rendering them more easily ingested. Bacteriophage may, however, also slightly increase the ability of leukocytes to ingest homologous bacteria. The action of bacteriophage adsorbed to leukocytes appears uncertain.

Phagocytosis has been reported at temperatures from 0° to 60° C. Most investigators agree that the rate of phagocytosis increases with rising temperature to 40° or 44° C. Ellingson and Clark found that 38° to 40° C. was optimal for phagocytosis of staphylococci in human blood.<sup>14</sup> Fenn noted that increasing temperature affected ingestion of quartz or carbon particles in the presence of serum more greatly below 30° C. than above and concluded that the temperature effect was associated with the viscosity of the leukocytes, which was less at higher temperatures.<sup>17</sup>

The effect of viscosity on rate of phagocytosis is further indicated by observations that polymorphonuclear leukocytes are less viscous than macrophages and phagocytize particles at a higher rate.

It is obvious that the various factors which affect phagocytic ingestion either modify the surface properties of the phagocyte and/or the particle, or modify the consistency of the phagocyte. Ingestion appears to be a phenomenon of surface spreading, that is, spreading of the phagocyte surface over that of the object being ingested. Vacuoles are not formed as an integral part of the process. Any vacuoles which appear are digestive in nature and develop subsequent to ingestion. Sensitization of the particle by immune or normal serum is the usual means of modifying surface properties. Ions present in the medium may favor or hinder such sensitization.

**Surface Phagocytosis.** Ingestion of bacteria in the absence of detectable antibody was reported by Wood in 1946 in the course of attempts

to determine the mechanism of rapid recovery of pneumonia patients treated with sulfonamides and antibiotics.<sup>54</sup> He discovered that bacteria may be mobilized on tissue surfaces and ingested before antibody appears in the circulation. This phenomenon was called *surface phagocytosis*. Leukocytes in the lungs of rats ingested virulent, encapsulated pneumococci after trapping them against the alveolar walls or against other leukocytes. This process was also demonstrated *in vitro* by microscopic

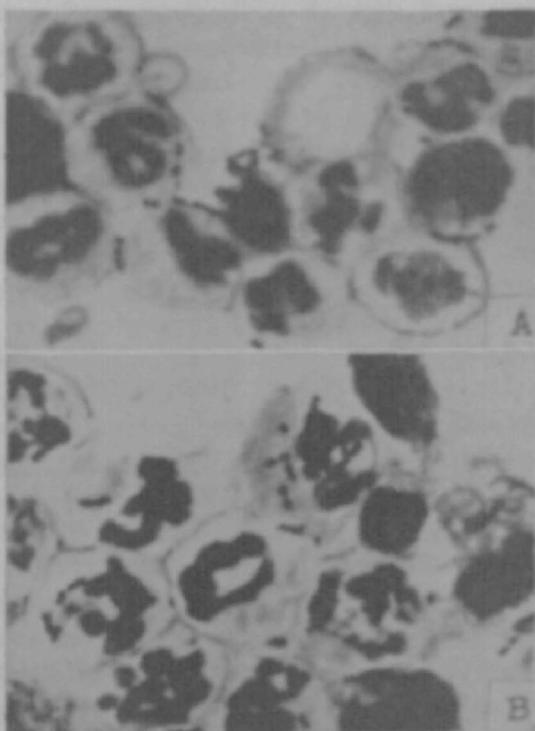


FIGURE 42. A. Failure of phagocytes to engulf encapsulated pneumococci on glass surface. Many pneumococci are in contact with the surfaces of the cells, but none has been ingested.

B. Phagocytosis of pneumococci on surface of moistened filter paper. Many organisms are within the cytoplasm of the phagocytes. (From Wood *et al.*, 1946. *Jour. Exp. Med.* 84, 387.)

examination of mixtures of thrice washed leukocytes and pneumococci upon a variety of substrates. Phagocytosis occurred when the substrates consisted of tissues, either living or killed by formaldehyde or heat, or "rough" materials such as filter paper, cloth and fiber glass (Figure 42).<sup>54</sup> Phagocytosis in the absence of serum components did not occur upon "smooth" substances like glass, paraffin, cellophane or mucus on glass. The nature of the available surface was therefore an important factor in phagocytosis when serum was not present.

These observations are contrary to the concept that encapsulated viru-

lent bacteria are phagocytized only after previous sensitization with specific antibody. Phagocytes within the body operate under conditions vastly different from those in the test tube. The occurrence of surface phagocytosis does not preclude the participation of antibody in recovery from pneumonia or in phagocytosis within the body. Antibody, when present, presumably agglutinates and immobilizes the organisms on the cell surfaces, thus checking spread of the infection. It also probably opsonizes the bacteria, particularly those floating free in edema fluid within the alveoli. The accumulation of antibody in sufficient concentration normally requires several days after pneumococcal infection, and the disease may be terminated one way or the other before antibody can play a role.

Wood demonstrated that surface phagocytosis also occurs in infections caused by *Klebsiella pneumoniae*, streptococci and staphylococci.<sup>42, 52, 56</sup> It may constitute an important early defense in all inflammatory processes.

Berry and Spies suggested that contact with a suitable rough menstruum such as capillary endothelium or filter paper causes a local reduction in surface energy of the phagocyte which permits ingestion of bacteria.<sup>1</sup>

#### TYPES OF PHAGOCYTOSIS

Contact between phagocytic cells and particles suspended in a fluid medium is subject to chance and is influenced by agitation. Contact upon a solid substrate is established by ameboid migration of the phagocyte, which may be either random or directed by chemotactic attraction toward substances diffusing from the particle.

Three principal types of phagocytosis are distinguished:

(1) *Spontaneous Phagocytosis.* Relatively few phagocytes participate and few bacteria are ingested; neither antibody nor complement is required. *Surface phagocytosis* appears to be spontaneous phagocytosis occurring upon suitable rough surfaces, either *in vivo* or *in vitro*.

(2) *Normal Phagocytosis.* Marked phagocytosis occurs; complement and/or "normal" antibody may participate.

(3) *Immune Phagocytosis.* Very active phagocytosis takes place; specific antibody is required and complement accelerates phagocytosis.

#### DISPOSAL OF INGESTED BACTERIA

Ingestion of bacteria by phagocytes is often followed by death and destruction of the bacteria. Most gram positive cocci are digested intracellularly. The phagocytic destruction of pneumococci and streptococci has been known for many years. Wood found that pneumococci eventually disappeared entirely from the leukocytes which ingested them, whether or not antibody was present.<sup>54</sup> Robertson and van Sant observed that dog macrophages possessed greater ability than polymorphonuclear

leukocytes to digest type 1 pneumococci.<sup>38</sup> The difference was marked in mixtures containing low concentrations of specific antibody, but serums of high titer induced more nearly equal digestion by the two kinds of phagocyte.

Toxigenic staphylococci are not killed by leukocytes, although they may be readily ingested. There is some evidence that gonococci survive, at least for a time, within phagocytic cells. Viable *Hemophilus influenzae* have been detected in pus cells from spinal fluid. The dissociative state of some organisms is apparently correlated with their susceptibility to intracellular digestion. Rough forms of *Salmonella schottmüller* and *Shigella dysenteriae* have been found susceptible to digestion, the smooth forms being resistant.

Delay between the ingestion of bacteria and their destruction may protect the organisms against lysis by antiserum and permit their multiplication and eventual spread about the body. Rous and Jones reported that intracellular typhoid bacteria resisted the lethal effect of potassium cyanide.<sup>40</sup> Acid-fast bacteria, particularly *Mycobacterium tuberculosis*, are often rapidly ingested by polymorphonuclear leukocytes,<sup>31</sup> but survive and resist digestion and are transported via the blood to other parts of the body. The organisms are toxic to the leukocytes and eventually kill them, whereupon fresh local sites of infection are established. The tuberculosis organism is ingested more slowly by macrophages but may be lysed by them. Intracellular digestion is accelerated when immune serum is present at the time of ingestion.

The mechanism of intracellular digestion is not known with certainty. Extracts of leukocytic exudates contain substances called *leukins*, which are bactericidal for certain microorganisms. These and various proteolytic, lipolytic and other enzymes probably play a role in the killing and disintegration of ingested bacteria.

#### PHAGOCYTOSIS IN RELATION TO DISEASE

Phagocytosis plays a significant role in recovery from pneumonia. It is also an important means of combating other pyogenic infections. Hemolytic streptococci are phagocytized and are then destroyed within the phagocytic cells. Urethral smears in gonorrhea and spinal fluids in meningitis often show large numbers of bacteria ingested by neutrophiles. Acute infections are usually characterized by a high percentage of neutrophiles. Lymphocytes increase in areas of chronic inflammation, and monocytes appear in late stages of inflammation and are typical of exudates in recovery from pyogenic infections.

Phagocytosis is undoubtedly important in the response to other types of infectious agents. The evidence in spirochete infections is conflicting. Several investigators reported indications of active phagocytosis in syphilis

and relapsing fever,<sup>1</sup> but Chesney stated that he had never seen a single instance of phagocytosis of *Treponema pallidum* in human syphilis or in experimental rabbit infections.<sup>9</sup> Moreover, Stavitsky failed to find phagocytosis in experimental leptospirosis, either in exudates, *in vitro* mixtures or stained tissue sections.<sup>48</sup>

Phagocytosis of certain viruses has been noted, but it is doubtful that the viruses are always destroyed. Merling observed ingestion of vaccinia virus by darkfield examination, but the intracellular virus survived and formed colonies, and the phagocytic cells eventually died.<sup>80</sup> Sabin also concluded that immune serum did not necessarily opsonize vaccinia or promote virus destruction by the leukocytes.<sup>41</sup>

Some strains of bacteria are less susceptible to ingestion than others. For example, Hale and Smith found that coagulase positive staphylococci were less easily ingested than coagulase negative strains in the presence of coagulable plasma.<sup>20</sup> They suggested that formation of a fibrin envelope around the organisms either prevented combination with antibody or nullified the surface changes produced by antibody.

Certain types of antibody appear to be necessary for opsonization of some bacteria, such as the typhoid organism. Delaunay found that phagocytosis of *Salmonella typhosa* was enhanced by O antibody but not by H antibody.<sup>13</sup> Bhatnagar reported that inagglutinable strains were highly resistant to ingestion by leukocytes,<sup>8</sup> and Felix and Bhatnagar found that Vi antibody exerted a powerful phagocytosis-promoting action on Vi strains but had no effect on strains lacking Vi antigen.<sup>15</sup>

**Measurement of Opsonizing Antibody.** Wright developed a procedure which was used for many years to determine the opsonizing power of patients' serums. Serum from the patient was compared with pooled serum from a number of supposedly normal individuals. Equal quantities of leukocytes from a normal person, serum and bacterial emulsion were mixed in capillary tubes and incubated. Smears were stained and the phagocytic index, or average number of bacteria ingested per leukocyte, was determined for the normal pool and for the patient's serum. The *opsonic index* of the patient's serum was calculated by dividing its phagocytic index by the normal phagocytic index.

Opsonic index determinations require careful attention to details. The nature of the normal serums used for reference is important. An individual who has recently suffered infection with the organism in question is likely to possess opsonizing antibody. It is therefore necessary to use the serums of at least five presumably normal individuals. Patients' serums may also vary from day to day or even within the same day. Repeated tests are sometimes advisable. The degree of mixing of reagents during incubation markedly affects the extent of phagocytosis. Wright's technique did not specify uniform and constant mixing. Shaking and rotating machines were soon introduced and gave more consistent results. Immune

serums and old serums sometimes produce a prozone in which the undiluted serum appears to have no opsonic action. Some investigators therefore employ serial dilutions of serum and determine the highest dilution producing phagocytosis. The age of the bacteria is important, particularly in tests with streptococci and pneumococci. Young encapsulated cells are resistant to phagocytosis. Cultures of uniform age, generally twenty-four hours, should always be used.

Wright used the opsonic index to determine the course of vaccine therapy best suited to an individual case of disease. An opsonic index higher than 1.0 denotes greater opsonizing power than that of normal individuals, and is found in recuperating patients. An index less than 1.0 occurs in patients with overwhelming infection, particularly if repeated determinations reveal a continued drop. A decreased opsonic index may also follow injection of antigen and may be accompanied by aggravation of symptoms. This reaction is called the *negative phase*. The index then rises to its former level or even higher as antibody is produced, and at the same time symptoms abate.

Wright was mainly responsible for introduction of vaccine therapy of certain infectious diseases. This treatment, when properly controlled by opsonic index determinations, sometimes hastens recovery of chronic infections but is of little use in acute infections.

Victor and co-workers recently described an opsonin titration in which the antibody concentration was determined by serial dilutions of plasma.<sup>44</sup> The cells from heparinized blood were washed six times with tenfold volumes of Krebs-gelatin solution and mixed with equal volumes of plasma dilutions and bacterial suspension. Mixtures were incubated thirty minutes at 37° C. while slowly rotating. Smears from each mixture were examined to determine the percentage of neutrophiles which had ingested bacteria. A "unit" of opsonin was that quantity which promoted phagocytosis by 94 to 100 per cent of the cells. Brucella opsonin titers of persons presumably never exposed to this organism were not more than 1.0. Titers from 1.0 to 100 were found in well persons who did not have histories of clinical brucellosis but had been exposed to the organism and gave positive skin or agglutination tests. Individuals recovered from brucellosis also possessed opsonin titers between 1.0 and 100. Titers ranged from 1.0 to 1,000,000 in clinical cases of the disease. An opsonin titer of 1,000 appeared to be diagnostic of active infection.

Opsonic studies in brucellosis have also been made for several years by the *opsonocytophagic test*. Fresh citrated patient's blood is mixed with an equal volume of a heavy suspension of smooth Brucella cells. The mixture is incubated without agitation in a waterbath at 37° C. for thirty minutes. Stained smears are examined and the degree of phagocytosis by polymorphonuclear leukocytes is classified according to the number of bacteria ingested:

No phagocytosis.....	No bacteria ingested
Slight phagocytosis.....	1 to 20 bacteria ingested
Moderate phagocytosis .....	21 to 40 bacteria ingested
Marked phagocytosis.....	More than 40 bacteria ingested

Individuals whose specimens are negative or slightly phagocytic are probably susceptible to brucellosis.<sup>5</sup> Marked phagocytosis in over 40 per cent of the leukocytes indicates a high level of immunity, as in a convalescent case or recovered individual. Intermediate levels of phagocytic activity are found in persons who are not immune but may have the disease. The opsonocytophagic test apparently measures the same antibody as the agglutinin test.

A somewhat similar opsonic test was used by Kendrick in studying the immune response to pertussis vaccine.<sup>26</sup> Opsonocytophagic tests indicated the persistence of high titers at least four years after immunization of babies. "Booster" inoculations increased the average titers by a factor of 1.6. Placental transfer of pertussis antibodies was also investigated. Babies of immunized mothers possessed nearly three times as much opsonin as those of nonimmunized mothers. The blood of immunized mothers contained twice as much opsonizing antibody as that of nonimmunized mothers. Average opsonin titers in each group of babies was lower than in the corresponding groups of mothers. Cravitz and Williams found a high correlation between opsonizing antibody and a history of pertussis.<sup>11</sup> Opsonic activity in immunized children paralleled agglutinin activity determined by a microscopic test. In children who had recovered from active infection the opsonic test was about 50 per cent stronger than the agglutination reaction.

The mode of action of the sulfonamide drugs was for several years the subject of considerable controversy. Some investigators maintained that the drugs enhanced phagocytic activity. Dilute solutions of sulfapyridine were reported to promote phagocytosis of *Streptococcus viridans*, *Staphylococcus aureus* and noncapsulated *Diplococcus pneumoniae*. Some of these organisms, when grown in a sulfonamide solution, underwent smooth to rough transformation and became more phagocytizable. Furthermore, phagocytosis of *Clostridium perfringens* and *Cl. sordellii* was better in wounds treated locally with sulfathiazole than in untreated wounds. Welch and co-workers found that sulfanilamide apparently stimulated the defense mechanism of guinea pigs infected with *Brucella abortus* by increasing the production of specific opsonins and therefore enhanced phagocytosis.<sup>41</sup> Improved phagocytosis was also noted in cases of human brucellosis treated with sulfanilamide.<sup>47</sup> The consensus is that the principal role of sulfonamides is to retard or prevent bacterial multiplication. Wood stated that in pneumonia caused by pneumococci, *Klebsiella pneumoniae*, staphylococci and streptococci, chemotherapeutic agents are

only bacteriostatic in the concentrations usually administered, or at least do not kill the organisms consistently.<sup>58</sup> Final destruction of the bacteria depends upon the host defenses, particularly phagocytosis.

Indiscriminate use of antibiotics and sulfonamides is not necessarily sound from the immunologic point of view. Suppression of growth of the pathogen and its consequent normal phagocytosis eliminates the opportunity for the patient to acquire active immunity. He is therefore susceptible to reinfection and a second attack may be caused by a strain resistant to chemotherapy.

#### PHAGOCYTOSIS IN THE EVALUATION OF ANTISEPTICS AND ANTIBIOTICS

Phenol coefficients or other indexes of germicidal activity provide only part of the information needed to evaluate antiseptics and antibiotics. Chemicals which are toxic to bacteria also possess toxicity for human tissue. Complete evaluation necessitates determination of a concentration in which the substance inhibits or kills bacteria but does not damage human tissue.

Salle devised a toxicity test in which the highest dilution of antiseptic preventing growth of embryonic chick heart tissue was determined. Bronfenbrenner proposed a similar test employing oxygen consumption of adult mouse liver cells. Both procedures are too cumbersome for routine use.

Fleming studied the bactericidal power of mixtures containing whole blood and various concentrations of phenol and found two ranges of phenol concentration within which bacteria were destroyed.<sup>19</sup> High concentrations were directly bactericidal and at the same time were toxic to phagocytes. Low concentrations of phenol were nongermicidal *per se* and were also nontoxic to phagocytes, which therefore could ingest the bacteria. Intermediate concentrations of the chemical inhibited phagocytosis but had no effect upon the bacteria.

Welch and Hunter employed a phagocytic system with *S. aureus* to evaluate the toxicity of various common antiseptics and antibiotics.<sup>40</sup> Human blood and guinea pig blood were satisfactory sources of leukocytes. A suspension of the test organism was artificially sensitized by incubating thirty minutes with an equal volume of 1 per cent chrome alum, washed two times with saline, and diluted to about 500,000,000 staphylococci per milliliter. Mixtures containing dilutions of the antiseptic, citrated guinea pig blood and antigen suspension were rotated at four revolutions per minute for thirty minutes at 37° C. Stained smears were examined and the degree of phagocytosis was classified as in the opsonocytophagic test for brucellosis. A sample protocol is shown in Table 61.<sup>40</sup> Phagocytosis decreased as the dilution of antiseptic decreased.

Table 61. Toxicity of an Antiseptic Tested by Phagocytosis Inhibition

DILUTION OF ANTISEPTIC	DEGREE OF PHAGOCYTOSIS			
	Negative	Slight	Moderate	Marked
Per cent				
0	24	10	14	52
1:2900	20	8	28	44
1:2600	44	20	16	20
1:2300	68	8	8	16
1:2000	80	16	4	0
1:1900*	100	0	0	0
1:1800	100	0	0	0

\* Greatest dilution completely inhibiting phagocytosis (toxic endpoint) = 1 1900.  
(From Welch and Hunter,<sup>40</sup> by permission.)

The greatest dilution which completely inhibited phagocytic activity was called the "toxic endpoint" of the antiseptic.

Toxicity tests were used in conjunction with a bactericidal test in which staphylococci and dilutions of antiseptic in the presence of 10 per cent blood were incubated for thirty minutes and tested for bactericidal action. The toxicity index of a chemical was calculated by dividing the highest dilution inhibiting phagocytosis by the highest bactericidal dilution. Theoretically the smaller the index the more satisfactory the antiseptic if it possesses sufficient bactericidal power.

Welch concluded that the antiseptics tested inhibited phagocytosis by acting on the humoral rather than on the cellular elements of blood.<sup>47</sup> Inhibition of phagocytosis by penicillin sodium appeared to be caused by an osmotic pressure effect.<sup>48</sup> Sixty lots of the drug were examined and the weight of the preparations which completely inhibited phagocytosis was approximately the same, although the inhibitory dosages varied from 1200 units to 16,000 units. The toxic concentrations were greater than those employed in ordinary clinical use of penicillin.

The phagocytic inhibition test was subsequently modified to show the toxicity of antiseptics by determining the quantity required to kill leukocytes.<sup>50</sup> Antiseptic dilutions were mixed with human blood and rotated on the mixing machine for ten minutes. Citrated saline was added to stop toxic action of the antiseptic and the mixture was centrifuged. The supernatant fluid was removed and the activity of the sedimented cells tested by addition of artificially sensitized bacteria and fresh human plasma. These mixtures were rotated for twenty minutes at 37° C., and the degree of phagocytosis in stained smears was determined as in the first method. Higher concentrations of antiseptic were usually required to kill the leukocytes than to inhibit their action. Either method indicated the relative toxicity of a group of antiseptics. The second procedure pro-

vided a better measure of absolute toxicity: actual destruction of human cells.

### References

1. Berry and Spies, 1949. Medicine 28, 239.
2. Berry, Starr and Haller, 1949. Jour. Bact. 57, 603.
3. Bhatnagar, 1935. Brit. Jour. Exp. Path. 16, 375.
4. Boivin and Delaunay, 1948. Exposé ann. Biochem. Med. 8, 31.
5. Borman and West, 1950. In *Diagnostic Procedures and Reagents*, 3rd ed., American Public Health Association, New York.
6. Bull, 1914. Jour. Exp. Med. 20, 237.
7. Bull, 1915. Jour. Exp. Med. 22, 457, 466, 475, 484.
8. Bull, 1916. Jour. Exp. Med. 24, 7, 25.
9. Chesney. Cited by Stavitsky, 1945.
10. Cowie and Chapin, 1907-08. Jour. Med. Res. 17, 57, 95, 213.
11. Cravitz and Williams, 1946. Jour. Pediatrics 28, 172.
12. Delaunay, 1940-41. Rev. d'Immunol. 6, 307.
13. Delaunay, 1943. Compt. rend. Soc. de Biol. 137, 425.
14. Ellingson and Clark, 1942. Jour. Immunol. 43, 54.
15. Felix and Bhatnagar, 1935. Brit. Jour. Exp. Path. 16, 423.
16. Fenn, 1921. Jour. Gen. Physiol. 3, 439, 465, 575.
17. Fenn, 1922. Jour. Gen. Physiol. 4, 331.
18. Fenn, 1923. Jour. Gen. Physiol. 5, 311.
19. Fleming, 1928. Proc. Roy. Soc. Med. 21, 25.
20. Hale and Smith, 1945. Brit. Jour. Exp. Path. 26, 209.
21. Hamburger, 1912. *Physikalisch-chemische Untersuchungen über Phagozyten*, Wiesbaden. Cited by Mudd *et al.* (1934).
22. Hamburger, 1927. In Uhlenhuth, *Handb. biol. Arbeits.*, IV, 4, 953.
23. Hanks, 1940. Jour. Immunol. 38, 159.
24. Heikonen and Ruediger, 1905. Jour. Infect. Dis. 2, 128.
25. Kendrick, Thompson and Elderling, 1945. Amer. Jour. Dis. Child. 70, 25.
26. Leishman, 1902. Brit. Med. Jour. I, 73.
27. McKendrick, 1913-14. *Science Progress in the 20th Century* 8, 497.
28. Maaløe, 1947. Acta Path. et Microbiol. Scand. 24, 33.
29. Menken, 1950. *Newer Concepts of Inflammation*, Charles C Thomas, Springfield, Ill.
30. Merling, 1945. Jour. Path. Bact. 57, 21.
31. Middlebrook and Freund, 1952. In Dubos, *Bacterial and Mycotic Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
32. Mudd, Lucké, McCutcheon and Strumia, 1929. Jour. Exp. Med. 49, 779.
33. Mudd, McCutcheon and Lucké, 1934. Physiol. Rev. 14, 210.
34. Nelson, 1928. Jour. Immunol. 15, 43.
35. Neufeld and Rimpau, 1904. Dtsch. med. Wschr. 30, 1458.
36. Neufeld and Rimpau, 1905. Ztschr. Hyg. Infectkr. 51, 283.
37. Ponder and MacLeod, 1938. Jour. Exp. Med. 67, 839.
38. Robertson and Van Sant, 1939. Jour. Immunol. 37, 571.
39. Rosenthal, 1909. Zentralbl. f. Bakter. IR. 42, 177.
40. Rous and Jones, 1916. Jour. Exp. Med. 23, 601.
41. Sabin, 1935. Brit. Jour. Exp. Path. 16, 158.
42. Smith and Wood, 1947. Jour. Exp. Med. 86, 257.
43. Stavitsky, 1945. Jour. Immunol. 51, 397.
44. Victor *et al.*, 1952. Jour. Amer. Med. Assoc. 149, 809.
45. Ward and Enders, 1933. Jour. Exp. Med. 57, 527.
46. Ward and Enders, 1933. Jour. Exp. Med. 57, 541.
47. Welch, 1939. Jour. Bact. 37, 109.
48. Welch, Davis and Price, 1945. Jour. Immunol. 51, 1.

49. Welch and Hunter, 1940. Amer. Jour. Pub. Health, *30*, 129.
50. Welch, Slocum and Hunter, 1942. Jour. Lab. Clin. Med. *27*, 1432.
51. Welch, Wentworth and Mickle, 1938. Jour. Amer. Med. Assoc. *111*, 226.
52. Wood and Smith, 1947. Science *106*, 86.
53. Wood and Smith, 1947. Trans. Assoc. Amer. Physicians *60*, 77.
54. Wood, Smith and Watson, 1946. Jour. Exp. Med. *84*, 387.
55. Wood, Smith and Watson, 1949. Science *110*, 187.
56. Wright, 1927. Jour. Path. Bact. *30*, 185.
57. Wright and Douglas, 1903. Proc. Roy. Soc. Ser. B. Biol. Sci. *72*, 364.
58. Zinsser, Enders and Fothergill, 1940. *Immunity, Principles and Applications in Medicine and Public Health*, The Macmillan Co., New York.

## Chapter 12

### CYTOLYSIS AND COMPLEMENT FIXATION

THE BACTERICIDAL AND CYTOLYSIS REACTIONS commanded the attention of many early investigators, including Nuttall, Behring, Pfeiffer, Metchnikoff, Bordet and Ehrlich. Their work established the general principles of the mechanism of complement reactions.

#### CYTOLYSIS

**Bactericidal Properties of Blood.** It was demonstrated in 1888 that defibrinated blood of a variety of animals was lethal to anthrax bacilli.<sup>22</sup> Known numbers of bacteria were added to blood specimens and the survivors were counted at intervals. Not more than five organisms remained viable after one hour in a suspension originally containing nearly 16,000 bacilli. The bactericidal power of blood was destroyed at 52° to 55° C. in forty-five minutes and also gradually diminished as the blood aged.

The ability of blood to kill bacteria was found to vary according to the organisms employed and the immune or nonimmune state of the animal supplying the blood. Bactericidal substances were present in serum as well as in whole blood, and their action could be demonstrated both *in vivo* and *in vitro*. Serum from an immunized guinea pig killed *Vibrio metchnikovi*, whereas serum from a normal animal did not. Pneumococci, however, were not killed by immune guinea pig serum. The bactericidal substances of serum were assumed to protect the animal body against fatal infections and were called *alexines* by Buchner.

**Bacteriolysis.** Bacteriolysis was described by Pfeiffer in 1894.<sup>23</sup> He introduced lethal doses of *V. cholerae* into the peritoneal cavities of normal and immune guinea pigs and withdrew samples of peritoneal fluid for microscopic examination at intervals of a few minutes. Specimens from normal animals contained considerable numbers of morphologically

typical bacteria which multiplied extensively and killed the animals. Peritoneal fluids of immune guinea pigs a few minutes after injection yielded irregularly staining, almost spherical organisms. They disappeared completely within twenty minutes to one hour, evidently having undergone lysis (dissolution). These animals survived. Lysis and protection were specific, since other vibrios were not lysed in animals immunized against the cholera organism, nor were the animals protected against heterologous bacteria.

The protective power of one animal could be transferred to another *via* the serum. Normal guinea pigs were injected with serum from an immune animal and given a dose of cholera bacteria which would have killed an unprotected animal. Bacteriolysis occurred and the animals survived. Lysis and protection were demonstrated even when the injected immune serum had been heated at 55° or 60° C. This fact appeared most remarkable in the light of previous observations that the *in vitro* bactericidal action of blood was destroyed at 52° to 55° C.

The Pfeiffer reaction was then demonstrated in hanging drop preparations. Cholera vibrios mixed with fresh anticholera serum were promptly lysed. Heated cholera immune serum did not dissolve the homologous organisms. Lysis did occur, however, if a small amount of peritoneal exudate or of blood serum from a normal guinea pig was added to the heated immune serum. Normal serum alone was incapable of inducing lysis, so it was concluded that two reagents were necessary. (1) a heat resistant substance found only in immune serum and (2) a thermolabile component of both fresh immune serum and normal serum.

**Hemolysis.** Lysis by fresh homologous antiserum or by heated antiserum in the presence of normal serum is not limited to bacteria. Hemolysis, or the dissolution of erythrocytes, was demonstrated by use of rabbit red blood cells and antiserum prepared against them in guinea pigs. The antierythrocyte serum was capable of agglutinating homologous blood cells and also dissolving them.<sup>1</sup> Heating at 55° C. for thirty minutes destroyed the lytic property of the immune serum, as in bacteriolysis, and such inactivated serum was reactivated by addition of normal guinea pig or rabbit serum. Hemolysis also was specific; only rabbit cells were lysed by antiserum for rabbit erythrocytes. Lysis was demonstrated *in vivo* by introducing heated immune serum and rabbit red cells into the peritoneal cavity of a normal guinea pig, as had been done with *V. cholerae*.

The specificity of lysis is associated with the thermostable substance produced by immunization. Bordet designated it the "substance sensitivatrice" because it sensitized cells to the action of the thermolabile material, alexine, found in normal (as well as immune) serum.

The mechanism of cytolysis has been studied principally with hemolytic systems because serum hemolysis is easily performed in test tubes and a

positive result is readily distinguishable. Hemolysis consists of laking of erythrocytes, the hemoglobin being liberated and imparting to the fluid a bright red color. The colorless *stomata* or "shadows" are intact but can be seen only by microscopic examination. The erythrocytes remain undissolved in negative reactions and settle to the bottom of the liquid.

**Mechanism of Cytolysis.** An experiment demonstrating the action of the principal reagents in cytolysis is represented diagrammatically in

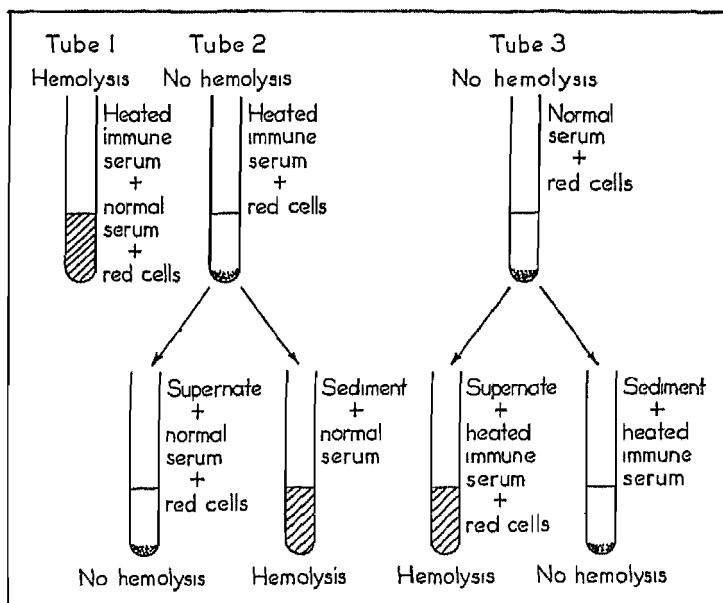


FIGURE 43. Hemolysis. Both antibody (in heated immune serum) and complement (in normal serum) are necessary. Tubes 2 and 3 and tests on their supernates and sediments demonstrate that antibody first combines with antigen, and then complement causes lysis.

Figure 43. Early investigators employed beef erythrocytes as antigen. Homologous immune serum was prepared in goats and was heated at 56° C. to "inactivate" the thermolabile component. Normal serum was secured from unimmunized goats. Similar experiments can be performed with erythrocytes and serums from other species; for example, sheep erythrocytes, anti-sheep rabbit serum and normal guinea pig serum.

The mixture of heated immune serum, normal serum and red blood cells (tube 1 in Figure 43) was completely lysed after forty-five to sixty minutes at 37° C., whereas mixtures lacking one of these reagents (tubes 2 and 3) showed no hemolysis. Supernatant fluid from tube 2, which had contained only heated immune serum and erythrocytes, was transferred to a separate tube; normal serum and red cells were added. Lack of

hemolysis indicated that antibody had been removed from the heated immune serum by previous treatment with red cells. This was confirmed by adding normal serum to the sedimented erythrocytes which had been exposed to heated immune serum, whereupon hemolysis took place. The supernatant liquid from the mixture of normal serum and red cells (tube 3) was treated with heated immune serum and red cells, and the resulting hemolysis showed that the normal serum component essential for hemolysis did not combine with red cells in the absence of antibody. This conclusion was verified by adding heated immune serum to the red cells which had been exposed to normal serum. No hemolysis occurred. Ehrlich concluded from such experiments that antibody combines with erythrocytes first and only then can the normal serum component react and cause hemolysis. He pictured the sensitizing antibody as possessing two combining groups or receptors and called it *amboceptor*; he applied the name *complement* to the thermolabile constituent of serum.

The same mechanism applies to bacteriolysis. It is now known that amboceptor may often be demonstrated by agglutination or precipitation, but the usual practice is to limit "amboceptor" to antibody for red blood cells. Another widely employed term for this antibody is *hemolysin*; some authorities consider this a misnomer because the effective hemolyzing agent appears to be complement.

#### FIXATION OF COMPLEMENT

Complement from different species is not necessarily identical, although from most animals it exhibits many similarities in properties and behavior. Guinea pig complement is one of the most active and is widely employed. It combines not only with erythrocyte-antibody complexes but also with many other antigen-antibody complexes.

Bordet and Gengou devised the complement fixation test in 1901.<sup>3</sup> They observed that bacteriolysis does not always take place when complement is present in bacteria-antibody mixtures. Even when bacteriolysis does occur, it is difficult or impossible to determine its extent with accuracy. Moreover, mixtures which appear completely lysed may still contain viable bacteria, so it is ordinarily necessary to resort to plate counts. It can also be shown that soluble antigens such as egg albumin, when mixed with their homologous antibodies, are capable of reacting with complement. In such mixtures there may be no visible endpoint or other evidence of reaction.

It was early found that when an antigen reacts with its antibody in the presence of complement, the complement is combined or "fixed" by the antigen-antibody complex and can no longer react with other antigen-antibody systems. As an indicator of the presence and relative amount of uncombined (unfixed) complement, Bordet and Gengou suggested the

use of erythrocytes sensitized with homologous antibodies. Lysis of the erythrocytes constituted evidence of unfixed complement; conversely, lack of hemolysis indicated that complement had completely reacted with the test antigen and antibody. First experiments were conducted with the bubonic plague, anthrax, typhoid and proteus bacteria and their respective antiserums. Soluble antigens were soon found to behave in the same manner. The complement fixation reaction is highly specific and very sensitive. It is used in several diagnostic procedures for the detection of antibody in patients' serums and for the identification of proteins and other antigenic substances.

#### COMPLEMENT

Complement is a substance in fresh blood serum or plasma which is not increased on immunization and which combines with antigen-antibody complexes.

The exact chemical nature and mode of action of complement are still uncertain in spite of more than a half century of study.<sup>24</sup> It has been considered at various times to be a lipase, a protease, a peptidase, an intracellular catalyst, a simple chemical like oleic acid, a lipid, a colloidal attribute of fresh serum. Activities attributed to it include: (1) killing and in some cases lysis of certain bacteria sensitized with homologous antibody, (2) hemolysis of sensitized red blood cells, (3) opsonization of bacteria in the absence of immune serum, (4) activation of thermostable opsonins, (5) combination with antigen-antibody systems even in the absence of visible reaction (complement fixation). Complement has also been implicated in numerous other physiologic processes.

**Sources of Complement.** Complement is normally present in the blood of most vertebrate animals. It is mainly globulin and may be derived from the same cells as other serum globulins, especially certain cells of the liver.<sup>6, 7</sup>

Many animals possess fairly strong complement demonstrable by one or more methods. Even cold-blooded animals such as the frog and the carp contain complement similar to that of the guinea pig. However, the complement of one species or even of one individual may differ from that of another species or individual. Some specimens react with certain antigen-antibody complexes but not with others. Ehrlich and Morgenroth noted as early as 1901 that complements from a number of animal species differ in their ability to hemolyze beef erythrocytes sensitized by anti-serums produced in different animals (Table 62).<sup>8</sup> Guinea pig, rabbit and human complements behave differently in bactericidal tests with *Hemophilus influenzae* (Table 63).<sup>9</sup> Most of these differences cannot be explained by present knowledge.

**Instability of Complement.** Complement is highly unstable. Inactiva-

Table 62. Effect of Source of Complement on Hemolysis of Beef Erythrocytes Sensitized by Two Different Anti-Beef Immune Serums<sup>8</sup>

SOURCE OF COMPLEMENT	SOURCE OF IMMUNE SERUM	
	Rabbit	Goose
Hemolysis		
Guinea pig	+	+
Rabbit	+	+
Rat	+	+
Hen	+	+
Goat	+	-
Pigeon	-	+
Horse	-	-

Table 63. Effect of Source of Complement on Bactericidal Action of Three Anti-Hemophilus influenzae Serums

SOURCE OF COMPLEMENT	SOURCE OF ANTISERUM		
	Guinea pig	Rabbit	Horse
Killing of <i>H. influenzae</i>			
Guinea pig	+	-	-
Rabbit	+	+	-
Human	+	+	+

(From Dingle et al.,<sup>6</sup> by permission.)

Table 64. Time Required for Thermal Inactivation of Complement<sup>18</sup>

TEMPERATURE	TIME
51° C.	35 minutes
53° C.	14 minutes
55° C.	12 minutes
57° C.	8 minutes
59° C.	4 minutes
61° C.	2 minutes

tion by mild heat is its most distinctive property. Data reported by Mawaring in 1906 (Table 64)<sup>18</sup> show the inverse correlation between temperature and the time required for complete loss of activity and indicate that at 53° C. less than fifteen minutes is necessary to inactivate. In routine work serums are heated for thirty minutes at 56° C. to deprive them of complement activity.

Aging, especially at room temperature or higher, leads to almost complete deterioration within a day or two, and even in the refrigerator considerable loss of activity occurs within three or four days.

Complement which has been inactivated by heat may regain part of its activity upon standing at temperatures between 7° and 37° C. Highest activity is reacquired in about twenty-four hours (Table 65).<sup>10</sup> Spontaneous reactivation is important in the repeated testing of clinical and other serum specimens and necessitates reinactivation. Heated complement may also be completely reactivated by adding about 10 per cent of fresh complement.

*Table 65. Spontaneous Reactivation of Complement<sup>10</sup>*

(Complement diluted 1:10 was heated to 56° C. for 7 minutes and was then tested at intervals for hemolytic activity against beef erythrocytes sensitized with homologous amboceptor.)

INTERVAL BETWEEN INACTIVATION AND TESI	HOLMOLYSIS AT 11 R			
	10 min.	20 min.	30 min.	40 min.
None	0%	20%	40%	70%
1.5 hours	0	30	60	80
24 hours	20	70	80	100
48 hours	10	40	70	—

Violent agitation, such as shaking a 1:10 dilution of fresh serum for twenty to twenty-five minutes, also inactivates complement. Moreover, complement is destroyed or inactivated by acids and alkalies, proteolytic enzymes, ether, chloroform, alcohol, bile salts and soaps.

Complement is probably colloidal because of its protein (globulin) nature. Many agents which inactivate complement are known to affect the colloidal character of proteins. Alteration in colloidal properties may partially explain the instability of complement but is probably not the sole factor, because the biologic activity of antibody protein, which is also globulin, is stable indefinitely.

Many particulate or colloidal substances are deleterious to complement. Adsorption with casein, kieselguhr or charcoal removes the activity from normal serum, as do tissue cells or extracts, yeast cells and many kinds of unsensitized bacteria. The latter are particularly important when they occur as contaminants of antiseraums employed in complement fixation as in the Wassermann test for syphilis, because their anticomplementary action (nonspecific adsorption of complement) may give falsely positive results unless proper controls are included in the test. Some serums develop anticomplementary properties on aging; even serums which have been inactivated at 56° C. may become anticomplementary after standing a few hours or days. Anticomplementary properties may be destroyed by heating at 56° C. for thirty minutes. In the case of a previously inactivated serum, the second heating obviously does not necessarily destroy complement.

The lability of complement necessitates extreme care to maintain it as

highly active as possible. Routine use of complement requires bleeding animals almost daily to provide a fresh supply of normal serum. Bleeding and subsequent operations are performed carefully to avoid tissue damage and hemolysis which introduce anticomplementary effects. Serum is removed from the clot and stored at a temperature near 0° C. Dilutions are made preferably in ice-cold saline and with a minimum of vigorous agitation. All glassware must be chemically clean, although not necessarily sterile.

Complement may be preserved for long periods by lyophilization and for varying intervals by freezing and storing at low temperature. The lower the temperature, the longer the activity will remain high. Considerable activity persists after several months at -15° C.

**Chemistry of Complement.**<sup>24</sup> A hint of the complexity of complement was given by early experiments in which fresh normal serum was dialyzed against distilled water until a precipitate formed. Neither the precipitate, redissolved in saline, nor the remaining liquid possessed complement activity when tested separately. Full activity was restored, however, when the two fractions were recombined. It appeared that the simple process of removing salts split complement into two fractions, both of which were necessary for activity. The precipitate, insoluble in water, but soluble in saline, was considered to be a globulin, and the water-soluble fraction in the supernate was thought to be an albumin. Further investigation revealed that the albumin could not combine with a sensitized antigen until the globulin had reacted. The globulin was therefore called *midpiece* and the albumin *endpiece*. In cytolysis reactions midpiece first combines with the sensitized cells, after which endpiece reacts with midpiece and catalyzes dissolution of the cells. The water-soluble material has now been found to be a muco-euglobulin rather than an albumin.

Fractions containing midpiece and endpiece can also be secured by treating normal serum with carbon dioxide water or very dilute hydrochloric acid. Both fractions are highly thermolabile and are inactivated within a few minutes at 56° C. These fractions are not pure chemical entities. Other methods of chemical separation together with inactivation have shown that complement is composed of four functionally distinct portions designated respectively C'1, C'2, C'3 and C'4. C'1 is present in midpiece, C'2 in endpiece; both are thermolabile.

C'3 and C'4 occur in both midpiece and endpiece and are relatively thermostable. Their presence in serum can be demonstrated by specific inactivation. The complement activity of normal serum is destroyed by yeast or by zymosan, an insoluble carbohydrate derived from yeast. Activity is restored by addition of normal serum which has been heated at 56° C. for thirty minutes. The substance inactivated by yeast and replaced by heated serum is the "third component" or C'3. A temperature

of 62° C. for thirty minutes is required for inactivation. C'3 may also be removed from serum by cobra venom or dilute formaldehyde.

Dilute ammonia or other amino compounds capable of reacting with carbonyl groups (=CO) destroy the thermostable component, C'4. Serum in which this component has been inactivated regains activity upon addition of heated normal serum, but the component replaced is not the same as C'3. The "fourth component" is apparently a carbohydrate containing reactive carbonyl groups, or else it is associated with a glycoprotein. Heating at 66° C. for thirty minutes is necessary for inactivation. It is quite stable in alkaline solutions but unstable in slightly acid solutions, whereas C'3 is fairly stable in weak acid and very unstable in weakly alkaline solutions.

The major portion of C'3 and a small amount of the C'4 activity of serum is associated with midpiece, the water-soluble fraction. Most of the C'4 and a small part of the C'3 activity is associated with the water-soluble endpiece.

*Table 66. Properties of Purified Components of Guinea Pig Complement*

	EUGLOBULIN	MUCO-EUGLOBULIN
Complement components	C'1	C'2 and C'4
Protein nitrogen	16.3%	14.2%
Carbohydrate	2.7%	10.3%
Optical rotation, [α] <sub>D</sub>	-28.7°	-192.5°
Apparent isoelectric point (pH)	5.2-5.4	6.3-6.4
Per cent of total serum protein	0.6%	0.18%
Temperature of inactivation in 30 min.	50° C.	C'2: 50° C. C'4: 66° C.

(From Pillement, Ecker, Oncley and Cohn,<sup>25</sup> by permission.)

C'1 is the only component which has been isolated in pure or nearly pure form. A muco-euglobulin possessing both C'2 and C'4 activity has been secured from guinea pig serum. C'3 has not yet been obtained in a sufficiently purified form to permit identification, although it appears to be a lipoprotein. Some of the chemical and physical properties of the other components are summarized in Table 66.<sup>25</sup>

Certain differences in activity of complements from various species are attributable to differing proportions of the four components in these animals. Hegedus and Greiner showed that the net titer of a complement serum is limited by the component present in the least amount.<sup>11</sup> A reaction which requires the cooperation of all four components, such as hemolysis and bactericidal action, cannot occur if one component is missing, even though the other three components are present in abundance. Determination of the titer of any one component is made by supplying an excess of the other three. This is accomplished with various serums (e.g., guinea

pig or human) in which certain components have been inactivated. Titers obtained in a recent study are cited in Table 67.<sup>27</sup> Inconsistent results are sometimes caused by slight variations in technique and individual differences in the animals tested.

*Table 67. Titers of the Components of Complement in Various Animal Serums*

(Results are expressed as mean titers (units per ml.) of four or more samples or pools of serum from each animal species. Serums were tested with sheep red blood cells and rabbit anti-sheep amboceptor.)

SPECIES	C'1 <sup>a</sup>	C'1	C'2	C'3	C'4
Guinea pig	625	3750	750	1000	8000
Human	100	4000	225	500	1750
Hamster	225	1750	210	325	625
Swine	153	1720	120	3670	95
Rabbit	60	150	68	625	55
Rat	150	600	110	1600	210
Mouse	10	390	10	50	10
Horse	10	500	18	125	10
Beef	15	500	10	175	10
Sheep	10	1380	10	20	10
Deer	10	100	15	250	50

\* Hemolytic titer of whole complement for sensitized sheep RBC

(From Rice and Crumson,<sup>27</sup> by permission.)

The superiority of guinea pig complement for hemolysis of sensitized sheep erythrocytes is clearly evident from Table 67, in confirmation of the practical experience of all investigators. C'2 and C'3 were the critical or limiting components in guinea pig serums. C'2 was the smallest fraction in the human, hamster and rat serums tested. The complement activity of swine and rabbit serums was limited by their C'2 and C'4 content. Serums of several species were poorly hemolytic for sheep cells but nevertheless possessed considerable amounts of one or more complement components.

Complement concentrations were formerly determined only by the dilution method. This is still the procedure used in most routine and research work. In 1941, Heidelberger applied the principles he had successfully used in quantitative antibody determination to the analysis of antigen-antibody aggregates which had been allowed to react with complement.<sup>12</sup> Soluble antigens (pneumococcal polysaccharides or egg albumin) were allowed to react with homologous rabbit antisera in the presence of complement. Control mixtures received heat inactivated complement instead of normal complement. After reaction, the nitrogen content of the precipitates was determined quantitatively. The difference between precipitates obtained with normal complement and those obtained with inactivated complement represented *complement combining component* (midpiece). Large volumes of complement serum contributed

proportionately less nitrogen to the precipitates than small volumes, which suggested a solubility effect associated with the greater amounts of reagent. The results were therefore roughly extrapolated to zero volume to obtain an estimate of the true amount of complement combining component. Guinea pig serum contained 0.04 to 0.06 milligram of complement combining nitrogen per milliliter, which corresponds to 0.25 to 0.40 milligram of protein, or approximately 0.4 to 0.7 per cent of the total serum protein. Human serum contained 0.19 to 0.32 milligram of complement combining protein per milliliter. These figures do not include the total protein in complement but are assumed to represent only C'1 or midpiece. The muco-euglobulin (C'2-C'4) is probably about one-third of this amount (see Table 66), and C'3 is not known.

The properties of the four components of complement may be summarized as follows:

C'1 is a euglobulin which constitutes about 0.6 per cent of the total protein in guinea pig or human serum. It is readily inactivated by heat and is unstable at neutral reaction but somewhat more stable between pH 5.4 and 6.0. It contributes a greater weight than any of the other components when complement combines with specific precipitates.

C'2 and C'4 activity appears to be associated with a single molecule of muco-euglobulin, the C'2 portion of which is readily inactivated by heat. C'2 is the limiting component of many serums. The muco-euglobulin comprises about 0.2 per cent of guinea pig serum protein. C'4 is the most thermostable component but is destroyed by ammonia or primary amines. It is evidently a carbohydrate portion of the muco-euglobulin.

C'3 is relatively resistant to heat but is the first component to disappear on standing at room temperature. It is removed from serum by washed yeast cells or zymosan. This treatment removes 0.02 to 0.03 milligram of nitrogen from the serum, but it is not certain that all of this is C'3. The anticomplementary action of unsensitized bacteria consists of adsorption of this component.

#### MECHANISM OF COMPLEMENT REACTIONS

**First Stage: Fixation.** The first stage of complement reactions is the union or fixation of complement by a sensitized antigen. Whereas the reaction between antigens and antibodies is practically instantaneous, combination with complement is less rapid. The rate of fixation may be slower with particulate antigens such as bacteria than with soluble antigens. Great excess of antigen or antibody markedly inhibits the speed and extent of fixation. Low temperatures are often considered preferable because greater fixation is obtained with many antigens at 0° to 6° C. than at 37° C., although the rate of reaction may be slower. Primary incubation of syphilis antigen-antibody-complement mixtures overnight at refriger-

ator temperature gives higher titers than 37° C. incubation for one to two hours. Use of high temperatures is limited by thermal inactivation of complement.

The various components of complement are not necessarily fixed to the same extent. C'1, C'2 and C'4 are apparently fixed completely by antigen-antibody precipitates. The fixation of C'3 is questionable; apparently it does not combine but plays an essential catalytic role in the second stage of reaction: cytolysis or bactericidal action. Lack of this component does not interfere with the fixation of complement, but such deficient complement cannot induce hemolysis or other detectable effects.

Heidelberger found that the amount of complement fixed was of the same general order of magnitude as the quantity of antibody combined<sup>13</sup> and that in order to bind more complement, additional antibody was required. Fixation of complement was relatively little influenced by quantities of antigen above a necessary minimum. Whether or not visible precipitation occurred seemed to exert relatively little effect on the amount of complement combined, as shown by indirect tests in which free complement was detected by a hemolytic reaction. This indicated a chemical rather than a physical explanation of complement fixation. Pillemer believes that the specificity of complement fixation also implies a chemical reaction.<sup>24</sup>

Heidelberger's proposed mechanism of complement fixation is an extension of his hypothesis of precipitation. He assumed that the combining component of complement is capable of loose, easily dissociable union with dissolved antibody. Molecules of complement are surrounded by antibody molecules when reacting with an antigen-antibody system, and the weak combination between complement and antibody becomes more stable. Stabilization results from one or more of the following causes: (1) attraction of ionized groups of opposite sign, (2) hydrogen bonding, (3) spatial accommodation of large groups on complement and antibody and (4) possession by complement of more than one group capable of reacting with antibody molecules. This hypothesis ascribes the major burden in the firm linkage of complement with antigen-antibody aggregates to the network of antibody molecules, although it is not denied that antigen also may play a part. A pictorial representation of Heidelberger's concept is shown in Figure 44.<sup>18</sup>

**Second Stage: Cytolysis.** The amount of complement required for complete lysis of a given number of erythrocytes is inversely related to the amount of amoebocyte (hemolysin) provided. In 1941, Heidelberger and his co-workers published data showing the quantitative relationship between the reagents (Table 68).<sup>18</sup> The amount of complement required decreased as the available amoebocyte increased. Below a certain minimum of each reagent, no amount of the other sufficed to cause hemolysis, as previously illustrated graphically by Gordon *et al.* (Figure 45).<sup>9</sup> Comple-

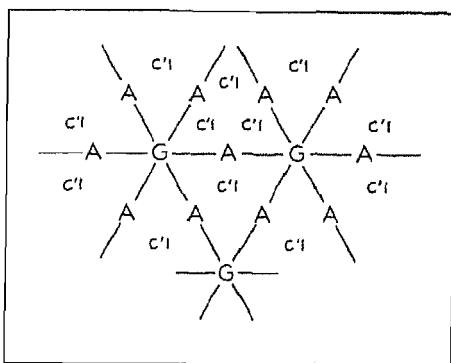


FIGURE 44. Heidelberger's concept of the structure of an antigen-antibody complex in which complement is fixed. (Redrawn from Heidelberger *et al.*, 1941. *Jour. Exp. Med.* 73, 695.)

*Table 68. Relationship between Amounts of Amboceptor and Complement Required for Complete Lysis of Sheep Erythrocytes*

AMBOCEPTOR NITROGEN	COMPLEMENT (C'1) NITROGEN
0.008 µg.	0.30 µg.
0.015 µg.	0.20 µg.
0.020 µg.	0.13 µg.

(From Heidelberger, Weil and Treffers,<sup>13</sup> by permission)

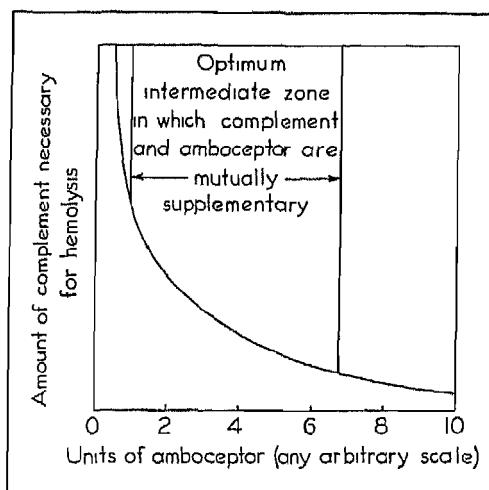


FIGURE 45. Mutually supplementary role of complement and amboceptor in hemolysis (diagrammatic). (From Gordon *et al.*, 1926. *Biochem. Jour.* 20, 1028.)

ment and amboceptor are mutually supplementary within limits and may be used in widely varying ratio.

The percentage of hemolysis of an erythrocyte suspension can be determined by colorimetric methods and used to measure the effect of various amounts of complement when constant quantities of amboceptor and erythrocytes are present. The results of such experiments yield an S-shaped curve (Figure 46).<sup>31</sup> The relationship between complement and

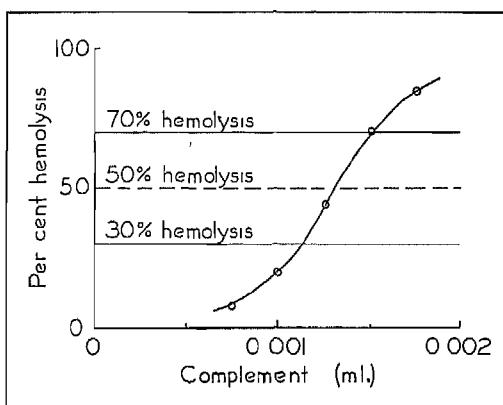


FIGURE 46. S-shaped curve showing the relationship between amount of complement and per cent hemolysis of sensitized red blood cells. Between 30 per cent and 70 per cent hemolysis the curve is almost linear. (Redrawn from Wadsworth. *Standard Methods*, 3rd ed., 1947, Williams & Wilkins Co., Baltimore.)

percentage of hemolysis is almost linear from 30 to 70 per cent lysis. Above this range each added increment of complement produces less effect, and relatively enormous doses are required for complete hemolysis. It was customary for many years to use 100 per cent hemolysis as the endpoint in titrations, and in fact this is still the situation in the majority of laboratories. Greater precision is obtained with a 50 per cent endpoint because the curve of hemolysis is steeper in this region. Simple procedures are available to estimate the 50 per cent endpoint and are gradually being adopted.

The speed of hemolysis depends on the amounts of the various reagents, the total volume of the test, the temperature of incubation and other factors. Complete hemolysis usually occurs within fifteen to sixty minutes. Increasing the volume of liquid reduces the apparent titer of the reagents. Recent data of Mayer and associates indicated that 0.0037 milliliter of a specimen of complement sufficed to produce 50 per cent lysis of 500,000,000 sensitized red blood cells when the reaction was conducted in a total volume of 2.0 milliliters, whereas 0.0093 milliliter of complement was required when the total volume was made up with buffered

saline to 7.5 milliliters.<sup>20</sup> It should be noted, however, that the final dilution of complement in the 7.5 milliliter volume was nearly 50 per cent greater than in the 2.0 milliliter volume, so the amount of lysis per milliliter of undiluted complement was actually greater when the reaction was performed in the larger volume.

Mayer *et al.* also studied the effect of temperature, pH and salt concentration on the hemolytic activity of guinea pig complement.<sup>20</sup> Slightly higher titers were obtained at 32.4° and 35.3° C. than at 37° C. Complement activity at 39° C. was about 8 per cent lower than at 37° C. The optimum pH for hemolysis was between 7.4 and 7.6, but satisfactory results were obtained as low as pH 6.9. Titers in 0.91 per cent NaCl were 17.5 per cent lower than in 0.85 per cent NaCl. The presence and concentration of Mg<sup>++</sup> ions was found to be important. Insufficient magnesium is present in hemolytic tests as ordinarily performed, but addition of only 0.005 per cent MgCl<sub>2</sub> almost doubled the complement titers. Seventy-five milligrams of MgCl<sub>2</sub> per liter of saline is usually sufficient to give maximum titers. The role of Ca<sup>++</sup> ions in lysis is controversial. Mayer *et al.* concluded that it may be essential but that its action is much less pronounced than that of Mg<sup>++</sup>.

Heidelberger's quantitative data indicated that 400 to 600 molecules of rabbit amboceptor sensitized a single sheep red blood cell for lysis by guinea pig complement.<sup>13, 16</sup> This amount of amboceptor was estimated to occupy 0.01 to 0.3 per cent of the surface area of the cell. Associated with the amboceptor were 17,000 to 32,000 molecules of complement combining component. It is apparent that the erythrocyte is not completely "coated" with either antibody or complement and that only very small areas or "key spots" are concerned in hemolysis.

The C'3 component of complement seems to behave as an enzymic catalyst and initiates the reaction leading to release of hemoglobin after the other components have combined with the sensitized cell. The stroma (supporting structure) of the cell is not destroyed. It has been suggested that the cell wall permeability is so increased that simple osmotic forces no longer operate, and hemoglobin diffuses into the surrounding medium.

#### COMPLEMENT FIXATION TESTS

The reagents in complement fixation unite as indicated in Figure 47. Antigen and antibody form a complex which reacts with complement during primary incubation. There is usually no visible evidence of reaction at this point. The hemolytic system (amboceptor and red blood cells) is then added, but hemolysis cannot occur if complement has already been "fixed" by the antigen-antibody complex. In the absence of sufficient antibody or antigen, some or all of the complement remains unfixed after primary incubation and hemolyzes the sensitized red cells.

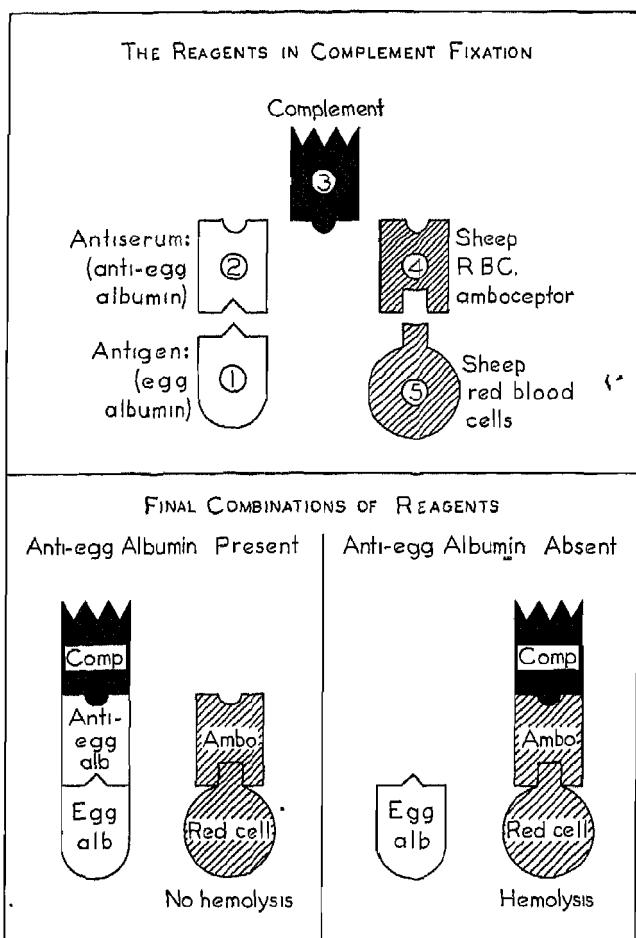


FIGURE 47. The reagents in complement fixation with egg albumin. The order in which they are used is indicated by encircled figures. Quantities are so adjusted that in an actual test no hemolysis occurs when anti-egg albumin is present. When it is absent, complement is free to combine with the sensitized red cells and hemolysis results.

All reagents are used in constant amounts except one, the unknown, of which varying dilutions are employed. The highest dilution of this reagent which produces a given degree of complement fixation represents the unit or titer. The success of the test depends upon complete fixation of complement in the presence of adequate amounts of all reagents and complete hemolysis in the absence of the unknown. The known reagents must therefore be carefully prepared and titrated in order to balance one another. For example, there must be just sufficient amboceptor to sensitize the cells and just enough complement to lyse the sensitized erythrocytes.

Quantitative details of the procedure vary from one laboratory to another, but certain general principles are usually followed.

**Preparation of Reagents.** Amboceptor is prepared by injecting rabbits with thoroughly washed red blood cells until a sufficiently high titer has been developed. The rabbit serum contains amboceptor. It is often preserved by adding an equal volume of neutral glycerine. Amboceptor is very stable and will keep for years.

Complement is secured in the form of guinea pig serum. Complement from each of several animals should be titrated individually, and serums of high titer may be pooled. Complement should not be kept more than twenty-four hours because of loss of titer.

Erythrocytes from the sheep are most widely used. Blood is taken into citrate or other anticoagulant or is defibrinated by shaking with glass beads. The cells should be fresh or preserved in such manner as not to be too fragile; otherwise spontaneous hemolysis will occur. Each day the cells to be used are washed three or more times by centrifuging with five to ten volumes of saline. Measured amounts of the sedimented cells are suspended in saline for titration of the other reagents and for the final test. Two per cent or 5 per cent suspensions are commonly employed.

Amboceptor is titrated by mixing various dilutions with constant amounts of red cell suspension and a more or less arbitrary dose of complement selected by trial and error or by experience. Mixtures are incubated in a waterbath at 37° C. for thirty minutes. The highest dilution of amboceptor producing complete hemolysis is called *one unit*. One to four units are usually employed in subsequent titrations and tests, according to the procedure being followed. The amboceptor solution is prepared to contain the required dose in a constant definite volume (e.g., 0.1 or 0.5 milliliter). Amboceptor needs to be titrated only occasionally because of its stability.

Complement must be titrated each day. Closely spaced volumes of a moderate dilution (1:10 to 1:40) of guinea pig serum are pipetted into a series of tubes, together with sufficient saline to make a constant volume. The tubes are incubated at 37° C. for thirty minutes, after which sensitized red cells are added. (Sensitized red cells are prepared by mixing equal volumes of cell suspension and the predetermined dilution of amboceptor.) After secondary incubation at 37° C. for thirty minutes the smallest amount of complement which produces complete hemolysis is noted. This constitutes *one unit* of complement. Preincubation of complement alone is important because this reagent is so readily inactivated by heat and because two periods of incubation are required in the final test. The complement titration should therefore be performed in a similar manner.

The dose of complement employed in a complement fixation test varies according to the particular application of the reaction in question

and the experience of the investigator. For research it is advisable to use approximately one unit, but in this case careful work is required. More precise results can be obtained than by use of greater quantities of complement, but occasionally tests have to be repeated because the controls do not react properly. Diagnostic techniques, such as the Wassermann reaction, often employ two or more units and thus allow a greater margin of error. This use of the test is subject to inaccuracies resulting from large scale operation, speed and variations in the training and experience of technicians.

Soluble antigens (e.g., crystalline ovalbumin) may require no more preparation than dissolving in saline. Bacterial antigens are often prepared by extracting mass cultures, heating and aging.

With some systems, such as bacterial antigens and lipoid extracts of tissues, which are highly anticomplementary, both antigenicity and anticomplementary titrations are necessary to define the upper and lower limits of antigen dosage which may be employed in complement fixation tests. The antigenic titration is performed if a known positive homologous antiserum is available. An amount of antigen is determined which, combined with antiserum, just completely fixes the full dose of complement and therefore inhibits hemolysis. This represents the smallest quantity of antigen which can be expected to give satisfactory results in the final test.

The anticomplementary titration of antigen is performed by incubating various amounts of antigen with the dose of complement and subsequently adding the hemolytic system. The anticomplementary unit is the smallest amount of antigen which slightly inhibits hemolysis. The dose employed in the final test is some amount between the antigenic unit and the anticomplementary unit.

The preceding discussion has been based upon the 100 per cent hemolysis or inhibition endpoint, but the same general principles apply to the 50 per cent endpoint, although the details of technique differ. Exactly 50 per cent hemolysis is rarely encountered, but two or three determinations bracketing this value permit it to be calculated by interpolation, or it may be estimated from a single colorimetric determination by an appropriate arithmetic factor.<sup>16, 19</sup>

**Titration of an Antigen or an Antiserum.** Complement fixation tests are sometimes performed with dilutions of antiserum, sometimes with dilutions of antigen. In general, soluble antigens such as egg albumin are diluted serially and tested against antiserum either undiluted or diluted only slightly (e.g., 1·5). Bacterial antigens and lipoid tissue extracts are usually employed in constant amounts and mixed with serial dilutions of patient's serum or rabbit antiserum. In either case, the serum is inactivated to destroy normal complement, which might upset the balance of reagents and yield falsely negative results.

The experimental procedure and results of a complement fixation test

with egg albumin antigen are indicated in Table 69. Dilutions of the antigen are prepared and 0.1 milliliter of each is placed in the tubes indicated. Rabbit anti-egg albumin serum (inactivated) and complement are then added. The control tubes (6 to 9) lack one or more reagents, so sufficient saline is employed to give a constant final volume. All tubes are incubated for one-half to one hour at 37° C. or overnight in the refrigerator. The hemolytic system is added and the tubes reincubated at 37° C. The duration of secondary incubation may be judged by observation of the hemolytic control tube. Five to ten minutes after this is completely lysed the tests may be read.

Table 69. Complement Fixation by Egg Albumin and Homologous Rabbit Antibody

TUBE	EGG ALBUMIN (0.1 ml.)	ANTI-EGG ALBUMIN SERUM (undil.)	COMPLEMENT DILUTION	SALINE		AMBOCEP- TATOR DILUTION	RHEE R.B.C.		RESULTS COMPLE- MENT FIXATION†
1	1:100	0.1 ml.	0.1 ml.	—		0.1 ml.	0.1 ml.		++++
2	1:1000	0.1	0.1	—		0.1	0.1		+++
3	1:10,000	0.1	0.1	—		0.1	0.1		+++
4	1:100,000	0.1	0.1	—		0.1	0.1		+
5	1:1,000,000	0.1	0.1	—		0.1	0.1		-
6*	—	0.1	0.1	0.1 ml.	Primary incubation: 37°C., Waterbath, 30-35 min.	0.1	0.1	Secondary incubation: 37°C., Waterbath, 15 min.	—
7*	1:100	—	0.1	0.1		0.1	0.1		-
8*	—	—	0.1	0.2		0.1	0.1		-
9*	—	—	—	0.4		—	0.1		+++

\* Controls:

Tube 6 = Antiserum control

Tube 7 = Antigen control

Tube 8 = Hemolytic control

Tube 9 = Corpuscle control

† + + + + = Complete fixation of complement (i.e., no hemolysis)

- = No fixation of complement

(i.e., complete hemolysis)

Results are recorded in terms of fixation of complement. A tube showing no hemolysis indicates complete fixation, which is recorded as + + + +. The anti-egg albumin serum illustrated fixed complement partially after reaction with egg albumin diluted 1:100,000, which may be considered the complement fixing titer of the serum.

The four controls are absolutely essential. The *antiserum control* is used to show that the antiserum under test is not anticomplementary. When serial dilutions of antiserum are tested, a single serum control containing the largest amount of serum employed in any of the tubes usually suffices, although occasionally a control is necessary for each dilution. The *antigen control* is required to demonstrate that the antigen is not anticomplementary. As in the case of the serum control, this tube usually receives the largest amount of antigen employed in any of the actual tests. The *hemolytic control* indicates that the complement and amboceptor are active in the dosages employed. The *corpuscle control* shows that the red cells are not too fragile and that hemolysis in the test

proper actually indicates a deficiency of the reagent being titrated. The first three controls should be completely hemolyzed, and the fourth should be unhemolyzed. When reacting correctly, the controls prove that the results of the test are significant insofar as the preparation of the reagents is concerned.

#### APPLICATIONS OF COMPLEMENT FIXATION

The most widely publicized application of complement fixation is the Wassermann test and its various modifications used in the laboratory diagnosis of syphilis. Apparently the antibody-like substance or "reagin" detected in patients' serum is the same as that which reacts in the Kahn and other precipitation tests for this disease. The test antigen is usually prepared in a similar manner, from beef heart. The purified material, cardiolipin, has recently been introduced into routine use in many laboratories.

The test is performed with one to three dilutions of patient's serum or spinal fluid and a constant amount of antigen. Best results are obtained when primary incubation is carried out in the refrigerator overnight. After the hemolytic system is added secondary incubation is at 37° C.

The nature of the Wassermann reaction is the subject of considerable debate. It has been contended that since the test antigen is derived from normal tissues, any reaction between it and patient's serum is nonspecific and hence has no relation to antibodies against the syphilis spirochete. Kahn proposed that, in the presence of syphilis infection, some of the host's own lipids or other components become auto-antigenic.<sup>17</sup> Whatever the origin of reagin, many years of intensive use indicate that the complement fixation test for syphilis is satisfactorily specific. It should be noted, however, that a few other diseases give positive results in the Wassermann test for varying, but usually short, intervals.

Complement fixation has been used more or less successfully in the laboratory diagnosis of other bacterial diseases such as gonorrhea, tuberculosis, pertussis, glanders and white diarrhea of chicks. The bacterial antigens are for the most part highly anticomplementary and must be carefully prepared.

Diagnostic complement fixation tests have been used experimentally in some parasitic infestations, such as trichinosis. Preparation of the antigen may be the most difficult part of the procedure. Trichina larvae, separated from the flesh of infested rats, are extracted with alkaline saline, and the extract, further treated, constitutes the test reagent.

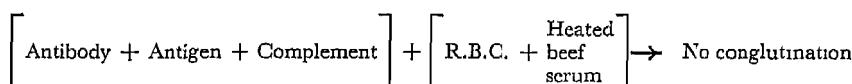
Positive complement fixation with possible diagnostic application has been obtained in helminth diseases like schistosomiasis and echinococcosis, in protozoan infections such as malaria, amebiasis and leishmaniasis, and in fungal infections with Coccidioides and Blastomyces.

Most viral and rickettsial infections give positive complement fixation reactions, but methods have not been developed in all cases to the point where they can be used for routine diagnosis, although they are reliable in the hands of experts. Antigens are usually prepared from infected mouse brains or chick embryos. Various solvents such as ether or benzene are employed to remove interfering lipids, and particles containing the antigens are separated from coarse tissue materials by differential centrifugation.

#### CONGLUTINATIVE COMPLEMENT-ABSORPTION TEST

In 1906 Bordet and Gay<sup>2</sup> and also Muir and Browning<sup>21</sup> observed that erythrocytes sensitized with heated normal beef serum were aggregated by the fresh serum of certain animals. This phenomenon was dependent upon complement present in the fresh serum and was called *conglutination* by Bordet and Streng to distinguish it from agglutination, which did not require the cooperation of complement.<sup>4</sup> Streng showed that two essential substances were supplied by heated beef serum: (1) a natural antierythrocyte antibody and (2) a clumping substance termed *conglutinin*.<sup>20</sup> Streng reported in 1929 that conglutination may be inhibited by the adsorption of complement to an antigen-antibody complex in essentially the same manner as hemolysis is inhibited in the ordinary complement fixation test.<sup>30</sup>

Horse serum is the usual source of complement. Antigen, antibody and complement are incubated together, and then sheep red blood cells sensitized with heated beef serum are added. After incubation for fifteen minutes at 37° C. the tubes are centrifuged and the cells resuspended for reading. The mechanism of conglutination inhibition may be summarized as follows:



Complement is fixed or adsorbed to the antigen-antibody complex and is hence unavailable to aggregate the red blood cells sensitized by heated beef serum. In the absence of antibody or antigen, complement remains free and aggregates or conglutinates the erythrocytes.

The procedure has not yet received wide application, although it is said to be more highly sensitive than hemolytic complement fixation in certain instances. Apparently it was first employed diagnostically by Hole and Coombs to detect antibodies in the serums of ponies convalescent from glanders.<sup>15</sup> Its use in the diagnosis of brucellosis of cattle has been studied by Rice and Avery.<sup>26</sup> Wolfe and Kornfeld and others have

adapted conglutination absorption to the investigation of rickettsial and viral diseases, including Q fever, psittacosis, lymphogranuloma venereum, vaccinia and influenza.<sup>14, 28, 32</sup>

### References

1. Bordet, 1909. *Studies in Immunity*, John Wiley & Sons, New York.
2. Bordet and Gay, 1906. Ann. Inst. Pasteur 20, 467.
3. Bordet and Gengou, 1901. Ann. Inst. Pasteur 15, 289.
4. Bordet and Streng, 1909. Zentralbl. f. Bakt. IO. 49, 260.
5. Dick, 1913. Jour. Infect. Dis. 12, 111.
6. Dingle, Fothergill and Chandler, 1938. Jour. Immunol. 34, 357.
7. Ehrlich and Morgenroth, 1900. Berl. klin. Wschr. 37, 453, 681.
8. Ehrlich and Morgenroth, 1901. Berl. klin. Wschr. 38, 598.
9. Gordon, Whitehead and Wormall, 1926. Biochem. Jour. 20, 1028, 1036.
10. Gramenitzki, 1912. Biochem. Ztschr. 38, 501.
11. Hegedus and Greiner, 1937. Ztschr. f. Immunitätsforsch. 92, 1.
12. Heidelberger, 1941. Jour. Exp. Med. 73, 681.
13. Heidelberger, 1941. Jour. Exp. Med. 73, 695.
14. Hilleman, Haig and Helmold, 1951. Jour. Immunol. 66, 115.
15. Hole and Coombs, 1947. Jour. Hyg. 45, 497.
16. Kabat and Mayer, 1948. *Experimental Immunoochemistry*, Charles C Thomas, Springfield, Ill.
17. Kahn and McDermott, 1949. Amer. Jour. Clin. Path. 19, 401.
18. Manwaring, 1906. Trans. Chic. Path. Soc. 6, 425.
19. Mayer, Eaton and Heidelberger, 1946. Jour. Immunol. 53, 31.
20. Mayer, Osler, Bier and Heidelberger, 1946. Jour. Exp. Med. 84, 535.
21. Muir and Browning, 1906. Jour. Hyg. 6, 20.
22. Nuttall, 1888. Ztschr. Hyg. Infektkr. 4, 353.
23. Pfeiffer, 1894. Ztschr. Hyg. Infektkr. 18, 1.
24. Pillemer, 1943. Chem. Rev. 33, 1.
25. Pillemer, Ecker, Oncley and Cohn, 1941. Jour. Exp. Med. 74, 297.
26. Rice and Avery, 1950. Amer. Jour. Vet. Res. 11, 98.
27. Rice and Crowson, 1950. Jour. Immunol. 65, 201.
28. Stoker, Coombs and Bedson, 1950. Brit. Jour. Exp. Path. 31, 217.
29. Streng, 1909. Zentralbl. f. Bakt. IO. 50, 47.
30. Streng, 1929. In Kolle, Kraus and Uhlenhuth, *Handbuch der pathogenen Mikroorganismen*, 3rd ed., 2, 1117. Fischer, Jena.
31. Wadsworth, 1947. *Standard Methods*, 3rd ed., Williams & Wilkins Co., Baltimore.
32. Wolfe and Kornfeld, 1948. Proc. Soc. Exp. Biol. 69, 251.

## Chapter 13

### ANTIVIRAL IMMUNITY

#### THE NATURE OF VIRUSES AND VIRAL DISEASE

VIRUSES ARE DEFINED by Rivers as "a heterogeneous group of infectious agents which are smaller than ordinary bacteria and require susceptible host cells for multiplication and activity."<sup>17</sup> They represent the most highly specialized infectious agents known and apparently constitute one end of the series of intergrading forms which continues through the somewhat larger Rickettsiae to the still larger and relatively unspecialized bacteria.

Viruses range in size from about ten millimicrons (0.01 micron) to approximately 300 millimicrons (0.3 micron). The smallest are therefore comparable in dimensions to large protein molecules, whereas the largest are larger than the smallest bacteria. The shapes of viruses include spherical or ovoid forms, cubes or parallelopipeds, rods and "tadpoles." Viruses also vary widely in composition. The tobacco mosaic virus, which is of intermediate size, appears to be composed solely of nucleoprotein. The considerably larger virus of vaccinia (cowpox) contains lipid, carbohydrate, thymonucleic acid, several serologically distinct proteins, copper, biotin and riboflavin. Despite its complexity, it apparently performs no independent metabolic function in the absence of living susceptible host cells.

**Parasitic Behavior of Viruses.** Strict parasitism is one of the outstanding characteristics of viruses and is important in understanding the nature of viral disease and the host response. Whether viruses are living or non-living is immaterial. Limitation of the term "parasite" to living agents is principally a matter of usage. Viruses unquestionably "reproduce" or are replicated in some manner within and at the expense of susceptible host cells. These cells consequently suffer damage or derangement of their physiologic functions and an abnormal or disease condition ensues.

One of the first noted abnormalities of cells infected by viruses was the appearance of *inclusion bodies* demonstrable by suitable staining.

These bodies have been found either in the cytoplasm or in the nucleus or in both. They are characteristic of certain diseases such as vaccinia, varicella (chickenpox) and variola (smallpox). Inclusion bodies of some of the larger viruses apparently consist in part of "colonies" of virus particles or *elementary bodies*.

Animal viruses in general have strong affinities for specific tissues. *Dermotropic* viruses, which parasitize epithelial surfaces of the body, include those of smallpox, cowpox and trachoma. *Neurotropic* viruses have a predilection for nerve tissue. Among the more widely known are the viruses which cause rabies, poliomyelitis and the encephalitides. *Viscerotrophic* viruses attack the abdominal viscera, particularly the liver, or induce generalized infection. Certain strains of the yellow fever virus are typical of this group. The fourth category consists of *pneumotrophic* viruses, which cause diseases of the lungs such as influenza. Some authors also refer to *pantropic* viruses, which have affinity for tissues derived from all three embryonal layers.

Tissue specificities are not absolute; primary infection usually involves the tissues indicated, but secondary invasion of other cells may occur. It is possible to alter the tissue specificity of certain viruses by appropriate manipulation. Viscerotrophic strains of yellow fever virus, for example, have been transformed by serial intracerebral passage in mice into neurotropic strains which produced fatal encephalitis in suitable experimental animals.

**Response of the Host to Viral Invasion.** The pathologic response to viral invasion is proliferation or degeneration of host cells; in some cases proliferation is followed by degeneration. The Rous sarcoma, Shope papilloma and herpes simplex viruses characteristically cause proliferative changes with hyperplasia of the invaded cells. The cells are relatively undamaged, but their rate of multiplication is stimulated and virus particles are passed to the daughter cells. Proliferation accelerates and an abnormal growth (e.g., cold sore, wart) results. Rabies and poliomyelitis cause degenerative changes. They infect nerve cells, and the resulting tissue death leads to paralysis. The course of yellow fever and foot and mouth disease is so rapid that there is no time for hyperplasia to occur.

Cellular multiplication is stimulated if the cells affected are capable of multiplying and if the disease is sufficiently protracted. Necrosis or destruction often follows stimulation, accompanied or succeeded by inflammation of neighboring tissues. The predominance of hyperplasia or of necrosis is determined by the balance of stimulating and destructive tendencies of the virus. Death and lysis of the cells occur if the action of the virus is rapid or if the affected tissues are incapable of multiplication (e.g., nerves).

It is not certain whether viruses are inherently toxic or whether toxins result from the interaction of virus and host cells. Purified influenza virus

and certain viruses of the lymphogranuloma-psittacosis group produce illness and death so rapidly when injected into experimental animals that they seem to possess toxic properties. The toxic agents have not, however, been separated from the viral particles.

#### IMMUNITY IN VIRAL DISEASES

Early investigations of antiviral immunity consisted almost entirely of neutralization and immunity tests in experimental animals. Such *in vivo* procedures have much to recommend them because they measure antibody actually concerned with protection, whereas it is not always certain that antibody determined *in vitro* is effective within the animal body. The use of *in vivo* techniques was dictated by the difficulty of securing suitable antigens for test tube experiments because of the intracellularly parasitic nature of viruses. First test tube methods included the complement-fixation reaction employing extracts of infected organs as antigens. Other procedures have since been developed.

**Natural Resistance to Viruses.** Natural resistance to viral disease is associated with such factors as species, age, sex, nutrition, genetics, climate and other environmental factors, and the presence of concurrent infection by other agents. Specific antibody appears to play little role.

Experimental inoculation of certain viruses such as the stomatitis virus into animals of various ages indicates that young animals possess non-specific characteristics which make them less resistant than older animals. The relative resistance of older animals may be caused by cellular changes accompanying maturation or increased responsiveness of the antibody-producing mechanism or both.

Olitsky and his colleagues obtained evidence indicating the formation of "barriers" against virus that increase with the age of the host.<sup>16</sup> Such barriers resist the passage of virus from the portals of entry to susceptible tissue. They consist of specific tissues which lie in the path of the virus and in which the virus does not multiply. The location of the barriers has been determined in the case of a few neurotropic viruses. The stomatitis virus inoculated into the eye of resistant older mice cannot pass the retina; when inoculated intramuscularly the virus encounters a barrier in the muscle or myoneural junction; following intranasal instillation its progress is blocked in the anterior olfactory area of the brain. The barrier to eastern and western equine encephalomyelitis viruses in older mice and guinea pigs is in the blood vessels. Both of these viruses are neurotropic. Typical infection follows intracerebral inoculation into either old or young animals. Resistance of non-nervous tissue to these viruses therefore increases with age.

Age may be a factor in determining the immune response of experi-

mental animals. Production of antibody against equine encephalomyelitis virus is rapid and strong in mature mice but slow and weak in infant mice.

The role of host nutrition in susceptibility to viral disease emphasizes the complete dependence of these parasitic forms on the host cells. Many viruses attack healthy, well nourished individuals as frequently as those suffering from malnutrition. Good health confers no protection against influenza, measles, chickenpox and smallpox. Malnourished or partially starved mice have been found less susceptible than normal animals to certain encephalitis viruses. Evidently viruses multiply better in healthy cells than in those which have been damaged by lack of proper nutrients or by other agents.<sup>17</sup>

Some plant, animal or bacterial viruses inhibit the simultaneous infection of their host cells by certain other viruses<sup>20</sup> A neurotropic yellow fever virus protects monkeys against an otherwise fatal dose of viscerotropic yellow fever virus given at the same time. The neurotropic form produces only transient fever, whereas the viscerotropic strain alone causes death. The protection afforded by the neurotropic virus against the lethal viscerotropic virus is known as the *interference phenomenon*. In like manner, Rift Valley fever virus suppresses infection of monkeys by the more highly pathogenic yellow fever virus. These viruses are immunologically unrelated, so it is evident that specific antibody is not the cause of interference. Schlesinger stated that the interfering virus usually possesses either a quantitative advantage or a head start and that interference is probably the result of competition for cellular constituents which are available in limited amounts.<sup>20</sup> The interference phenomenon may thus be correlated in part with host nutrition.

**Actively Acquired Antiviral Immunity.** Immunity following frank or subclinical cases of viral disease may be relatively permanent, as in the case of yellow fever and smallpox, but there are a number of diseases such as the common cold, influenza and dengue fever which produce only transient immunity. Burnet considers that two conditions must be fulfilled in order that prolonged strong immunity may follow a case of viral disease: first, the virus must be distributed *via* the blood stream, thus providing an effective stimulus to the whole antibody-producing mechanism; secondly, upon reinfection, the virus must be exposed to circulating antibody before it reaches susceptible cells.<sup>3, 5</sup> These conditions are met in yellow fever. The blood may contain ten billion infective doses of virus per milliliter at the height of the disease. This provides excellent opportunity for stimulation of the antibody-producing mechanism. Reinfection occurs by the bite of a mosquito and the virus immediately encounters circulating antibody.

The same conditions of permanent immunity are probably also fulfilled in smallpox, chickenpox, measles and German measles. These diseases are characterized by focal infections of mucosal surfaces, but symptoms are

preceded and accompanied by general distribution of virus in the blood. Hence, primary infection gives opportunity for antibody formation, and virus may react with antibody in the event of reinfection.

Immunity in dengue fever is only temporary. There is some question regarding the concentration of virus present in the blood at any one time.

It has been postulated that continued presence of antibody following recovery from virus infection is the result of frequent exposure which causes repeated mild and unrecognized infections. This might apply to such common childhood diseases as measles, German measles and chicken-pox, which recur in epidemics every two or three years, clinical cases being confined to succeeding groups of young, previously unexposed children.

Maintenance of immunity has also been explained by the persistence of undetected virus within the body. This is known as *infection or persistence immunity*. Failure to find such virus is no proof of its absence because techniques for virus detection are not sufficiently sensitive to permit isolation of single infective doses with certainty. Furthermore, if antibody is present in the host the virus may not multiply when removed to a test animal or chick embryo.

Solid and lasting immunity does not necessarily accompany persistence of virus within a host. The virus may be inactive but may become activated under the influence of some abnormal physiologic condition within the body. Herpes simplex illustrates this situation. The virus is apparently acquired by the majority of persons during infancy and remains in the body throughout life. It is activated under the stimulus of mild fever, as in the common cold, and causes typical vesicles (cold sores) on the mucous membranes, particularly of the lips.

The lack of permanent immunity following influenza or the common cold illustrates inadequate stimulation of the antibody-producing mechanism. The lesions of these diseases are relatively superficial, occurring on the mucous membranes of the upper respiratory tract. The viruses do not penetrate deeply into the body and apparently do not normally enter the blood stream. Consequently, there is little if any opportunity for antibody production.

**Artificial Active Immunization.** Artificial active immunization against viruses is accomplished by at least three different methods. The oldest procedure on record consisted of administration of *virulent virus by an unnatural route*. Before the Christian era, the Chinese practiced "variolation" as a means of preventing fatal smallpox. Pustular material from patients with mild cases of the disease was inoculated into the skin. A mild infection resulted, usually followed by permanent immunity. This method of immunization is dangerous because of the possibility that a serious or even fatal case may develop; furthermore, as practiced before the nineteenth century, there was also likelihood of concurrent infection

caused by bacteria in the pus used as inoculum. This type of immunization is not practiced at present in man but is used experimentally in animals. The viruses of stomatitis and foot and mouth disease injected into the footpads of guinea pigs normally produce local vesicular lesions. Guinea pigs inoculated intradermally with these viruses into areas other than the footpads display no signs of local or generalized illness but develop immunity to challenge injections into the footpads.

*Living modified viruses* are used in immunization against rabies, yellow fever, dengue fever, cattle plague and other diseases. Rabies virus as encountered in naturally infected animals and man (so-called *street-virus*) is characterized by a long and varied incubation period. The virus is almost always found in the central nervous system of rabid dogs, usually gains access to and multiplies in the salivary glands, and may be present in certain other non-nervous tissues. Serial intracerebral passage in rabbits markedly increases its speed of multiplication in the brain but decreases its invasiveness for the salivary gland. After a number of passages the incubation period following intracerebral inoculation becomes short and constant, and the virus is no longer able to multiply in non-nervous tissue such as the salivary gland. This modified virus is known as *fixed virus*. Pasteur discovered that the virulence of fixed virus in dried brain and spinal cord decreased during storage. Virus dried for fourteen days produced no untoward effects in humans injected subcutaneously. A series of injections of progressively fresher virus-containing tissue conferred a high degree of immunity upon man.

The Pasteur procedure is still employed, although in most countries the vaccine is prepared by different methods. The "Pasteur treatment" of humans exposed to rabies is actually a prophylactic rather than a therapeutic measure. Its success depends upon the lengthy incubation following infection with street-virus, usually one to three months but sometimes five or more months. Fixed virus inoculated in a series of seven or fourteen subcutaneous injections provides an immediate antigenic stimulus and usually prevents the appearance of symptoms caused by the original infecting virus.

Immunity against yellow fever is conferred by inoculation of a virus altered by numerous serial passages through tissue cultures.<sup>28</sup> The parent virus, known as the Asibi strain, was maintained in tissue cultures containing mouse embryonic tissue and possessed high neurotropic as well as viscerotropic properties. Theiler and Smith cultivated this virus in tissue cultures containing minced chick embryos from which the head and spinal cord were removed so that minimal amounts of nervous tissue were present, and found that both viscerotropic and neurotropic properties decreased markedly. The resulting strain, known as virus 17D, produced only a slight febrile reaction in humans, and yellow fever antibodies were present two to four weeks after inoculation.<sup>29</sup>

Immunity against many viruses is apparently acquired only by natural or artificial infection. Therefore, it was long believed that immunization with *killed viruses* would never be successful. However, certain viruses treated with formalin, phenol, ultraviolet light or other agents induce some degree of resistance or of antibody formation detectable by neutralization and immunity tests. It may be debated whether such treatment actually kills all the virus, but numerous observations indicate that adequate concentrations or dosages of the lethal agent, applied for a sufficient time, destroy the infectivity of the virus preparations. Most authors, perhaps from deference to the early controversy regarding the living or nonliving nature of viruses, employ the term *inactivated virus* rather than killed virus. Olitsky and Casals reported that adequate doses of formalized equine encephalomyelitis virus caused as great antibody response in mice as active virus although the formalized preparations contained no viable particles.<sup>16</sup> Formalin inactivated influenza virus was used by Salk, Menke and Francis and by others with some success for immunization of humans.<sup>19</sup> Immunity against this disease lasts only a few months, and a protective level of resistance can be maintained only by repeated inoculations. Levinson *et al.* employed ultraviolet irradiation for the preparation of highly antigenic but completely killed vaccines for rabies and St. Louis encephalitis viruses.<sup>12</sup> Poliomyelitis virus killed by the same method was found antigenic for mice.<sup>15</sup>

Salk<sup>18a</sup> immunized humans with formalin inactivated poliomyelitis virus from monkey kidney tissue cultures. His results were sufficiently promising to warrant a large scale field trial, which was conducted in the summer of 1954.<sup>7a</sup> The vaccine contained poliomyelitis virus of Types I, II and III, and was administered in three intramuscular injections of one milliliter each at zero, one and five weeks, respectively.

Two types of study were employed. In the "observed control study," 221,998 children in the second grade at school were vaccinated with the Salk vaccine, and 725,173 first and third grade children in the same schools were kept under observation as controls. In the "placebo control study," children in the first three grades were combined. One group, comprising 200,745 individuals, received vaccine; a control group of 201,229 children received a solution of similar appearance lacking poliomyelitis virus and kidney protein. Children inoculated with vaccine and with placebo were observed for possible untoward reactions to the inocula. The occurrence and severity of poliomyelitis in all children was studied in detail. In addition, antibody titers in sample populations were determined.

Untoward reactions were no more frequent in vaccinated children than in those who received the placebo. Vaccination did not affect the incidence of nonparalytic poliomyelitis but caused significant decrease in paralytic poliomyelitis. The paralytic poliomyelitis case rate per 100,000 children was 48.0 in the total control groups and 16.8 in the vaccinated

groups. Vaccination therefore reduced the paralytic poliomyelitis case rate by 65 per cent.

Cases in which the diagnosis of poliomyelitis was confirmed by laboratory examination provided the most impressive data, although the numbers of cases were small, particularly in the placebo study. In this group there were ten cases of spinal or bulbospinal poliomyelitis among vaccinated children and sixty-eight among control children, figures which indicated that the vaccine decreased these forms of the disease by 80 to 90 per cent. Vaccination appeared to be less effective against disease caused by Type I virus than that caused by Type II and Type III virus.

Antibody determinations showed that certain lots of vaccine possessed greater antigenicity than others. However, there was not clear-cut correlation between the antigenicity of the vaccines measured in this way and the reduction of paralytic poliomyelitis. In fact, two lots of vaccine classified as poorly antigenic decreased this type of poliomyelitis as much as three lots of good antigenicity.

**Passive Immunity against Viruses.** Passive immunization against viruses is effective only if sufficient doses of antiserum are given either before exposure to the virus or within a very short interval after exposure or experimental introduction of the virus. Andrewes found that antivaccinal serum introduced into the shaved skin of a rabbit prevented formation of a local lesion following subsequent inoculation of vaccinia virus.<sup>1</sup> However, immune serum introduced only five minutes after intradermal injection of the virus did not prevent a lesion, no matter how much serum was employed. Virus diseases resemble bacterial toxemias with respect to the speed with which antiserum treatment must be instituted. Serum is ineffective after the virus has invaded susceptible cells and thereby has become protected against antibody. The only role which antibody then plays is to limit the infection to the initial site.

Combined active-passive immunization of animals and humans is practiced to a limited extent. Bodian found that human gamma globulin administered to chimpanzees together with active poliomyelitis virus reduced the risk of paralysis but permitted the animals to develop active immunity.<sup>2</sup> Before introduction of strain 17D for immunization of man against yellow fever, the French neurotropic strain was widely used but occasioned a relatively high percentage of untoward reactions. Reactions were reduced, however, by injecting human or animal immune serum prior to the yellow fever virus. Human gamma globulin is used at present to modify the course of disease in persons exposed to measles. Carefully controlled doses of gamma globulin permit the appearance of mild symptoms, but the patient acquires active immunity from the infection. Larger doses may prevent all symptoms and eliminate development of active immunity.

Sabin reported successful nasal prophylaxis in animals by instillation of

antiserum against viruses which progress along the nasal pathway.<sup>18</sup> Guinea pigs were protected against pseudorabies and eastern equine encephalomyelitis viruses introduced by the nasal route for at least five hours after antiserum treatment. Smorodintsev *et al.* in Russia claimed prevention of influenza in man by nasal instillation of antiserum,<sup>26</sup> but attempts in this country have not been so successful.<sup>11</sup>

**Mode of Action of Antiviral Antibodies.** Antibodies may protect cells against the entry of virus, they may inhibit or kill the virus, or they may enhance phagocytosis.

Early reports indicated that mixtures of virus (e.g., vaccinia) and antiserum which produced no signs of disease when tested by animal inoculation regained virus activity when diluted with saline. McKee and Hale, however, questioned the actual "neutrality" of such mixtures.<sup>14</sup> They worked with an influenza virus suspension having a mouse infectivity titer greater than 1,000,000. A 1:1250 dilution of this suspension was apparently neutralized in one hour by homologous antiserum diluted 1:500, when judged by survival of mice inoculated intranasally with 0.1 milliliter amounts. However, these mice contained living virus as shown by serial passage. Suspensions of their lung tissues were prepared and inoculated into a second group of mice, all of which died. Complete survival of the second group of mice was not obtained until active virus diluted 1:2000 was employed with 1:500 antiserum. This mixture was termed *absolutely neutral* in contrast to the first, which was called *apparently neutral*. Much previous work had been done with apparently neutral mixtures.

McKee and Hale were unable to reactivate the virus in absolutely neutral mixtures by dilution. Reactivation could be accomplished by use of a concentrated suspension of heat inactivated (57° C. for seventy minutes) influenza virus. Active virus and antibody in absolutely neutral proportions were incubated together for one hour. Heated virus was then added and after two additional hours inoculations were made. All animals died. It was further found that virus overneutralized with 500 times the requisite amount of antibody could still be reactivated by this method. Evidently dissociation occurred and set free some active virus, the antibody released being taken up by inactive virus.

It can be concluded that neutralization of virus suspensions by antibody does not necessarily kill the virus. Whatever the action of antibody, it is evidently exerted before the virus particles enter susceptible cells; in some way it prevents entrance or alters the virus so that intracellular multiplication cannot occur.

Schlesinger and his colleagues reported that immunity apparently inhibits multiplication of equine encephalomyelitis virus in guinea pigs but does not prevent invasion.<sup>21, 22</sup> Intracerebral injection of active virus elicited a febrile reaction in both immune and nonimmune animals. Im-

mune guinea pigs recovered from the fever after twenty-four to thirty hours; fever continued in nonimmune animals until prostration occurred. Brain lesions in both groups of animals indicated that invasion had taken place, but remission of fever showed that the infection was abortive in immune animals.

Burnet cited the contrasting action of antibody in yellow fever, influenza and herpes simplex.<sup>4</sup> The blood of an individual immune to yellow fever contains a relatively high concentration of antibody. Virus introduced into such a person encounters the full antibody content of the blood before reaching suitable susceptible tissues where multiplication can occur. Influenza virus spreads from cell to cell in the film of liquid on the surface of the respiratory mucosa. Animal experiments show that the degree of resistance to infection is directly correlated with the amount of antibody in this liquid film. Herpes simplex virus is constantly latent within susceptible cells and can multiply within these cells although antibody might be present in the blood. The infection does not usually progress beyond its initial local site, however, which indicates that antibody may prevent extension from one cell to another.

It is evident that there is more than one mode of action of viral antibodies. Their action depends upon the nature of the virus and the type and physiologic condition of the host cells.

#### SEROLOGIC REACTIONS IN VIRAL DISEASES

Neutralization, protection and complement fixation tests have long been used in the study of viral diseases. Serologic reactions are employed for detection of viral antibodies in normal populations and in the serum of patients, for study of the response to prophylactic immunization and for identification and classification of viruses. *In vitro* tests are more rapid and less expensive than *in vivo* methods. Attempts are usually made to correlate test tube reactions with neutralization or immunity tests, which better indicate actual resistance to disease.

The serologic investigation of viruses received a great impetus when it was discovered that many could be cultivated in the developing chick embryo. Previously it had been necessary to employ animals, some of them expensive (e.g., monkeys). The technique of egg inoculation is relatively simple, although care and practice are required. Fertile eggs (Figure 48)<sup>8</sup> which have been incubated for a suitable length of time are cleansed and disinfected, an opening is made through the shell with a dental drill or small grinding wheel, and the desired site is inoculated by hypodermic needle. The opening is then closed with sterile paraffin, Scotch tape, or a cover glass sealed with paraffin. Embryonated eggs provide adequate quantities of virus material for serologic as well as animal inoculation experiments. Animals are still required as sources of certain

viruses to be employed for particular purposes: the mouse brain is a common source of neurotropic virus for neutralization and complement fixation.

**Serum Neutralization.** Sternberg first employed the serum neutralization reaction in 1892 in a study of vaccinia.<sup>27</sup> Mixtures of serum and infectious material are inoculated into susceptible animals, and the host response is compared with that of control animals, which receive only the virus.

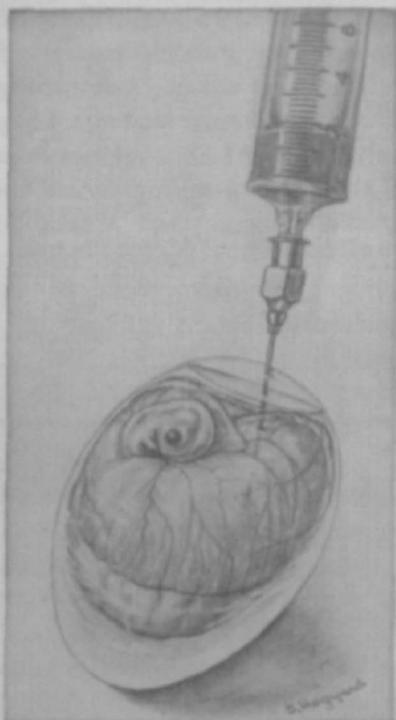


FIGURE 48. Inoculation of yolk sac of chick embryo through small hole drilled in end of shell. The hole will then be sealed and the embryo incubated. (From Therapeutic Notes, October 1942, Parke, Davis & Company.)

Details of technique vary with the kind of virus, the susceptible hosts and other factors, but the general principles of serum neutralization are illustrated by a procedure in use with certain neurotropic viruses. Virus is obtained by emulsifying infected mouse brains in inactivated rabbit serum and removing tissue by centrifugation at moderate speed. This preparation can be preserved with practically unchanged infective titer for considerable periods by freezing and storing at the temperature of dry ice (approximately  $-70^{\circ}$  C.).

Decimal dilutions of virus are mixed with equal volumes of undiluted test serum and incubated at  $37^{\circ}$  C. for two hours. Control mixtures of

virus dilutions and known negative serum are similarly treated. (With certain viruses more satisfactory results are obtained when *serum* dilutions are employed and a constant amount of virus.) Groups of five mice are injected intracerebrally with 0.03 milliliter doses of each mixture. Deaths are recorded during a period which depends upon the neurotropic virus employed. Ten days of observation are sufficient with the equine encephalitis viruses, whereas fourteen days are necessary with St. Louis encephalitis. Titration of three test serums is illustrated in Table 70.<sup>24</sup> The lethal dose for 50 per cent of a group of animals ( $LD_{50}$ ) is calculated by the method of Reed and Muench, previously described (page 192). These titers are conveniently expressed as logarithms of the virus dilution. The *neutralization index* is determined by comparing the  $LD_{50}$  of virus treated with antiserum and the  $LD_{50}$  of virus in control mixtures. The logarithm of the  $LD_{50}$  of virus mixed with serum C (Table 70) was 6.0 and the corresponding figure for the control was 8.0.

Table 70. Example of Virus Neutralization by Antiserum

MIXTURE	FINAL DILUTION OF VIRUS						LOG OF $LD_{50}$	NEUTRAL- IZATION INDEX	RESULT: VIRUS NEUTRAL- IZATION
	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$	$10^{-8}$	$10^{-9}$			
Control	..	5/5	5/5	4/5*	3/5	0/5	8.0	<10	Negative
Serum A	5/5	5/5	5/5	3/5	..	..	7.0+	32	Equivocal
Serum B	5/5	5/5	3/5	2/5	..	..	6.5	100	Positive
Serum C	5/5	4/5*	3/5	0/5	..	..	6.0		

\* The fraction 4/5 indicates that 4 of 5 inoculated animals died within a designated period (e.g., 10 days).

(From Smadel,<sup>24</sup> by permission. Copyright by The National Foundation for Infantile Paralysis, Inc.)

These logarithms represent dilutions of 1/1,000,000 and 1/100,000,000, respectively. Serum C therefore neutralized 100 times the amount of virus required to kill 50 per cent of the control mice.

Other criteria of infection may be used instead of death of the experimental animal. Unneutralized vaccinia virus is detected in rabbits by formation of a lesion at the site of dermal inoculation. Neutralization of mumps virus can be tested in chick embryos, which are examined after incubation for complement fixing antigen.

The mechanism of virus neutralization is not known with certainty. Several authors have recently emphasized the similarity of all neutralization reactions whether involving bacterial toxins or viruses. Virus-neutralizing antibodies are not always identical with antibodies detected *in vitro*. This has been shown with vaccinia, lymphocytic choriomeningitis and possibly with influenza.<sup>7, 25, 30</sup> Complement may participate in neutralization of viruses. Western equine encephalomyelitis antiserum

has been reported to lose its neutralizing power gradually at 4° C. and rapidly at 56° C., the activity of heated serum being restored by addition of complement. The C'1 fraction is less important than the other three components of complement. Heat inactivated mumps serum is also restored to activity by fresh normal serum.

The presence of virus-neutralizing antibody in a single specimen of patient's serum is often little indication of actual infection by the virus. The incidence in so-called normal populations of neutralizing antibody for certain viruses is very high: St. Louis encephalitis, lymphocytic choriomeningitis, poliomyelitis and others. These antibodies may result from previous cases or unrecognized infections with the same or closely related viruses or from vaccination. For diagnostic purposes at least two serum specimens should be examined from each patient. The first is obtained during the acute phase of the disease or as early as feasible and the second during convalescence. Neutralizing antibodies are determined in these paired specimens, and, barring an anamnestic reaction, a higher titer in the second specimen than in the first indicates that the patient was probably infected by the virus in question.

Neutralizing antibodies develop rapidly in some diseases but in others they are not detectable for months. Many poliomyelitis patients never acquire such antibody, and, conversely, the disease occurs in some persons who already possess antibody in their serums. Most of these contradictory observations still await explanation.

**Immunity Tests.** Immunity tests are performed by inoculating challenge doses of virus into immune animals, whereas in neutralization tests normal animals are injected with virus-antisera mixtures. In either case the effects observed (survival or death, paralysis, etc.) are the same. An immunity test may be used to assist the diagnosis of viral disease. Animals which have recovered from the disease in question are inoculated with known viruses; those which survive were presumably infected with the virus to which they possess immunity.

Cross immunity experiments can be employed to study serologic relationships between viruses in the same way that cross agglutination is used with bacteria. For example, it can be shown by cross immunity tests, in confirmation of cross neutralization, that eastern and western equine encephalomyelitis viruses are serologically distinct.<sup>16</sup> Animals recovered from infection or artificially immunized with eastern equine encephalomyelitis virus possess no immunity against western equine encephalomyelitis and *vice versa*.

The cross immunity test is particularly valuable when there is no satisfactory *in vitro* reaction and when neutralization tests are technically difficult or impossible. Studies of the common cold are hampered by lack of suitable experimental animals for large scale investigations. Experiments

with human volunteers indicate little if any cross immunizing relationship between the cold virus and the virus of primary atypical pneumonia.

/ **Complement Fixation with Viral Antigens.** Complement fixation is the most widely employed *in vitro* test with viruses. Preparation of satisfactory antigens is the major problem because virus is necessarily secured from living tissue, which is likely to contribute anticomplementary and nonspecific properties to the reagent. These properties are removed to a greater or lesser extent by extraction of infected tissues with benzene, acetone-ether and other lipid solvents, precipitation of nonspecific proteins with protamine, and ultracentrifugation.

Complement fixation has been successfully employed in the laboratory diagnosis of many of the viral encephalitides, smallpox, herpes, influenza, mumps, yellow fever, psittacosis and lymphogranuloma venereum. Accurate and painstaking technique is required to secure reliable results. This factor limits widespread diagnostic use of complement fixation and often restricts it to research applications.

It is impossible to generalize regarding the significance of complement fixation titers. Loring and co-workers reported fair agreement between the results of neutralization and complement fixation tests in poliomyelitis.<sup>13</sup> Similarly influenza neutralizing and complement fixing antibodies appear and disappear at about the same rate. On the other hand, yellow fever protective and complement fixing antibodies are not identical. Protective antibodies appear first and persist longer, perhaps throughout the remainder of life. Neutralizing antibodies for most of the encephalitis and other neurotropic viruses usually appear within two weeks of clinical onset and may remain in the circulation for years. Complement fixing antibodies appear later and are more transient, disappearing often within a few weeks or months, and always within three years. Lymphocytic choriomeningitis is a striking exception; complement fixing antibodies appear first and may start to decline before neutralizing antibodies are detectable.

/ **Agglutination and Precipitation with Viral Antigens.** Agglutination is restricted to some of the larger viruses like psittacosis, lymphogranuloma venereum and vaccinia. Precipitation is demonstrable with soluble antigenic materials derived from certain viruses such as vaccinia.

A recently developed rapid slide agglutination test promises to be of use in the diagnosis of psittacosis. The virus is cultivated in chick embryos. Allantoic fluid is harvested, inactivated with 0.4 per cent formalin and centrifuged to deposit the elementary bodies, which are resuspended in buffered saline. Gross particles are allowed to settle and the dense supernate constitutes the antigen for a slide test similar to that employed in rapid identification of bacteria or blood cells.

The antigen for vaccinia agglutination consists of elementary bodies

from the skin of rabbits inoculated with the virus. The viral particles are purified by differential centrifugation and washed in dilute buffer. In these agglutination tests either the antigen or the antiserum is kept constant and the other reagent varied, depending upon whether the antibody or antigen titer is desired. Tubes are incubated at 50° C. overnight.

Inert or nonspecific particulate agents have been employed to adsorb viruses from crude suspensions for use as test antigens in agglutination. Such materials include bacteria, blood cells, collodion and insoluble dyes. Bentonite, a colloidal aluminum silicate, possesses greater stability and uniformity than collodion particles and has recently been used in a diagnostic rapid flocculation test for trichinosis; apparently it has not been tried with viruses.

Some of the larger viruses resemble bacteria in being composed of several antigenic fractions, each capable of inducing specific antibody formation. The virus of vaccinia is one of the most thoroughly studied and best known. Vaccinia elementary bodies are agglutinated by at least four different antibodies in convalescent serum: the corresponding antigens are designated L, S, NP, and X. The L and S antigens are portions of a protein having a molecular weight of 240,000 found on the surface of the vaccinia particle.<sup>23</sup> The LS molecule precipitates in either L or S antiserum. The precipitability of fraction L is destroyed by heat but not by chymotrypsin.

The NP fraction is an immunologically specific antigenic nucleoprotein found in the surface of vaccinia particles and comprises about one-half of the substance of the particles. It may be secured by dilute alkali extraction of elementary bodies. Rabbits injected with the extract produce specific NP precipitins.

Antibodies against the other known fraction, X, agglutinate the virus elementary bodies. The nature of this agglutinin and its relation to other antibodies have not yet been determined.

The close relationship between vaccinia (cowpox) and variola (smallpox) has been recognized since Jenner's classic discovery (1798) that infection with vaccinia protected man against variola. The immunologic relationship has been repeatedly confirmed by cross neutralization and by *in vitro* methods (complement fixation, precipitation, etc.). The LS antigen is common to and specific for vaccinia and variola. It appears to be the same, whether derived from humans infected with smallpox or from animals infected with vaccinia. Antigen NP has not been sought in the virus of variola. The hemagglutinin of vaccinia (see page 266) is closely related to that of variola. As yet the antigenic fraction of vaccinia which elicits protective or neutralizing antibody is unknown; it does not seem to be any of those just discussed.

Agglutination and precipitation are useful in study of the serologic properties of certain viruses but so far possess only limited value in

laboratory diagnosis. Improvements in the preparation of test antigens may increase their diagnostic utility.

/ **Hirst Hemagglutination Inhibition Test.** Hirst reported in 1941 that chicken or human erythrocytes are agglutinated by influenza virus.<sup>9</sup> Several other viruses have since been found to agglutinate the erythrocytes of various animals.

Chemical receptors on the surfaces of the red cells are believed to participate in viral hemagglutination. Each virus particle possesses several receptors, and the virus acts as a "bridge" attracting and holding adjacent red cells in a manner similar to that postulated by the lattice hypothesis of agglutination by specific antibody. The attraction between red cells treated with virus is much weaker than the forces in antigen-antibody agglutination. Cells aggregated by virus are easily dispersed by slight shaking.

Influenza virus is almost completely adsorbed by appropriate red cells within a few minutes. However, the adsorbed virus spontaneously elutes from the cells within a short time. Within an hour or two the virus reappears in the solution if the mixture is kept at 37° C. with intermittent shaking, and the red cells cease to be agglutinated. The eluted virus is unaltered and can combine with fresh red cells. Cells from which virus has been eluted are incapable of combining with more of the same virus but can still react with certain other viruses. Changes in their surface properties are indicated by a marked alteration of their electrophoretic mobility. Moreover, immunologic tests of cells from which virus has been removed reveal the presence of a new antigen, presumably on the cell surface.

The hemagglutinin of vaccinia virus is apparently soluble because the virus may be deposited by centrifugation leaving hemagglutinin in the supernatant fluid. Vaccinia hemagglutinin seems to be a phospholipid resembling lecithin. The hemagglutinins of certain viruses of small particle size are closely associated with or are part of the virus particle. These viruses include Japanese and St. Louis encephalitis, pneumonia virus of mice, and foot and mouth disease.

The hypothesis has been advanced that a virus possesses an enzyme which can attack a surface component or receptor group of the red blood cells.<sup>10</sup> These receptors are probably mucoproteins. The virus presumably adsorbs to the mucoprotein, attacks it and is subsequently released. The surface properties of the erythrocytes are changed as a result of this action. Support for this concept is derived from observations that *Vibrio cholerae* and many other bacteria produce an enzyme which destroys the virus receptors of erythrocytes.<sup>8</sup>

Viral hemagglutination is important serologically because it can be specifically inhibited by antiviral immune serum. Viral antibody can therefore be detected and titrated by its ability to prevent agglutination

of red cells by virus. Dilutions of the inactivated test serum are mixed with virus suspension and washed erythrocytes. Test tubes containing the mixtures are observed without agitation after one to two hours at room temperature, the time of incubation depending upon the virus employed. Failure of hemagglutination constitutes a positive test for antibody in the various serum dilutions. Unagglutinated cells slide down the sides of the tube or settle directly to the bottom and form a compact circular "button" with smooth, sharply defined edges. Agglutinated cells stick to the sides of the bowl at the bottom of the tube or form a ragged edge around the "button" of unagglutinated cells.

Hemagglutination inhibition has been employed in the study of relationships between viruses and in fundamental investigations on the nature of virus action. Viruses which cause hemagglutination and hence might be used in inhibition tests include influenza, swine influenza, mumps, vaccinia, smallpox, Newcastle disease and several others. Different viruses do not agglutinate all kinds of erythrocytes. Human and chicken red cells are agglutinated by influenza A and B but not by the swine influenza virus, whereas ferret erythrocytes are agglutinated by all three of these viruses.

The vaccinia hemagglutination inhibiting antibody is apparently not the same as antibody detected by neutralization tests. On the other hand, there is fairly close relationship between influenza hemagglutination inhibiting, neutralizing, and complement fixing antibodies.

### References

1. Andrewes, 1929. Jour. Path. Bact. 32, 265.
2. Bodian, 1951. Amer. Jour. Hyg. 54, 132.
3. Burnet, 1945. *Virus as Organism*, Harvard University Press, Cambridge, Mass.
4. Burnet, 1950. Lancet 1, 1059.
5. Burnet, 1951. Bull. Johns Hopkins Hosp. 88, 119.
6. Burnet and Stone, 1947. Aust. Jour. Exp. Biol. Med. Sci. 25, 227.
7. Craigie, 1939. In Doerr and Hallauer, *Handbuch der Virusforschung*, 2, Julius Springer, Wien.
- 7a. Francis, 1955. *Evaluation of 1954 Field Trial of Poliomyelitis Vaccine, Summary Report*, J. W. Edwards, Ann Arbor, Mich.
8. Frobisher, 1953. *Fundamentals of Microbiology*, 5th ed., W. B. Saunders Co., Philadelphia.
9. Hirst, 1941. Science 94, 22.
10. Hirst, 1952. In Rivers, *Viral and Rickettsial Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
11. Kreuger *et al.*, 1944. Amer. Jour. Med. Sci. 207, 40.
12. Levinson, Milzer, Shaughnessy, Neal and Oppenheimer, 1945. Jour. Immunol. 50, 317.
13. Loring, Raffel and Anderson, 1947. Proc. Soc. Exp. Biol. 66, 385.
14. McKee and Hale, 1946. Jour. Immunol. 54, 233.
15. Milzer, Oppenheimer and Levinson, 1944. Jour. Amer. Med. Assoc. 125, 704.
16. Olitsky and Casals, 1945. Bull. N. Y. Acad. Med. 21, 356.
17. Rivers, 1952. In Rivers, *Viral and Rickettsial Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.

18. Sabin, 1936. *Jour. Exp. Med.* **63**, 863.
- 18a. Salk, 1953. *Jour. Amer. Med. Assoc.* **151**, 1081.
19. Salk, Menke and Francis, 1945. *Amer. Jour. Hyg.* **42**, 57.
20. Schlesinger, 1952. In Rivers, *Viral and Rickettsial Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
21. Schlesinger, Olitsky and Morgan, 1943. *Proc. Soc. Exp. Biol.* **54**, 272.
22. Schlesinger, Olitsky and Morgan, 1944. *Jour. Exp. Med.* **80**, 197.
23. Shedlovsky and Smadel, 1942. *Jour. Exp. Med.* **75**, 165.
24. Smadel, 1952. In Rivers, *Viral and Rickettsial Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
25. Smadel and Wall, 1940. *Jour. Exp. Med.* **72**, 389.
26. Smorodintsev, Gulamow and Tschalkina, 1940. *Ztschr. klin. Med.* **138**, 756.
27. Sternberg, 1892. *Trans. Assoc. Amer. Physicians* **7**, 68.
28. Theiler and Smith, 1937. *Jour. Exp. Med.* **65**, 767.
29. Theiler and Smith, 1937. *Jour. Exp. Med.* **65**, 787.
30. Walker and Horsfall, 1950. *Jour. Exp. Med.* **91**, 65.

## Chapter 14

### ALLERGY

THE TERMS ALLERGY AND HYPERSENSITIVITY are often used synonymously, although strictly speaking hypersensitivity is only one phase of allergy. Allergy was originally defined as *the altered reactivity of an individual toward a specific substance, usually resulting from prior experience with the same or a chemically related substance.* This definition, which is adhered to in Europe, includes decreased capacity to react (hypoergy) as well as increased capacity to react (hyperergy) and also the customary responses to natural or artificial immunization. Hypersensitivity is essentially equivalent to hyperergy.

Probably no medical term is more misused by laymen than the word allergy. It is popularly, and to some extent professionally, employed to designate any naturally occurring unpleasant or harmful responsiveness of man to a considerable variety of agents. In the narrow sense which is usually implied, allergy should be limited to hypersensitivity toward substances usually antigenic in nature or capable of specifically influencing the antigenicity of protein. It will be used both with the original meaning and in this more restricted aspect. The specific implication in any given instance will be apparent from the context.

This chapter deals only with hypersensitivity reactions. Hypersensitivity is acquired by prior contact with the inciting agent. As a result of this experience the animal or human becomes hypersensitive to that agent and reacts more or less violently when reexposed to the same material at a later date.

Substances which induce hypersensitivity differ widely in chemical nature. Some are frankly antigenic, such as foreign serum and egg albumin. Others, like plant pollens, possess feeble antigenicity. Still others are completely nonantigenic *per se*, but may combine with and alter the specificity of proteins. Certain low molecular weight drugs are of this type. In view of the heterogeneity of the agents which produce hypersensitivity, the term *allergen* is employed as a general designation.

Sensitization, or induction of the hypersensitive state, requires one or more exposures to the allergen, followed by a latent period. After the latent period a reaction is elicited by another exposure to the allergen. The nature of the hypersensitivity acquired is determined by the chemical composition of the allergen, the sensitizing route (injection, ingestion, inhalation, etc.), the physiology and anatomy of the host and other factors. The specific reaction observed depends upon the type of hypersensitivity and the mode of contact with the eliciting dose.

#### TYPES OF HYPERSENSITIVITY

Hypersensitivity reactions are conveniently and logically divided into two categories, depending upon the speed with which a hypersensitive individual displays a detectable response to the inciting agent.

"Immediate" or "early" hypersensitivities are those in which the response occurs soon after application or adequate absorption of the appropriate material. Such reactions are found in anaphylaxis, which is usually experimentally induced, and in the clinical conditions of asthma, hay fever, "hives," certain unusual drug sensitivities and gastrointestinal disturbances. In many instances circulating antibodies are demonstrable.

Hypersensitive reactions of the "delayed" type become apparent only after several hours, and the affected sites show progressive changes for two or three days or even longer. These reactions are common following exposure to poison ivy and poison oak, certain drugs and various microbial products such as tuberculin. This form of hypersensitivity is sometimes known as the "tuberculin" type. Circulating antibodies are rarely if ever found.

#### *"Immediate" Hypersensitivities*

The "immediate" hypersensitivities possess several characteristics in common. (1) Sensitive individuals usually respond within a few minutes to the eliciting dose of allergen. (2) Circulating antibodies of some type are demonstrable and sensitivity may be transferred passively to normal individuals or animals by injection of serum from a hypersensitive individual or animal. (3) Following union of antigen or allergen with antibody *in vivo*, the basic reaction appears in most if not all cases to consist of release of histamine or a histamine-like substance from some combined form in body cells, with subsequent dilatation of blood vessels and possibly contraction of smooth muscles. Succeeding events are determined in part by the location of the affected vessels and muscles. "Immediate" hypersensitivities include anaphylaxis, the Arthus reaction, evanescent allergic inflammation and the so-called atopic sensitivities or "spontaneous"

allergies characterized by the presence of a peculiar thermolabile antibody ("reagin") and an hereditary tendency.

**Anaphylaxis. First Observations.** Anaphylaxis was apparently first reported in 1839 by Magendie, who noted the sudden death of animals repeatedly injected with egg albumin.<sup>27</sup> Several other investigators also observed a similar phenomenon during the nineteenth century, but no systematic study was made until Richet and his collaborators (1898-1902) rediscovered the phenomenon.<sup>31</sup> They attempted to immunize dogs by repeated injections of eel serum or an extract of sea anemone tentacles, both of which are toxic. When the animals were reinjected about three weeks later, even with doses sublethal for a normal animal, they became violently ill and often died. Instead of producing a protective immunity, the series of injections induced an unusually sensitive state in the dogs, so that amounts too small to affect normal animals caused a rapidly fatal response. Since this situation appeared to represent the reverse of prophylaxis, Richet designated it *anaphylaxis*. The reaction is known as anaphylactic shock.

Following a communication by Theobald Smith to Ehrlich in 1905, the guinea pig became the animal of choice for anaphylactic experiments because it was more easily sensitized and shocked than other common experimental animals. Smith observed that guinea pigs prepared by injections of diphtheria toxin-antitoxin mixtures promptly displayed fatal anaphylactic shock when reinjected several weeks later with normal horse serum. Otto, a student of Ehrlich, found that sensitization was independent of the toxin or antitoxin content of the injected material and was induced by horse serum alone.<sup>30</sup> He also noted that sensitized guinea pigs which were given a number of very small injections of horse serum withstood subsequent doses which ordinarily produced fatal anaphylaxis. Such animals were said to be in an "anti-anaphylactic" or "desensitized" condition.

Rosenau and Anderson (1906-1907) sensitized guinea pigs with as little as a millionth of a milliliter of horse serum, although larger amounts gave more consistent results.<sup>30</sup> They also found that anaphylaxis possessed the same specificity as precipitation or other serologic reactions and that sensitivity could be transferred passively from mother to offspring.

It thus became apparent that anaphylaxis was an *in vivo* antigen-antibody reaction. It may be defined as an acute systemic reaction of hypersensitive animals, elicited by reinjection with the material to which they are sensitive. It is principally an experimental phenomenon demonstrated in laboratory animals, although anaphylactic reactions occasionally occur in man. Each animal species exhibits its own characteristic anaphylactic syndrome.

**Active Anaphylaxis.** Active anaphylaxis resembles active immunity

in that sensitivity is developed by the same animal in which anaphylactic shock is later demonstrated. It is contrasted with passive anaphylaxis, in which sensitization is accomplished by transfer of serum.

Production of active anaphylaxis requires three definite steps. First is the *sensitizing injection*. This is followed by the *incubation* or *latent period* leading to the sensitized state, and finally by the *eliciting* or *shocking injection*.

Active sensitivity is produced by injection of any antigenic substance such as a complete protein or protein complex. The route of administration is relatively immaterial: any parenteral injection may be employed. Intraperitoneal or subcutaneous injections are most often employed with guinea pigs. Feeding is not usually successful, although it has been said that about 50 per cent of guinea pigs may be sensitized to certain proteins by this method.

The sensitizing dose depends upon the antigen and the animal. A single injection of a soluble foreign protein suffices for the guinea pig, and the amount may be very small. Customary doses of horse serum range from 0.0001 to 0.25 milliliter. Egg albumin usually sensitizes in doses of 0.0001 to 1 milligram. Guinea pigs are rendered sensitive less easily by means of bacterial proteins, pollens and some other vegetable proteins, of which several preparatory injections on consecutive days are often necessary. Dogs usually require more than one injection of any antigenic substance, and rabbits must almost always be injected repeatedly.

Following the last sensitizing injection, an incubation period of at least ten to twenty-one days must elapse before typical anaphylactic shock can be demonstrated. Once established, the sensitive state may persist for months or sometimes even years. Mild but definite reactions have been elicited eight months after sensitization of guinea pigs with horse serum.

The specificity of the anaphylactic reaction is of the same order as that of *in vitro* serologic tests. Hence, the material used for the shocking injection is the same as or is closely related to that employed for sensitization. Certain separated complex haptens, such as bacterial polysaccharides, elicit typical shock as well as the complete antigen of which the hapten was originally a part. Simple haptens, however, have the reverse effect and specifically inhibit shock following subsequent injection of the original complete antigen or complex hapten. Landsteiner employed anaphylactic methods in studying the specificity of azoprotein antigens (see page 32).

Shocking injections are best given by a route which ensures rapid absorption and almost immediate contact with sensitized cells. For this reason intravenous injections are preferred. The reactions after intraperitoneal injections are usually delayed in onset for fifteen to thirty minutes.

The shock dose is generally larger than the sensitizing dose, although

not necessarily so. For maximum effect it should provide an appreciable concentration in the blood within a period of thirty to sixty seconds. Guinea pigs sensitive to ovalbumin are thrown into shock by 0.1 to 10 milligrams of the same material.

*Symptom Complex in Anaphylaxis.* Certain symptoms of anaphylactic shock are common to almost all animals; these include decreased blood pressure, body temperature, and number of circulating leukocytes, and often decreased blood coagulability, probably caused by liver damage which is believed to liberate heparin, an anticoagulant. These symptoms appear to be associated with attachment of leukocytes and platelets to the endothelium of small blood vessels, liberation of histamine, contraction of smooth muscles, arteriolar and venous spasm often followed by dilatation of the vessels, and rapid occurrence of edema. In addition to the foregoing general responses there are special features by which anaphylactic shock in one species differs from that in another but which are constant within the species.

Within a minute after intravenous administration of an adequate shocking dose to a sensitive guinea pig, the animal becomes restless, its hair bristles, it coughs and scratches its face, and its respiration is slow and labored. Convulsions and death follow, often within two to four minutes after injection. Immediate postmortem examination reveals the heart still beating and active intestinal peristalsis. The lungs are fully inflated, and there may be evidence of visceral congestion.

In the rabbit, systemic anaphylaxis does not always occur. When fatal shock does take place, the preliminary reaction consists of irregular respiration succeeded by panting. The ears become hyperemic and then blanched. The animal collapses and lies outstretched, gives a few convulsive movements associated with passage of feces and urine, and dies suddenly with head thrown back and eyes protruding. At autopsy the right side of the heart is found greatly dilated, and the inferior vena cava, portal vein and liver are engorged with blood.

Preliminary signs of anaphylaxis in the dog consist of restlessness and excitement, followed by vomiting and passage of urine and feces, the latter often bloody. The animal then collapses in a state of marked muscular weakness, its respiration is slowed, and it becomes comatose and exhibits epileptiform convulsions. Death may occur in one to several hours. Autopsy discloses a greatly distended and engorged liver, which often contains as much as 60 per cent of the animal's blood. The animal literally bleeds to death with the blood collected within the vessels of its own liver, a process accompanied by complete collapse of the systemic vascular system. If shock is prolonged, there may be considerable congestion of the gastrointestinal tract.

The symptoms of anaphylactic shock in these three animals share features referable to the distribution of prominent areas of smooth muscle.

The bronchial musculature of the guinea pig and the muscles of the rabbit's pulmonary arterioles are unusually well developed. The principal musculature of the intestines is also of the smooth type. In the guinea pig, death is the result of suffocation, even though the lungs are fully inflated. Contraction of the bronchial muscles prevents exhalation. Coughing is associated with attempts to secure oxygen. Engorgement of the right side of the rabbit heart is caused by contraction of the pulmonary arterioles, which prevents passage of blood through the lung to the left side. Death is attributed to heart failure. Defecation, a common reaction in anaphylaxis, is a natural consequence of the hyperactivity of the smooth muscle of the intestine wall. It should be pointed out that recent observations indicate that massive edema may play a greater role than was formerly suspected in certain symptoms of anaphylactic shock: for example, in the occlusion of guinea pig bronchioles.

Engorgement of the dog liver during shock has been attributed to constriction of the hepatic blood vessels, although this mechanism has not been proved. On the contrary, it appears that capillary dilatation and injury to liver cells with resulting edema may retard the flow of blood through this organ. Manwaring *et al.* observed contraction of various smooth muscles *in vivo* during anaphylaxis.<sup>29</sup> These muscles included those of the uterus, intestines, urinary bladder and bronchial system. Symptoms of anaphylaxis were prevented by excluding the liver from the circulation. Furthermore, by joining the liver of a sensitized dog to the circulation of a normal animal, reactions were induced in the latter when the sensitive dog was shocked. These facts indicate that some substance released from the liver during anaphylaxis and distributed about the body *via* the blood stream stimulates contraction of smooth muscles and causes certain other damage. The active material has been shown to be histamine, long known as a powerful stimulant of smooth muscle activity. It causes contraction of visceral muscles, dilates capillaries, and stimulates salivary, lacrimal, pancreatic, intestinal and gastric secretions. The dog liver is rich in histamine and releases it during shock.

*Passive Sensitization.* As early as 1907, Otto showed that it is possible to sensitize a normal guinea pig passively by injection of serum from a sensitized guinea pig. The recipient undergoes a shock reaction typical of its species when homologous antigen is administered. In most cases an incubation period of six to twenty-four hours must elapse before introduction of the shocking dose of antigen. Moreover, passive sensitization of guinea pigs can be accomplished by injection of antiserum from animals, even of certain other species, which have been immunized against soluble antigens. Sensitizing efficacy is directly related to the precipitin titer of the serum; a small amount of high titer anti-egg albumin rabbit serum sensitizes a guinea pig to a shocking dose of egg albumin injected a few hours later.

These observations demonstrate clearly that anaphylaxis is an antigen-antibody reaction. Passive transfer of sensitivity shows that one participating reagent (now identified as antibody) is present in the circulating blood. Furthermore, the fact that an antiprotein serum can sensitize a normal animal to the homologous antigen indicates that one and the same antibody may be responsible for precipitation and anaphylaxis. The incubation period in passive sensitization is frequently explained as the interval required for antibody to attach to or become intimately associated with certain cells (or possibly all cells) of the animal body.

*Desensitization.* A hypersensitive animal which is given several very small (subshocking) subcutaneous injections of antigen at closely spaced intervals (e.g., one-half hour) may then be able to tolerate an ordinarily shocking dose without severe reaction. Such an animal is said to be *desensitized*. The total *in vivo* antigen-antibody reaction is apparently so prolonged that little tissue damage occurs; moreover, histamine may be detoxified almost as rapidly as it is liberated and may never accumulate in sufficient concentration to produce a violent reaction. Desensitization is only temporary, lasting no more than a few days or weeks.

The appearance of hypersensitivity is delayed two to four weeks in a guinea pig which receives a sensitizing dose of soluble antigen considerably larger than required for normal anaphylactic sensitization, or in an animal given several smaller preparatory injections. The animal is said to be in an "antianaphylactic" or refractory state at the end of the ordinary three week incubation period. This situation has been attributed to the presence of too much antibody, because passive transfer experiments reveal the presence of sensitizing antibody in such a refractory animal. Circulating antibody might then protect against anaphylaxis, presumably by combining with antigen before it can reach sensitive cells. The whole truth is probably much more complex than this simple statement.

*In vitro Anaphylaxis.* Schultz and Dale found that when strips of intestinal or uterine muscle were excised from sensitized guinea pigs and suspended in a suitable physiologic saline solution, the muscle contracted strongly upon addition of homologous antigen to the solution.<sup>12, 41</sup> This response displays typical antigen-antibody specificity and can be used to distinguish between different proteins. The Schultz-Dale reaction is also extremely sensitive.

The animal which donates the muscle tissue may be sensitized either actively or passively. Several investigators were also able to sensitize excised normal uterine tissue by suspending it in antiserum. The tissue was washed after several hours and treated with antigen, whereupon typical contraction occurred.

In the Schultz-Dale test a latent period of a few seconds usually follows addition of antigen to the bath before contraction of the sensitive muscle

tissue. The tissue remains contracted for some time (Figure 49) and gradually relaxes. If the saline solution is then removed and replaced by fresh solution, addition of more antigen produces no further contraction. This constitutes *in vitro* evidence of desensitization. The muscle is still physiologically capable of contracting, however, and responds typically to the addition of histamine, acetylcholine, pilocarpine or other appropriate reagents. Desensitization therefore involves only the antigen-antibody mechanism and has nothing to do with the contractility of the muscle.

*Quantitative Studies of Anaphylaxis.* The application of quantitative methods to serology made possible precise studies of anaphylaxis by Kabat and others.<sup>17, 20, 21</sup> However, quantitative determination of antibody

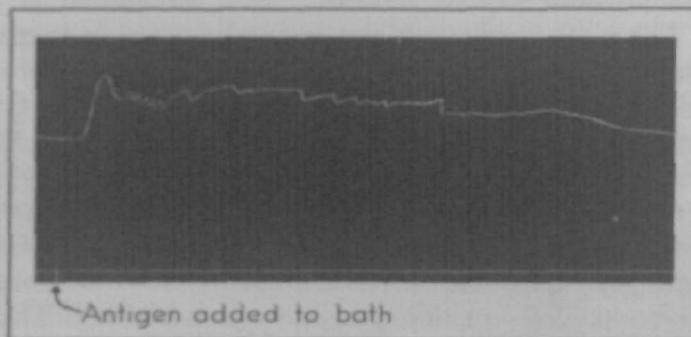


FIGURE 49. Kymograph record of the contraction of an intestinal strip from a guinea pig sensitized with egg albumin. Contraction occurred a few seconds after addition of antigen to the bath, and the muscle did not completely relax for many minutes.

which is "fixed" to tissues is as yet impossible, so it was not feasible to work extensively with active sensitization. Instead, experimental animals were sensitized passively with known amounts of antiserum containing predetermined quantities of precipitating antibody. Approximately 0.2 milligram of rabbit antiovalbumin sufficed to prepare guinea pigs weighing 250 grams for uniformly fatal anaphylaxis when challenged with one milligram of ovalbumin intravenously (Table 71).<sup>21</sup> Doses of antibody as small as 0.012 milligram sensitized two out of four animals so that they reacted slightly to one milligram of antigen. Only two-thirds of the guinea pigs prepared with 0.07 milligram of antibody were killed by one milligram or ten milligrams of antigen (Table 72).<sup>21</sup> All animals sensitized with 0.375 milligrams of antibody were killed by one milligram of antigen, but only 60 per cent of those shocked with 0.1 milligram.

It is apparent that a certain minimal concentration of antibody was required to sensitize an animal. Administration of more antibody led to more severe reactions or a greater percentage of deaths. Moreover, with sufficient antibody an increased dose of antigen produced a higher proportion of fatal reactions.

*Mechanism of Anaphylactic Shock.* Anaphylaxis is a consequence of *in vivo* antigen-antibody interaction, which apparently damages tissues, especially the vascular endothelium. The events following tissue damage are determined in part by the fact of damage itself and in part—perhaps principally—by materials leaving the damaged tissues.

Table 71. Passive Sensitization of Guinea Pigs with Various Amounts of Rabbit Anti-egg Albumin

(Guinea pigs shocked with 1 mg. egg albumin intravenously 48 hours after sensitizing injection.)

ANTIBODY INJECTED	NO. OF GUINEA PIGS USED	ANAPHYLAXIS			
		Fatal	Severe	Slight	None
0.012 mg.	4			2	2
0.024	4		1	3	
0.047	4		3	1	
0.071	6	4	2		
0.144; 0.150	5	3	2		
0.213; 0.225	6	6			

(From Kabat and Landow,<sup>21</sup> by permission.)

Table 72. Effect of Shocking Dose of Antigen (Egg Albumin) on Fatal Anaphylaxis in Guinea Pigs Passively Sensitized with Homologous Rabbit Antiserum

(Each group of guinea pigs consisted of 3 to 8 animals.)

ANTI-EGG ALBUMIN (mg.)	EGG ALBUMIN (mg.)			
	0.01	0.1	1	10
Deaths (per cent)				
0.071	0	20	67	67
0.375	0	60	100	67

(From Kabat and Landow,<sup>21</sup> by permission.)

The evidence for participation of both antigen and antibody in anaphylaxis may be summarized as follows:

- (1) Only substances which are complete antigens or complex haptens can be used to induce anaphylactic shock.
- (2) The incubation period in active anaphylaxis is similar to that in antibody formation.
- (3) Antibody can be demonstrated in the serums of sensitized animals by use of suitable techniques.
- (4) Sensitivity can be transferred passively by means of serum or its globulin fractions from a hypersensitive or an immune animal.
- (5) The ability of serum to confer passive sensitivity is roughly proportional to its precipitin content.

(6) The specificity of anaphylaxis is the same as that of the *in vitro* antigen-antibody reactions.

Anaphylaxis was early explained on the basis of a cellular hypothesis according to which antigen reacts with "sessile" antibody attached to body cells. Antigen-antibody reaction was assumed to initiate cellular disturbances which induced the characteristic syndrome of anaphylactic shock. Later evidence indicated that a toxic substance liberated into the blood stream might be the immediate cause of symptoms, so the humoral hypothesis developed. It was postulated that antigen and antibody reacted in the circulating blood and that the resulting complex was split by proteolysis, perhaps through the action of complement, to yield an unknown toxic substance, "anaphylatoxin." When it was found that non-protein antigens such as bacterial polysaccharides can shock suitably sensitized animals, it was proposed that the host's own proteins constitute the substrate for anaphylatoxin formation. It will be recalled that Keysser and Wassermann, Jobling and Peterson, and others (see page 53) reported that injection of foreign normal serum, after incubation with barium sulfate, starch, agar, kaolin or other substances, induced reactions in normal animals which were similar in many respects to those of anaphylactic shock. Moreover, peptone injected intravenously induces an excellent imitation of anaphylactic shock. These phenomena are called *anaphylactoid* reactions, because they resemble anaphylaxis clinically but do not appear to have an antigen-antibody mechanism. However, there may be some similarity in cause which is not immediately apparent.

The present concept of the mechanism of anaphylaxis again suggests a cellular site of reaction between antigen and antibody, although no actual evidence exists for the attachment of antibody to body cells, despite its probability. The incubation period required for passive sensitization is considered indicative of such attachment by some investigators, who assume that it takes place during this interval of a few hours. The situation is confused, however, by reports of almost immediate passive sensitization, and experiments have been described in which typical reactions were obtained when antigen and antibody were injected almost simultaneously. In such cases, much larger amounts of immune serum than usual were required. It is of course conceivable that one reagent or the other may attach rapidly to at least some body cells. The phenomenon of *reversed passive anaphylaxis* is demonstrated by injecting antigen first and antibody some hours later, whereupon typical anaphylactic shock is produced. Hence, antibody need not always be "fixed" to cells before antigen is administered.

Further indication of a cellular site of interaction between antigen and antibody is provided by the observation that a sensitized animal may be shocked even though its own blood has been replaced by that of a normal animal.<sup>28</sup> The Schultz-Dale reaction, performed with tissues washed as free from serum as possible, indicates that antibody is closely

associated with the reacting tissues. It has not been unequivocally demonstrated, however, that such tissues are completely devoid of free antibody.

Most available evidence supports the conclusion that during anaphylactic shock some substance is released from the cells upon which antigen-antibody reaction takes place and that this substance is transported in the circulating blood to muscles and other tissues, which are then stimulated to their characteristic reaction. The possible role of histamine as this toxic substance was suggested by Lewis in 1927.<sup>24</sup> Histamine is widely distributed throughout all tissues in the body, presumably bound to protein. Its concentration differs in the various organs. It is particularly abundant in the aorta, lungs, uterus and seminal vesicles of the guinea pig; in the dog, its concentration is high in the liver; its principal source in the rabbit is the white blood cells and platelets. The pharmacologic action of histamine has already been mentioned (page 274). It is of interest that the susceptibility of various species of animals to anaphylactic shock usually parallels their susceptibility to histamine. The lethal dose of this chemical injected intravenously into the guinea pig, rabbit and dog is between 0.3 and 3.0 milligrams per kilogram of body weight, whereas the corresponding dose for the rat, which is quite resistant to anaphylaxis, is 170 to 500 milligrams.<sup>8</sup> Animals which have been pretreated to increase their resistance to histamine are also often more resistant to anaphylactic shock. Moreover, tissues which are most active in anaphylactic shock are usually most sensitive to histamine. Recent work indicates that various antihistaminic compounds may also inhibit anaphylaxis. Finally, substances chemically and pharmacologically resembling histamine have been detected in the circulation of animals during shock. Failure to find histamine, as reported by a number of investigators, may be attributed to its rapid detoxification in the blood.

There is also evidence contradictory to the histamine hypothesis. Histaminase, an enzyme which inactivates histamine and which is obtained from extracts of the kidney and intestinal mucosa, does not protect against anaphylactic shock.<sup>1, 38</sup> Furthermore, a substance liberated from the guinea pig lung during anaphylaxis is capable of stimulating contraction in the rat uterus, although the latter organ does not respond to histamine by contraction.<sup>6</sup>

These observations seem damaging to the histamine hypothesis, but the weight of evidence justifies the present conclusion that histamine or some substance having many of the same pharmacologic properties is liberated from tissues damaged by associated antigen-antibody reaction. It has been suggested that different substances are liberated from different organs (choline or acetylcholine, epinephrine, serotonin and potassium ions, for example)<sup>8</sup> and that the various shock organs are stimulated by these diverse substances. There is also evidence that a proteolytic enzyme is activated or set free during anaphylactic and anaphylactoid shock,

possibly by disturbance of the balance between the enzyme and a normal enzyme inhibitor. The significance of this is not clear.

In summary, anaphylaxis appears to be the consequence of an antigen-antibody reaction occurring on or in living cells. The cells are damaged and liberate a pharmacologically active substance having many of the properties of histamine. This substance is then transported, if necessary, to histamine-sensitive cells and tissues in which it induces a characteristic response.

**The Arthus Reaction.** Hypersensitivity in the rabbit was first observed by Arthus (1903) and consisted of a local rather than a general reaction.<sup>2</sup> Rabbits which received *subcutaneous* injections of horse serum at appropriate intervals showed no untoward response to early injections, but later injections produced local infiltration which developed into necrosis and abscess formation at the site of inoculation. This reaction became known as the Arthus phenomenon. Injections are now commonly made by the *intracutaneous* route rather than *subcutaneously* because the response is more striking and more easily observed.

Macroscopic evidence of reaction may not be apparent for several hours, but microscopic changes begin within one hour. Stetson reported marked but transient leukopenia within fifteen minutes after intradermal injection of antigen; white blood cells and platelets adhered to the endothelium of small blood vessels at the site of injection, where they formed thrombi, possibly aided by a layer of fibrin on the vessel walls.<sup>42</sup> Gerlach found that within an hour swelling of connective tissue fibrils and compression of blood vessels occurred at the site of *subcutaneous* injection, accompanied by immobilization of leukocytes and massive edema.<sup>18</sup> Twenty-four hours after injection there was histologic evidence of necrosis of the arterioles and resulting hemorrhage, together with local tissue degeneration, inflammation and edema.

The intensity of the Arthus reaction in actively sensitized rabbits varies with the concentration of antibody in the serum. Tissue culture experiments have indicated that body cells of the hypersensitive animal are apparently sensitized by antibodies in their environment and are not intrinsically reactive. The Arthus phenomenon is not limited to skin reactions; inflammation and necrosis have been induced in the lung, heart, kidney, peritoneum, testis, brain and joints.

*Reversed Arthus reactions* are produced in rabbits by injecting protein intravenously and homologous antiserum by the *intracutaneous* route either immediately or at any time within the next three or four days. The intensity of local reaction at the site of antiserum injection diminishes as the interval between injections increases. Reversed Arthus reactions may not be as severe as direct reactions, often resulting only in severe local edema.

*Passive Arthus sensitivity* can be induced in normal rabbits by injection

of serum from other rabbits sensitized or immunized against foreign proteins. When the antiserum is injected intracutaneously and is followed one-half hour later by antigen intravenously, the reaction occurs at the site of antiserum injection. Antigen administered intracutaneously into the site which originally received antiserum produces a reaction in that location. Antiserum injected intraperitoneally or intravenously sensitizes the entire animal skin so that subsequent intracutaneous injection of antigen elicits a reaction at the site of antigen injection.

The amount of antibody necessary to produce Arthus sensitivity in the rabbit has been determined by quantitative methods. Culbertson elicited mild reactions in rabbits containing 0.5 milligram antiovalbumin protein per milliliter of serum; 0.75 to 1.0 milligram antiovalbumin per milliliter was required for deep necrosis.<sup>11</sup> Fischel and Kabat studied passive Arthus sensitization in the rabbit and found that minimal reactions were obtained when 0.15 milligram of antiovalbumin was injected intracutaneously, followed one-half hour later by ovalbumin.<sup>16</sup> Maximal reactions were produced with 1.4 milligrams of antibody protein. Benacerraf and Kabat reported that approximately the same amount of antibody was required to sensitize guinea pigs passively.<sup>8</sup>

It will be noted that no latent or incubation period is needed for passive Arthus sensitization, whereas such an interval is often necessary for passive anaphylactic sensitization. Both types of reaction may be demonstrated in the same animal some hours after antiserum injection. Furthermore, passive Arthus sensitization requires several times as much antibody as fatal anaphylactic sensitization.

Arthus reactions can be elicited in other animals than the rabbit and guinea pig. The phenomenon has also been produced in the horse, monkey and man. Reactions in man occasionally occur during the course of repeated serum inoculations.

The basic cause of the Arthus phenomenon is an antigen-antibody reaction occurring locally, but the chain of events leading to the detectable response is not known. Raffel stated that when sufficient concentrations of precipitating antibody and homologous antigen react in a tissue containing blood vessels, the resulting precipitate *per se* seems to damage the blood vessel walls.<sup>88</sup> He did not postulate the liberation of histamine or its participation in subsequent events. Chase, on the other hand, implied the possibility of a histamine mechanism.<sup>8</sup> Rabbit white blood cells or platelets are rich in histamine and apparently liberate considerable quantities into the plasma in anaphylactic shock. As noted previously, these formed blood elements accumulate at the site of injection in the early stages of the Arthus reaction. The interaction of antigen and antibody in their presence may cause the release of histamine and the consequent local changes.

**Evanescent Allergic Inflammation.** Evanescent cutaneous reactions

may be produced in certain hypersensitive animals and humans when the appropriate test antigens are injected intracutaneously. Some investigators believe that such reactions are mild Arthus responses, but this point is not certain. Gerlach concluded from histologic observations that the tissue alterations are similar to the Arthus reaction in the rabbit and differ from it only in degree.<sup>18</sup>

An actively sensitized guinea pig reacts to intracutaneous injection of antigen within five to fifteen minutes. The test area swells and develops erythema and edema of varying intensity, increasing to a maximum within thirty minutes to a few hours, after which the reaction fades and disappears in two to four hours. Further sensitizing injections in the guinea pig cause a higher degree of hypersensitivity in which the reaction is less transient. Eventually a state of Arthus sensitivity may be attained. The evanescent reaction is therefore apparently associated with a lower concentration of antibody and a lesser degree of sensitization than the Arthus phenomenon.

Under certain conditions evanescent reactivity can be passively transferred by serum from previously immunized or sensitized rabbits or guinea pigs to normal guinea pigs. Chase considered that these passively transferred reactions may often be feeble Arthus reactions, but not always.<sup>8</sup> Sometimes the relationship between antibody and tissue appears to be more intimate; moreover, the latent period following serum injection, the immediate circumscribed reaction to antigen and the rapid fading indicate an anaphylactic type of reaction which occurs within the skin rather than systemically.

Immediate cutaneous reactions in man are more pronounced than in lower animals. They can be elicited in such allergic conditions as hay fever and sensitivity to foreign serums and foodstuffs. The marked responsiveness of man is probably associated with a form of antibody which is retained in the skin for some time, a high degree of cutaneous reactivity to histamine and possession of a well developed superficial lymphatic system.

The reaction in man is known as the "triple response" or "wheal and flare." Lewis attributed this response to a histamine-like compound ("H-substance") and perhaps other materials liberated from the site of cellular injury caused by antigen-antibody reaction or by direct injury.<sup>24</sup> Three successive phases follow diffusion of "H-substance" from the site of injury: (1) erythema caused by local dilatation of capillaries, (2) a spreading flare or flush resulting from widespread arteriolar dilatation produced by a local nerve reflex, and (3) formation of a wheal or area of circumscribed edema attributed to increased permeability of the endothelium of small blood vessels. The same sequence of events is produced by intracutaneous injection of histamine into the human. The triple response starts almost immediately after injection of histamine into a

normal individual or of antigen into a sensitive individual, reaches a maximum and usually begins to fade within fifteen to sixty minutes. The skin may soon regain its normal appearance.

**"Spontaneous" Hypersensitivities.** "Spontaneous" hypersensitivities include many of the disease conditions popularly known as allergies, in the more restricted sense. Various manifestations of allergy have been known since ancient Greek and Roman times. Galen, a second century Roman physician, applied the term *idiopathy* to abnormal conditions in which one individual reacts differently from most of his fellows to certain agents in his environment (e.g., "rose fever"). In 1831 Elliotson, in London, sought the cause of the seasonal rhinitis now known as hay fever in the pollens of grasses; and in 1837 Blackley, a physician of Manchester, England, who suffered from seasonal attacks of hay fever and asthma, reported tests performed on himself with about one hundred pollens. He was apparently the first to test the skin and mucous membranes with the suspected causes of allergic disorders. He also correlated his findings with the seasonal incidence of the pollens to which he was sensitive by daily counts of pollen grains on slides exposed to the air. His work was confirmed by Dunbar in 1903,<sup>15</sup> and interest in hay fever increased.

Allergens, the agents which incite allergic reactions, do not all possess obvious antigenicity. The body areas affected are sometimes known as *shock organs* or *shock tissues*. Different allergens commonly encounter different organs or tissues in the course of normal exposure, so a variety of clinical forms of allergy are known. Included among the spontaneous allergies are hay fever, asthma, urticaria, angioedema and probably infantile eczema. Serum allergy may occur in individuals "spontaneously" sensitized to foreign animal proteins such as horse danders, but also frequently develops as a result of obvious active sensitization with foreign serum. In addition, there are a number of other allergic conditions in which the reaction is delayed and some allergic disorders which do not fit clearly into either category. Delayed and intermediate hypersensitivities will be discussed separately.

*Hay Fever* is characterized by watery exudation from the mucous membranes of the upper respiratory tract and conjunctivae, the natural consequences of which include violent and often protracted sneezing, nasal discharge and lacrimation. The allergen is some agent in the surrounding atmosphere which is either inhaled or comes into direct contact with exposed mucous membranes; plant pollens are most commonly incriminated, but house dust, mold spores and various other substances may also produce the symptoms of hay fever.

*Asthma* is a condition of recurrent or paroxysmal difficulty in breathing, with a wheezing or whistling respiration produced by obstruction of the smaller bronchioles. It is either allergic or nonallergic. The nonallergic

type, sometimes called "intrinsic asthma," is produced by unknown factors within the subject. Allergic asthma may be caused by the same inhaled substances as hay fever, together with certain drugs and biologicals inhaled by laboratory workers. Various ingested foods may also incite asthmatic attacks, among them eggs, wheat and milk.

*Urticaria* or hives is a skin disorder characterized by the appearance of crops of intensely itching wheals or welts with raised, often white centers surrounded by an area of erythema. They are usually distributed widely over the body surface and tend to disappear in one or two days. Allergic urticaria is generally caused by ingestion of the allergen or more rarely by inhalation or contact. Common food allergens include strawberries, citrus fruits, fish and shellfish, eggs, tomatoes and chocolate.

*Angioedema* is similar to urticaria, but the lesions are larger and more restricted in distribution. They often occur about the head and neck.

*Infantile Eczema* consists of reddened vesicular or blister-like lesions of the face and neck and the skin inside the elbow and knee joints. The lesions ooze and crust over. The usual allergens are thought to be foods, particularly eggs. There is some question whether infantile eczema is actually an allergy of the immediate type.

*Serum Allergy* occurs in individuals with a high "natural" sensitivity to foreign animal proteins or in individuals who have previously received foreign serum injections. "Horse asthmatics" are unusually apt to display allergic reactions when injected with horse serum. These persons are sensitive to horse danders and other proteins, probably as a result of hereditary predisposition combined with previous close contact. Even a small amount of horse serum may provoke an immediate violent reaction.

*Serum Sickness* is closely related to serum allergy, although the relationship is not immediately apparent because the reaction is usually delayed eight to twelve days after serum injection. Serum sickness is characterized by general swelling of lymph nodes, and is accompanied by an itching, urticarial or erythematous eruption and often edema of the eyelids, face and ankles. In severe cases fever and pain in the joints are also noted. The average duration of symptoms is two days, but they may persist as long as two weeks. The occurrence of serum sickness depends upon co-existence within the body of foreign serum proteins and antibodies produced against these proteins. The interval of eight to twelve days before symptoms appear provides opportunity for antibodies to be manufactured. These then react with the persisting foreign proteins to produce the symptoms described. As the concentration of antibody increases, the reaction becomes more violent. Continued production of antibody hastens disappearance of the foreign protein, whereupon the reaction subsides. A second injection of the same foreign serum after a considerable interval, when antibody is still present but at a low level, provokes a similar

sequence of events three to five days after the injection. This is the "accelerated" reaction, reminiscent of the "recall" phenomenon in immunized animals reinjected with antigen after a rest period. Rejection of an individual who is in a *high state of sensitivity* elicits an "immediate" reaction consisting of local response within a few minutes and also generalized reactions within a few minutes to twelve hours. Such reactions, occurring almost exclusively in horse asthmatics, are often severe and sometimes fatal. Death may occur in a few minutes or after as much as twenty-four hours. This is a typical anaphylactic reaction.

The antibodies which participate in serum allergy include both precipitins and reagins (see below). Anaphylactic sensitivity has been transferred from highly sensitive humans to guinea pigs. Sensitivity to foreign serum proteins can be detected in the human by an intracutaneous skin test with diluted foreign serum. A positive reaction, noted after ten to sixty minutes, consists of a local wheal and erythema. Cutaneous sensitivity is a danger signal and indicates the strong probability of a serious reaction if the foreign serum is administered to the patient.

**Role of Antibodies in Allergies of the Immediate Type.** Three types of antibodies have been found in individuals with allergies of the immediate type. Precipitating antibodies are present in some humans and animals. A "skin sensitizing" antibody, *reagin* (not to be confused with syphilitic reagin), occurs particularly in humans with certain allergic sensitivities. Reagins do not precipitate homologous allergens under any conditions yet found. The third type of antibody, which has been termed "blocking," "inhibiting" or "neutralizing" antibody, is found under certain circumstances and can be detected only by *in vivo* tests.

*Precipitating Antibodies* are largely responsible for anaphylactic and Arthus reactions and may also be concerned in serum allergy and certain food sensitivities.

*Reagins* are a special class of antibodies which have particular affinity for human skin and appear to be responsible for evanescent cutaneous reactions. Serums containing reagins only do not precipitate with the corresponding allergens. Reagins can be transferred passively to human skin and to the nasal, ophthalmic and intestinal mucous membranes. Human reagins can rarely produce passive anaphylactic or skin sensitivity in guinea pigs.

Passive transfer of reagins was first demonstrated by Prausnitz and Küstner in 1921, when they injected serum from Küstner, who was sensitive to fish, into the skin of Prausnitz.<sup>32</sup> Injection of the same site with a protein fish extract a few hours later elicited a prompt and evanescent "wheal and erythema" reaction. The *Prausnitz-Küstner* test is employed in the diagnosis of human allergies; it is useful in cases in which the patient might react violently to a test allergen, although at present the fear of virus transmission by passively transferred serum is reducing use

of the test. The so-called P-K test is performed by injecting a small amount of patient's serum into the skin of a normal person and following it twenty-four to forty-eight hours later with the allergen in question introduced into the same area and also into an untreated control area. A positive reaction appears in ten to fifteen minutes, and the control should show no change. Positive, although weaker, reactions can be secured when reagin and allergen are injected together into normal human skin.

Reagins possess several properties which distinguish them from precipitating antibodies. They are thermolabile, being destroyed at 56° C. in one-half to eight hours or at 60° C. in one-half to one hour. They do not seem to pass the placental barrier, so cutaneous sensitivity of this type is not congenitally acquired. Finally, their affinity for cutaneous tissues is marked. Passive skin sensitization may persist for at least forty-five days.

Reagins are found in humans with hay fever and asthma caused by pollens and animal danders, in some kinds of eczema and in some food sensitivities. They do not occur in urticaria or angioedema. They are often produced after injection of horse serum and in 40 per cent of individuals who receive "booster" injections of diphtheria toxoid. They may be present even though no clinical disease is apparent; for example, positive skin tests are sometimes obtained with foods which the patient can eat without allergic symptoms. This curious situation may reflect differing sensitivities of the various shock tissues (e.g., skin vs. intestinal mucosa).

"Spontaneous" allergic disorders in which reagins are demonstrable tend to follow family lines. The role of heredity appeared so striking that Coca subdivided allergies on this basis. Those with marked familial incidence he called "atopic" and others "nonatopic." Heredity does not determine the particular kind of sensitivity displayed, but rather a predisposition toward hypersensitization of some sort. Hereditary factors may possibly influence the nature of the shock tissue and/or the capacity to produce reagin, but in general the particular sensitivity developed is determined by the individual's experience. About 10 per cent of humans possess "spontaneous" allergies of one kind or another.

"Blocking Antibodies" were discovered by Cooke *et al.*<sup>9</sup> and Loveless<sup>25</sup> in the serums of allergic individuals undergoing desensitization by repeated injections of minute amounts of their allergens. Clinical improvement was not correlated with a decrease of circulating reagins. Instead, the injections caused the formation of a thermostable antibody which did not sensitize the skin. Normal individuals similarly injected produced the same thermostable antibody but no reagin.<sup>10, 26</sup>

The thermostable antibody readily and specifically combines with the allergen and "neutralizes" it so that the mixture does not produce a skin

test in a sensitive subject. It will be recalled that reagin does not "neutralize" its allergen and that both materials injected together may produce a detectable skin reaction. The thermostable antibody passes the placenta. It resembles precipitating antibody by leaving a site of cutaneous injection readily (within a few hours). However, it fails to precipitate *in vitro* with the corresponding allergen and can therefore be demonstrated only *in vivo*.

**Hay Fever.** Seasonal hay fever is caused principally by pollens. Pollens of trees are usually responsible in the spring; in the summer, the grasses such as timothy, rye and June grass are involved; ragweed and various other weeds are common sources of the offending pollens in the fall. Diagnosis is often based on the seasonal incidence of the disease, and the specific incitant is determined by correlation of symptoms with pollination periods of plants in the locality.

Plant pollen contains at least two allergenic agents: a water-soluble component is the chief cause of hay fever; the other substance is oily or insoluble and produces pollen dermatitis. The water-soluble material is easily extracted in alkaline solution and is used in skin tests for sensitivity. Small superficial scratches made with a sharp instrument are covered with minute amounts of the test liquids. If the reagent is in powdered form, the powder is mixed in a drop of tenth normal sodium hydroxide over the scratch. Reactions appear in ten to thirty minutes. Skin tests, although not always reliable, are useful to confirm a diagnosis based on seasonal incidence of the disease.

Specific prophylaxis sometimes attempted consists of desensitization (more correctly called "hyposensitization") before the pollen season starts. The patient receives a series of gradually increasing, very minute doses of an extract of the pollen to which he is sensitive. Injections are timed to culminate just before the beginning of the pollen season.

Local reactions to the injections are common. Systemic or constitutional symptoms occur more frequently in pollen therapy than in any other kind of allergic treatment and usually appear within one to twenty minutes. General reactions are controlled by application of a tourniquet above the site of injection to reduce entrance of allergen into the circulation and by administration of adrenalin or ephedrin.

The results of prophylactic treatment are variable. It has been reported that complete or partial relief may be expected in about 85 per cent of patients. Permanent and complete remission may never be obtained, the patients returning year after year for preseasonal injections. In some cases, treatment for two or three seasons leads to remission and in others treatment for five to ten seasons accomplishes this result.

So-called perennial hay fever occurs throughout the year and bears a clinical resemblance to seasonal hay fever. It is caused by a variety of inhaled or ingested materials such as animal danders, vegetable powders

(orris root, kapok), house dust, foods (milk, eggs, shellfish) and drugs (quinine, aspirin, iodine). In a considerable percentage of cases the cause cannot be ascertained.

Specific treatment includes avoidance of the allergen if possible and attempted desensitization against allergens which cannot be eliminated. Desensitization must be resorted to in cases of allergy to dust.

**Allergic Asthma.** Allergic asthma may be seasonal or perennial and is incited by substances which are inhaled or ingested. It is caused by many of the same allergens as hay fever, but the shock organs are located in the bronchi instead of the nasal mucous membranes.

Determination of the specific cause of allergic asthma follows the same pattern as that of hay fever. The history of the patient, including other allergies in himself or his family, may be significant. Skin tests are sometimes performed in an attempt to confirm deductions from the history and physical examination. These tests are positive with 50 to 65 per cent of asthmatic patients. Sensitivities to more than one allergen are common. Negative results are useful because they help to exclude an allergic cause of disease. Another diagnostic procedure, which is applicable to many other hypersensitivities, consists of deliberate elimination followed by diagnostic trial. If, for example, a patient has symptoms while at home but not when taken into the hospital for examination and treatment, it is probable that the causative agent is environmental. He may then be exposed to house dust, animal danders and other possible allergens to determine whether, under controlled conditions, they induce attacks of asthma. It is possible in like manner to ascertain whether some particular food in the customary diet is at fault.

Treatment of allergic asthma is similar to that of hay fever. Avoidance of the allergen is always the most desirable method when feasible. Some materials, such as certain foods or drugs, can usually be avoided without too much difficulty. House dust and pollens, on the other hand, are practically inescapable. In these cases specific desensitization may be attempted as already described, with about the same anticipated effectiveness. For temporary relief of severe symptoms physicians often employ adrenalin, epinephrine or ephedrine.

**Food Allergies.** *Food Sensitivities* are often the cause of eczema, urticaria (hives) and angioedema, in addition to the hay fever and asthmatic reactions already mentioned. In a very severe case, an extremely small amount of the allergenic food taken into the mouth may cause immediate edema and swelling of the lips and tongue so that the patient has difficulty in swallowing. Ordinary urticarial and eczematous reactions are caused by foods which pass through the intestinal wall and are carried by the blood stream to the skin, which is the shock organ. Asthma may be induced by foods which reach the bronchioles, either *via* the

blood stream following ingestion or by inhalation (e.g., wheat flour in a bakery).

*Gastrointestinal Allergy* is food allergy in which vomiting, cramps and diarrhea follow ingestion of the allergen. The symptoms result from strong contractions of the pylorus and intestinal muscles and from marked edema of the intestinal mucosa. Intestinal reactions may be attributed to unusual sensitivity of this organ or to concentrated exposure to the allergen.

Skin tests are sometimes positive in food allergies but are frequently negative. The Prausnitz-Küstner reaction may be successfully used to determine the allergen responsible for cases of exquisite sensitivity in which direct skin tests might be dangerous. In any event, skin tests provide chiefly contributory evidence regarding the inciting agent, because many patients give positive reactions with foods to which they are not clinically sensitive or fail to react with foods to which they are clinically sensitive. Therapeutic trial is therefore the most accurate diagnostic procedure. The subject is first allowed a very simple, nonallergenic diet, and then gradually one or more of the suspected foods is added at intervals until typical reactions are obtained.

Avoidance of the responsible food is the most desirable method of treatment. Occasionally this is almost impossible, as in the case of an infant reactive to milk, eggs or wheat. In these cases desensitization is sometimes attempted, either parenterally or orally; in the latter method very small amounts of the food are given repeatedly in pills or capsules until a tolerance is developed. The recently introduced hormones, ACTH (pituitary adrenocorticotropic hormone) and cortisone (compound E of the adrenal cortex) have been found partially or completely to relieve symptoms of allergic asthma, hay fever and eczema, and the gastrointestinal allergies.

#### *"Delayed" Hypersensitivities*

"Delayed" hypersensitivities are characterized by the slowness with which symptoms become apparent after exposure of a sensitive individual to the inciting allergen, several days usually being required. Moreover, circulating antibodies are not customarily demonstrable, and passive transfer of hypersensitivity by means of serum is impossible. However, in many instances sensitivity has been transferred by use of cells (leukocytes or plasma cells) from the sensitive individual. There are two principal clinical types of "delayed" hypersensitivity: allergy of infection and contact allergy.

**Allergy of Infection.** The classic and probably most thoroughly studied example of infection allergy is that involving the tuberculosis organism or some of its products. Even though many other kinds of

bacteria, as well as viruses, fungi and various animal parasites, induce allergic states, the phenomenon is often designated the *tuberculin type* of allergy.

About sixty years ago Koch demonstrated that guinea pigs infected two or more weeks previously with *Mycobacterium tuberculosis* possess an unusual reactivity toward this organism.<sup>22</sup> Subcutaneous reinjection of tuberculosis organisms into such animals in an attempt to induce superinfection produces, at the site of second injection, massive inflammation which appears after several hours and progresses in intensity for two or three days. The area becomes walled off and eventually necrotic, and sloughing occurs. This is known as the *Koch phenomenon*. Normal guinea pigs, similarly injected, develop progressive tuberculosis.

The specific inflammatory change upon reinjection can be brought about by dead as well as living cells; furthermore, tuberculin (a bacteria-free extract prepared by heating and concentrating a broth culture of the organisms) also produces a similar reaction. Larger doses of tuberculin injected subcutaneously into infected animals are rapidly absorbed and induce a delayed severe or even lethal shock, the "systemic reaction." Two types of lesion are seen at autopsy: (1) *local* hemorrhage and edema at the site of injection, the regional lymph nodes being enlarged and discolored; and (2) *focal* inflammation of the previously existing tuberculous lesions, with tremendously dilated capillaries and large numbers of leukocytes. Toxemia and death of the animals is apparently associated with development of the focal lesions. Tuberculin in the customary doses produces no untoward reactions in normal guinea pigs.

These observations antedated the early scientific work on anaphylaxis and were not understood until about 1903, when von Pirquet suggested that the reaction to tuberculin is another example of sensitization. This hypothesis led him in 1907 to propose the cutaneous tuberculin test which has since been widely used in the detection of individuals having tuberculous disease.<sup>48</sup>

Tuberculin injected into the skin of a tuberculous animal causes no apparent reaction for a few hours. Local inflammation gradually appears and increases in intensity. Edema and a firmly indurated swelling develop, and the reaction progresses to a maximum at between fifteen and forty-eight hours. The inflammation slowly fades, often with sloughing, but discoloration may be apparent for several weeks. A similar reaction occurs in man. If the dose of tuberculin is large or if the degree of sensitivity is great, the lesion becomes necrotic.

The delayed response of the tuberculous animal to injection of *M. tuberculosis* or tuberculin does not represent an actual latent period in which the host tissues are unaffected. Microscopic study shows an immediate cellular reaction, which does not become macroscopically evident until a few hours later. The epithelial layer of the skin is the primary

site of injury, and polymorphonuclear cells accumulate quickly. By six hours after the injection, however, mononuclear cells appear and they ultimately predominate.

A normal animal injected with tuberculosis organisms develops local inflammation and hypersensitivity beginning within a period of anywhere from a very few days to several weeks, depending upon the number and virulence of the bacteria and upon various host factors. Coincident with and indicative of acquisition of hypersensitivity, the animal will respond positively to a tuberculin test.

The clinical signs of reaction to tuberculin depend in part upon the route by which the tuberculoproteins are introduced into the sensitive individual or animal. The reactions which have been described are elicited by dermal or intracutaneous inoculation. When 0.1 milligram of tuberculin is injected subcutaneously or by some other route permitting fairly rapid absorption into the blood stream, a general or systemic reaction is evoked in a hypersensitive individual. The systemic response consists of malaise, fever, headache, pains in the joints and back, loss of appetite and prostration. It is of interest that the same symptoms are characteristic of serum sickness.

In tuberculous infection, hypersensitivity is established early, before signs of disease are apparent. The relationship between hypersensitivity and clinical symptoms of tuberculosis is not clear, however; this subject has been debated for many years.

The tuberculin type of reaction in a sensitive animal is elicited only by protein or certain intermediate products of protein degradation; apparently it is not evoked by carbohydrates or lipids. However, injection of the isolated tuberculoproteins into a normal animal does not stimulate development of tuberculin type sensitivity; rather, it may induce anaphylactic and Arthus sensitivity, as is true of most proteins. Until recently it was believed that only intact, whole bacteria, either alive or dead, are capable of establishing the tuberculin type of sensitivity. Raffel reported that a lipopolysaccharide-containing wax from tuberculosis organisms, in conjunction with tuberculoprotein, induced typical delayed tuberculin hypersensitivity.<sup>33, 34, 35</sup> Injection of the same lipid wax together with egg albumin caused the development of delayed sensitivity to egg albumin. Raffel therefore suggested that the biologic activity of the lipid may be general.

Despite failure to transfer tuberculin sensitivity by means of serum, the participation of antibody is indicated by (1) the specificity of the reaction, (2) the rapid and marked increase in sensitivity following reinfection at a considerable interval after primary infection (specific anamnestic reaction), (3) specific reduction of sensitivity after a single large dose of tuberculin has produced severe constitutional reactions, and (4) the adjuvant effect of paraffin oil upon the development of tuberculin

type hypersensitivity. The tuberculin antibody may be intimately associated with certain cells or tissues. This conclusion is supported by passive transfer of tuberculin sensitivity from hypersensitive guinea pigs to normal animals by means of living leukocytes from peritoneal exudates, lymph nodes, spleen or blood.<sup>7</sup> Such passive hypersensitivity is of short duration but possesses the essential features of actively acquired sensitivity. Furthermore, blood and tissue cells from tuberculous animals, repeatedly washed with Locke solution and suspended in plasma, lose their mobility and die quickly when tuberculin is added; cells from normal animals, on the contrary, survive similar treatment.<sup>37</sup>

Hypersensitivity has been demonstrated in a number of bacterial and nonbacterial infections. Sensitivity of the tuberculin type is produced in animals or man by group A hemolytic streptococci, viridans streptococci, *Salmonella typhosa* (tested with *typhoidin*), pneumococci, *Pfeifferella mallei* (*mallein* test), Brucella species (*brucellergin*, a nucleoprotein extract, and *brucellin*, a culture filtrate), *Pasteurella tularensis*, and *Mycobacterium leprae* (*lepromin*). Delayed tuberculin type reactions can be elicited in patients by preparations derived from certain fungi. Three diseases in which such tests have been employed are coccidioidomycosis (*coccidioidin*), histoplasmosis (*histoplasmin*), and trichophytosis (*trichophytin*).

One of the first discovered hypersensitivity reactions induced by an infectious agent was the response of immunized humans to revaccination against smallpox. Jenner observed in 1798 that individuals previously immunized with cowpox vaccine and later inoculated with the same material or with smallpox virus developed a small local redness which appeared within forty-eight to seventy-two hours and faded quickly. Hooker later showed that heated virus also elicited a similar delayed reaction.<sup>19</sup> Skin tests have been applied in two other virus infections, mumps and lymphogranuloma inguinale (*Frei* test).

Diagnostic skin reactions of the *immediate* type are used in certain parasite infestations: schistosomiasis, filariasis, trichiniasis, echinococcosis disease and ascaris infestation. In the protozoan disease leishmaniasis, a tuberculin type skin reaction is elicited by an appropriate extract of the organisms.

In most of the foregoing illustrations in which the response is of the tuberculin type, the reaction is less striking than in tuberculosis itself, which has been cited by Boyd as "one of the more extreme examples of bacterial allergy."<sup>4</sup>

The significance of a positive skin test for hypersensitivity to a microbial cause of disease varies with the particular infection and must be determined separately for each disease. It does not in all instances indicate that the host is currently infected with the organism in question. The tuberculin test, for example, is often positive in an individual who

no longer has active tuberculous lesions. In fact, at one time nearly all persons reaching the age of twenty gave a positive reaction, although obviously they were not all tuberculous at the time. X-ray and other examinations revealed that all had previously been infected and that the lesions had healed but had left the specific sensitivity to tuberculin. In very young children a positive tuberculin test is indicative of active infection. A negative test is significant in older persons because it eliminates the possibility of active tuberculosis, either past or present, except when the patient is in the terminal stages of the disease; such patients may give a negative test, perhaps as a result of desensitization by the great amount of tuberculoprotein in the body.

The manner in which delayed or tuberculin type hypersensitivity is induced is not known with certainty. Infection or injection of whole bacteria usually, although not always, stimulates this type of sensitivity. Microbial extracts, particularly those containing polysaccharide bacterial components, give rise to anaphylactic sensitivity. Delayed type sensitivities are often related to bacterial proteins. This is not an invariable rule, however. Ghase considers it more reasonable to assume that the responsible microorganism is carried into a site which becomes a special environment as the host cells mobilize; the type of sensitivity which develops depends upon the way in which the antigenic material is dealt with, this being in some manner different from the process characteristic of the usual antibody-producing sites.<sup>8</sup>

There is evidence supporting the hypothesis that the type of sensitivity produced depends upon the site into which antigen is introduced and the local cellular response. Injections into the skin are most successful in inducing sensitivities of the delayed type. It has already been noted that normal guinea pigs develop anaphylactic hypersensitivity ten days or more after receiving a single subcutaneous injection of egg albumin. When a guinea pig is rendered tuberculous and egg albumin is injected into its focus of infection, it develops both delayed and anaphylactic sensitivity to egg albumin.<sup>13</sup> The tuberculous lesion apparently plays some role in determining the kind of sensitivity produced. Similar results have been obtained with other proteins introduced into tuberculous lesions. Moreover, injections of proteins together with dead tuberculosis organisms and an adjuvant induces delayed type hypersensitivity against the proteins. The effectiveness of intracutaneous injections in stimulating delayed sensitivity seems to indicate some mechanism involving a local or focal cellular reaction, perhaps located in the regional lymph nodes. It has been proposed that the antibodies produced are of a special sort having affinity not only for the specific antigen which stimulated their formation but also for tissue cells and that their affinity for tissues prevents their general distribution and detection in the blood.

**Contact Dermatitis.** Contact dermatitis, including certain forms of

*drug allergy*, results solely from contact of the skin with the incitant. Symptoms appear after some delay and consist of inflammatory rashes or eczematic eruptions, often vesicular.

The causative agents are many and varied. They include metals like nickel and mercury; relatively simple compounds such as formaldehyde, picric acid, 2,4-dinitrochlorobenzene and para-phenylene-diamine (often used in partially oxidized form in dyeing furs); orris root, cosmetics and insect powders; salvarsan; novocaine; various dyes; and fat-soluble plant extractives such as urushiol, the active principle of poison ivy, and substances from poison sumac, poison oak and other plants.

Many if not all of these materials are nonantigenic in themselves. For many years, therefore, it was believed that contact dermatitis was separate and distinct from those hypersensitivities in which the allergens are complex, protein-containing substances. An explanation of the sensitizing powers of these simple allergens was provided by the work of Landsteiner (page 31 ff.) and others showing that the specificity of proteins may be altered by coupling them with low molecular weight substances such as arsanilic acid. It is now generally agreed that simple compounds can combine, either directly or through some intermediate reactions, with various body proteins, which thereby acquire antigenicity for the same animal. It is obvious that their specificity as proteins is somewhat changed by the new radical which has entered into their composition. Antibodies (and perhaps reagins) produced in response to such "derivative antigens" may react specifically with the added radicals, which behave as haptens.

Although unsensitized individuals may be exposed without ill effects to large quantities of the specific materials, after sensitization has been established even very minute amounts induce the characteristic response. Heredity, frequency and mode of contact and the chemical properties of the sensitizing agent are important factors determining the probability of sensitization.

In both the human and the guinea pig, dermal sensitivity of the delayed type (contact dermatitis) is established in five to twenty days by repeated cutaneous or intracutaneous applications of the allergenic chemical. The visible dermatitis reaction is elicited by cutaneous application of the allergen. This type of sensitivity cannot be transferred passively by serum but may be transferred by means of washed living white blood cells, of which the lymphocytes seem to be most important.<sup>28</sup>

The same active sensitizing procedure produces two different parallel sensitivities: the anaphylactic type and the contact dermatitis type. Certain substances produce chiefly anaphylactic sensitivity; others cause both types and still others principally delayed sensitivity. Anaphylactic sensitivity can be shown in guinea pigs by intravenous injection of a protein conjugate of the allergen, by passive anaphylactic sensitization

with serum from a sensitive animal, and by elicitation of the Prausnitz-Kustner effect using serum from a sensitive animal to produce a local area of sensitization in which an immediate reaction can be produced by a protein conjugate of the allergen.

Diagnosis of contact dermatitis is aided by the usual occurrence of eruptions on exposed surfaces of the body (i.e., not protected by clothing), because direct contact is the only way in which reaction is induced. The presence of vesicles and the absence of a personal or family history of allergy strengthen the diagnosis. Contact dermatitis is more common among men than women and children, inasmuch as many of the responsible agents are used in industry or various trades: paints, lacquers and metallic substances, for example.

The specific cause of a given case may be indicated by the patient's history. The "patch" test is also used in an attempt to identify the incitant. The test is performed by placing upon the skin a small piece (one square centimeter or less) of linen or blotting paper soaked in a solution of the test reagent or coated with an ointment containing it. This is covered with waterproofed cellophane to prevent drying and is held in position with adhesive tape. The patch is removed after twenty-four hours, and the area is observed daily for as long as two weeks because reactions sometimes develop slowly. A positive test consists of an area of erythema in which vesicles of various size may be present. The most significant reaction is one which closely resembles the patient's original lesion. In a negative test there is no change at the site of the patch.

Specific treatment depends upon knowledge of the causative agent. Avoidance is always recommended but is sometimes impractical because this form of allergy is so frequently occupational in nature. In such a case, change of occupation may be the only alternative. Desensitization usually has chance of success only if the excitant is oily in character: for example, plant or pollen oils. Considerable work with poison ivy desensitization has yielded only indeterminate results; avoidance is still the best method of prevention.

**Physical Allergy.** Reactions to physical agents such as heat and cold and to certain radiations have been called allergies because their clinical symptoms are so similar to those of some of the accepted allergies and because reagins have been demonstrated in patients' serums. No one can say with assurance exactly how physical allergy is induced, although it can be supposed that these stimuli act on certain body substances, producing materials which react with the hypersensitivity apparatus.

In one form of physical allergy immediate urticarial symptoms appear at the site of contact or exposure to cold, light or other stimuli. The other form consists of a reflex-like reaction in which symptoms appear in other parts of the body than that exposed. This phenomenon is illustrated by the asthma or coryza with which certain individuals respond

to a sudden local or general change in temperature, sneezing and a watery nasal discharge sometimes follow chilling of the feet or some other part of the body.

#### ALLERGY AND IMMUNITY

There is no general agreement about the relationship between allergy (in the narrow sense) and immunity. Schick considered allergy to be the basis of immunity and immunity to be the beneficial effect of a preceding allergic state.<sup>40</sup> Similarly, Dienes and Mallory stated that the tuberculin type of sensitivity represents the first stage of every immune response to injected protein.<sup>14</sup>

It has been seen that microbial causes of disease may incite one or both of two responses in their hosts: (1) the formation of antibodies, some of which may be protective in one way or another, and (2) the development of hypersensitivity or allergy to some cellular constituents. The hypersensitivity in turn may be of the immediate type or of the delayed type.

Both types of hypersensitivity can be engendered by infection with or injection of pneumococci. An immediate skin response is elicited by the capsular polysaccharide of this organism, although this is not the allergic reaction characteristically associated with pneumococci or other infectious agents. Pneumococcal nucleoprotein incites a reaction of the delayed type. However, protection against pneumococci is not afforded by antiprotein antibodies, so it appears that the relationship between allergy and immunity is not clear in the case of this organism.

A hypersensitive state can be induced in experimental animals by injections of a purified protein from *M. tuberculosis* together with a nonantigenic lipoidal substance, but such animals are without resistance to subsequent infection by the organisms. Moreover, animals which have been immunized with B.C.G. possess both resistance to infection and sensitivity to tuberculin, but the sensitivity can be abolished without destroying the immunity. Therefore, in tuberculosis also there seems to be no correlation between sensitivity and immunity.

Obviously, however, immunity and allergy possess numerous points of similarity. Both are induced by antigenic substances or by compounds which may combine with and modify proteins, both states develop only after an incubation period, and both display the same kind of specificity. These facts and other evidence previously mentioned indicate the participation of antibodies of some kind. The differences between immunity and allergy may therefore be determined by differences in the site and manner of formation of these antibodies, the nature of the antibodies and their behavior and place of action, or any combination of these factors. Actually, these various factors may be interdependent; for

example, the delayed or tuberculin type of sensitivity, as already pointed out, may be associated with a peculiar sort of antibody produced in special loci within the body and not necessarily possessing demonstrable activity in recovery from infection. In tuberculosis, the tuberculin reaction is independent of the presence in the serum of antibodies reactive in agglutination, complement fixation and phagocytosis. The role of circulating antibodies in immunity against or recovery from this disease is not known.

Burnet noted that allergy of infection, as evidenced by skin reactivity, is particularly typical of diseases with subacute or chronic lesions, such as brucellosis and mumps.<sup>5</sup> Inflammatory cells which accumulate at the site of contact with the sensitizing agent include macrophages, endothelial cells, primitive mesenchymal cells, lymphocytes and plasma cells, some of which have a possible role in formation of circulating antibody. Burnet considered that "all the evidence is compatible with the view that the sensitizing antibody is produced within this inflammatory cell complex." The nature of the antibody formed is determined partly by any preliminary modification which the inciting agent may undergo and partly by the kinds of cells available to produce antibody. Finally, he suggested that this type of antibody response to antigenic stimulus may represent a primitive process which, when further refined and adapted, evolves into the more highly specialized process of producing circulating antibody, as carried out in lymph nodes and spleen.

At the moment it is impossible to say whether immunity and allergy are part and parcel of the same phenomenon. However, it would not be particularly surprising to find, eventually, that these various responses of the body to foreign substances represent merely different "trials and errors" in the slow process of evolution. Certainly they will continue to provide investigators with fundamental as well as practical problems for many years.

### References

1. Alexander and Bolton, 1940. *Jour. Immunol.* **39**, 457.
2. Arthus and Breton, 1903. *Compt. rend. Soc. de Biol.* **55**, 1478.
3. Benacerraf and Kabat, 1950. *Jour. Immunol.* **64**, 1.
4. Boyd, 1947. *Fundamentals of Immunology*, 2nd ed., Interscience Publishers, Inc., New York.
5. Burnet and Fenner, 1949. *The Production of Antibodies*, Macmillan & Co., Ltd., Melbourne.
6. Campbell and Nicoll, 1940. Cited by Chase, 1952.
7. Chase, 1945. *Proc. Soc. Exp. Biol.* **59**, 134.
8. Chase, 1952. In Dubos, *Bacterial and Mycotic Infections of Man*, 2nd ed., J. B. Lippincott Co., Philadelphia.
9. Cooke, Barnard, Hebard and Stull, 1935. *Jour. Exp. Med.* **62**, 733.
10. Cooke, Loveless and Stull, 1937. *Jour. Exp. Med.* **66**, 689.

11. Culbertson, 1935. Jour. Immunol. 29, 29.
12. Dale, 1913. Jour. Pharmacol. & Exp. Therap. 4, 167.
13. Dienes, 1929. Jour. Immunol. 17, 531.
14. Dienes and Mallory, 1932. Amer. Jour. Path. 8, 689.
15. Dunbar, 1903. Dtsch. med. Wschr. 29, 149.
16. Fischel and Kabat, 1947. Jour. Immunol. 55, 337.
17. Follensby and Hooker, 1944. Jour. Immunol. 49, 353.
18. Gerlach, 1923. Virchows Arch. f. path. Anat. 247, 294.
19. Hooker, 1929. Jour. Infect. Dis. 45, 255.
20. Kabat and Boldt, 1944. Jour. Immunol. 48, 181.
21. Kabat and Landow, 1942. Jour. Immunol. 44, 69.
22. Koch, 1891. Dtsch. med. Wschr. 17, 101, 1189.
23. Landsteiner and Chase, 1942. Proc. Soc. Exp. Biol. 49, 688.
24. Lewis, 1927. *The Blood Vessels of the Human Skin and Their Responses*, Shaw & Sons, Ltd., London.
25. Loveless, 1940. Jour. Immunol. 38, 25.
26. Loveless, 1942. Jour. Immunol. 44, 1.
27. Magendie, 1839. Cited by Morgenroth, 1906. *Collected Studies in Immunity*, John Wiley & Sons, New York.
28. Manwaring, 1910. Ztschr. f. Immunitätsforsch. 8, 1.
29. Manwaring, Hosepian, Enright and Porter, 1925. Jour. Immunol. 10, 567.
30. Otto, 1906. In Von Leuthold, *Gedenkschrift I*, 153.
31. Portier and Richet, 1902. Compt. rend. Soc. de Biol. 54, 170.
32. Prausnitz and Küstner, 1921. Zentralbl. f. Bakter. IO. 86, 160.
33. Raffel, 1946. Amer. Rev. Tuberc. 54, 564.
34. Raffel, 1948. Jour. Infect. Dis. 82, 267.
35. Raffel, 1950. Experientia 6, 410.
36. Raffel, 1953. *Immunity, Hypersensitivity, Serology*, Appleton-Century-Crofts, New York.
37. Rich and Lewis, 1932. Bull. Johns Hopkins Hosp. 50, 115.
38. Rose and Browne, 1941. Jour. Immunol. 41, 409.
39. Rosenau and Anderson, 1907. U. S. P. H. S. Hyg. Lab. Bull. 36.
40. Schick, 1947. Jour. Mt. Sinai Hosp. 14, 585.
41. Schultz, 1910. Jour. Pharmacol & Exp. Therap. 1, 549.
42. Stetson, 1951. Jour. Exp. Med. 94, 347.
43. Von Pirquet, 1907. Berl. klin. Wschr. 44, 644, 699; *Klinische Studien über Vakzination und vakzinale Allergie*, Deutike, Leipzig u. Wien.

## APPENDIX

### EXPERIMENTS IN SEROLOGY

**I. Introduction.** The first requirement for success in serologic work is the ability to follow instructions and exercise good technique. The student should become familiar with each experiment before he begins work and should then concentrate on the required manipulations. With adequate practice and constant attention to details nearly everyone can acquire sufficient facility to work rapidly and accurately and obtain proper results.

The aims of laboratory experiments in serology are twofold: first, to provide familiarity with various technical procedures, and secondly, to illustrate fundamental principles. The student must develop skill in injecting and bleeding laboratory animals and handling pipettes and other serologic equipment. He should learn how to set up and read the various tests and should understand the basic principles of the reactions and factors which affect their outcome. He should also learn the importance and significance of proper controls.

Serologic work requires clean equipment, although strictly aseptic technique is not always necessary. However, the materials are often dangerous because of their infectious nature and must be handled carefully and disposed of properly to avoid serious accident.

The procedures outlined in the following sections are intended as experiments or demonstrations for teaching purposes and are not to be considered "standard methods." In many particulars they are subject to considerable variation, according to the preference of the instructor and his experience with the materials available. He will usually find that preparation for each experiment requires one or more "trial runs" to establish a suitable choice of reagents or range of concentrations which can be expected to yield the desired result. No attempt is made to indicate all the possible experimental variations.

The methods suggested in the first few sections are described in considerable detail; later, when the student should have mastered most of the fundamental techniques, directions are given in briefer form.

**II. Preparation of Immunizing Materials.** *1. Bacterins.* Bacterins are suspensions of bacteria used for stimulating the production of antibodies in humans and animals for prophylactic or therapeutic purposes, or for the production of antiserums. A suitably prepared bacterin contains a definite number of bacterial cells (e.g., 500,000,000 or 1,000,-000,000 per ml.) and is usually sterile. Preparation of a bacterin includes the following steps: (a) mass cultivation of the bacteria, (b) suspension in saline and dilution to the requisite concentration, and (c) sterilization and preservation.

a. Inoculate two to five slants of a suitable agar medium heavily with the organism to be employed and incubate under proper conditions to secure heavy growth. *Salmonella typhosa*, for example, grows well on nutrient or tryptose agar in 12 to 18 hours at 32° to 37° C.

b. Add approximately 2 ml. of saline (0.85 per cent NaCl) to each slant and gently loosen the bacterial growth with a sterile loop. Gram stain the suspensions, pool those which appear pure in a sterile test tube containing glass beads, and shake well to break up clumps.

c. Several methods are available for counting the bacteria in a suspension. The Wright method is widely used.

(1) Prepare a capillary pipette by drawing an 8 inch piece of 7 mm. glass tubing and breaking the capillary so that the constricted portion is about 3 inches long. Mark the pipette approximately 1 inch from its tip.

(2) Using a rubber bulb, draw 4 per cent sodium citrate solution to the mark and let in a bubble of air.

(3) Take up blood from a finger prick to the mark and let in another bubble of air.

(4) Draw up the bacterial suspension to the mark.

(5) Expel the contents of the pipette onto a clean microscope slide and mix by alternately aspirating into the pipette and expelling five or six times.

(6) Deposit a drop on one end of each of two or three microscopic slides. Using another slide as a spreader, prepare thin films as illustrated in Figure 50. Smears covering the entire slide are desirable. Hold the spreader at a fairly acute angle, draw it from position 1 to position 2, and after the blood-bacteria mixture has spread by capillarity push it rapidly to position 3 and remove. Thicker smears are produced when the spreader is held at a less acute angle.

(7) Stain the dried smears by the Wright procedure. Flood with Wright's stain and after one minute add an equal amount of distilled water. When a greenish metallic scum or sheen appears on the surface (usually 2 to 3 minutes), wash with running water until the smear appears pink.

(8) Count the bacteria and red blood corpuscles separately in several oil immersion fields until about 200 corpuscles have been counted. As-

suming that normal blood contains 5,000,000,000 R.B.C. per ml., calculate by simple proportion the number of bacteria per ml. of the stock suspension.

d. Calculate the amount of stock suspension required to prepare 30 ml. of bacterin containing one billion cells per ml. as follows:

$$\text{ml. stock to use} = \frac{30 \times 1,000,000,000}{\text{bacteria/ml. in stock suspension}}$$

e. Pipette the required amount of stock suspension into a sterile vaccine bottle (e.g., Army Medical School type), add sufficient preservative to yield the desired final concentration, and make up to 30 ml. with saline. Cap the container with a sterile vaccine bottle stopper. Merthiolate (1:10,000) is a good general sterilizing and preserving agent, although

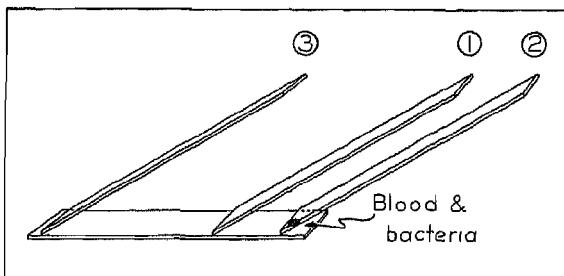


FIGURE 50. Preparation of blood-bacteria smear. Start with spreader slide at 1, draw it to position 2 and let drop of blood and bacteria spread, then push spreader quickly to 3 and remove

formalin (0.5 per cent) is preferred for certain purposes. Some bacterins are sterilized by heating in a waterbath for 30 minutes at a temperature between 55° and 65° C.

f. Test the bacterin for sterility. Heated preparations may be tested immediately, chemically disinfected bacterins after one or more days. Using a sterile syringe and needle withdraw a sample of the bacterin and inoculate suitable broth and agar media. If no growth appears in 4 days the preparation is considered ready for use.

g. Label the bottle showing the name of the organism, the name of the person who prepared the bacterin, and the date. Store in the refrigerator when not in use.

*2. Erythrocytes.* A 50 per cent suspension of washed red blood cells is satisfactory for production of antiserum in experimental animals.

a. Draw blood of the desired species aseptically. Defibrinate it by shaking with glass beads or use one of the following to prevent clotting: sodium citrate (3 mg. per ml. of blood), potassium oxalate (2 mg. per ml.), or heparin (0.1 mg. per ml.). Blood which must be stored may be preserved in the refrigerator for as long as 10 weeks with an equal volume of Alsever's solution;

Glucose	2.05%
Sodium citrate	0.80%
Sodium chloride	0.42%
Citric acid	0.055%

b. To prepare cells for injection, centrifuge the desired quantity of blood at moderate speed, remove the supernatant liquid by aspiration with a capillary pipette attached to a water pump, and wash the cells three times by suspending in 0.9 per cent NaCl, centrifuging and discarding the supernatant liquid. After the final centrifugation suspend the cells in an equal volume of 0.9 per cent NaCl. (Saline containing 0.9 per cent NaCl, rather than 0.85 per cent NaCl, is often used with red blood cells because it is more nearly isotonic with the cell contents.)

3. *Protein Solutions.* Solutions containing about 1 per cent protein are satisfactory for inoculation into laboratory animals. Serum diluted 1:7 with saline contains about this concentration of protein. Dissolve egg albumin and other dried proteins by adding a little saline and mixing with a glass rod to make a thick paste; dilute the paste gradually. Stirring is preferable to shaking, which creates a troublesome foam. Remove undissolved particles by centrifugation or by filtration through paper. Add a preservative if desired.

4. *Tissue Extracts.* Guinea pig tissue is used in the production of Forssman antiserums. Trim fat from the kidneys, wash them several times in saline to remove free blood, and weigh. Cut the tissue finely with scissors and grind in a mortar with sterile sand. Add saline to give a 20 per cent suspension and centrifuge. Transfer the supernatant fluid to a sterile vaccine bottle, add Merthiolate to a final concentration of 1:10,000, and stopper. Store the extract in the refrigerator.

5. *Use of Adjuvants.* Aluminum hydroxide and paraffin oil are sometimes incorporated into materials for animal inoculation. They reduce the number of injections required and often increase the antibody response.

A. ALUM PRECIPITATION. Prepare 50 ml. of the bacterin or other immunizing agent and its preservative (if any) in double strength. Add 2.33 ml. of 10 per cent AlCl<sub>3</sub> and sufficient 20 per cent NaOH to bring the reaction to pH 7. Dilute to 100 ml. with saline.

B. OIL-IN-WATER EMULSION. Prepare the immunizing material and preservative in double strength. Sterilize good quality light paraffin oil and Arlacel A (Atlas Powder Co., Wilmington, Delaware) by autoclaving. Mix 8.5 volumes of the paraffin oil with 1.5 volumes of Arlacel A, using a Waring Blender if available, or aspirating it repeatedly into a syringe without needle. Add 10 volumes of antigen and mix as before. Test the emulsion by allowing a drop to fall from an applicator stick onto the surface of water. If the drop remains perfectly formed and does not spread over the surface, the emulsion is ready for use.

An additional adjuvant effect is sometimes provided by incorporation of killed acid-fast bacteria. Dried *Mycobacterium tuberculosis* or *M. butyricum* which have been killed by heat (70° C. for 30 minutes) are added to the paraffin oil at the rate of 10 mg. per 100 ml. and thoroughly mixed by grinding in a mortar. Arlacel A and antigen are then added as described above.

**III. Inoculation and Bleeding of Animals.** Laboratory animals are usually as cooperative as humans if treated properly. There should be no confusion or unnecessary noise in the operating room. Animals should be handled firmly but gently, and the student should know beforehand what he is going to do so that he will not have to fumble around unnecessarily and irritate the animal.

Observe animals closely during the period of experimentation for signs of an unfavorable reaction to the inoculated material. Such signs include loss of weight resulting from loss of appetite, listlessness and apathy, and fever. If an animal which is under immunization shows such signs, suspend injections until its condition improves, or decrease the doses.

Table 73. Sizes of Hypodermic Needles for Various Purposes

PURPOSE	GAUGE	LENGTH
Intravenous injection of rabbits	25-27	1/2 in.
Subcutaneous injections	23-26	3/4-1 in.
Intraperitoneal injections	20-24	1 in.
Bleeding rabbits from the heart	18	1 1/2-2 in.
Bleeding guinea pigs from the heart	22	1-1 1/2 in.

Syringes of 1 ml. (tuberculin), 2 ml., 5 ml., 10 ml., 20 ml., and 50 ml. capacity are required. In addition, adapters for connecting hypodermic needles to rubber tubing are useful. The size of needle varies with the intended use (see Table 73).

Sterilize syringes and needles by autoclaving or by boiling for 20 minutes. It is advisable to keep on hand a reserve supply of sterile needles. They may be slipped into pieces of 6 or 7 mm. glass tubing slightly longer than the needles, placed point down in plugged test tubes, and sterilized in the autoclave. Check needles for sharpness before sterilization. Examine them with a hand lens, and test by drawing them between the fingers. Animals respond very poorly to barbed and dull needles. Needles can be sharpened, if not in too bad condition, with a very fine sharpening stone, or they may be "touched up" by rubbing on a flat glass surface.

Before filling a syringe from a vaccine bottle disinfect the stopper (e.g., with 70 per cent alcohol). Retract the plunger of the syringe to the same volume as the vaccine to be withdrawn, insert the needle

through the thin central portion of the stopper, and force air from the syringe into the bottle. Invert bottle and syringe, and with the bottle uppermost take slightly more than the desired quantity of vaccine into the syringe. Return entrapped air bubbles to the bottle and bring the plunger to the required volume.

After each use a syringe and needle must be thoroughly cleaned. If it is undesirable (because of the virulence of the inoculum) to separate the barrel and plunger immediately, withdraw the plunger almost completely from the barrel to prevent sticking. Boil syringes and needles contaminated with infectious material before cleaning. All equipment used with blood must be thoroughly rinsed with water immediately—otherwise it will have to be soaked for some time in a detergent to loosen and remove clotted blood.

1. *Rabbits.* Young adult rabbits (2 to 4 kg.) are usually preferred for production of antiserums. Inoculations may be intravenous, intra-peritoneal or subcutaneous; the intravenous route is commonly employed.

A variety of schedules is used for routine production of antibacterial serums in rabbits. One which gives satisfactory results with killed suspensions of gram negative rod bacteria consists of the following intravenous injections:

1st day: 0.1 ml.  
4th day: 0.3 ml.  
8th day: 0.5 ml.  
11th day: 1.0 ml.  
15th day: 2.0 ml.

Serum of high titer is usually secured 3 to 5 days after the last injection.

Closely spaced intravenous injections of living bacteria are satisfactory with many kinds of organisms. Stock saline suspensions having the turbidity of an agglutination test antigen (see page 308) are employed. Fresh stock suspensions are prepared as indicated below and refrigerated when not in use. The injection schedule is as follows:

1st day: 0.1 ml. fresh stock suspension diluted 1:100  
2nd day: 0.2 ml. suspension diluted 1:100  
3rd day: 0.3 ml. suspension diluted 1:100  
4th day: 0.1 ml. fresh stock suspension undiluted  
5th day: 0.2 ml. suspension undiluted  
6th day: 0.3 ml. suspension undiluted  
11th day: 0.5 ml. fresh stock suspension undiluted  
16th day: trial titration; if titer is satisfactory, bleed from the heart;  
otherwise inject 0.5 ml. fresh undiluted stock suspension and titrate after an additional five days.,

Prepare anti-erythrocyte serums in rabbits by a series of twice weekly

intravenous injections of 1 ml. of 50 per cent suspension of washed red blood cells. Three or four days after the fourth injection titrate the animal's serum, and if the titer is not satisfactory give additional inoculations.

Inject 1 per cent protein solutions intravenously once or twice a week; the number of injections is determined by the results of trial titrations of the animal's serum. After the first two weeks, make injections following a rest period of a week or more by the intraperitoneal route to reduce the likelihood of anaphylactic shock. Intravenous injections usually consist of 1 ml. amounts; intraperitoneal doses may be 2 to 3 ml.

One or two 0.5 ml. subcutaneous injections of antigens emulsified in paraffin oil usually suffice to produce antiserums of high titer. Three or four weeks after the first injection perform a trial titration, and if the antibody titer is not as high as desired give a second injection.

A. INTRAVENOUS INJECTION. The marginal vein of the rabbit ear is readily accessible. It is located at the outer edge of the dorsal side of the ear. Reserve one ear for injections, the other for bleeding. The first injection of a series is always made as near the tip of the ear as possible, succeeding injections being made closer toward the animal's head, so that scar tissue will not prevent injected material from entering the circulation.

Hold the animal on the lap facing the operator. Shave the skin over the vein with a sharp razor blade and rub vigorously with 70 per cent alcohol. A right-handed person usually finds it more convenient to inject the animal's left ear. Hold the ear with the left hand so that the middle finger supports the area to be injected, the little finger holding the tip of the ear down out of the way. Insert the inoculating needle, bevel up, in the direction of blood flow (toward the heart) through the skin and into the vein at a very acute angle (almost parallel) to the vein so that it does not pass completely through. Inject the inoculum slowly. When the vein is entered correctly the inoculum can be seen passing toward the heart as it partially replaces the blood. If the needle is not within the vein, the antigen will produce a blanched, raised area in the neighboring tissue. Gentle massage should be used to force the material out of the needle puncture, and injection should be attempted at another site. After the inoculation is completed, firmly apply cotton moistened with alcohol and withdraw the needle. After a few moments remove the cotton. If bleeding occurs, replace the cotton and hold for several minutes.

B. SUBCUTANEOUS INJECTION. The back is a convenient site for subcutaneous inoculation. Clip and shave the hair on one side of the back and disinfect the area with alcohol. Pinch up a fold of skin between the thumb and forefinger, insert the inoculating needle into the ridge between the finger and thumb for about one-half inch, and release the skin. Inject the inoculum slowly; a raised bleb shows that the injection was properly made. Wash the area again with alcohol.

C. INTRAPERITONEAL INJECTION. Have an assistant hold the animal firmly, head down. Clip the hair in the median abdominal line and disinfect with alcohol. Pinch up a fold of skin and peritoneum between the thumb and forefinger, insert the needle into the ridge of skin and through the fold of peritoneum, release the peritoneum and skin and make the injection. There should be no bleb of injected fluid. Wash the area with alcohol.

D. BLEEDING FROM THE EAR VEIN. Small amounts of blood are easily secured from the marginal vein of the ear, and with patience even as much as 50 ml. may be taken. Usually the vein is punctured and the blood allowed to flow, although a syringe and needle are sometimes employed. The first bleeding of a series is made from a site near the base of the ear, succeeding punctures being made more and more distal from the head.

Hold the animal on the lap facing away from the operator. Rub the ear vigorously to promote strong circulation of blood, and if necessary place a drop of xylol on the tip of the ear to produce mild inflammation. If xylol is used, it must be removed before the animal is returned to its cage, lest prolonged inflammation cause permanent damage and destroy the future usefulness of the ear. Xylol is removed by washing at least three times with liberal amounts of alcohol, wiping each time with cotton.

Shave the area to be punctured and wash with alcohol. Rub petrolatum over both sides of the ear to prevent blood from sticking to the fur. Place an artery clamp (Dieffenbach) over the vein proximal to the site to be punctured. Hold the ear so that the middle finger of the left hand supports the site of puncture, and make a short quick jab with a sharp instrument; this may be a piece of broken microscope slide or capillary pipette, or a metal lancet. Some operators make a small snip with scissors or a scalpel or razor blade. Collect the desired amount of blood in a centrifuge tube, then move the artery clamp distal to the puncture and hold dry absorbent cotton firmly over the puncture until bleeding stops.

Allow the tube of blood to stand at room temperature until clotting occurs. Then loosen the clot from the wall of the tube with a fine glass rod. The tube may be centrifuged immediately to throw down the clot or refrigerated overnight to allow the clot to retract and express the clear, straw colored serum. Remove the serum carefully with a pipette. A rubber tube with mouthpiece or a rubber bulb on the pipette permits close observation while the serum is withdrawn.

E. BLEEDING FROM THE HEART. Large quantities of blood are secured from the heart or from the jugular vein. Cardiac bleeding is usually simpler and can be repeatedly performed on the same animal if proper technique is used. Blood is drawn into a large syringe, or the needle may be attached by rubber tubing to a bottle fitted with a suction tube. If an

assistant is available, anesthesia is not necessary, although it is usually helpful. Ether is often used, but great care must be exercised because rabbits are easily killed by an overdose. Nembutal or another barbiturate is frequently preferred. The dose by intravenous injection is about 1 ml. of a 4 per cent solution of Nembutal per kg. of body weight, or the solution may be injected until the animal no longer gives an eye reflex. An animal anesthetized with Nembutal remains asleep for about two hours.

Clip the area over the sternum and disinfect with alcohol and tincture of iodine. Locate the area of maximum pulsation, usually about midway of the sternum and slightly to the left, and insert the needle between two ribs, advancing it in a straight line toward the right shoulder. When the heart is entered, blood will appear in the syringe or tubing connected to the needle, and may be slowly aspirated. Fifty milliliters may safely be removed from an average rabbit; 80 to 120 ml. can usually be secured when it is not intended to save the animal. If difficulty is experienced in finding the heart, withdraw the needle almost completely and reinsert in another direction. Twisting the needle within the pericardial cavity is likely to tear the heart and cause immediate death.

After the blood has clotted, loosen the clot from the wall of the container with a glass rod and refrigerate for 24 to 48 hours. The clot usually retracts sufficiently to permit removal of clear serum by pipette. The last serum withdrawn may contain red blood cells and require centrifugation.

Serum is stored in glass bottles in the refrigerator and can be preserved with Merthiolate (1:10,000) or phenol (0.5 per cent). A stock 1 per cent solution of Merthiolate is prepared by adding 1 gm. of the dry powder to 100 ml. of 1.4 per cent borax. Add this solution at the rate of 1 ml. per 100 ml. of serum. Phenol dissolved in ether (equal parts by weight) is used in the proportion of 0.9 ml. per 100 ml. serum.

*2. Guinea Pigs.* Subcutaneous and intraperitoneal inoculations of guinea pigs are most commonly used. The procedures are very similar to those employed with rabbits.

A single operator can bleed guinea pigs from the heart if they are anesthetized. Ether is satisfactory. Hold an ether cone over the animal's nose until it stops kicking. Lay the animal on its back with the cone partially over its head, disinfect the area over the heart, insert the needle between two ribs at the point of maximum pulsation and advance it slowly, exerting slight negative pressure with the plunger of the syringe. As soon as the heart is entered, blood will appear in the syringe. Average guinea pigs yield about 5 ml. of blood without ill effects. If subjected to ether for only a few minutes they recover quickly.

*3. Mice.* Intraperitoneal injections are usually employed with mice and can be done without assistance. Hold the mouse by its tail with the

right hand and let it try to run away. Grasp it firmly between the ears with the thumb and forefinger of the left hand and turn it over. Use the little finger of the left hand to hold the tail down and keep the body taut. Disinfect the abdomen with 70 per cent alcohol. Hold the animal's head down so that the intestines fall forward, and make the injection in the posterior region of the abdomen. A quick jab of the needle is usually followed by sudden contraction and relaxation of the abdominal wall, which indicates that the peritoneum has been entered.

**IV. Agglutination.** Agglutination tests are performed with several kinds of antigens. Methods for preparing test antigens and setting up tests will be described.

*1. Tube Agglutination.* Tube agglutination tests are performed by mixing a constant amount of antigen suspension with increasing dilutions of antiserum, incubating, and reading the degree of clumping in each tube.

A. PREPARATION OF TEST ANTIGENS. For most purposes a bacterial culture is grown on a slant of suitable agar medium inoculated heavily in a zig-zag manner with a loop or slightly bent needle. After incubation the organisms are washed off with saline and diluted to a turbidity which can best be determined by trial. The suspension should be sufficiently dense so that when added to serum in the actual test a faint but definite turbidity is evident. At a distance of six or eight feet from a north window the strips of wood separating the panes are just visible through the suspension contained in an ordinary culture tube.

Test antigens for flagellar agglutination are either living or killed with formalin (0.5 per cent). Prepare somatic antigens of motile bacteria by heating heavy suspensions at 100° C. for 30 minutes before diluting with saline to the proper turbidity. Suspend nonmotile bacteria in saline containing 0.5 per cent formalin. Living or formalized (0.5 per cent) organisms are used for Vi agglutination.

B. SETTING UP THE TEST. Arrange in a serologic rack enough agglutination tubes ( $11 \times 100$  mm.) to exceed the expected titer of the serum and add one tube for a control. Pipette saline into each tube, 0.9 ml. into the first tube and 0.5 ml. into each of the others. Carefully measure 0.1 ml. of antiserum into the first tube with a 1 ml. serologic pipette (graduated to the tip). Mix the contents by aspirating most of it into the pipette and blowing out three to five times. This tube contains a 1:10 dilution of the serum. With the same pipette, transfer 0.5 ml. to the second tube, mix as before, and continue transfers in like manner to and including the next to the last tube. Discard 0.5 ml. from this tube. Each tube contains one-half the concentration of antiserum in the tube preceding (i.e., the dilution series is as follows: 1:10, 1:20, 1:40 . . .). Reserve the last tube as a control. Carefully measure 0.1 ml. of test antigen with a gradu-

ated pipette into each tube including the control. Shake the rack thoroughly and incubate.

It is frequently necessary to prepare more than one identical series of dilutions of the same antiserum. This is most conveniently done, and with greatest accuracy, by preparing all series of dilutions simultaneously. If only four or five such series are required, "master dilutions" can be prepared in the front row of agglutination tubes and pipetted into the other rows ("internal master dilutions"). When more rows of dilutions are needed, "external master dilutions" are prepared in larger test tubes. The use of master dilutions can best be illustrated by a diagram (Figure

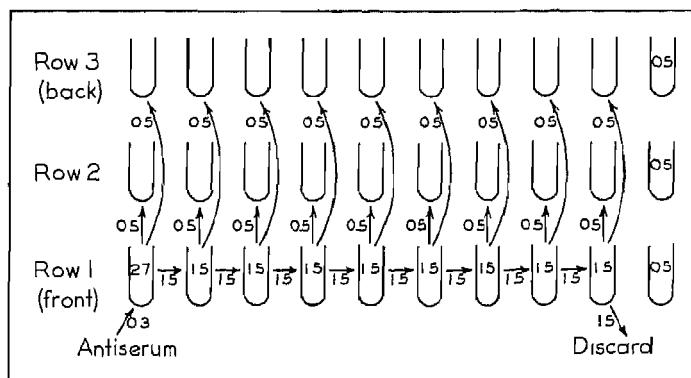


FIGURE 51. Preparation of triplicate simultaneous serial dilutions of antiserum using "internal" master dilutions. Figures within tubes indicate the amount of saline (ml.) initially added. Figures accompanying arrows represent the volumes (ml.) transferred. (Dilution series, 1:10, 1:20, 1:40 . . . 1:2,560, Control.)

51). Dispense saline into tubes in the front row and into the last tube of each row as indicated by the figures within the various tubes. Add antiserum to the first tube in the front row and mix. Pipette 0.5 ml. quantities into the two tubes behind it, and 1.5 ml. into the second tube of the front row. Continue mixing and transfers through the next to last tubes, and discard 1.5 ml. as indicated. Then add appropriate antigens to all tubes in each row.

C. INCUBATION. There is not complete uniformity with regard to incubation time and temperature for various kinds of agglutination. However, it is suggested that flagellar agglutination tests be incubated 2 hours at 37° C., read, reincubated for 12 to 18 hours at 55° C., and read again. Take the highest reading as the flagellar agglutinin titer of the serum. Incubate somatic agglutination tests 12 to 18 hours at 55° C. Tests for Vi agglutinin are incubated 2 hours at 37° C., read, and kept at room or refrigerator temperature overnight before final reading. In

this case also the highest value obtained is considered the titer of the serum.

D. READING AGGLUTINATION TESTS. A good light source (for example, a gooseneck lamp) and a dark background are desirable for reading agglutination tests. Examine each tube first without shaking; then shake or flip to suspend the sediment. A 4+ (++++) reaction is one in which all cells are clumped at the bottom of the tube and usually resuspend as distinct granules, flakes or flocculent masses in an otherwise clear fluid. In a negative (0 or -) reaction there is no clumping except normal sedimentation of individual cells, so shaking the tube causes little if any change in turbidity. Intermediate degrees of agglutination are graded 3+, 2+ and 1+. Somatic agglutination usually produces fine granules, whereas flagellar agglutination usually appears in the form of flocculent masses, but all gradations between these extremes may be observed. Considerable practice is necessary to develop facility and confidence in reading tests.

2. *Microscopic Slide Agglutination.* The formation of clumps of agglutinated bacteria can be observed directly with the microscope when antiserum diluted sufficiently is mixed with homologous bacteria. Employ a broth culture or saline suspension of a motile organism such as *S. typhosa*. Dilute the corresponding antiserum to about  $\frac{1}{10}$  or  $\frac{1}{20}$  of its titer as determined by tube agglutination (for example, a serum having a tube titer of 10,240 or 20,480 can be used in a dilution of 1:1000). Mix on a cover glass one small (2 mm.) loopful each of the bacteria and the antiserum dilution and prepare a hanging drop, sealing it with Vaseline. Observe with the high dry objective of the microscope for one hour or longer and note the gradual cessation of motility and formation of loose clumps. It is desirable also to prepare a control slide consisting of bacteria and saline, and compare the two preparations.

3. *Macroscopic Slide Agglutination.* The identification of unknown bacteria, particularly members of the Enterobacteriaceae, is often hastened by macroscopic slide agglutination. Antiseraums diluted only slightly (e.g., 1:5 or 1:10, according to their titers) are employed and are mixed with very heavy suspensions of the bacteria. Suspend the growth from a heavily inoculated agar slant evenly in about 2 ml. of saline. Mix one small drop (e.g., from a capillary pipette) of diluted antiserum with an equal amount of bacterial suspension within a paraffin or wax pencil ring on a microscope slide or other glass surface, and rock and tilt the slide for one to five minutes. Agglutination is shown as fine or coarse granulation. Always include a control consisting of saline and bacterial suspension for each organism tested.

This procedure is adaptable to large scale operation. Several bacteria can be tested against one or more antiseraums simultaneously. Plates of window glass can be employed, and a simple device for making rings with melted paraffin can be fashioned from a piece of wire bent into a ring.

about three-quarters of an inch in diameter, with a handle several inches long. The wire ring should be wrapped with string. In use, it is dipped into the melted paraffin and then touched momentarily to the glass plate, where the paraffin cools and hardens almost immediately. After the required number of horizontal and vertical rows of rings have been prepared, each bacterial antigen (for example) is placed in the rings of a horizontal row, and each antiserum in the rings of a vertical row. A piece of glass 4½ inches by 6 inches is large enough to test six organisms in each of eight antiserums.

**V. Hemagglutination: Blood Grouping.** It is customary to determine the blood group and Rh type of persons who are to receive blood transfusions and of prospective donors. Test serums should be secured from a reliable laboratory.

*1. Determination of ABO Group.* Slide agglutination is considered less delicate than test tube methods and is more likely to show rouleaux formation, but it is commonly used because it is more rapid and convenient and less equipment is required.

Collect a large drop of blood from the finger in 1 ml. of 0.9 per cent NaCl in a small test tube. Divide a microscope slide in half with a marking pencil. Place a drop of anti-A serum in the left half of the slide, and a drop of anti-B serum in the right half. Add a drop of the blood cell suspension to each half of the slide but separate from the serum. Mix serum and cells with a wooden applicator or toothpick, using a fresh stick for each serum. Rock and tilt the slide to hasten agglutination; reaction will usually be apparent within 30 seconds to 2 minutes. Occasionally the reaction in anti-A will be slow because cells of subgroup A<sub>2</sub> react less strongly, so the slide should be kept 20 minutes before being discarded. Microscopic examination helps to decide some doubtful cases and to distinguish agglutination from rouleaux formation. The blood group of the cells is determined from reactions in the two serums as follows:

AGGLUTINATION OF R.B.C. IN		
ANTI-A	ANTI-B	CELLS OF GROUP
-	-	O
+	-	A
-	+	B
+	+	AB

*2. Determination of Rh Type.* Most Rh typing serums in use today require fresh whole blood or oxalated blood rather than saline suspensions of erythrocytes because they contain albumin agglutinins, which will not react properly in the absence of protein or certain other substances. The directions supplied with the testing serum must be followed carefully. One procedure used to determine the Rh<sub>0</sub>(D) type of red blood cells will be described.

Warm a microscope slide on a substage lamp and collect on it a large drop of blood directly from the finger. Add a drop of anti-Rh<sub>0</sub> (anti-D) serum and mix with an applicator stick, spreading the mixture over an area about  $\frac{3}{4} \times 1\frac{1}{2}$  inches. Keep the slide warm ( $37^{\circ}$  to  $45^{\circ}$  C.) and tilt it back and forth. Look for clumping detectable with the naked eye. Positive reactions begin to appear in 30 seconds and should be complete in 2 minutes. Do not examine slides with the microscope or after drying.

Additional serums are required to determine the other Rh types.

*3. Cross Match (Direct Compatibility) Tests.* Cross match tests are used directly before transfusion to determine that the blood of the donor is compatible with that of the recipient; otherwise transfusion reactions might occur even though the two bloods are of the same group because of other intragroup factors. The test with recipient's serum and donor's cells is most important, but many laboratories also include a test with patient's cells and donor's serum.

Slide tests are commonly performed. Secure specimens of venous blood from the recipient and from the prospective donor, allow them to clot and remove the serums. Also prepare R.B.C. suspensions by collecting a drop of blood from each individual in 1 ml. of saline. Mix one drop of recipient's serum and one drop of donor's cell suspension on the left half of a slide as in blood group determination, and mix donor's serum and recipient's cells on the right half of the slide. Cover the slide with half a Petri dish to retard drying, and read after 15 and 30 minutes. If the cells at the left agglutinate, the prospective donor is unsuitable for the patient in question, because his erythrocytes would be promptly agglutinated by antibodies in the circulation of the recipient. The cells at the right will not agglutinate if the two blood specimens are perfectly compatible.

Tube tests of compatibility may be more reliable than slide tests. A complete test includes mixtures containing 2 drops each of (a) recipient's serum and donor's cells and (b) donor's serum and recipient's cells, together with control tubes containing each serum and its own cells. The cells are often suspended in 20 to 30 per cent bovine albumin to detect albumin agglutinins. Centrifuge at moderate speed for 3 minutes, shake to resuspend the cells, and read. There should be no agglutination in any tube if the donor's blood is compatible with that of the recipient.

**VI. Adsorption of Agglutinins.** Adsorption of agglutinins is used to compare the antigenic structures of two or more bacteria. A laboratory demonstration of the principles of adsorption can be performed with a mixture of antisera against unrelated bacteria (e.g., *S. typhosa* and *Escherichia coli*), which is adsorbed with each organism separately, followed by titration of residual antibodies for each organism. A more practical demonstration consists of the reciprocal adsorption of antisera for two related bacteria, such as *S. typhosa* (antigenic formula: 9,12:d;---)

and *S. pullorum* (antigenic formula: 9,12---:---). A suggested procedure for this experiment will be described.

1. *Preparation of Adsorbing Antigens.* Inoculate four thick (30 to 40 ml.) tryptose agar plates heavily all over the surface with *S. typhosa*, and four plates with *S. pullorum*. Employ cotton swabs for this purpose, and secure the inoculum by removing the entire growth from one slant culture for each plate. Incubate the plates at 37° C. for 24 to 48 hours. Add about 2 ml. of saline to each plate and gently loosen the growth with a bent glass rod. Use a capillary pipette with rubber bulb to pool the thick suspensions in centrifuge tubes so that there are two tubes for each organism. Centrifuge at high speed to pack the cells. Remove and discard the supernatant fluid, using a capillary pipette.

2. *Adsorption.* Prepare 4 ml. each of 1:20 dilutions of antityphoid and antipullorum serums. Add 2 ml. of diluted antipullorum serum to one tube of *S. typhosa* cells, and 2 ml. of diluted antityphoid serum to one tube of *S. pullorum* cells. Suspend the cells in the serums by means of a wire loop, and incubate the tubes in a waterbath at 37° C. for 30 minutes to 1 hour. Centrifuge the two tubes at high speed. Carefully remove the antipullorum serum with a capillary pipette and transfer it to the other tube of packed *S. typhosa* cells. Similarly transfer the antityphoid serum to the other tube of *S. pullorum*. Resuspend the cells as before, incubate at 37° C. for 30 minutes to 1 hour, and centrifuge. Remove the serums to appropriately marked test tubes. These are known as "adsorbed serums."

3. *Titration of Unadsorbed and Adsorbed Serums.* Prepare simultaneous duplicate serial dilutions of the original unadsorbed 1:20 anti-typhoid and antipullorum serums, using internal master dilutions. Start with a 1:40 dilution (tube 1 in the front row receives 1 ml. of saline and 1 ml. of 1:20 antiserum), and continue through as many dilutions as needed to indicate the titers of the serums. Prepare similar dilutions in duplicate of the adsorbed serums. Eight rows of tubes in all will be required.

Prepare test antigens of the usual turbidity for agglutination from 24 hour tryptose agar slants of the two organisms. Add typhoid antigen to all tubes in one row of each unadsorbed and each adsorbed serum, and pullorum antigen to the remaining rows of tubes. Incubate 2 hours at 37° C., read, reincubate overnight at 55° C., and read again. Tabulate the results in a form like that of Table 74.

VII. **Precipitation.** Two of the many methods for demonstrating precipitation will be described. The original papers of Heidelberger and colleagues and the book by Kabat and Mayer (*Experimental Immunochemistry*, Charles C Thomas, Springfield, Ill., 1948) should be consulted for the quantitative method of determining precipitin.

1. *Ring (Interfacial) Test.* This sensitive precipitin test is used to

detect and identify proteins and other antigenic materials. The reagents are carefully added to small test tubes so that they do not mix but form a sharp interface. Undiluted antiserum constitutes the bottom layer in each tube, with serial dilutions of antigen forming the upper layer. Reaction is rapid with antiseraums of high potency. For this experiment any antigenic protein such as egg albumin or animal serum may be employed, together with homologous antiserum.

*Table 74. Form for a Reciprocal Adsorption Experiment*

ANTISERUM	ADSORBING ANTIGEN	TEST ANTIGEN	INCUBA-TION	SERUM DILUTION				CONTROL (saline)
				1.40	1.80	1:160	etc.	
Anti-typhoid	None	Salmonella typhosa	37° C.					
			55° C.					
		Salmonella pullorum	37° C.					
			55° C.					
	None	Salmonella typhosa	37° C.					
			55° C.					
		Salmonella pullorum	37° C.					
			55° C.					
Anti-pullorum	None	Salmonella typhosa	37° C.					
			55° C.					
		Salmonella pullorum	37° C.					
			55° C.					
	Anti-typhoid	Salmonella pullorum	37° C.					
			55° C.					
		Salmonella typhosa	37° C.					
			55° C.					
Anti-pullorum	Anti-typhoid	Salmonella typhosa	37° C.					
			55° C.					
		Salmonella pullorum	37° C.					
			55° C.					

Use a pipette with capillary tip to place undiluted antiserum in each of a series of precipitin tubes (about 5 mm. inside diameter) to a depth of 6 to 10 mm. The exact amount is not critical, but the quantities should be reasonably uniform. In a separate series of larger test tubes prepare decimal dilutions (1:10, 1:100, 1:1000 . . .) of the antigen solution (e.g., 0.9 ml. saline + 0.1 ml. antigen, etc.). With a capillary pipette and rubber bulb, transfer antigen dilutions (and saline for a control tube) to the precipitin

tubes containing antiserum. Insert the tip of the pipette into the precipitin tube and touch it to the inside wall a few millimeters above the antiserum. Carefully expel antigen so that it runs slowly down and forms a layer over the serum, withdrawing the pipette at the same rate. The layer of antigen should be one to two times as deep as that of antiserum. The same pipette may be used throughout provided that the control tube (saline over antiserum) is prepared first, the *most dilute* antigen tube next, and so on.

Examine the tubes after 10 and 30 minutes and 1 and 2 hours at room temperature. They may also be refrigerated overnight and observed again. The first sign of reaction is a thin line of white precipitate between the layers, which gradually broadens and increases in intensity. The precipitate later falls to the bottom of the tube and may be dislodged by flipping, as in reading agglutination tests. Grading the reactions (+, ++ . . .) is difficult, but after some experience a fairly uniform system may be evolved. The titer is indicated by the reciprocal of the highest antigen dilution which gives a detectable reaction.

*Table 75. Plan of a Precipitin Optimal Ratio Experiment*

ANTI-EGG ALBUMIN	DILUTIONS OF 1% EGG ALBUMIN							
	1:5	1:10	1:20	1:40	1:80	1:160	1:320	etc.
1:2.5								
1:5								
1:10								

**2. Optimal Ratio Titration.** When antiserum and antigen are mixed, each lot of antiserum reacts most rapidly with its antigen when the two reagents are in a certain definite proportion. This may be demonstrated with a purified protein such as recrystallized ovalbumin (egg albumin) and anti-ovalbumin rabbit serum. Antigen dilutions to be employed must be determined by preliminary rough titration with each antiserum.

Ovalbumin can be recrystallized by the method described by Kabat and Mayer (*op. cit.*). A 1 per cent solution in saline is a convenient working stock dilution.

Prepare triplicate simultaneous serial dilutions of 1 per cent egg albumin in 0.5 ml. amounts in agglutination tubes. Start with 1:5, for example, and continue through 10 to 12 tubes (see Table 75). Prepare external master dilutions of anti-ovalbumin: 1:2.5, 1:5 and 1:10. To each egg albumin dilution in the front row add 0.5 ml. of 1:2.5 anti-ovalbumin; to each tube in the middle row add 1:5 antiserum; and to the last row add 1:10 antiserum. Note the time antiserum was added to each row. Place

the rack of tubes in the waterbath at 37° C. and observe the tubes every few minutes. Note the tube in each row in which opalescence or other evidence of reaction first appears. At the end of 1 and 2 hours grade the amount of precipitate in each tube. Refrigerate overnight and regrade the amounts of precipitate. Compare the dilutions of antigen which reacted most rapidly and most strongly with each dilution of antiserum.

**VIII. Hemolysis.** The necessity for both complement and amboceptor in hemolytic reactions and the order in which they combine with erythrocytes can be demonstrated by a simple experiment. Preparation of the red blood cell suspension and preliminary titrations will first be described.

It is important that all reagents used in complement reactions, including saline, red cells, amboceptor and complement, be kept in the refrigerator except when actually being used.

*1. Red Blood Cell Suspension.* Sheep red blood cells are usually employed in hemolytic and complement fixation experiments. Either defibrinated or citrated blood secured from the jugular vein of a sheep (or purchased from a reliable laboratory) is satisfactory. Dilute sufficient blood for the day's work with 5 to 10 volumes of saline (0.9 per cent NaCl) and centrifuge at moderate speed just long enough to deposit the cells. Discard the supernatant liquid, resuspend the cells in saline, and centrifuge again. Repeat this process twice more. Measure the final deposit of packed cells and dilute with 50 volumes of saline. This provides a 2 per cent suspension of red blood cells. (Some laboratories prefer a 5 per cent suspension.) The suspension must always be shaken immediately before it is used, because erythrocytes settle rapidly in saline.

*2. Amboceptor Titration.* Sheep amboceptor is prepared by immunizing rabbits with sheep erythrocytes; it may be purchased from various laboratories. It is very stable, so it needs to be titrated only occasionally. Amboceptor is usually preserved with an equal volume of neutral glycerine, a fact which must be taken into account when preparing dilutions (e.g., 1:500 amboceptor may be prepared by adding 0.2 ml. of preserved amboceptor to 49.8 ml. saline).

Complement will also be required. Guinea pig serum is ordinarily used. Fresh serum can be secured by bleeding at least three males or non-pregnant females from the heart under light ether anesthesia (or without anesthetic if an assistant is available), and removing and pooling the serums after clotting has occurred. Serum from each animal should be tested individually for hemolytic activity, although in practice this is not always done. Complement can be preserved for some time in a freezer. It can also be purchased in dehydrated form, in which case it is reconstituted according to directions supplied.

Table 76 illustrates the titration of amboceptor. Pipette diminishing amounts of amboceptor to the bottoms of agglutination tubes, using a 1 ml. serologic pipette graduated in hundredths. Add increasing amounts

of saline, followed by complement diluted 1:20 and the red cell suspension. Shake the rack of tubes just sufficiently to disperse the cells uniformly and place in a 37° C. waterbath for 30 minutes. Read the degree of hemolysis in each tube. Complete hemolysis (++++) consists of apparent dissolution of all cells, the hemoglobin imparting a clear red color to the liquid. The smallest amount of amboceptor yielding complete hemolysis is considered the unit of amboceptor. For many purposes two

Table 76. Amboceptor Titration

TUBE	AMBOCEPTOR (1:500)	SALINE	COMPLEMENT (1:20)	R.B.C. (2%)	
1	0.10 ml.	0.20 ml.	0.1 ml.	0.1 ml.	
2	0.09 ml.	0.21 ml.	0.1 ml.	0.1 ml.	
3	0.08 ml.	0.22 ml.	0.1 ml.	0.1 ml.	
4	0.07 ml.	0.23 ml.	0.1 ml.	0.1 ml.	
5	0.06 ml.	0.24 ml.	0.1 ml.	0.1 ml.	
6	0.05 ml.	0.25 ml.	0.1 ml.	0.1 ml.	
7	0.04 ml.	0.26 ml.	0.1 ml.	0.1 ml.	
8	0.03 ml.	0.27 ml.	0.1 ml.	0.1 ml.	
9	0.02 ml.	0.28 ml.	0.1 ml.	0.1 ml.	
10	0.01 ml.	0.29 ml.	0.1 ml.	0.1 ml.	
11	0.00 ml.	0.30 ml.	0.1 ml.	0.1 ml.	

Waterbath at 37° C. for 30 min.

Table 77. Complement Titration

TUBE	COMPLEMENT (1:40)	SALINE	AMBOCEPTOR (2 units)	R. B.C. (2%)	
1	0.13 ml.	0.17 ml.	0.1 ml	0.1 ml.	
2	0.12 ml.	0.18 ml.	0.1 ml.	0.1 ml.	
3	0.11 ml.	0.19 ml.	0.1 ml.	0.1 ml.	
4	0.10 ml.	0.20 ml.	0.1 ml.	0.1 ml.	
5	0.09 ml.	0.21 ml.	0.1 ml.	0.1 ml.	
6	0.08 ml.	0.22 ml.	0.1 ml.	0.1 ml.	
7	0.07 ml.	0.23 ml.	0.1 ml.	0.1 ml.	
8	0.06 ml.	0.24 ml.	0.1 ml.	0.1 ml.	
9	0.05 ml.	0.25 ml.	0.1 ml.	0.1 ml.	
10	0.04 ml.	0.26 ml.	0.1 ml.	0.1 ml.	
11	0.03 ml.	0.27 ml.	0.1 ml.	0.1 ml.	
12	0.02 ml.	0.28 ml.	0.1 ml.	0.1 ml.	

Waterbath at 37° C. for 30 min.

units are employed, the serum being diluted so that they are contained in a volume of 0.1 ml. For example, if the unit were 0.04 ml. of 1:500 amboceptor, two units would be 0.08 ml. of 1:500 or 0.1 ml. of 1:625 amboceptor.

3. Complement Titration. Complement is very unstable and must be titrated each day. The procedure is illustrated in Table 77. Decreasing amounts of complement diluted 1:40 are pipetted into agglutination tubes, together with increasing amounts of saline. One-tenth milliliter of amo-

ceptor containing two units is added to each tube, followed by red cells. Hemolysis is read after 30 minutes in the 37° C. waterbath. It is sometimes desirable, as in preparation for complement fixation tests, to allow a period of primary incubation of the complement-saline mixtures before addition of amboceptor and red cells, because this more nearly duplicates the conditions in the final test. The smallest amount of complement yielding complete hemolysis constitutes one unit. Two units are often employed, and the guinea pig serum is diluted to contain this quantity in 0.1 ml.

*Table 78. Participation of Complement and Amboceptor in Hemolysis*

TUBE	COMPLEMENT (2 units)	SALINE	AMBOCEPTOR (2 units)	R.B.C. (2%)
A	—	0.3 ml.	0.1 ml.	0.1 ml
B	0.1 ml.	0.3 ml.	—	0.1 ml.
C	0.1 ml.	0.2 ml.	0.1 ml.	0.1 ml.
D	—	0.4 ml.	—	0.1 ml.

*4. Role of Amboceptor and Complement in Hemolysis.* The role of the two principal reagents in hemolysis can be shown by the following simple experiment:

a. Set up four agglutination tubes as shown in Table 78, adding the reagents column by column. Shake and incubate in the waterbath at 37° C. for 30 minutes. Read hemolysis. This part of the experiment demonstrates that both complement and amboceptor are necessary for hemolysis.

*Table 79. The Order with Which Complement and Amboceptor Unite with Red Blood Cells*

TUBE	COMPLEMENT (2 units)	SALINE	AMBOCEPTOR (2 units)	R.B.C. (2%)
A (R.B.C.)	0.1 ml.	0.6 ml.	—	—
A <sub>s</sub> (supernate)	0.1 ml.	—	—	0.1 ml.
B (R.B.C.)	—	0.6 ml.	0.1 ml.	—
B <sub>s</sub> (supernate)	—	—	0.1 ml.	0.1 ml.

b. Centrifuge tubes A and B at moderate speed. Carefully transfer the supernatant liquids to clean agglutination tubes and label these A<sub>s</sub> and B<sub>s</sub> respectively. Treat tubes A, A<sub>s</sub>, B and B<sub>s</sub> as indicated in Table 79. Shake and incubate at 37° C. as before. Read hemolysis. From the results it is possible to decide in what order complement and amboceptor combine with red cells.

**IX. Bacteriolysis.** Bacteriolysis can be demonstrated with some strains

of *S. typhosa* and *Vibrio cholerae*. With most samples of complement a dilution of 1:20 or 1:25 gives satisfactory results in the procedure outlined, together with a 1:500 dilution of antibacterial serum having an agglutinin titer of about 10,000. Use young (5 to 6 hour) nutrient broth cultures grown at 37° C.

Prepare four Wassermann tubes (13 × 100 mm.) as indicated in Table 80. Mix the contents of the tubes by flipping and incubate in the waterbath at 37° C. for 2 hours. At the end of each 30 minutes observe the tubes with the naked eye and prepare a smear from each and stain 15 seconds with gentian violet. Examine with the oil immersion objective.

*Table 80. Bacteriolysis*

TUBE	COMPLEMENT (1:20)	ANTISERUM (1.500)	SALINE	YOUNG BROTH CULTURE
1	—	—	1 0 ml.	0.5 ml
2	—	0.5 ml	0.5 ml	0.5 ml
3	0.5 ml.	—	0.5 ml	0.5 ml
4	0.5 ml.	0.5 ml.	—	0.5 ml

Agglutination may be noted in one or two tubes and bacteriolysis in one tube. Bacteriolysis is shown by irregular staining and bloated or otherwise abnormal cells, and ultimately by disappearance of formed cells. Occasionally complete dissolution is apparent upon macroscopic inspection of the tube.

**X. Complement Fixation.** Complement fixation is conveniently demonstrated with a protein-antiprotein system, such as crystallized egg albumin or foreign animal serum. The procedure with egg albumin will be outlined.

The 1 per cent solution of egg albumin employed in the precipitin test will be satisfactory. Inactivate anti-egg albumin serum by heating in a waterbath at 56° C. for 30 minutes shortly before using. Employ two periods of incubation in titrating complement (see page 318 and Table 77): incubate the complement-saline mixtures at 37° C. for 1 hour, add amboceptor and sheep cells, and return to the 37° C. waterbath for an additional 30 minutes. Prepare sensitized R.B.C. by mixing equal volumes of 2 per cent R.B.C. and amboceptor diluted to contain two units in 0.1 ml. Keep them cold until needed.

The complement fixation test is set up as illustrated in Table 81. Prepare decimal dilutions from the stock 1 per cent egg albumin solution in any convenient amounts. The actual test is performed in agglutination tubes. Pipette egg albumin dilutions into these tubes as indicated, using a single pipette and beginning with the greatest dilution, rinsing the pipette in each succeeding dilution before transferring. Note that tube 6 (antigen

control) receives 0.1 ml. of the lowest dilution employed in any of the tests. Add antiserum, complement, and saline where needed, mix by shaking the rack, and incubate for 1 hour before adding the sensitized red blood cells. Shake sufficiently to suspend the cells evenly and reincubate. Read the tests 5 to 10 minutes after the cells in tube 8 (hemolytic control) are completely lysed. Tubes 6 through 9 are controls and must always be included in a complement fixation test. There should be complete hemolysis in all except tube 9, which should show no hemolysis. Complete hemolysis in the test indicates no fixation of complement, and is recorded by the symbol — or 0. Absence of hemolysis represents complete fixation, and is designated + + + +.

*Table 81. Complement Fixation*

TUBE	EGG ALBUMIN (0.1 ml.)	INACTIVATED ANTI-EGG ALBUMIN SERUM (1:5)	COMPLE- MENT (2 units)	SALINE	SENSITIZED R.B.C. (2%)	Waterbath at 37° C. for 1 hour	Waterbath at 37° C. for 15 to 30 minutes
1	1:1000	0.1 ml.	0.1 ml.	—	0.2 ml.		
2	1:10,000	0.1 ml.	0.1 ml.	—	0.2 ml.		
3	1:100,000	0.1 ml.	0.1 ml.	—	0.2 ml.		
4	1:1,000,000	0.1 ml.	0.1 ml.	—	0.2 ml.		
5	1:10,000,000	0.1 ml.	0.1 ml.	—	0.2 ml.		
6	1:1000	—	0.1 ml.	0.1 ml.	0.2 ml		
7	—	0.1 ml.	0.1 ml.	0.1 ml.	0.2 ml.		
8	—	—	0.1 ml.	0.2 ml.	0.2 ml.		
9	—	—	—	0.3 ml.	0.2 ml.		

Complement fixation is most widely used in various modifications of the Wassermann test for syphilis. Details of the procedure vary from one laboratory to another; for example, in some laboratories the final total volume of reagents is 0.5 ml., and in others it is 3.0 ml. Specific methods can be found in such references as Wadsworth, Standard Methods, 3rd ed., 1947, Williams and Wilkins Co., Baltimore; and Kolmer, Spaulding and Robinson, Approved Laboratory Technic, 5th ed., 1951, Appleton-Century-Crofts, New York.

**XI. Phagocytosis.** Phagocytosis can be shown by use of artificially opsonized staphylococci. Normal or immune opsonins are demonstrated in suitable human or animal serums.

*1. Phagocytosis of Artificially Opsonized Staphylococci.* The antigen is prepared from well grown, heavily inoculated nutrient or tryptose agar slants of *Staphylococcus aureus*. Wash the organisms from each slant with about 2 ml. of saline. Pool and add an equal volume of 1 per cent chrome alum in saline. Incubate 2 hours at 37° C. Centrifuge at high speed

to pack the cells, discard the supernatant liquid, and wash the cells twice by centrifugation with saline. Resuspend the organisms in saline to a concentration of about 500,000,000 cells per ml.

In an agglutination tube mix 0.2 ml. heparinized human blood and 0.2 ml. staphylococcus antigen. Incubate in the waterbath at 37° C. for 30 minutes. With a capillary pipette transfer single drops to the ends of several microscope slides, smear with a spreader slide as in preparing blood films, and stain by the Wright method. Examine with the oil immersion objective and count the bacteria ingested by each of the first 25 polymorphonuclear leukocytes encountered. The average number of bacteria ingested is the phagocytic index.

2. *Opsonizing Antibodies in Serum.* Opsonins for Brucella or *S. aureus* can be detected in suitable human or animal serums, if available. The pooled serums of at least five apparently normal individuals should be used as a control.

Cultivate the organisms on an appropriate agar medium and suspend them in saline. Wash the cells three times by centrifugation with saline, and finally resuspend them to a concentration of about one billion bacteria per ml. in saline, or preferably in Krebs-gelatin solution, the composition of which follows:

Gelatin (6% in 0.9% NaCl)	50 parts
0.9% NaCl	50 parts
1.15% KCl	4 parts
1.22% CaCl <sub>2</sub>	3 parts
2.11% KH <sub>2</sub> PO <sub>4</sub>	1 part
3.82% MgSO <sub>4</sub> ·7H <sub>2</sub> O	1 part
5% NaHCO <sub>3</sub>	4.6 parts

Mix in an agglutination tube 0.1 ml. of heparinized human blood, 0.1 ml. of patient's serum, and 0.1 ml. of bacterial suspension. Prepare a similar (control) tube containing 0.1 ml. quantities of heparinized human blood, pooled normal serum and bacterial suspension. Incubate at 37° C. in the waterbath for 30 minutes. Make smears from each tube by the usual blood smear technique, stain by the Wright method, and examine with the oil immersion objective. Determine the phagocytic index of each preparation. Calculate the opsonic index of the patient's serum by dividing its phagocytic index by that of the normal serum.

**XII. Toxin-Antitoxin Reactions.** Laboratory demonstrations of the action of toxins and antitoxins depend upon the nature and properties of the materials available. Tetanus toxin, toxoid and antitoxin can be used to demonstrate determination of the M.L.D. of toxin, neutralization of toxin by antitoxin and immunization by toxoid. Similar experiments can also be arranged with diphtherial products. Either mice or guinea pigs may be employed with tetanus toxin. Mice are preferable from the standpoint of

expense and offer the advantage that statistically better experiments can be arranged without too great cost. Guinea pigs are usually employed with diphtheria toxin. It should be pointed out that in the titration of diphtheria and tetanus toxins and antitoxins for control purposes guinea pigs are always used.

1. *M.L.D. of Tetanus Toxin.* Unless the potency of the toxin is known, preliminary trial with widely spaced doses will be necessary. Prepare decimal dilutions of toxin from 1:100 to 1:1,000,000 using a diluent consisting of 0.5 per cent NaCl containing 1 per cent peptone to increase the stability of the toxin. Inject pairs of mice (approximately 20 gms. in weight) intramuscularly in the right hind leg with 0.5 ml. amounts of the various toxin dilutions. Observe the mice at frequent intervals during 5 days, noting the time when paralysis and/or death occurs in each animal.

*Table 82. Death Time of Mice and Dosage of Tetanus Toxin*

*T = death time (hours)*

T	NO. OF M.L.D. <sup>1</sup>	T	NO. OF M.L.D.	T	NO. OF M.L.D.
12	282	60	2 3	108	1 10
18	45	66	2 0	114	1 05
24	18	72	1.74	120	1.00
30	9 8	78	1.55	126	0.96
36	6.3	84	1 41	132	0.93
42	4 5	90	1 30	138	0 90
48	3 4	96	1.21	144	0 87
54	2 8	102	1 15	150	0.85

\* 1 M.L.D. of tetanus toxin is defined for this table as the dose which kills a 20 gram mouse in 120 hours.

(Modified from Ipsen, 1941. *Arch. Exp. Path. Pharmak* 197, 536.)

Determine the approximate M.L.D. of the toxin from the average death time of the pair of mice which received the smallest fatal dose by reference to Table 82. For example, if both mice injected with 0.5 ml. of 1:100,000 toxin survived, and the mice injected with 1:10,000 toxin died after 40 and 44 hours, respectively, the 1:10,000 toxin contained about 4.5 M.L.D. One M.L.D. is therefore 0.5 ml. of 1:45,000 toxin.

Prepare 3 toxin dilutions, one consisting of the estimated M.L.D. dilution and the others differing by about 10 per cent above and below, and repeat the inoculations and observations using 10 mice for each dose. In the above example, dilutions of 1:40,000, 1:45,000 and 1:50,000 might be employed.

2. *Neutralization of Toxin by Antitoxin.* Antitoxin of known potency can usually be secured because it is very stable. Dilute tetanus antitoxin to contain 0.4 antitoxic unit per ml. Prepare a dilution of the tetanus toxin previously titrated in saline-peptone so that each milliliter contains 10,000 M.L.D. For example, if 1 M.L.D. is represented by 0.5 ml. of 1:45,000 toxin, 10,000 M.L.D. is contained in 1.0 ml. of 1:9 toxin.

Into each of 6 test tubes or shell vials pipette 1.0 ml. of the antitoxin dilution, add saline-peptone as indicated in Table 83, and then toxin, mixing the contents of each tube immediately. Allow the mixtures to stand 1 hour at room temperature, inject 0.5 ml. amounts of each intramuscularly into two or more mice as before, and record reactions during the next five days. From the observed death times calculate the indicated free toxin (in M.L.D. units) in as many mixtures as possible, and subtract from the corresponding doses of toxin injected to determine the quantity presumably neutralized by 0.1 unit of antitoxin.

Table 83. Neutralization of Tetanus Toxin by Antitoxin

ANTITOXIN (1 : ) (1 ml. = 0.4 unit)	SALINE- PEPTONE	TOXIN (1 : ) (1 ml. = 10,000 M.L.D.)	DOSE PER MOUSE (0.5 ml.)	
			ANTITOXIN	TOXIN
1.0 ml.	0.92 ml.	0.08 ml	0.1 unit	200 M.L.D.
1.0 ml.	0.87 ml.	0.13 ml.	0.1 unit	325 M.L.D.
1.0 ml.	0.80 ml.	0.20 ml.	0.1 unit	500 M.L.D.
1.0 ml.	0.68 ml.	0.32 ml.	0.1 unit	800 M.L.D.
1.0 ml.	0.50 ml.	0.50 ml.	0.1 unit	1250 M.L.D.
1.0 ml.	0.00 ml.	1.00 ml.	0.1 unit	2500 M.L.D.

3. Immunizing Action of Toxoid. Inject each of four mice subcutaneously in the back with a dose of tetanus toxoid containing 20 flocculating units (Lf). Inject an equal number of mice with 2 Lf of toxoid. After 14 days challenge all mice by intramuscular injection of 10 M.L.D. of tetanus toxin, and at the same time inject a third equal group of (unimmunized) mice with the same dose of toxin, as a control. Observe the animals during five days and compare the survival times of mice immunized with the two dosages of toxoid.

4. Flocculation of Toxin or Toxoid by Antitoxin. Flocculation can be demonstrated with diphtherial or tetanal antitoxin and homologous toxin or toxoid. Varying amounts of antitoxin are mixed with a constant amount of toxin or toxoid, incubated, and observed constantly. The first mixture which shows visible flocculation is called the *indicating mixture* and is in general neutral when tested by animal inoculation. Toxin mixtures on one side of the indicating mixture are toxic and on the other side are antitoxic. The relationship between toxin and antitoxin in the indicating mixture is expressed by the equation:

$$\text{ml. antitoxin} \times \text{antitoxic units/ml.} = \text{ml. toxin} \times \text{Lf units/ml.}$$

Thus, if the indicating mixture consisted of 1 ml. of toxin and 0.01 ml. of an antitoxin which possessed a potency of 1,000 units per ml., the toxin contained 10 Lf units per ml.

Use an antitoxin of known potency. Unless the approximate Lf value of the toxin or toxoid is known, preliminary titration over a wide range

will be necessary to establish the zone in which an endpoint can be expected. Measure antitoxin into agglutination or Wassermann tubes, the amounts varying from tube to tube by increments of 25 to 30 per cent (e.g., 0.010 ml., 0.013 ml., 0.016 ml., 0.020 ml., 0.025 ml., etc.). Add toxin in constant amounts of 1 to 4 ml. rapidly to each tube. Shake the rack of tubes and incubate in a constant temperature waterbath at about 42° C. Cloudiness and a fine granular precipitate may appear within a very few minutes in certain tubes, and eventually flocculation should occur in one or more tubes. The flocculation time may vary from a few minutes to several hours. Toxoids and some toxins, particularly those with a low Lf value, flocculate slowly and may require a higher incubation temperature. Calculate the approximate Lf value of the toxin or toxoid from the equation above.

If toxin, rather than toxoid, is employed, conclude the experiment by centrifuging the indicating mixture and one or two mixtures on either side and injecting the supernatant liquids into experimental animals to test for residual toxicity.

**XIII. Mouse Protection Tests.** Protection tests in mice are readily performed using *S. typhosa* and antityphoid serum, either from rabbits or from humans who have received typhoid immunization. One group of mice is inoculated with the immune serum and an equal number of mice with normal serum of the same species. One hour later each group of animals is divided into smaller groups containing two or more mice, which are then inoculated with graded doses of living typhoid bacteria suspended in mucin. The animals are observed during the next six days and deaths are noted. The LD<sub>50</sub> values calculated for each type of serum treatment provide an indication of the protective potency of the immune serum under the conditions of the experiment.

Twenty-eight mice are needed for the protection test. Inoculate 14 mice intraperitoneally with 0.5 ml. amounts of immune serum; similarly inoculate the remaining mice with normal serum.

Suspend the growth from a heavily inoculated tryptose agar slant of *S. typhosa* which has been incubated 18 to 20 hours at 37° C. in 10 ml. sterile saline. Prepare serial decimal dilutions in sterile saline from 1:10 to 1:100,000,000. Plate 1 ml. quantities of the 1:10,000,000 and 1:100,000,000 dilutions for a count of the viable bacteria in the suspension.

Pipette 4.5 ml. of sterile 5 per cent mucin into each of 7 shell vials or other suitable containers of 10 to 15 ml. capacity. [To prepare the mucin solution, suspend 5 gms. of mucin (e.g., Granular Mucin, Type 1701-W, The Wilson Laboratories, Chicago) in 100 ml. distilled water, heat in a boiling water bath to aid solution, filter through several thicknesses of cheesecloth, and sterilize by steaming 30 minutes on each of three successive days.] To each vial add 0.5 ml. of one bacterial dilution from 1:10 to 1:10,000,000, thus giving final dilutions in mucin from 1:100 to

1:100,000,000. Use a separate pipette for preparing each mucin dilution, and mix the bacteria and mucin thoroughly.

One hour after the serum injections inoculate each mouse intraperitoneally with 0.5 ml. of one of the mucin dilutions of typhoid bacteria. A syringe fitted with a 1 inch 22 to 24 gauge needle will be found useful for this purpose, because the inoculum is quite viscous. Pick the mice by pairs at random and inoculate two from each serum treatment with each dosage of bacteria. Keep each pair of mice in a separate cage or jar and observe at frequent intervals during the next six days, recording the time of death of each animal. At the end of the period of observation tabulate the results (see Table 84) and calculate the LD<sub>50</sub> of typhoid bacteria for

*Table 84. Mouse Protection Test*

BACTERIAL DILUTION IN MUCIN (0.5 ml.)	NUMBER OF BACTERIA INJECTED	NORMAL SERUM (0.5 ml.)		IMMUNE SERUM (0.5 ml.)	
		TIME OF DEATH (hours)	TIME OF DEATH (hours)	TIME OF DEATH (hours)	TIME OF DEATH (hours)
1:100					
1:1000					
1:10,000					
1:100,000					
1:1,000,000					
1:10,000,000					
1:100,000,000					

animals treated with normal serum and compare with a similar figure for animals treated with immune serum.

It may also be of interest to perform agglutination tests to confirm the absence (or low normal titer) of typhoid agglutinins in the normal serum, in contrast to the immune serum.

**XIV. Anaphylactic Shock.** It is difficult to give detailed directions for anaphylactic experiments because of differences in the materials available and the natural biologic variation of animals. Until familiarity with these variations has been gained, experiments should be planned in duplicate or triplicate to increase the chance of providing a satisfactory demonstration.

*1. Active Anaphylaxis.* Sensitize one or more guinea pigs by intraperitoneal injection of 1 to 5 mg. of foreign protein contained in about 1 ml. of saline. Horse serum or egg albumin is satisfactory for this purpose. Three weeks later administer the shocking injection of the same

protein. The dose should be about ten times that of the sensitizing injection and should be introduced directly into the blood stream, either intravenously or by cardiac puncture. Intracardial injections without anesthetic are not difficult if the animal is properly held on its back with its front legs stretched forward and outward. Insert the needle as in bleeding from the heart, and when gentle aspiration brings blood into the syringe, inject the contents into the heart. In a typical anaphylactic reaction the animal begins to cough and gasp for breath within a few moments, may scratch its face with its forepaws and make convulsive movements. In a fatal reaction the animal collapses and dies, often within two to four minutes. Immediately perform an autopsy, noting especially the continued heart beat and intestinal peristalsis and the inflated condition of the lungs.

2. *Passive Anaphylaxis.* Guinea pigs can be sensitized passively by means of rabbit precipitating antiserum. The dosage depends upon the titer of antibodies in the antiserum and must be determined by trial for each serum. As little as 0.1 ml. of high titer antiserum may suffice. Inject the antiserum intraperitoneally and allow about one day to elapse before attempting to elicit anaphylactic shock. The shocking material is the antigen homologous to the antiserum (i.e., egg albumin will produce shock in a guinea pig passively sensitized with rabbit anti-egg albumin serum), and the dose should be the same as used to elicit active anaphylaxis. If the animal dies perform an autopsy and look for the usual signs.

3. *Histamine Shock.* Inject a guinea pig intracardially with about 0.25 mg. (1 ml. of 1:4,000 solution) of histamine in saline. The response may be indistinguishable from fatal anaphylaxis. Perform an autopsy and observe the customary signs.

4. *Action of Antibistamines.* Sensitize guinea pigs as usual and wait three weeks. Demonstrate the hypersensitive condition of at least one animal by inducing anaphylactic shock. Inject an antihistaminic drug intraperitoneally into one or more of the remaining sensitive animals. The dose required may vary from 1 to 10 mg. per kg. and may be dissolved in a small amount of saline. After 15 to 30 minutes inject a shocking dose of antigen intracardially. The anaphylactic reaction may be prevented entirely, may be mild and nonfatal, or may consist of greatly delayed death (e.g., one-half hour).

5. *Shock Induced by Anti-Kidney Serum.* A reaction resembling anaphylactic shock in guinea pigs can be induced by injecting rabbit anti-guinea pig kidney serum. The dosage of the serum available will have to be determined by trial; 3 ml. of a potent serum is satisfactory. Intracardial injection of the antiserum may be followed by most of the signs of anaphylactic shock, including death within two or three minutes. Autopsy the animal as usual.

# INDEX

- A and B substances, use in transfusion, 183  
A antigens of *E. coli*, 146  
*A<sub>1</sub>* blood subgroup, 171  
*A<sub>2</sub>* blood subgroup, 171  
A substance, composition of, 170  
    distribution of, 171  
    reaction with pneumococcal antibodies, 45  
    sources, 45  
Acid radicals, effect on specificity of azoproteins, 33-38  
Acids, effect on antigenicity of proteins, 42  
Acidfast bacteria, adjuvant effect of, 86  
    use as adjuvant, 303  
Acquired immunity, 8  
    factors affecting, 8  
    origin of, 8  
ACTH, use in allergies, 289  
Active anaphylaxis, 271-274  
    demonstration of, 325-326  
Active immunity, 8  
    origin of, 8-10  
Active immunization, antiviral, 255-258  
"Active patches" of proteins, 107  
Adjuvants, 26, 86, 302-303  
    effect on antigenicity of pollen extracts, 121  
    mode of action, 87  
Adler and Humphries, 110  
Adrenal cortical hormones, 77  
    effect on antibody titers, 85  
    on lymphoid cells, 85  
Adsorption, differential, 158  
    nature of, 112-113  
    of agglutinins, 312-313  
    of antibodies, 27, 157  
    reciprocal, 158-161  
Adsorption hypothesis of antigen-antibody reaction, 112  
Adsorption test, 27-28  
*Aerobacter aerogenes*, agglutination of, 110  
Agar, antibodies against, 141  
Age, host, effect on resistance to viruses, 253  
Agglutination, 17  
    applications of, 150 ff.  
Agglutination, diagrammatic representation of, 114  
    effect of pH on, 109  
    of temperature on, 110  
factors affecting, 108-111  
flagellar, 145, 148-149  
    effect of temperature on, 105  
identification and classification of bacteria by, 154-156  
incubation temperature and time, 145  
lattice hypothesis of, 148-150  
macroscopic slide test, 144, 310-311  
mechanism of, 118, 147-150  
microscopic slide test, 143-144, 310  
mixed aggregates in, 147-148  
of antigen-coated inert particles, 125-127  
of mucoid bacteria, 110, 146  
optimal temperature for, 110  
photomicrographs of, 148-149  
prozone in, 96-97  
quantitative, procedure for, 146-147  
role of electrolytes in, 96, 108-109  
    of lipids in, 46  
somatic, 145, 149  
spontaneous, of bacteria, 161  
tube test, 144-146, 308-310  
two-stage nature of, 96-97  
V<sub>1</sub>, 149, 308-309  
viral, 264-266  
Agglutination test(s), centrifugation of, 146  
    incubation of, 309  
    procedure, 18, 143-147  
    reading of, 310  
Agglutinin(s), adsorption of, 312-313  
alumin, Rh, 177  
alpha, 158  
cold, 105, 153-154  
production of, 141-143  
quantitative determination of, 146-147  
saline, Rh, 177  
Agglutinins and precipitins, identity of, 147  
Agglutinoids, 97  
Albumin agglutinins, Rh, 177  
Albumin(s), electrophoretic mobility of, 59  
    molecular weights of, 64-65  
per cent in normal serum, 60

- Alcohol precipitation of proteins, 66-69  
 Alcoholic extracts, antigenicity of, 46  
 Alexander, 93  
 Alexine(s), 229, 230  
 Alkalies, effect on antigenicity of proteins, 42  
 Allergens, antigenicity of, 269, 283, 294  
 Allergic asthma, 288  
 Allergic reactions, following immunization, 208  
 Allergy and immunity, 296-297  
 Allergy(ies), definition of, 269  
     drug, 294  
     evanescent inflammation, 281-283  
     food, 288-289  
     gastrointestinal, 289  
     immediate, types of antibodies in, 285-287  
     of infection, 289-293  
     physical, 295-296  
     precipitins in, 121, 126  
     role of heredity in, 286  
     serum, 284  
         tuberculin type, 290-293  
 Alpha agglutinin, 158  
 Alpha-beta variation of *Salmonella*, 164  
 Alsever's solution, 301-302  
 Alternation hypothesis. See *Lattice hypothesis*.  
 Alum precipitated toxoids, use in immunization, 10  
 Alum precipitation of vaccines, 302  
 Amboceptor(s), 111, 232  
     preparation of, 245  
     quantitative relations in hemolysis, 240-243  
     titration of, 245, 316-317  
 Amino acids, terminal, effect on specificity of azoproteins, 36-38  
 p-Aminobenzene arsonic acid (atoxyl), 31  
 Ammonia, inactivation of C<sub>4</sub> by, 237  
 Amphibia, serologic relationships between, 136  
 Anamnestic reaction, 89, 150  
     nonspecific, 84  
     specific, 84  
 Anaphylactic sensitivity, production of, by tuberculoproteins, 291  
 Anaphylactic shock, caused by antitoxin injection, 194  
     common symptoms of, 273  
     demonstration of, 325-326  
     inhibition by antihistamines, 279  
     mechanism of, 273-274, 277-280  
     role of histamine in, 279  
     symptoms in dogs, 273  
         in guinea pigs, 21, 273, 326  
         in rabbits, 273  
 Anaphylactoid reactions, 278  
 Anaphylatoxin, 52, 278  
 Anaphylaxis, 271 ff.  
     active, 271-274  
         incubation period, 272  
         sensitization, 272  
         shocking injection, 272-273  
     cellular hypothesis of, 278  
     definition of, 271  
     desensitization in, 275  
     duration of sensitivity, 272  
     during immunization of animals, 26  
     first observations of, 271  
     humoral hypothesis of, 278  
     in man, 284-285  
     in study of serologic specificity, 32  
     *in vitro*, 275-276  
     passive, 21  
         sensitization, 274  
         sensitizing dose in, 276-277  
         shocking dose in, 276-277  
     passive transfer of sensitivity, 271  
     procedure for demonstrating, 21, 325-326  
     quantitative studies of, 276-277  
     reversed passive, 278  
     sensitization, dose required for, 271, 272  
     specificity of, 271  
         symptoms in guinea pig, 21, 273, 326  
 Andrewes, 162, 258  
 Anesthesia of rabbits, 307  
 Angioedema, 284, 288  
 Animal organs, alcoholic extracts, antigenicity of, 46  
 Animal serums, precipitin reactions of, 134-137  
 Anthrax, precipitin test for, 129  
     resistance of sheep to, 8  
 Anthropology, blood groups and, 183-184  
 Anti-A hemagglutinins, occurrence of, 169  
     production of, 86  
 Anti-B hemagglutinins, occurrence of, 169  
     production of, 86  
 Anti-anaphylaxis, 271, 275  
 Antibacterial antibodies, electrophoretic properties of, 61  
 Antibacterial serums, treatment of disease by, 11  
 Antibody(ies), adsorption of, 27, 157  
     anti-A and anti-B, rate of production of, 86  
     anti-agar, 141  
     antigenic specificity of, 72  
     antiviral, appearance of, 264  
         mode of action of, 259-260

- Antibody(ies), antiviral, titration of, 261 ff.  
by hemagglutination inhibition, 266-267  
as proteins, 56  
as species proteins, 72  
blocking, in allergies, 286  
Rh, 177  
chemical nature of, 12, 56  
complete, Rh, 177  
concentration in immunized rabbits, 82  
definition, 16  
demonstration of, 17  
denaturation of antigen by, 113  
detection of, in patients' serums, 150-154  
determination of, quantitative, 101-103  
effect of enzymes on, 56  
of heat on, 56  
on viruses, 259-260  
electrophoretic properties of, 61  
elution from agglutinates, 110  
first order, 111  
flagellar, 145  
flocculating, 129  
function of, 12, 17  
heterogeneity of, 94  
effect of number of injections on, 87  
factors affecting, 87-88  
heterophile, 47  
horse, electrophoretic properties of, 61  
human species specific, rate of production of, 86  
immune, titer of, 5  
incomplete, 97  
Rh, 177-178  
inhibiting, 97  
isophile, 48  
meningococcal, concentration in human serum, 147  
molecular sizes of, 66  
weights of, 64-65  
natural, 4  
anti-erythrocyte, 6  
in lower animals, 6  
in man, 6  
origin of, 5  
titer of, 5  
neutralization of virus by, 259  
neutralizing, in virus diseases, 263  
normal, 131, 150, 152, 158, 169  
Forssman, 153  
hemagglutinins, 110  
origin of, 12  
participation in immunity, 8  
in tuberculin sensitivity, 291-292  
persistence after secondary stimuli, 82-83  
pertussis, placental transfer of, 224  
Antibody(ies), physical heterogeneity of, 88  
plasma fractions (Cohn) and, 69  
pneumococcal, purification of, 71  
precipitation by alcohol, 67  
protective, antiviral, 264  
purification of, 70-71  
purified, properties of, 71  
quantitative determination of, 82, 127  
rabbit anti-egg albumin, molecular weight of, 103  
rabbit, electrophoretic properties of, 61  
rate of adsorption of, 104  
role in phagocytosis, 214  
in surface phagocytosis, 220  
second order, 111  
skin sensitizing, 285-286  
site of production of, 297  
solubility of, 114  
somatic, 145  
somatic and flagellar, thermostability of, 56  
third order, 111  
types in immediate allergies, 285-287  
typhoid, effect on phagocytosis, 222  
valence of, 91, 114, 117  
Antibody-dye complex as specific stain for antigen, 92  
Antibody formation, against multiple antigens, 85  
Buchner's hypothesis of, 89  
by R-E system, histologic studies of, 75  
effect of benzene injection on, 75  
of exsanguination and transfusion on, 74  
of injection route on, 77, 78  
of injuring R-E cells on, 75  
of R-E blockade on, 75  
of removing organs on, 74  
of X-rays on, 75  
Ehrlich's hypothesis of, 89  
in liver and spleen, 92  
in lymph nodes, 76, 77, 78  
in plasma cells, 76  
in skin, 76  
in spleen, 77, 78  
in tissue cultures, 76  
*in vitro*, 91  
liver as possible site of, 74  
lymph node transfer and, 78  
mechanism of, 88 ff.  
modified enzyme hypothesis of, 93  
participation of R-E system in, 75  
role of lymphocytes in, 76, 77  
of mitochondria in, 92  
site of, 74 ff.  
spleen as site of, 74

- Antibody formation, spleen transfer and, 78  
 template hypothesis of, 89-93
- Antibody globulin, relation to normal globulin, 71-72
- Antibody production, individual variation in, 39-40, 194  
 against particulate antigens, 80  
 against soluble antigens, 80-81  
 antiviral, effect of host age on, 253-254  
 effect of age on, 10, 253-254  
   of antigen dosage, 81  
   of series of injections on, 78-79  
 factors affecting, 78 ff.  
 phases of, 79  
 pneumococcal, in man, 81  
 rate of, 75  
 relationship to amount of antigen injected, 89  
 response to single injection, 79-84
- Antibody titers, decrease in, 81  
 effect of adjuvants on, 86-87  
   of bleeding on, 85  
 factors affecting, after immunization, 79  
 in patients' serums, 150-154  
 rising, during disease, 150, 152
- Anticoagulants, for blood, 12, 301
- Anticomplementary action, 235
- Anticomplementary serums, 235
- Anticomplementary titration of antigen, 246
- Antigen(s), azoprotein, 31-38  
 antigenicity of, 91  
 bacterial, 26  
   flagellar, 160-165, 308  
   for immunization, 141-142, 300-301  
   nomenclature of, 156 ff.  
   somatic, 160-165, 308  
 blocking, 24  
 Bovine, 50-52  
 Buchbinder, 48  
 classes of, 24  
 common, in *Proteus* and *Rickettsiae*, 152  
 definition, 15, 24, 53  
 derivative, in contact allergy, 294  
 detection of, 24  
   in mixtures, 124  
 disappearance from blood, 82  
 effect on synthesis of globulins, 89 ff.  
 flagellar, 145  
   group (phase 2), 162  
   preparation of, 308  
   specific (phase 1), 162  
 human species, in R.B.C., 170  
 infectious mononucleosis, 49  
 labeled with  $I^{121}$ , 82
- Antigen(s), labeled, accumulation in organs, 75  
 distribution of, 92  
 molecular weights of, 25  
 multiple, antibody response to, 85  
 nature of, effect on antibody formation, 80-81  
 number of determinant groups, 107  
 persistence in animal, 88, 92, 94  
 polyhaptenic, 114  
 polysaccharide, 42 ff.  
 properties of, 24 ff.  
 serum, genic control of, 137  
 serum sickness, 49  
 somatic, 145  
   preparation of, 308  
   thermolability of, 41  
 specificity of, 26  
 syphilis, 130-131  
 titration of, anticomplementary, 246  
 valence of, 114  
 $V_1$ , preparation of, 308  
 Wassermann, 46
- Antigen-antibody-complement complex, structure of, 241
- Antigen-antibody complexes, dissociation of, 70-71, 105
- Antigen-antibody compounds, solubility of, 117
- Antigen-antibody precipitates, composition of, 102-103  
 molecular compositions of, 103
- Antigen:antibody ratio, effect on precipitation, 101
- Antigen-antibody reaction(s), adsorption hypothesis of, 112  
 effect of shaking on, 111  
 factors affecting rate of, 104-105  
 first stage of, 104  
 intermolecular forces and, 105-106  
 lattice hypothesis of, 113-117  
 mass action hypothesis of, 112  
 mechanism of, 118  
 occlusion hypothesis of, 117  
 quantitative hypothesis of, 115  
 reversibility of, 112, 116  
 second stage of, 104  
 side-chain hypothesis of, 111
- Antigen-antibody union, forces in, 105-106
- Antigenic analysis, 156 ff.
- Antigenic formulas of *Salmonellas*, 163-164
- Antigenic structure of bacteria, 26-28  
   of vaccinia and variola, 265
- Antigenic variation, induced, 165-167
- Antigenicity, factors determining, 25  
   *in vitro*, 15  
 molecular surface and, 25

- Antigenicity, of blood cells, effect of zoologic relationship on, 39  
of histones, 38  
of pollen extracts, effect of adjuvants on, 121  
of proteins, effect of acids and alkalies on, 42  
of coagulation on, 41  
of denaturation on, 40-41  
of heat on, 41  
of hydrolysis on, 41-42  
polysaccharides and lipids, 38 ff.  
of xanthoproteins, 42
- Antihistamines, inhibition of anaphylactic shock by, 279, 326
- Antihuman serum, precipitin reactions with, 133-136  
preparation of, 132
- Antiserum(s), effects of heat and age on, 97  
polyvalent, precipitation of individual antigens by, 57  
production of, antigen dosages for, 16  
choice of animal for, 16  
procedure for, 16, 304-308  
routes of injection for, 16
- Antiserum-agar, selection of serologic variants by, 162-163, 166
- Antitoxic immunity, effectiveness of, 205
- Antitoxin(s), carbohydrate content of, 195  
digested by enzymes, 195  
diphtheria, intracutaneous titration of, 198  
persistence in humans, 81  
effect of adjuvants on persistence of, 87  
of temperature on, 196  
electrophoretic properties of, 61-62  
homogeneity of, 124  
horse, precipitation by anti-horse precipitin, 72  
mechanism of neutralization by, 202  
molecular weight of, 64-65  
neutralization of toxin by, 322-323  
passive immunization with, 206 ff.  
plasma fraction (Cohn) and, 69  
preparation and purification of, 193-195  
precipitation (flocculation) of, 128-129  
purification of, 71, 195  
quantitative determination of, 128-129
- Ramon titration of, 101, 198-199  
rate of absorption of, 207  
stability of, 196  
standard units of, 196  
standardization of, 195-199
- tetanus, 85
- Antitoxin(s), therapeutic use of, 10  
titration of, 19, 196-199
- Antitoxin molecules, structure of, 195
- Antitoxin production, immunizing procedure for, 194-195  
latent period in, 80-81  
selection of animals for, 194
- Antiviral antibodies, mode of action of, 259-260
- Antiviral immunity, actively acquired, 254-258  
duration of, 254
- A. P. T., diphtheria, immunization with, 204  
local retention in guinea pigs, 204  
tetanus, immunization with, 206
- Arkwright, 3
- Aromatic amino acids, role in specificity and antigenicity, 38-39
- Arrhenius and Madsen, 112
- Arthus, 280
- Arthus reaction, 280-281  
comparison with anaphylaxis, 281  
mechanism of, 281  
passive, 280-281  
quantitative studies of, 281  
relation to evanescent allergic inflammation, 282  
reversed, 280
- Arthus sensitivity, production by tuberculoproteins, 291
- Artificial opsonization, 225, 320-321
- Ascoli, 123
- Asthma, 283-284  
allergic, 288
- Atopic allergies, 286
- Atoxyl, 31  
azoprotein, formation of, 32
- Atypical pneumonia, cold agglutinins in, 154
- Auto-antibodies, 25
- Auto-antigenicity, 248
- Autohemagglutinins, 110
- Autoimmunization, 25  
with organ extracts, 46
- Autolysis, 2, 3
- Avery, 43, 167
- Avidity of toxins and toxoids, 202
- Azoproteins, 31 ff.  
antigenicity of, 91  
formation of, 32  
serologic reactions of, 31  
specificity of, 31 ff.  
effect of chain length on, 36-37  
of halogens on, 33-34  
of methyl radicals on, 33-34  
of nitro groups on, 34  
of spatial configuration on, 33-36  
of strong acids on, 33-38

- Azoproteins, specificity of, effect of taric acids on, 35-36  
of terminal amino acids on, 36-38  
use in quantitative studies, 102
- B antigens of *E. coli*, 146
- B substance, composition of, 170  
distribution of, 171
- Bacillus anthracis*, attenuation of, 3  
Forssman antigen in, 47  
killing of, by blood, 229  
nonsusceptibility to, 5  
polysaccharide haptene of, 45
- Bacteria, antigenic structures of, 156 ff.  
classification of, 154 ff.
- Bacterial antigens, 26
- Bacterins. See also *Vaccines*.  
effect of heat on, 9  
preparation of, 141-142, 300-301  
sterilization and preservation of, 301
- Bacteriolysis, 19  
demonstration of, 318-319  
*in vitro*, 230  
*in vivo*, 229-230
- Bacteriophage, effect on phagocytosis, 218
- Bacteriotropin, 213-214  
identity with other antibodies, 213-214
- Barr and Gleany, 79
- Basophiles, source of, 14
- Benacerraf and Kabat, 281
- Bentonite, use in precipitin test, 126
- Benzene, injected, effect on antibody formation, 75
- Berger and Scholer, 46
- Bergmann, 108
- Berry, 217, 220
- Bhatnagar, 222
- Bieling, 84
- Biochemical classification of bacteria, 155
- Bird serums, precipitin test with, 135 ff
- Bjorneboe, 85
- Blackley, 283
- Bleeding, effect on antibody titers, 85  
of animals, 16  
for serum production, 121  
of rabbits, 306-307
- Blocking antibody(ies), in allergies, 286  
Rh, 177
- Blocking antigens, 24
- Blocking test, Rh, 177
- Blood, anticoagulants for, 12, 301  
bactericidal action of, 229  
composition of, 12 ff.  
human, individuality of, 179
- Blood cell antigens, in lower animals, 183-185
- Blood-clearing mechanism, 75, 210, 212
- Blood compatibility tests, 312
- Blood group A, subgroups of, 171
- Blood grouping tests, 170, 311-312
- Blood groups, 169 ff.  
distribution of, 170, 184  
inheritance of, 172  
of corpses, 182  
of primates, 183  
permanence of, 171
- Blood group substances, 170-171  
secretion of, 171
- Blood proteins, alcohol precipitation of, 66-69  
electrophoretic properties of, 67 ff.  
sizes of, 65-66
- Blood smears, 300-301
- Blood staining, Wright procedure, 300
- Blood stains, group of, 181-182  
identification of, 123, 131-132
- Blood transfusion, 169, 179-180  
Rh factor and, 174
- Blood types, MNS and P, 172-173
- Blood typing, medico-legal applications of, 180-182  
Rh, 179
- Blood volume, 12
- Bodian, 258
- Boivin, 50
- Boivin and Delaunay, 216
- Boivin antigens, 50-52  
immunogenicity of, 51  
toxicity of, 50
- Booster injection(s), 84  
pertussis, effect of, 224  
Schick test as, 205  
tetanus, 206
- Bordet, 112, 120, 133, 147, 210, 230, 249
- Bordet and Gengou, 232
- Botulinum toxin. See *Clostridium botulinum*.
- Botulism, prophylaxis and therapy of, 207-208
- Bowen, 124
- Boyd, 42, 104, 106, 117, 149, 202, 292
- Boyd and Hooker, 117
- Boyden, 99, 136, 137
- Boyden and Noble, 136
- Bozicevich, 126
- Breinl and Haurowitz, 90
- Brucella antibodies in milk, detection of, 151
- Brucellergin, 292
- Brucellin, 292
- Brucellosis, opsonin determinations in, 223-224
- Bruner and Edwards, 165-166
- Bubonic plague, agglutinin titers in, 151
- Buchbinder, 48
- Buchner, 89, 229

- Buehler, Schantz and Lamanna, 188  
Bull, 15, 212  
Burnet, 77, 82, 93, 254, 260, 297
- C'1, 236-240  
C'2, 236-240  
C'3, 236-240  
    role in hemolysis, 243  
C'4, 236-240  
Calcium salts, effects on phagocytosis, 211, 217-218  
Calmette and Massol, 198  
Cannon and Marshall, 126  
Capsular polysaccharides, of *E. coli*, 146  
    of pneumococci, 43  
Capsules, 2  
    antigenicity of, 45  
    antiphagocytic nature of, 210  
    Quellung with homologous antiserum, 130  
    relation to virulence, 3  
Cardiolipin, 131, 248  
Carrel and Ingebrigsten, 76  
Catalysis and antibody formation, 93  
Cattle, blood types of, 185  
CDE (Rh) blood factors, 175  
Cell receptors, 89  
Cellular hypothesis of anaphylaxis, 278  
Cephalin, role in agglutination and precipitation, 46  
Chain length, effect on specificity of azoproteins, 36-37  
Chase, 78, 281, 282, 293  
Chemotaxis, factors affecting, 216  
Chemotherapy, effects on phagocytosis, 224  
Chesney, 222  
Chick embryos, cultivation of viruses in, 260-261  
Chicken cholera, attenuation of, 3  
Chimpanzees, blood groups of, 183  
Cholera, natural antibodies against, 6  
Cholesterol, antigenicity of, 45  
*Clostridium botulinum*, exotoxin of, 2  
    chemical properties of, 188  
    M.L.D. of, 187, 190, 191  
    pharmacologic action of, 190  
    site of action of, 187  
*Clostridium perfringens*, exotoxin of, 2  
    M.L.D. of, 187  
    pharmacologic action of, 190  
    lecithinase of, 190  
    spreading factors of, 2  
*Clostridium tetani*, exotoxin of, 2  
    M.L.D. of, 187, 191  
    pharmacologic action of, 190  
    site of action of, 187  
Clupein phenylisocyanate, antigenicity of, 25
- Coagulation, effect on antigenicity of proteins, 41  
Cobra venom, inactivation of C'3 by, 237  
Coca, 286  
Coca and Kelley, 97  
Coccidioidin, 292  
Cocco-antiseraums, 133  
Cohn, 66, 67  
Cold agglutinins, 105, 110, 153-154  
Cold hemagglutination test, procedure for, 154  
Coleman, 151  
Collagenase, 2  
Collodion particles, coated, use in precipitin test, 126  
Combination immunization, 45-46  
Combining power, 15  
    of iodoprotein, 31  
Complement, 19, 111, 232  
    chemical nature of, 235  
    chemistry of, 236-239  
    definition, 233  
    deterioration of, 234  
    dialysis of, 236  
    effect of colloidal substances on, 235  
        of species on, 233, 234  
    fixation of, 232-233, 239-240  
    fractionation of, 236-239  
    inactivated, reactivation of, 235  
    inactivation of, 231, 234-235, 319  
    instability of, 233-236  
    nature and activity of, 233  
    postulated role in toxic reactions, 52-53  
    precautions in handling, 236  
    preparation and titration of, 245, 316-318  
    preservation of, 236, 316  
    quantitative determination of, 238  
    quantitative relations in hemolysis, 240-243  
    relationship to opsonin, 213  
    role in anaphylaxis, 278  
        in conglutination, 249  
        in phagocytosis, 214, 215  
        in virus neutralization, 262-263  
    sources of, 233  
    thermolability of, 233-234  
    titration of, 238
- Complement combining component (midpiece), 238-239
- Complement components, determination of titers of, 237-238  
    fixation of, 240  
    properties of, 237, 239  
    titers of, in various serums, 238
- Complement fixation, 19  
    applications of, 248-249  
    by specific precipitate, 20

- Complement fixation, effect of antibody amount, 240  
     of temperature on, 239-240  
     mechanism of, 20, 240, 243-244  
     rate of, 239  
     titration of antigen or antiserum, 246-248  
     with viruses, 264
- Complement fixation tests, 319-320  
     procedure for, 20, 244 ff.
- Complement reactions, cytolysis, 240-248  
     first stage of, 239-240  
     mechanism of, 239-243  
     second stage of, 240-243
- Complement titer, net, of serum, 237, 238
- Complement-antigen-antibody complex, structure of, 241
- Complementarity of antigen and antibody, 106
- Complete antibodies, Rh, 177
- Complex haptenes, definition, 53
- Components, of complement, 236-240
- Conglutination, 249
- Conglutinative complement-absorption test, 249-250
- Conglutinin, 249
- Conjugated antigens, 31. See also *Azo-proteins*.
- Contact dermatitis, 293-295  
     transfer of sensitivity, 294
- Control tube, in agglutination tests, 144
- Controls, in forensic tests, 132
- Cooke, 286
- Coombs test, for Rh antibodies, 177-178
- Coons, 92
- Cooper, 124
- Corpse, blood groups of, 182
- Cortisone, use of, in allergies, 289
- Corynebacterium diphtheriae*, exotoxin of, 2
- Coulomb attraction, 106
- Cowie and Chapin, 213
- Cravitz and Williams, 224
- Cromwell, 104
- Cross-agglutination, 28, 157
- Cross immunity test, in virus diseases, 263
- Cross match blood tests, 180, 312
- Cross reactions, nonreciprocal, 157
- Cryptagglutinoids, 177
- Culbertson, 125, 281
- Cumley, Irwin and Cole, 136
- Cytolysis, 19, 229 ff., 240-243  
     mechanism of, 231-232
- DAKIN, 42
- Danysz, 198, 199
- Danysz phenomenon, 199-200
- Dawson and Sia, 167
- Dean, 22
- Dean and Webb, 80
- Dean and Webb titration, 100, 125
- Dean, Taylor and Adair, 57
- DeFalco, 136
- Delaunay, 222
- Delayed hypersensitivities, 270, 289 ff.  
     production of, role of lipid wax in, 291
- Denaturation, of antigen by antibody, 113  
     of proteins, 40-41
- Denys and Leclef, 211
- Derivative antigens, in contact allergy, 294
- Dermatitis, contact, 293-295
- Desensitization, anaphylactic, 271, 275, 276  
     in food allergies, 289  
     in hay fever, 287
- Desoxyribonucleic acid, 167
- Determinant radicals, 31, 105  
     minimum size and number, 106
- Deutsch, 74
- Dextrans, antigenicity of, 42
- Dialysis of complement, 236  
     of serum proteins, 66
- Diapadesis, 216
- Dick test, 203-204
- Dienes and Mallory, 296
- Diethylene glycol, extraction of bacteria by, 51
- Differential adsorption, 158
- Digestion, of bacteria, by phagocytes, 220-221
- 3,5-Di-iodotyrosine as simple haptene, 30
- Dilutions, master, of serum, 309
- Dingle, 234
- Diphtheria, duration of active immunity against, 205  
     homogeneity of toxins and antitoxins, 124  
     immunity against, in human populations, 203  
     immunity in, 6  
     immunization of humans against, 10, 204-205  
     *in vitro* virulence test, 199  
     prophylaxis and therapy of humans, 208  
     subclinical cases and active immunity, 8
- Diphtheria antitoxin, flocculation of, 128-129  
     persistence in humans, 81  
     purification of, 71  
     Ramon titration of, 101  
     standard, 196

- Diphtheria toxin, chemical properties of, 188  
L<sub>d</sub> dose of, 197  
M.L.D. of, 187, 191  
molecular compositions of precipitates, 103  
pharmacologic action of, 190  
site of action of, 187
- Diplococcus pneumoniae*, Forssman antigen in, 47  
normal antibodies against, 6
- Dissociation, bacterial, 3, 161-162
- Dissociation, of antigen-antibody complexes, 105  
of antigen-antibody precipitates, 109  
of toxin-antitoxin complexes, 201-202
- Distearyl-lecithin, antigenicity of, 46
- Dixon, 82
- Dose-response curve, 192
- Dreyer and Waller, 84
- Drug allergy, 294
- Dunbar, 283
- Dye-protein antigen, fate of, after injection, 75
- Dysentery, bacillary, agglutinin titers in, 151  
natural antibodies against, 6
- EAGLE, 72, 113
- Eaton, 124
- Ecker and Pillemer, 25
- Eczema, 288  
infantile, 284
- Edwards and Bruner, 164
- Eel serum, precipitation of, 120
- Egg, precipitins for, 121, 126
- Egg albumin(s). See also *Ovalbumin*.  
antigenicity of, 25  
complement fixation by, 247  
delayed sensitivity to, 291, 293  
hen and duck, precipitin tests with, 133  
molecular composition of precipitates, 103  
molecular weight of, 25, 103  
per cent in egg white, 125  
precipitation of, 97-98, 110  
precipitins for, 120  
quantitative precipitin tests with, 102  
specificity of, 57
- Ehrlich, 76
- Ehrlich, 89, 97, 111, 202, 232
- Ehrlich and Morgenroth, 233
- Electric charges, bacterial, effect on agglutination, 149
- Electrolytes, effect on surface tension and particle charge, 108
- Electrolytes, role in agglutination, 147-150  
in agglutination and precipitation, 96, 104, 108-109  
in antigen-antibody reactions, 112-113, 118
- Electrophoresis, methods of demonstrating, 57-62  
of antibodies, 61  
of proteins, 59
- Electrophoretic fractions of normal serum, 59
- Electrophoretic mobility, factors affecting, 57-61
- Elek, 199
- Elementary bodies, 252  
agglutination of, 264-265
- Ellingson and Clark, 218
- Elliottson, 283
- Endotoxins, antigenicity of, 50  
effect on diapedesis of leukocytes, 216  
extraction of, 50-52  
M.L.D. of, 187  
nature of, 2  
properties of, 50, 186-187  
source of, 2  
symptoms produced by, 2, 187
- "Endotoxins" from digested proteins, 53
- Endpiece, of complement, 236
- Enzyme adaptation, 93-94
- Enzyme(s), intracellular, replication of, 93  
viral, receptor-destroying, 266
- Eosinophiles, source of, 14
- Epitoxoid, 112, 202
- Equilibria, in antigen-antibody reactions, 116-117
- Equivalence zone, in flocculation reactions, 129  
in precipitation, 98, 103
- Ergophore groups, 111
- Erythroblastosis fetalis, 173-175
- Erythrocytes, antigens of, in lower animals, 183-185  
average life of, 13
- Forssman antigen in, 47
- human, heterophile antigens of, 47-50  
immunization with, 86  
number of serologic types, 179
- human species antigens in, 170
- normal size and number, 13
- preparation of, for hemolysis tests, 245, 316
- reagglutination of, 117
- source of, 13
- transfused, survival time of, 180
- trypsinized, in Rh testing, 177-178
- use in precipitin test, 127
- washed, 302

- Escherichia coli*, endotoxins of, 3, 50  
 K antigens of, 146  
 production of Vi antibodies by, 45  
 Vi antigen of, 44
- Euglobulin(s), electrophoretic properties of, 66  
 of complement, 237  
 precipitation by dialysis, 66  
 precipitation by sodium sulfate, 66  
 solubility of, 66
- Euglobulin fraction, antibody content of, 69
- Evanescence allergic inflammation, 281 ff.
- Evanescence allergic sensitivity, passive transfer of, 282
- Exotoxins, 2  
 affinity for body cells, 189  
 animal susceptibility to, 190-191  
 antigenicity of, 187  
 chemical properties of, 187 ff.  
 definition, 187  
 determination of potency of, 190-193  
 differences from endotoxins, 186-187  
 immunogenicity of, 9  
 incubation period after injection of, 189  
 M.L.D. of, 187, 191  
 mode of action of, 189-190  
 neutralization of, 19  
 properties of, 186-190  
 specificity of action of, 187
- Exotoxin and endotoxin, diphtherial, immunologic relationships between, 205
- Extracts, bacterial, antigenicity of, 121
- FELIX, 45
- Felix and Bhatnagar, 222
- Felton, 67
- Felton and Bailey, 56
- Fenn, 212, 214-215, 218
- Ferguson, 185
- Fibrinogen, electrophoretic mobility of, 60
- Filtrates, culture, precipitation of, 120
- Finland and Curnen, 48
- First order antibodies, 111
- Fischel and Kabat, 281
- Fisher-Race Rh nomenclature, 175
- Fixation of complement, 232-233
- Fixed macrophages, 210
- Flagellar agglutination, effect of temperature on, 105  
 mechanism of, 148-149  
 optimum temperature for, 110
- Flagellar agglutinin, plasma fraction (Cohn) and, 69
- Flagellar antibodies, thermostability of, 56
- Flagellar antigens, 145  
 group (phase 2), 162  
 of bacteria, 160 ff.  
 specific (phase 1), 162
- Flagellar test antigens, preparation of, 308
- Fleming, 225
- Flocculation, toxin-antitoxin, 198-199, 323-324
- Flocculation reaction, quantitative, 128-129
- Flocculation time, 100  
 factors affecting, 101
- Food allergy(ies), 15, 288-289
- Food sensitivities, 288
- Forces in antigen-antibody union, 105-106
- Forensic precipitin tests, 123, 131-133
- Form variation of O antigens, 161
- Formalin, effect on antiseraums, 71
- Forssman, 47
- Forssman antibody(ies), 47  
 in human serums, 48-49  
*in vivo* effect of, 48  
 normal, in humans, 153  
 production of, 47
- Forssman antigen, antigenicity of, 47  
 chemical and physical properties of, 47  
 distribution of, 30, 47  
 extraction of, 50  
 implantation in bacteria, 47  
 in human blood cells, 49
- Forssman haptene, relationship to A substance, 171
- Fractionation of complement, 236-239
- Framework hypothesis. See *Lattice hypothesis*.
- Frei test, 292
- Freund, 86
- Friedberger, 52
- Friedenthal, 136
- Frobisher, 199, 205
- Frogs, proteins in eggs, embryos and serum, 124
- GAMMA globulin. See  $\gamma$ -Globulin.
- Gastrointestinal allergy, 289
- Gelatin, antigenicity of, 38-39
- Genes, relationship to antigens, 185
- Genetic control of serum antigens, 137
- Genetics, erythrocyte antigens and, 184-185
- Gerlach, 280, 282
- Glandular fever. See *Infectious mononucleosis*.
- Glenny, 80, 85, 196
- $\alpha$ -Globulin, electrophoretic mobility of, 59

- $\alpha_1$ -Globulin, per cent in normal serum, 60  
 $\alpha_2$ -Globulin, per cent in normal serum, 60  
 $\beta$ -Globulin, electrophoretic mobility of, 59  
per cent in normal serum, 60  
 $\gamma$ -Globulin, as antibody, 62  
beef, persistence in mouse, 92  
electrophoretic mobility of, 59  
human, prophylaxis and therapy by, 258  
natural functions and clinical uses of, 68  
per cent in normal serum, 60  
Globulins, amount in serum, 13  
cross precipitin tests with, 133  
immune, function of, 13  
prevention and treatment of disease by, 11  
specificity of, 72  
molecular weight of, 13, 64-65  
normal, function of, 13  
normal vs. antibody, 71-72  
per cent in horse serum, 125  
separation from serum, 13  
synthesis of, 90-94  
site and mechanism of, 92  
Glucolipids of Proteus and Rickettsiae, 152  
Glucose, structural formula of, 43  
Glycogen(s), antigenicity of, 42  
effect of  $Al(OH)_3$  on, 121  
Goebel, 43, 48  
Gonadal tissues, antigenicity of, 25  
Gordon, 240, 241  
Gorillas, blood groups of, 183  
Graded response, 192  
Grains, serologic relationships of, 138-139  
Gram negative bacteria, endotoxins of, 50-52  
Granulocytes, 14  
Griffith, 166  
Group flagellar antigens (phase 2), 162  
Groups, blood, determination of, 170  
Guinea pig kidney, 24  
serum adsorption by, 153  
Guinea pig kidney antiserum, shock induced by, 326  
Guinea pig serum, C1 in, 239  
Guinea pig tissues, Forssman antigen in, 47  
Guinea pigs, injection and bleeding of, 307  
  
H antigens, 161  
Hale and Smith, 222  
Halogens, effect on specificity of azo-proteins, 33-34  
Hamburger, 212  
Haptene(s), complex, antigenicity of, 25  
definition, 24, 53  
definition, 15, 24  
nature of, 24  
polysaccharide, of *B. anthracis*, 45  
of *S. typhosa*, 45  
of *Sh. dysenteriae*, 45, 51  
simple, definition, 24, 53  
Haptophores, 111  
Harris, 78  
Hartley, 46  
Haurowitz, 92, 107  
Hay fever, 15, 283, 287-288  
early studies of, 283  
Healey and Pinfield, 200  
Heat, effect on antibody, 56  
of serologic reactions, 104  
Heated proteins, antigenicity and specificity of, 41  
Hegedes and Greiner, 237  
Heidelberger, 43, 71, 82, 87, 94, 101, 102, 104, 109, 115 ff., 127, 148, 238, 240, 241, 243  
Heidelberger and Kabat, 117, 146  
Hektoen, 74, 79, 85  
Hektoen and Ruediger, 218  
Hemagglutination, 169 ff.  
blood grouping, 311  
effect of temperature on, 110  
Hirst, 154  
in titration of Vi antibodies, 127  
viral, 266  
inhibition by immune serum, 266-267  
mechanism of, 266  
Hemagglutination inhibition test, viral, 154, 226-267  
Hemagglutinins, anti-A, in antisheep serums, 171  
cold, 153-154  
normal, in rabbits, 110  
sheep, in infectious mononucleosis, 153  
in serum sickness, 153  
vaccinia and variola, 265  
viral, nature of, 266  
Hemocyanin(s), antigenicity of, 25  
molecular composition of precipitates of, 103  
Hemocyanin-antihemocyanin, heat of reaction of, 104  
Hemocyanin-azo-p-aminobenzolic acid, 87  
Hemoglobins, serologic relationships among, 29

- Hemolysin(s). See also *Ambococeptor*.  
 antisheep, in anti-A serums, 171  
 production of, 49  
 bacterial, 2  
 rate of reaction with R.B.C., 104
- Hemolysins and hemagglutinins, anti-sheep, in infectious mononucleosis, 49  
 in serum sickness, 49
- Hemolysins vs. hemagglutinins, anti-sheep, 49
- Hemolysis, 19, 230  
 complement and amboceptor required for, 240-243  
 effect of Mg<sup>++</sup> on, 243  
 of NaCl on, 243  
 of pH on, 243  
 of reagent volumes on, 242-243  
 of temperature on, 243  
 endpoint(s), 50%, 246  
 50% and 100%, 242  
 mechanism of, 231  
 number of molecules of complement and amboceptor required for, 243  
 rate of, factors affecting, 242-243  
 S-curve of, 242
- Hemolysis test, procedure, 19, 316-318
- Hemolytic disease of the newborn, 174
- Hemophilus influenzae*, typing of, by Quellung, 130
- Heparin, as anticoagulant, 301
- Herdegen, Halbert and Mudd, 87
- Heredity, role of, in allergies, 286
- Herpes simplex, activation of, by fever, 255  
 immunity in, 260
- Heterogeneity of antibodies, 94  
 effect of number of injections on, 87  
 factors affecting, 87-88
- Heterophile antigen(s), Buchbinder, 48  
 definition, 47  
 of human erythrocytes and type 14 pneumococci, 48  
 of type 1 pneumococci, 48
- Hexuronic acids, 44
- Hirst, 266
- Hirst hemagglutination test, 154, 266-267
- Hirschfeld, 6
- Histamine, animal susceptibility to, 279  
 distribution of, 279  
 pharmacologic action of, 274  
 role in anaphylaxis, 274, 279  
 in Arthus reaction, 281  
 in hypersensitivity reactions, 270  
 in skin reactions, 282
- Histamine shock, demonstration of, 326
- Histones, antigenicity of, 38
- Histoplasmin, 292
- Hives (urticaria), 284, 288
- Hole and Coombs, 249
- Holtzman, 47
- Homogeneity of proteins, precipitation in study of, 124
- Hooker, 292
- Hooker and Boyd, 87, 107
- Horse serum, hypersensitivity to, 194
- Horsfall and Goodner, 46
- H-substance, 282
- Human serum, C1 in, 239
- Humoral hypothesis of anaphylaxis, 278
- Hyaluronidase, 2
- Hybrids, plant, serologic relationships of, 139  
 serologic relations to parents, 39, 136, 184
- Hydrogen bonds, 91, 106, 107  
 in complement fixation, 240
- Hydrolysis of proteins, effect on antigenicity, 41-42
- Hydrophobia, immunization against, 9
- Hyperergy, 269
- Hypersensitivity(ies), 15, 269  
 delayed, 270, 289 ff.  
 immediate, 270 ff.  
 common characteristics of, 270  
 precipitins in, 121, 126  
 production of, 269 ff.  
 spontaneous, 283 ff.  
 types of, 270 ff.  
 factors affecting, 293
- Hypodermic needles, care of, 303-304  
 sizes of, for various purposes, 303
- Hypoergy, 269
- IDIOPATHY, 283
- Immediate hypersensitivities, 270 ff.
- Immune antibodies vs. natural antibodies, 5
- Immune phagocytosis, 220
- Immunity, acquired, 8  
 active, 8  
 against viruses, 253 ff.  
 mechanism of, 260  
 antitoxic, effectiveness of, 205  
 antiviral, actively acquired, 254-258  
 conditions necessary for, 254  
 duration of, 254  
 infection or persistence, 255  
 diphtherial, duration of active, 205  
 passive, against viruses, 258-259  
 tetanial, 205-206
- Immunity and allergy, 296-297
- Immunity tests, in virus diseases, 263
- Immunization, 17  
 active-passive, 258  
 antityphoid, agglutinin titers after, 83  
 artificial active, antiviral, 255-258  
 effect of injection route on, 142

- Immunization, length of, effect on antibodies, 87  
of humans, against diphtheria, 204-205  
bacterial extracts and, 52  
multiple antigens in, 85  
of rabbits, 141-142  
passive, with antitoxin, 206-208  
prolonged, effect of, 31  
routes of injection, 26  
schedules for, 142-143  
with attenuated organisms, 9  
with bacterial extracts, 9  
with exotoxins, 9  
with killed organisms, 9  
with multiple antigens, 57
- Immunogenetics, 185
- Immunogenicity, definition, 22  
of endotoxins, 51
- Inactivation of complement, 231, 234, 319
- Inclusion bodies, 251-252
- Incomplete antibodies, 97  
Rh, 177-178
- Incubation period, after toxin injection, 188
- Indicating mixture, toxin-antitoxin, 323
- Individuality of human blood, 179
- Induced antigenic variations, 165-167
- Infantile eczema, 284
- Infection, allergy of, 289-293
- Infection immunity, antiviral, 255
- Infectious disease, nature of, 1
- Infectious mononucleosis, 153  
anamnestic reactions accompanying, 84  
sheep hemolysins and hemagglutinins in, 49  
syphilitic reagin in, 131
- Infectious mononucleosis antigen, 49
- Influenza, hemagglutination by, 266  
immunity in, 260  
immunization against, 87, 257
- Influenza antibodies, plasma fraction (Cohn) and, 69
- Ingested bacteria, disposal of, 220-221
- Ingestion, by phagocytes, factors affecting, 217-218  
methods of, 217  
by protozoa, 216-217
- Inheritance, of blood groups, 172  
of MN blood factors, 173  
of Rh factor, 174
- Inhibiting antibody, 97
- Inhibition phenomenon, in precipitation, 98
- Inhibition test(s), 24, 106, 171  
in blood grouping, 182
- Injected bacteria, fate of, 210, 212
- Injection(s), of animals, 303-308  
number of, effect on antibodies, 87
- Injection route, effect on antibody formation, 77, 78
- Insulin, precipitins for, 121, 126
- Interfacial precipitin test, 18, 123, 313-315
- Interference phenomenon, 254
- Intergrading forms, biologic, 251
- Intermolecular forces and antigen-antibody reaction, 105-106
- International blood groups, 170
- Intracellular enzymes, replication of, 93
- Invasiveness, 2
- In vitro* anaphylaxis, 275-276
- In vitro* virulence test, diphtheria, 199
- Iodo-ovalbumin, labeled with I<sup>131</sup>, persistence in rabbit, 92
- Iodoprotein, serologic reactions of, 30
- Ipsen, 191 ff., 322
- Irradiation, of viruses, 257
- Irwin, 136, 184
- Isoelectric point, of proteins, 59
- Isohemagglutination, 169 ff.
- Isohemagglutinins, 6, 110  
anamnestic increase of, 84  
human, molecular weights of, 65  
normal, appearance of, 172  
plasma fractions (Cohn) and, 69
- Isoimmunization, 26  
by Rh antigen, 174  
with organ extracts, 46
- Isophile antibodies, 48
- JANSKY, 169-170
- Jenner, 265, 292
- Jobling and Peterson, 53, 278
- K antigens, 146
- Kabat, 276, 277
- Kabat and Mayer, 65
- Kahn, 131, 248
- Kahn test, 130
- Kauffmann, 164
- Kauffmann-White Salmonella antigenic schema, 164
- Kendrick, 224
- Keysser and Wassermann, 53, 278
- Kidney antiserum, shock induced by, 326
- Kidney extracts, guinea pig, preparation of, 302
- Klebsiella pneumoniae*, agglutination of, 110  
capsules, antigenicity of, 45  
surface phagocytosis of, 220  
typing of, by Quellung, 130
- Koch, 290
- Koch phenomenon, 290
- Konigsberg Stammbaum, 138-139

- Kowarski, 138  
 Kraus, 120  
 Krebs-gelatin solution, 211, 321
- L antigens of *E. coli*, 146  
 $L_+$  dose, of diphtheria toxin, 197  
 of toxin, 202  
 $L_+$  and  $L_0$  doses of toxin, relationship between, 202  
 $L_0$  dose of toxin, 202  
 Lactose, structural formula of, 43  
 Lancefield test, 130  
 Landsteiner, 24, 31, 33, 36, 42, 46, 89, 103, 105, 150, 169, 183, 294  
 Landsteiner and Levine, 171, 172, 173  
 Landsteiner and Wiener, 174  
 Landy and Webster, 127  
 Latent period of antibody production, 79 ff.  
 Lattice hypothesis, of agglutination, 148–150  
 of antigen-antibody reaction, 113–117  
 of precipitation, 98  
 of viral hemagglutination, 154, 266
- $LD_{50}$ , calculation of, 192–193  
 definition of, 192  
 determination of, 325
- Lecithin, antigenicity of, 45  
 role in agglutination and precipitation, 46
- Lecithinase, of *Clostridium perfringens*, 190
- Lederberg, 164, 166
- Leishman, 212
- Length of immunization, effect on antibodies, 87
- Lens proteins, antigenicity of, 25
- Lepromin, 292
- Leprosy, syphilitic reagin in, 131
- Leukins, 221
- Leukocytes. See also *White blood cells*.  
 increase of, in infections, 221  
 ingestion by, 216–218  
 kinds and normal percentages, 14  
 migration of, 216  
 normal life of, 14  
 normal size and number, 13  
 polymorphonuclear, phagocytic activity of, 210  
 relation to antibody formation, 76–77  
 preparation of, for phagocytosis, 211
- Leukotaxine, nature and action of, 216
- Levine, 173, 175, 176
- Levine and Stetson, 174
- Levinson, 257
- Lewis, 279, 282
- Lf dose of toxin or toxoid, 198  
 determination of, 323–324
- Lipids, antigenicity of, 38, 45–46, 54  
 effect of kaolin on, 121  
 chemical nature of, 45  
 distribution of, 45  
 extraction of, 45  
 in syphilis test antigens, 130–131  
 role in agglutination and precipitation, 46  
 serologic role of, 46
- Lipopolysaccharides, antigenicity of, 48
- Liver, antibody content of, 74  
 as site of antibody formation, 92
- Loring, 264
- Loveless, 286
- Lymph nodes, antibody formation in, 76, 77, 78  
 response to regional injection of antigen, 76, 77  
 role in delayed type sensitivity, 293  
 transfer of, from immunized to normal animals, 78
- Lymphocytes, functions of, 15  
 life of, 77  
 role in antibody formation, 76, 77  
 source of, 14
- Lymphoid cells, effects of adrenal cortical hormones on, 85
- Lysis, 19
- McKEE and Hale, 259
- McKendrick, 212
- MacLeod and Pappenheimer, 191
- McMaster and Hudack, 76
- McMaster and Kruse, 92
- Maaløe, 212, 214
- Macrophages, 14, 210
- Macroscopic slide agglutination test(s), 144, 310–311
- Maier, 46
- Magendie, 271
- Magnesium ions, effect on hemolysis, 243
- Malaria, syphilitic reagin in, 131
- Mallein, 292
- Manwaring, 234, 274
- Marrack, 105, 113–114, 148
- Martin, 125, 126
- Mass action hypothesis, of antigen-antibody reaction, 112
- Maturation immunity, 6
- Mayer, 242–243
- Measles, immunity against, 89  
 use of gamma globulin in, 258
- Meats, identification of, by precipitin tests, 133
- Mechanism of antigen-antibody reactions, 118  
 of toxin-antitoxin reaction, 199–202
- Medico-legal blood typing, 180–182
- Menkin, 216

- Merling, 222  
Metchnikoff, 211  
Methyl radicals, effect on specificity of azoproteins, 33-34  
Meyer and Loewenthal, 76  
Mez and Ziegenspeck, 139  
Mice, injection of, 307-308  
Micrococci, spreading factors of, 2  
Microphages, 210  
Microscopic slide agglutination test, 143-144, 310  
Midpiece, of complement, 236  
Milk, Brucella antibodies in, 151  
    precipitation of, 120  
    proteins, serologic relationships of, 29  
Mineral oil. See also *Paraffin oil*.  
    adjuvant effect of, 86-87  
    persistence in local depot, 87  
Minimal lethal dose (M.L.D.), definition of, 192  
    determination of, 322  
    of exotoxins, 187, 191  
    vs. LD<sub>50</sub>, 193  
Mirror test, 158  
Mitochondria as sites of antibody formation, 92  
Mixed agglutination, 147-148  
MN blood factors, 172-173  
    occurrence of, 173  
Modified enzyme hypothesis of antibody formation, 93-94  
Molecular compositions of antigen-antibody complexes, 103  
Molecular weights, determination of, 62-66  
    of antibodies, 64-65  
    of exotoxins, 188  
    of proteins, 107  
    of serum proteins, 64-65  
Moloney test, 202  
Monkeys, blood groups of, 183  
    precipitin reactions of, with antihuman serum, 134-136  
Monocytes, source, 14  
Monospecific serums, 157  
Moody, 137  
Morgan and Partridge, 50-51  
Morphologic classification of bacteria, 155  
Moss, 169-170  
Mouse protection tests, 324-325  
Mucin, preparation of, 324  
Muco-euglobulin, of complement, 236, 237  
Mucoid organisms, agglutination of, 110, 146  
Mudd, 90, 217, 218  
Muether and MacDonald, 127  
Muir and Browning, 249  
Multiple antigens, antibody response to, 85  
Mummies, blood group substances in, 182  
Mumps, antibody, plasma fraction (Cohn) and, 69  
Muscle proteins, serologic reactions, 29  
*Mycobacterium tuberculosis*, adjuvant effect of, 86  
    resistance to phagocytic digestion, 14  
    survival in leukocytes, 221  
Myers, 120, 133  
NASAL prophylaxis, by antiserum, 258-259  
Native proteins, antigenicity of, 38-40  
Natural antibody(ies), 72. See also *Natural antibodies*.  
    origin of, 5  
    antierythrocyte, 6  
    isoagglutinins, 6  
Natural immunity, 4, 5  
    role of heredity in, 6  
Negative phase, 223  
Neisseria, endotoxins of, 50  
*Neisseria intracellularis*, typing of, by Quellung, 130  
Nembutal, dose of, for rabbits, 307  
Neufeld and Rimpau, 213  
Neufeld Quellungreaktion, 130  
Neurath, 66  
Neutralization, of toxins by antitoxins, 202, 322-323  
    of toxins, role of lipids in, 46  
    of viruses, by serum, 261-263  
    mechanism of, 262-263  
Neutralization index, viral, 262  
Neutralization method of precipitin titration, 125  
Neutrophiles, phagocytic activity of, 210  
Newborn, hemolytic disease of, 174  
Nitro groups, effect on specificity of azoproteins, 34  
Nogueira, 15  
Nonspecific protein therapy, 84  
Nonususceptibility, 4  
    accessory growth factors and, 5  
    body temperature and, 5  
    diet and, 5  
    of lower animals to human diseases, 4  
    of man to animal diseases, 4  
Normal antibody(ies), 131, 150, 152, 158, 169. See also *Natural antibodies*.  
    antisheep, in humans, 48  
Forssman, 153  
    occurrence, 6  
    precipitation test for, 18  
thermostability of, 213

- Normal phagocytosis, 220  
 Northrop, 71  
 Northrop and DeKruif, 108, 109, 147  
 Novy and DeKruif, 53  
 Number of determinant groups in antigens, 107  
 Nutrition, host, effect on resistance to viruses, 254  
 Nuttall, 133 ff.
- O antibody, effect on phagocytosis of *S. typhosa*, 222  
 O antigens, 161  
 O-inagglutinability, 146, 161  
 Obermayer and Pick, 30  
 Occlusion hypothesis of antigen-antibody reaction, 117  
 Oil-in-water emulsions, preparation of, 302  
 Old tuberculin, *S. marcescens* coated with, 127  
 Olitsky, 253  
 Olitsky and Casals, 257  
 Oncley, 65, 68, 69  
 Opsonic index, 19  
     determination of, 222–223, 321  
 Opsonic theory of phagocytosis, 210–211  
 Opsonin(s), 211  
     Brucella, 223–224  
     detection of, 321  
     measurement of, 222–224  
     relationship to complement, 213  
     thermolability of, 213  
 Opsonization, artificial, 225, 320–321  
 Opsonocytophagic test, 223–224  
 Optimal proportions, determination, 125  
     in toxin-antitoxin reaction, 199  
 Optimal ratio, constant antibody, 101  
     constant antigen, 101  
     of antiserum to antigen, 100  
     titration, procedure, 315–316  
 Organ specificity, 29  
 Osmotic pressure, effect on phagocytosis, 226  
 Osterman and Rettger, 110  
 Otto, 271, 274  
 Oudin, 124  
 Ourangs, blood groups of, 183  
 Ovalbumin. See also *Egg albumin*.  
     effect of phylogenetic relationships on cross reactions of, 39–40
- P blood factor, 173  
 Pangborn, 131  
 Pappenheimer, 66, 71, 124, 128, 190, 201  
 Pappenheimer and Robinson, 201  
*Paracolobactrum ballerup*, production of Vi antibodies with, 45, 127
- Paraffin oil. See also *Mineral oil*.  
     adjuvant effect of, 86–87  
     as adjuvant, 302  
 Parasitism, 251  
 Paratyphoid fever, agglutinins in, 150–151  
 Park and Schroder, 207  
 Partial antigen, 16  
 Passive anaphylaxis, 274–275  
     demonstration of, 326  
 Passive Arthus reaction, 280–281  
 Passive immunity, against viruses, 258–259  
     nature and duration, 10  
 Passive immunization, antitoxic, 206–208  
     uses of, 10  
 Passive sensitization, 21  
 Pasteur, 3, 256  
 Pasteur treatment for rabies, 256  
 Pasteurella, Forssman antigen in, 47  
 Patch test, in contact dermatitis, 295  
 Paternity, disputed, blood grouping and, 181–182  
 Pathogenicity, 1  
 Pauling, 90, 105–106, 114, 115  
 Peluffo, 146  
 Peptone shock, 278  
 Persistence immunity, antiviral, 255  
 Pertussis, opsonocytophagic tests in, 224  
 Petermann, 71  
 Petermann and Pappenheimer, 195  
 Pfeiffer, 229  
 pH, effect on serologic reactions, 109  
 Phagocytes, kinds of, 210  
 Phagocytic index, 212  
     determination of, 321  
 Phagocytic ingestion, factors affecting, 216–218  
 Phagocytosis, by granulocytes, 14  
     by R-E cells, 14  
     demonstration of, 320–321  
     disposal of ingested bacteria after, 220–221  
     effect(s) of bacteriophage on, 218  
       of calcium chloride on, 211  
       of chemotherapy on, 224  
       of immune serum on, 211, 213  
       of ions on, 217–218  
       of leukotaxine on, 216  
       of normal serum on, 213  
       of temperature on, 218  
     effectiveness in removing bacteria from blood, 15  
     factors affecting, 214–216  
     first stage of, 214–216  
     immune, 220  
     in daphnia, 211  
     inhibition of, by antiseptics and antibiotics, 225–227

- Phagocytosis, methods of studying, 211-212  
normal, 220  
of capsulated bacteria, 19  
of pneumococci, inhibition by homologous polysaccharide, 214  
of quartz and carbon particles, 214-216  
opsonic theory of, 210-211  
resistance of inagglutinable *S. typhosa* to, 222  
role in disease, 221-225  
of antibody in, 19, 214, 220  
of complement in, 214, 215  
of serum constituents in, 213-214  
second stage of, 216-218  
spontaneous, 213, 220  
surface, 218-220  
types of, 220  
use of, in evaluating antiseptics and antibiotics, 225-227
- Phagocytosis tests, procedure, 19, 320-321
- Pharmacologic action, of exotoxins, 190  
of histamine, 274
- Phase variation, induction of, 162 ff.  
of flagellar antigens, 162 ff.
- Phospholipid-protein-polysaccharide of *Sh. dysenteriae*, 51
- Photronreflectometer, 99
- Phylogenetic relationships, precipitation in study of, 124
- Phylogeny, and serologic specificity, 29  
and antigenicity of proteins, 39-40  
of amphibia, serologic study of, 136  
of primates, 183
- Physical allergy, 295-296
- Piper, 148-149
- Pillemer, 124, 188, 189, 237, 240
- von Pirquet, 290
- Plague, precipitin test for, in rats, 129
- Plants, serologic relationships of, 137-139
- Plasma, 12
- Plasma cells, antibody formation in, 76  
description, 77  
functions of, 15
- Plasma fractions (Cohn), alcohol precipitation of, 67-69  
electrophoretic properties of, 67-69  
influenza antibodies and, 69  
method of separation of, 67-69  
mumps antibody and, 69  
proportions in normal plasma, 68
- Plasma proteins, site and mechanism of synthesis of, 92
- Plasmagenes, 93
- Pneumococcal agglutinins and precipitins, identity of, 147
- Pneumococcal antibody(ies), molecular weights of, 64-65, 66  
production in man, 81
- Pneumococcal polysaccharide(s), 22, 24  
precipitation of, 56, 110, 125  
purification of antibodies for, 71  
quantitative studies of precipitation of, 102
- Pneumococcal precipitins, isoelectric point of, 71
- Pneumococcus(i), autolysis of, 3  
capsulated, surface phagocytosis of, 219-220  
capsulation and virulence, 3  
hypersensitivity to, 296  
immunization of humans with, 82  
injection with multiple types, 85  
reagglutination of, 117  
spreading factors of, 2  
types, specificity of, 43  
type I, heterophile antigen of, 48  
polysaccharide, antigenicity of, 44  
type 3, polysaccharide, molecular com-  
positions of precipitates, 103  
reaction with antibody, 116-117  
structure and molecular weight of, 44
- type 14, heterophile antigen of, 48
- type transformations of, 166-167
- typing of, by Quellung, 130
- Pneumonia, atypical, cold agglutinins in, 154
- Poison ivy, 294, 295
- Polar radicals, 105  
effect on solubility of antibody, 114  
role in agglutination, 148  
in antigen-antibody reactions, 117, 118
- Poliomyelitis, immunization against, 87, 257-258  
subclinical cases and active immunity, 8
- Pollen extracts, antigenicity of, 121
- Polymorphonuclear neutrophiles, sources and functions of, 14
- Polypeptides, capsular, 2
- Polysaccharide antigens, of *E. coli*, 146
- Polysaccharide(s), alteration during purification, 44  
antigenicity of, 38, 42-45, 54  
bacterial, 43-45  
resistance of, to digestion, 94
- capsular, 2
- pneumococcal, 24  
antigenicity of, 25  
immunization of humans with, 82  
reaction with antiserum, 22
- serologically active, 43-45
- structure of, 43

- Polysaccharide(s), use in quantitative studies, 102-103
- Polysaccharide-lipid-protein complexes, 2
- Polysaccharide-phospholipid complexes of bacteria, 50-52
- Polysaccharide - phospholipid - protein complex, antigenicity of, 50
- Polysaccharide-protein complexes, 43  
antigenicity of, 44  
of *S. typhosa*, antigenicity and toxicity of, 51
- Pope, 71, 124
- Potassium oxalate, as anticoagulant, 301
- Prausnitz and Kustner, 285
- Prausnitz-Küstner test, 285-286, 295  
in food allergies, 289
- Precipitate(s), amount of, factors affecting, 100  
composition of, 102-103  
dissociation of, 71  
molecular formulas of, 103  
quantitative analysis of, 101-103
- Precipitation, 18  
diagrammatic representation of, 115-116  
effect of pH on, 109  
of temperature on, 110  
equivalence zones in, 103  
factors affecting, 101, 108-111  
flocculation time in, 100  
in presence of unrelated proteins, 57  
in study of homogeneity of proteins, 124  
of phylogenetic relationships, 124  
inhibition phenomenon in, 98  
mechanism of, 118  
necessity for concentrated antibody in tests, 122  
of culture filtrates, 120  
photoelectric measurement of, 99-100  
ring or interfacial test, 18, 123, 313-315  
role of electrolytes in, 96, 108-109  
of lipids in, 46  
two-stage nature of, 96-97  
use of antigen-coated inert particles, 125-127
- viral, 264-266
- zone phenomenon in, 97-103
- Precipitation test(s), procedures, 18, 313-316
- Precipitin(s), 120  
anti-pollen, 121  
for insulin, 121, 126  
formation in man, 120  
in serum allergy, 285  
in serums of tuberculous patients, 127  
laboratory production of, 121  
passive sensitization by, 274
- Precipitin(s), production of, antigen doses for, 121  
role of, in allergies, 285
- Precipitin curve, quantitative, with horse antitoxic serum, 127-129  
with rabbit anti-egg albumin serum, 127-129
- Precipitin production, species differences, 121
- Precipitin reaction, phylogenetic applications of, 133-139
- Precipitin serums, antihuman, preparation of, 132
- Precipitin test(s), antiserum dilution method for, 125-126  
diagnostic, 129-131  
forensic, 131-133  
in identification of meats, 133  
in study of plant materials, 137-139  
in typing of bacteria, 130  
interfacial, 18, 123, 313-315  
neutralization method, 125  
optimal proportions method, 125  
optimal ratio titration, 315-316  
Oudin agar column method, 124  
procedures for, 121-129  
quantitative, 127-129  
ring (interfacial), 313-315  
simple mixtures, 122-123  
early use of, 133  
temperature for, 122  
with animal serums, 134-137  
with serum of Siberian mammoth, 136
- Precipitinogen, 120
- Preservation, of serum, 143, 307
- Prezone. See *Prozone*.
- Primates, blood groups of, 183  
lower, precipitin reactions of, 134-136
- Protamines, antigenicity of, 38
- Protection tests, 21  
mouse, 324-325
- Protective antibodies, antiviral, 264
- Protein(s), alkali-treated, antigenicity of, 42  
antigenicity of, 38-42  
effect of source on, 39-40  
blood, specificity of, 40  
chemically modified, specificity of, 30  
coagulated, antigenicity of, 41  
denatured, antigenicity and specificity of, 40-42  
determinant radicals of, 107  
effect on antigenicity of polysaccharides, 44, 51  
egg, serologic specificity of, 40  
heated, antigenicity and specificity of, 41

- Protein(s), homogeneity of, precipitation in study of, 124  
hydrolysis of, effect on antigenicity, 41-42  
identification of, 123  
isoelectric point of, 59  
lens, antigenicity of, 25  
milk, specificity of, 40  
molecular weights of, 107  
muscle, serologic reactions, 29  
native, antigenicity of, 38-40  
of *Sh. dysenteriae*, relation to *S. typhosa*, 51  
racemization of, 42  
seed, specificity of, 40  
serologic valences of, 107  
specificity of, 53-54  
structure of, 107  
synthesis of, 90-94
- Protein poisons, 52
- Proteoses, antigenicity of, 42
- Proteus X strains, 152
- Prototoxoid, 112, 202
- Prozone(s), factors affecting, 97  
hypotheses of, 97  
in agglutination, 96-97, 150  
in phagocytosis, 223  
in Widal test, 143
- Pseudoglobulin(s), antibody content of, 69  
electrophoretic properties of, 66  
precipitation by ammonium sulfate, 195  
separation from serum by dialysis, 66  
solubility of, 66
- Pseudoglobulin 1, precipitation by sodium sulfate, 66
- Pseudoglobulin 2, precipitation by sodium sulfate, 66
- Psittacosis, slide agglutination test in, 264
- QUANTITATIVE agglutination test, 146-147
- Quantitative analyses, of toxin-antitoxin precipitates, 200-201
- Quantitative antibody determination, 101-103
- Quantitative hypothesis of antigen-antibody reaction, 115
- Quantitative precipitin test, 127-129  
uses of, 129
- Quantitative studies, of anaphylaxis, 276-277  
of passive Arthus reaction, 281
- Quellungreaktion, 130  
of *E. coli*, 146
- RABBITS, anesthesia of, 307  
inbred, antibody production by, 6
- Rabbits, injection and bleeding of, 304-308  
systematic position of, 137
- Rabbit and hare serum proteins, serologic relationships of, 39
- Rabies, immunization against, 256
- Racemization of proteins, 42
- Raffel, 178, 281, 291
- Raistrick and Topley, 50
- Ramon, 198
- Ramon titration of antitoxin, 101, 198-199
- Rate of antigen-antibody reaction, 108
- R.B.C. See *Erythrocytes*.
- R-E system. See *Reticuloendothelial system*.
- Reagglutination, of sensitized bacteria, 117  
of sensitized R.B.C., 117
- Reagin(s), allergic, 270  
in serum allergy, 285  
occurrence of, 286  
passive transfer of, 285-286  
properties of, 285-286  
role of, in allergies, 285-286
- Reagin, syphilitic, 21, 248  
electrophoretic properties of, 61  
in other diseases, 131  
nature of, 131
- Receptor-destroying enzyme, viral, 266
- Receptors, viral, 266
- Reciprocal adsorption, 158-160  
procedure, 312-314
- Red blood cells. See *Erythrocytes*.
- Reed and Muench, 192
- Reimmunization, 84-85
- Replication of enzymes, 93
- Residue antigens, 43
- Resistance, 4, 6  
effect of previous disease, 8  
genetic control of, 7  
racial, 8  
to viruses, 253-254
- Reticuloendothelial system, 210  
blockade of, 75  
distribution of, 14  
effect of injury to, 75  
functions of, 14-15  
role in antibody formation, 75
- Reversed Arthus reactions, 280
- Reversed passive anaphylaxis, 278
- Reversibility, of antigen-antibody reaction, 116
- Reversion of agglutination, 105
- Rh antibody(ies), albumin, 177  
blocking, 177  
complete, 177  
cryptagglutinoids, 177  
incidence of, 175-176

- Rh antibody(ies), incomplete, 177–178  
 saline, 177  
 valence of, 178
- Rh antigen(s), incidence of, 175  
 in rhesus monkeys and humans, 174
- Rh factor(s), 173–179  
 nomenclature of, 175  
 role in erythroblastosis fetalis, 174  
 in transfusion reactions, 174
- Rh serums for typing, 178
- Rh subtypes, 175–176
- Rh typing, procedure, 179, 311–312
- Ribonuclease, antigenicity of, 25
- Rice and Avery, 249
- Rice and Crowson, 238
- Richel, 271
- Rickettsiae, complement fixation with, 249  
 glucolipids of, 152
- Rickettsial diseases, agglutination of *Proteus X* strains in, 152
- Ring (agglutination) test in brucellosis of cattle, 151
- Ring (precipitin) test, 18, 123, 313–315  
 forensic, 132–133  
 in identification of meats, 133  
 in thermoprecipitin reaction, 129  
 in typing streptococci, 130
- Rivers, 251
- Robertson and van Sant, 220
- Rodents, blood proteins of, serologic properties, 137
- Römer, 197–198
- Römer titration of diphtheria antitoxin, 198
- Rosenau and Anderson, 271
- Rough form(s), of bacteria, 161–162  
 of *Sb. dysenteriae*, 51
- Rous and Jones, 221
- Roux and Yersin, 204
- SABIN, 75, 152, 222, 258
- Sachs and Klopstock, 45
- Sachs, Klopstock and Weil, 46
- Saline, 18, 302
- Saline agglutinins, Rh, 177
- Saline-peptone, for diluting tetanus toxin, 322
- Salk, 257
- Salk vaccine, field trial of, 257–258
- Salmonella, antigenic structures of, 161–166  
 Forssman antigen in, 47
- Salmonella abony*, 166  
*anatum*, 165, 166  
*ballerup*, Vi antibodies in antiserum against, 45, 127  
*bispebjerg*, 164
- Salmonella breslau*, phagocytosis of, 212
- cambridge*, 164, 165
- cardiff*, 166
- champaign*, 161
- cholerae-suis*, H and O antigens of, 160
- dar-es-salaam*, 164
- enteritidis*, resistance of mice to, 7
- fayed*, 164
- glosti up*, 164
- illinois*, 164
- jerusalem*, 164
- manchester*, 164
- melleagris*, 164, 165, 166
- miami*, 164
- montevideo*, 164, 166
- new brunswick*, 165
- newington*, 165
- newport*, 162
- oranienburg*, 166
- paratyphi*, acquisition of Forssman antigen by, 47
- paratyphi A*, 163, 166
- paratyphi B*, 161, 162, 163, 166
- paratyphi C*, 162
- potsdam*, 164
- salinatis*, 164
- sundsvall*, 164
- thompson*, 166
- typhimurium*, 162, 163, 166  
 immunization of mice against, 17
- typhosa*, 3, 164, 165, 166  
 acquisition of Forssman antigen by, 47  
 antibody titers in rabbits injected with, 86  
 bacteriolysis of, 20  
 boiled, adsorption of Vi antibodies by, 45  
 Boivin antigen of, 50  
 flagellar agglutination of, 148–149  
 immunization of animals with, 17  
 normal antibodies against, 6  
 phagocytosis of, 15, 222  
 polysaccharide hapten of, 45  
 precipitation test with, 120  
 somatic agglutination of, 149  
 somatic antigen, composition of, 51  
 Vi agglutination of, 149  
 Vi antigen of, 44, 146
- wien*, 164
- worthington*, 164
- Salt agglutination, 108
- Sanger and Race, 173
- Scarlet fever, immunity against, in human populations, 203  
 subclinical cases and active immunity, 8
- Schick, 202, 296

- Schick reaction, significance of positive, 203  
Schick test, 202-203  
    as booster, 205  
Schiff and Boyd, 132, 182  
Schlepper, 46  
Schlesinger, 254, 259  
Schmidt, 109  
Schultz and Dale, 275  
*Schultz-Charlton test*, 204  
Schultz-Dale test, 275-276  
Second order antibodies, 111  
Second stage, of agglutination, 147-150  
    of antigen-antibody reaction, 113  
    specificity of, 117  
Secondary antigenic stimuli, effects of, 82-85  
Secondary response, 82-85  
    to pneumococcal polysaccharide, 82  
Secretors, 171  
Sedimentation constant ( $s_{20}$ ), 63-64  
Self-markers, 93  
Sensitivity, anaphylactic, production of, 293  
    tuberculin type, production of, 293  
    to various agents, 292  
Sensitization, allergic, 270  
Sensitizing antibody, site of production of, 297  
Serologic classification of bacteria, 155  
Serology, definition of, 17  
*Serratia marcescens*, use in precipitation test, 127  
Serum(s), 12  
    animal, precipitin reactions of, 134-137  
    anticomplementary, 235  
    bactericidal substances in, 229  
    bird, precipitin tests with, 135 ff.  
    dialysis of, 66  
    eel, precipitation of, 120  
    globulins in, 13  
    master dilutions of, preparation, 309  
    preparation of, 16  
    preparation and preservation of, 307  
    preservation of, 16, 143  
    Serum albumin, specificity of, 57  
    Serum allergy, 284  
    Serum antigens, genic control of, 137  
    Serum neutralization tests, 261 ff.  
    Serum proteins, alcohol precipitation of, 66-69  
        alterations in, during separation of, 70  
        difficulties in study of, 69-70  
        electrophoretic fractions, concentrations of, 60  
        electrophoretic homogeneity of, 61  
        electrophoretic properties of, 59 ff.  
        intergradations of, 70  
    Serum proteins, molecular weights of, 62 ff., 64-65  
        per cent in serum, 12  
        physical properties of, 57 ff.  
        precipitation by neutral salts, 66  
        salting out, 13  
        serologic relationships among, 29  
        site of synthesis of, 15  
        solubilities of, 66-70  
        specificity of, 29  
        terminology of, 69-70  
Serum sickness, 284-285  
    antigen of, 49  
    precipitins in, 121  
    sheep hemolysins and hemagglutinins in, 49  
Sevag, 93  
Shaking, effect on serologic reactions, 111  
Sheep R.B.C., for hemolysis, 316  
*Shigella dispar*, antigenic analysis of, 159  
    antigenic structure of, 28  
    cross agglutination of, 28  
*dysenteriae*, Boivin antigen of, 50  
    exotoxin of, 2  
    Forssman antigen in, 47  
    phospholipid-protein-polysaccharide of, 51  
    polysaccharide haptene of, 45  
    virulence of, 3  
*sonnei*, virulence of, 3  
Shock, induced by anti-kidney serum, 326  
Shock organs or tissues, 283  
Shocking injection, anaphylactic, 272-273  
Side-chain hypothesis, of antibody, 89  
    of antigen-antibody reaction, 111  
Simple haptenes, azo compounds as, 32  
    definition, 53  
Single factor serums, 144, 157  
Sizes, of determinant groups, 106  
    of serum proteins, 65-66  
Skin, antibody formation in, 76  
Skin reactions, in allergies, 282  
Skin test, for serum sensitivity, 285  
    in food allergies, 289  
    in leishmaniasis, 292  
    in parasitic infestations, 292  
Skin test dose, of scarlet fever toxin, 203  
Slide test(s), for blood grouping, 170  
    macroscopic, 131, 144  
    microscopic, 131, 143-144  
Smadel, 262  
Smallpox, immunization against, 255  
    revaccination against, 292  
Smith, Theobald, 271  
Smith and Marrack, 72

- Smith and Reagh, 159-160  
 Smorodintsev, 259  
 Sodium citrate, as anticoagulant, 301  
 Solubility, of antibody, 114  
     of serum proteins, 66-70  
 Soluble complexes, antigen-antibody, 98, 115  
 Somatic agglutination, mechanism of, 149  
     optimum temperature for, 110  
*Somatic agglutinin, plasma fraction* (Cohn) and, 69  
 Somatic antibodies, thermolability of, 56  
 Somatic antigen(s), 145  
     and Boivin antigens, relationship between, 51-52  
     endotoxins and, 50  
     of bacteria, 160-165  
     of *S. typhosa*, composition of, 51  
     thermolability of, 41  
 Somatic test antigens, preparation of, 308  
 Spatial configuration, effect on specificity of azoproteins, 33-36  
 Species specific antibodies, human, 86  
 Species specificity, 28  
 Specific (flagellar) antigens (phase 1), 162  
 Specificity, 16  
     chemical basis of, 40, 112  
     of antigen-antibody union, 105  
     of proteins, 53-54  
     effect of heat on, 41  
     of second stage of antigen-antibody reaction, 117  
     organ, 29  
     species, 28  
 Sperm, antigenicity of, 25  
 Spermocidin, 25  
 Spleen, antibody content of, 74  
     antibody formation in, 77, 78  
     as site of antibody formation, 92  
     role in antibody formation, 74  
     transfer of, from immunized to normal animals, 78  
 Spontaneous hypersensitivities, 283-285  
 Spontaneous phagocytosis, 213, 220  
 Spreading factors, 2  
 S-R variation, 161-162  
 Ss blood factors, 173  
 Stability of suspensions, 108  
 Stammbaum, Königsberg, 138-139  
 Staphylococci, coagulase negative, phagocytosis of, 222  
     surface phagocytosis of, 220  
*Staphylococcus aureus*, exotoxin of, 2  
     phagocytosis of, 19  
 Starches, antigenicity of, 42  
 Stavitsky, 78, 222  
 Steric hindrance, 117  
 Sterilization, of bacterins, 141-142  
 Sternberg, 261  
 Stetson, 280  
 Stimulin hypothesis of phagocytosis, 211  
 Street-virus, of rabies, 256  
 Streng, 249  
*Streptococcus*(i), exotoxin of, 2  
     mucoid, antigenicity of capsules of, 45  
     spreading factors of, 2  
     surface phagocytosis of, 220  
     toxin of, site of action of, 187  
     typing of, by precipitation, 130  
*Streptococcus viridans*, phagocytosis of, 15  
 Streptokinase, 2  
*Streptopelia*, serum antigens of, 136-137  
 Stuart, 6, 105, 153  
 Subgroups of blood group A, 171  
 Substance sensibilisatrice, 230  
 Surface phagocytosis, 218-220  
 Surface potential, effect on visible serologic reactions, 114  
 Surface tension, effects on phagocytosis, 217  
 Survival time of transfused erythrocytes, 180  
 Swine serum, effect on antigenicity of lipids, 45-46  
 Syntoxoid, 112  
*Syphilis*, complement fixation tests for, 248  
     diagnosis of, by precipitin test, 130-131  
     false positive tests for, 131  
 Syringes and needles, care of, 303-304  
 "T" component of serums, 61-62, 88  
 Tartaric acids, effect on specificity of azoproteins, 35-36  
 Taylor, Adair and Adair, 125  
 Tchistovitch, 120  
 Temperature, effect on amount of precipitate, 110  
     on antigen-antibody reaction, 104  
     on hemagglutination, 110  
     on phagocytosis, 218  
     on rate of agglutination, 110  
     on rate of precipitation, 110  
     for precipitin tests, 122  
 Template hypothesis of antibody formation, 89-93, 94  
 Teorell, 117  
 Test dose, of tetanus toxin, 197  
     relationship to M.I.D., 197  
 Tetanus, immunization against, 10, 205-206  
     prophylaxis and therapy of, 207  
 Tetanus antitoxin, 85  
     production of, in horses, 194-195  
     standard, 196  
     titration of, in mice, 196-197

- Tetanus toxin. See also *Clostridium tetani*,  
relationship between dose and death  
time in mice, 322
- Theiler and Smith, 256
- Thermoprecipitin test, 129
- Thiele and Embleton, 52
- Third order antibodies, 111
- Thyroxine as simple haptene, 31
- Tisarius, 59, 63
- Tissue cultures, antibody formation in,  
76
- Tissue extracts, lipid, 130  
preparation of, 302
- Tissue transplantation, 179
- Titer(s), 144  
of antibodies, 18, 79  
decrease in, 81
- Titration, anticomplementary, of anti-  
gen, 246
- Tobacco mosaic virus, antigenicity of,  
25
- Tomcsik and Schwarzwäiss, 49
- Topley, 81, 147
- Topley and Wilson, 24, 81, 83
- Toxicity, of viruses, 252-253  
determinations, factors affecting, 190-  
191
- Toxigenicity, 2
- $\alpha$ -Toxin of *Clostridium perfringens*, 2  
action of, 190
- Toxin(s), conversion to toxoid, 194  
diphtheria, L<sub>d</sub> dose of, 197  
homogeneity of, 124  
L<sub>d</sub> dose of, 202  
Lf dose of, 198  
L<sub>0</sub> dose of, 202  
multiple, antitoxin production against,  
85  
neutralization by antitoxins, 112, 322-  
323
- tetanus, dose of and death time of  
mice, 322  
nonsusceptibility to, 5  
test dose of, 197  
tissue affinity of, 207
- Toxin injection, incubation period after,  
188
- Toxin-antitoxin, diphtheria, immuniza-  
tion with, 204-205  
use in immunization, 10
- complexes, dissociation of, 201, 202  
flocculation of, 198-199, 323-324  
electrolyte and, 109
- flocules, enzymatic digestion of, 71
- precipitates, quantitative analyses of,  
200-201
- reaction(s), 321-324
- diphtheria, equations for, 201  
mechanism of, 199-202
- Toxoids, 112, 202  
alum precipitated, immunogenicity of,  
86
- diphtheria, immunization with, 204
- formation of, 187-188
- L<sub>d</sub> dose of, 199
- preparation of, 194
- properties of, 188
- tetanus, immunization of mice with,  
323  
immunization with, 206
- use in immunization, 10
- Toxone, 112, 202
- Toxophore groups, 111
- Transduction of *Salmonella* antigens, 164,  
166
- Transformation of pneumococcus types,  
166-167
- Transfusion, blood, 169, 179-180  
blood group substances in, 183  
reactions, 180  
role of Rh factor in, 174
- Transplantation of tissues, 179
- Trial bleeding, 143
- Trichina extract, 126
- Trichinosis, complement fixation in,  
248
- Trichloroacetic acid, extraction of bac-  
teria by, 50
- Trichophytin, 292
- Triple response, in evanescent allergic  
inflammation, 282
- Tube agglutination, 144-146
- Tuberculin, effect of, in normal animal,  
291  
in sensitive animal, 290-291  
precipitins for, 121, 126  
sensitivity, 290  
mechanism of, 292  
passive transfer of, 292  
production of, 291  
test, 290  
significance of, 292-293  
type of allergy, 290-293
- Tuberculoproteins, production of sensi-  
tivity against, 291
- Tuberculosis, hypersensitivity in, 291  
resistance to, 7
- Tularemia, agglutinin titers in, 151
- Two-stage nature of agglutination and  
precipitation, 96-97  
of phagocytosis, 214
- Typhoid fever, agglutinins in, 150-151  
laboratory diagnosis of, 143  
reimmunization against, 84
- Typhoidin, 292
- Typhus fever, Weil-Felix test for, 152
- Typing, serologic, of bacteria, 156

- Tyrosine, reaction with azo-compounds, 32  
reaction with iodine, 30-31
- UHLENHUTH, 134
- Ultracentrifugation, 62
- Undulant fever, significance of agglutination tests in, 150-151
- Unit, of amboceptor, 245  
of antitoxin, 196  
of complement, 245
- Unitarian hypothesis of antibodies, 22
- Universal donor, 180
- Universal recipient, 180
- Urticaria, 284, 288
- Urushiol, 294
- VACCINE(s), 141-142. See also *Bacterins*.  
Salk, 257-258  
sterilization and preservation of, 301
- Vaccine therapy, 223
- Vaccinia, agglutination and precipitation of, 264-265  
antigenic fractions of, 265  
hemagglutinin of, 265, 266
- Valence(s), of antibodies, 178  
of antigens and antibodies, 114  
of proteins, 107
- van der Scheer, 61, 62
- van der Waals force, 105-106
- Variation,  $\alpha$ - $\beta$ , of Salmonella, 164  
antigenic, induced, 165-167  
form, 161  
phase, 162 ff.  
S-R, 161-162  
Vi, 161
- Variola, antigenic fractions of, 265  
hemagglutinin of, 265
- Variolation, 255
- Vaughan, 52
- Vaughan's ferments (toxins), 52
- Vi agglutination, 146  
mechanism of, 149
- Vi agglutinins, significance in humans, 150
- Vi antibody(ies), effect on phagocytosis of *S. typhosa*, 222  
in immunized humans, 127  
production of, 45, 146  
titration of, 127
- Vi antigen, 146  
chemical nature of, 45  
extraction from bacteria, 45  
sources, 44  
stability of, 146  
thermostability of, 45  
use in immunization, 9
- Vi test antigens, preparation of, 308
- Vi variation, 161
- Vibrio cholerae*, bacteriolysis of, 20, 229-230  
precipitation test with, 120  
receptor destroying enzyme of, 266  
*metchnikovi*, killing of, by immune serum, 229
- Victor, 211, 223
- Viral antibodies, detection by hemagglutination inhibition, 154
- Virulence, 1  
alterations in, 3  
correlation with colony form, 161
- Virulence test, for diphtheria, *in vitro*, 199
- Virus(es), active immunity against, 254 ff.  
active immunization against, 255-258  
barriers to, 253  
cell degeneration caused by, 252  
cell proliferation caused by, 252  
complement fixation with, 249  
cultivation in chick embryos, 260-261  
fixed, of rabies, 256  
formalized, immunization by, 257-258  
hemagglutination by, 154  
immunity against, mechanism of, 260  
immunity tests, 263  
inactivated, immunization by, 257  
*in vitro* serologic reaction with, 264-267  
irradiated, immunization by, 257  
killed, immunization by, 257  
modification of, 256  
natural resistance to, 253-254  
nature of, 251  
neutralization of, by antibodies, 259  
mechanism of, 262-263  
parasitic nature of, 251  
passive immunity against, 258-259  
response of host to, 252-253  
serum inactivated, reactivation of, 259  
serum neutralization of, 261-263  
tissue affinities of, 252  
alteration in, 252  
toxicity of, 252-253
- Virus diseases, serologic diagnosis of, 263  
serologic reactions in, 260-267
- V-W transformation, 161
- WADSWORTH, 242
- Walsh and Montgomery, 173
- Wandering macrophages, 210
- Ward and Enders, 213, 214
- Wassermann and Schütze, 134
- Wassermann antigen, 46
- Wassermann reaction, in infectious mononucleosis, 84  
nature of, 248
- Wassermann test, 21, 248

- Webster, 7  
Weil, 46  
Weil and Sherman, 48  
Weil-Felix test, 152  
Welch, 224  
Welch and Hunter, 225-226  
Wells, 40  
Wheal and flare response, in evanescent allergic inflammation, 282  
Wheeler, 6  
White and Dougherty, 85  
White blood cells. See *Leukocytes*.  
Widal reaction, 97  
Widal test, 143  
Wiener, 175  
Wiener and Peters, 174  
Wilson and Miles, 100, 101  
Winkler and Westphal, 104  
Wolfe and Kornfeld, 249  
Wood, 218 ff., 224  
Wright, 222-223  
Wright and Douglas, 211, 212  
Wright method, of counting bacteria, 300-301  
X-RAYS, effect on antibody formation, 75  
Xanthoproteins, antigenicity of, 42  
YELLOW fever, immunity against, 89, 260  
immunization against, 9, 256  
ZADE, 139  
Zinder and Lederberg, 166  
Zinsser, 22, 43, 122  
Zinsser, Enders and Forhergill, 213, 214  
Zone phenomenon, 97-103  
Zwicker, 178  
Zymophore groups, 111  
Zymosan, inactivation of C3 by, 236