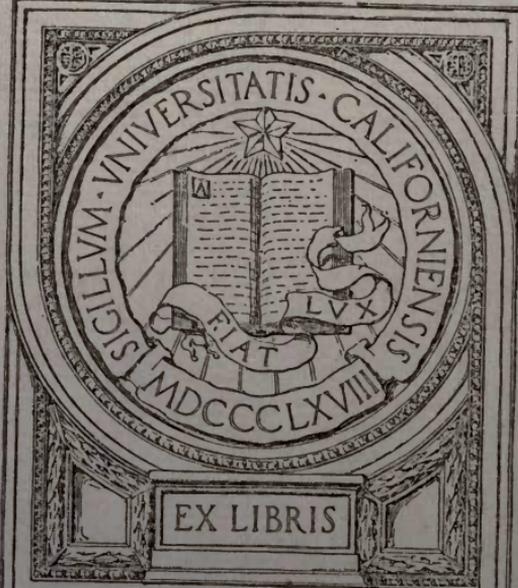




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# PROTEIN SPLIT PRODUCTS

IN

## RELATION TO IMMUNITY AND DISEASE

BY

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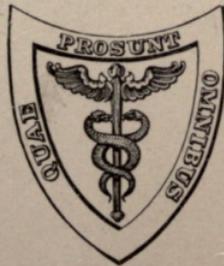
IN CHARGE OF THE TUBERCULOSIS WORK OF THE DETROIT BOARD OF HEALTH  
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AND

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## PREFACE

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THE investigations recorded in this volume, begun nearly fifteen years ago, were inaugurated in consequence of certain fundamental ideas or theories held by the writer, and these have directed and dominated all our labors along this line. The purpose of this work has been to solve scientific problems, rather than to discover practical applications. The latter, so far as they have in any way influenced our studies or even received our attention, have been only incidental. Quite naturally our theories have been more or less modified, and have developed as the work has progressed, but the essentials and fundamentals have not been materially altered. From time to time these theories have been given in more or less detail, notably in an address at the opening of the Medical Department of the University of Toronto in 1905 (*Canadian Jour. of Med. and Surg.*, xviii, 283) and in the Shattuck lecture for 1906 (*Boston Med. and Surg. Jour.*, clv, 215). However, it may be well to restate briefly the original conceptions which impelled us to begin and continue these studies.

The only essential and constant difference between living and non-living matter is that within the molecules of the former there is constant metabolism, while in the latter no such process operates. We are to conceive of the living molecules as made up of numerous atoms, and each atom surrounded by its electrons; atoms and elec-

trons in ceaseless motion, and groups of atoms being constantly cast out of the molecule and replaced by new groups split off from outside matter. As soon as a molecule becomes the seat of assimilation and excretion, it is no longer dead; it lives. As a result of assimilation it acquires the property of building up its own structure; then polymerization follows and reproduction in its simplest form begins. The one phenomenon always manifested by living matter, and never exhibited by non-living matter, is metabolism.

When matter becomes endowed with life it does not cease to be matter; it does not lose its inherent properties; it is not released from the laws that govern its structure, its attractions, and its motions. In studying living things it should be borne in mind that they are material in composition and subject to the fundamental laws that govern matter, and possessed of those properties essential to matter.

Matter is alive when it feeds and excretes. The living molecule not only absorbs; it assimilates. It chemically alters what it absorbs, and within limits, it may be altered by what it absorbs. Atomic groups taken into living molecules enter into new combinations. The living molecule is not stable, but is highly labile. Its composition is never constant, and it is never in a condition of equilibrium. It is in constant chemical reaction with outside matter. Apart from other matter it could not exist. There is a constant interchange of atoms between it and external matter. A condition, best designated as latent life, may exist without interchange of atoms. This is exemplified in spores, seeds, and ova. Matter existing in these forms may be awakened into activity by proper stimuli; active life begins with the interchange of atoms.

Why is there this constant change of atomic groups between the living molecule and outside matter? It is for the purpose of supplying the living molecule with energy. It is probable that in the absorption of energy by the living molecule, oxygen is released from its combination with carbon or hydrogen, and is attached to nitrogen, while in the liberation of energy the reverse takes place. Nitrogen seems to be the master element within the living molecule. It is by virtue of its chemism that groups are torn from non-living matter, taken into the living molecule, and assimilated by atomic rearrangement; and furthermore, it is on account of the lability of the compound thus formed that potential energy is converted into kinetic and work is accomplished. A nitrogen side-chain serves as a receptor and transmitter of oxygen, and thus the traffic in energy within the living molecule goes on rhythmically. It is not to be supposed that the nitrogen side-chain, which serves as the receptor and transmitter of oxygen, consists of so simple a body as nitrogen or nitrogen oxide, but it is probably a highly complex nitrogenous body in which the location of the nitrogen is central, as suggested by Allen. Nor is it probable that only oxygen is broken off from the pabulum, but substances containing this element. This is the way in which the living molecule keeps up its constant, rhythmic traffic in energy, absorbing heat by assimilation, and giving it off by dissociation. Each living molecule has not only one, but many of these nitrogenous groups that act as receptors. Moreover, metabolism within the molecule is not confined to the absorption of oxygen, and the casting out of non-nitrogenous products of combustion. The whole molecule is labile, and there is probably in every living molecule a nitrogenous, as well as a non-nitrogenous,

metabolism. Nitrogen absorbed with the oxygen is, in part at least, utilized in replacing the waste in this element, and the carbon brought into the molecule at the same time is in part detached by the free valences in the carbohydrate groups, and used to repair loss in this part of the molecular structure. In the living molecule it is probable that nitrogenous metabolism proceeds much more slowly than the carbon and hydrogen metabolism, but both move rhythmically, and the tempo depends upon the swing of the atomic groups that constitute the molecule, and this rate can be changed, hastened or retarded, by alterations, either physical or chemical, in the medium in which life resides. When the molecule is in active life its food is prepared for it by ferments, and it is quite certain that these ferments have their origin in the nitrogenous metabolism of the living molecule.

The keystone or archon of the protein molecule is our poison. It is common to all protein molecules. It is the primary group. One protein differs from another in the secondary and tertiary groups. Ordinary proteins are not poisonous, because in them the chemism of the primary group is satisfied by combination with secondary groups. Strip off the secondary groups and the primary becomes poisonous on account of the avidity with which it combines with the secondary groups of other molecules.

The specificity of proteins resides in the secondary groups of their molecules, and all specific protein reactions are due to these groups. This is true of agglutination, precipitin, and lytic reactions. Biological relationship between proteins is dependent upon the chemical structure of their molecules. The poisonous part of a protein is its primary group; the sensitizing part is found among the secondary

groups. The former is physiologically the same in all proteins. There probably are chemical differences in the primary groups of varied proteins, and it is possible that fine physiological differences may be detected by more careful study, but the primary group is the ring about which all proteins are built, or at least, it contains this ring; and just as innumerable compounds may be built with the benzol ring as a nucleus, so all proteins are constructed about a common centre. The secondary groups are not identical in any two kinds of proteins. There may be one or more common to the two, but in some respects there are differences.

The cell is not the unit of life; life is molecular. The cell is not only made up of protein molecules, but its form and function are determined by the chemical structure of its constituent molecules. The lines along which the spore, seed, or ovum develops are determined by the chemical structure of its proteins. Growth in other directions is impossible, and this accounts for stability in reproduction. However, gradual changes in the chemical structure of living proteins occur, and in these lies the basis of organic evolution.

The basic points of our theory, as stated above, will be in evidence throughout this volume. The experimental work devoted to the development of this theory could not have been done without the aid of able assistants who have devoted much time to it, and all without adequate reward. Besides those associated with me in the preparation of this volume, special mention is due Drs. Sybil May Wheeler and Mary Leach. The former gave eight years and the latter two years of most devoted and skilful service to the elaboration of the problems discussed here.

I regard the studies recorded in this volume as the mere beginnings of work which should be developed. I dare say that our record contains many imperfections and possibly some errors. Future studies will perfect the former and eliminate the latter. Attempts to solve the problems stated in this volume have occupied many years and filled them with the interest and pleasure that always come to those who seek to widen the fields of the known.

THE SENIOR AUTHOR.

ANN ARBOR, 1913.

# CONTENTS

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## CHAPTER I

### INTRODUCTION

Bacteria are particulate proteins; all true proteins contain a poisonous group; the chemical nucleus contains the poison; when proteins are disrupted the poisonous group may be set free; the pathogenicity of a bacterium is not determined by its capability of forming a poison, but is determined by its ability to grow and multiply in the animal body; any foreign protein which can grow and multiply in the body of a given animal is pathogenic to that animal; the infectious diseases result from the parenteral digestion of proteins; natural bacterial immunity, that which follows an infectious disease, and that induced by vaccination, result from inability of the organism to grow and multiply in the animal body; protein sensitization and bacterial immunity, apparently antipodal, are in reality identical; protein sensitization consists in the development of a new function in certain body cells—that of elaborating a specific, proteolytic ferment; a foreign protein introduced into the blood is distributed through the tissues; vaccines are protein sensitizers; toxin and bacterial immunities are different; the protein poison is not a toxin; it is not specific; it elaborates no antibody; it develops a specific ferment; different proteins tend to accumulate in predilection places; the symptoms of the infectious diseases are largely determined by the organ or tissues in which the foreign protein accumulates; the poison elaborated in all the infectious diseases is the same; when a cell in the animal body is permeated by a foreign protein, the former strives to elaborate a ferment by which the latter is destroyed; this we believe to be a biological law . . . . .

18

## CHAPTER II

### THE GROWTH OF MASSIVE CULTURES OF BACTERIA

The large tanks and the preparation of bacterial cellular substances

29

CONTENTS

CHAPTER III

PRELIMINARY EXPERIMENT OF BACTERIAL CELLULAR SUBSTANCES

They consist essentially of complex proteins, each of which contains a poisonous group . . . . . 37

CHAPTER IV

THE CHEMISTRY OF BACTERIAL CELLULAR SUBSTANCES

Their protein, nuclein, carbohydrate, fatty and amino constituents 52

CHAPTER V

THE CLEAVAGE OF PROTEINS WITH DILUTE ALKALI IN SOLUTION IN ABSOLUTE ALCOHOL

Bacterial, vegetable, and animal proteins can be split into poisonous and non-poisonous parts; the former are non-specific, the latter are specific; the exact nature of neither of these portions is yet known 95

CHAPTER VI

ACTION OF ANIMALS

The action of the living bacillus, of the dead bacillus, and of the poisonous split product . . . . . 119

CHAPTER VII

THE PRODUCTION OF ACTIVE IMMUNITY WITH THE SPLIT PRODUCTS OF THE COLON BACILLUS

The establishment of a certain degree of tolerance with the poisonous product; this is non-specific; the production of a mild degree of immunity with the non-poisonous product; this is specific . . 137

CHAPTER VIII

THE SPLIT PRODUCTS OF THE TUBERCLE BACILLUS AND THEIR EFFECTS ON ANIMALS

The cellular substance; the cell poison; the cell residue; the precipitate poison; the precipitate residue; the final filtrate; the action of these on animals; the effects of the tuberculo-poison; sensitization with tuberculo-protein; the relation of tuberculo-sensitization to immunity . . . . . 164

# CONTENTS

xi

## CHAPTER IX

### THE ANTHRAX PROTEIN

Literature, investigations; the anthrax cellular substance, like other proteins, contains a poison; sensitization with anthrax protein 189

## CHAPTER X

### THE CELLULAR SUBSTANCE OF THE PNEUMOCOCCUS

Difference in virulence in strains; properties and effects on animals of the cellular substance; the action of the poisonous portion; auto-lysis of the pneumococcus; sensitization with pneumococcus protein 205

## CHAPTER XI

### PROTEIN SENSITIZATION

Introduction; definition; the sensitizer; all true proteins sensitize; volatile sensitizers; the sensitizing group in the protein molecule; the effects on different animals; period of incubation; the anaphylactic state; the reinjection; symptoms; the mechanism of anaphylaxis; passive anaphylaxis; anti-anaphylaxis; the Arthus phenomenon; anaphylaxis and toxic sera; the toxogens; anaphylaxis *in vitro*; the poison;  $\beta$ -iminazolethylamin; the kyrins; anaphylatoxin; physiological action of the protein poison; general physiological action of proteins; sensitization is cellular; theories; theory of Friedberger; theory of Vaughan and Wheeler; theory of Nolf . . . . . 214

## CHAPTER XII

### PARENTERAL DIGESTION

The disposition of peptones; the fate of proteins introduced directly into the circulation; the poisonous action of proteins; egg-white injected into the stomach of a rabbit may be in part absorbed unchanged; egg-white injected into the rectum of a rabbit may be, in part at least, absorbed unchanged; egg-white injected into the peritoneal cavity of a rabbit may be absorbed unchanged; egg-white injected intravenously in a rabbit quickly disappears from the circulating blood; egg-white injected intravenously in a rabbit may be detected in the peritoneal cavity, in the bile, and in certain organs after it has disappeared from the circulating blood; the injection of a large amount of egg-white intravenously in a rabbit may prove fatal; the blood is a digestive fluid; proteolytic digestion in the blood is regulated by the accumulation of digestive products . 342

## CHAPTER XIII

## PROTEIN FEVER

The production of acute, intermittent, remittent, and continued fevers by the injection of foreign proteins; fever results from the parenteral digestion of proteins; the sources of fever in the parenteral digestion of proteins; fever *per se* is a beneficent process . . . 373

## CHAPTER XIV

## SPECIFIC FERMENTS OF THE CANCER CELL

Extra- and intracellular ferments; a ferment developed in animals by injections of cancer protein; the nature and action of this ferment 416

## CHAPTER XV

## THE PHENOMENA OF INFECTION

How bacteria grow; how bacteria cause disease; the phenomena of the period of incubation; the phenomena of the active period of the infectious diseases; the germicidal properties of the blood; the phenomena of tubercular infection; the tuberculin test; vaccines and sensitization; sensitization and idiosyncrasies to food and medicine . 436



# PROTEIN POISONS

## CHAPTER I INTRODUCTION

MANY years ago the senior contributor to this volume began a research on the chemistry of bacterial cellular substance. This work has grown and the progress made has been reported from time to time in current scientific and medical literature. Able assistants have rendered valuable service, and as the research has developed it has been correlated with that done along similar lines in other laboratories. We feel that the time has come when the more important facts ascertained along this and related lines, by all investigators, should be classified and proper deductions drawn from them. We are the more inclined to do this because we believe that the proper interpretation of the results obtained opens up a view of the etiology and development of both immunity and disease, which has hitherto not been appreciated. We have thought it best to state briefly in this introduction some of the most important points dwelt upon in the volume. We have done this somewhat dogmatically, hoping that they will impress the reader and hold his attention while they are more fully detailed in subsequent chapters.

1. *Bacteria are essentially particulate, specific proteins.* Bacteria are usually classified as microscopic plants, but we have sought diligently for the presence of cellulose in their structure, with uniformly negative results. We have shown that some bacteria, at least, contain two carbohy-

drates, but neither of these gives the reactions characteristic of cellulose. One of these is certainly a constituent of the nucleic acid group, having the same relation, or at least a similar relation, to the other members of this group as exists in the nucleins and nucleoproteins found in the vegetable and animal world. Our studies together with those of other investigators render it quite certain that bacterial cellular substance yields the nuclein bases on hydrolysis. The position of the second carbohydrate in the molecular structure has not been determined with certainty. It is thought possible that it is simply stored in the cell as a reserve food supply; but that this is not true is indicated by the fact that it cannot be removed by simple solvents, and that its separation is secured only after disruption of the molecular structure. We are inclined to the opinion, subject to change as the result of more exact knowledge, that the second carbohydrate group found in at least some bacteria is an essential constituent of the protein structure. Our work on the amino-acids, both mono- and di-amino, makes it certain that the greater part of the bacterial cell is made up of true proteins. We have not only isolated and identified many of the amino-acids, but we have shown that they exist in widely different proportions in different species of bacteria, just as they do in different proteins obtained from plants and animals. While fats and waxes are found in relatively large amount in certain bacteria, notably in bacillus tuberculosis, we see no reason for concluding that they are essential constituents of the living molecule. That they are specific products of the life activities of certain bacteria we are convinced, but we have seen no reason for believing that they are essential constituents of the bacterial molecules. We conclude that, chemically, bacteria, at least those with which we have worked, are nucleoproteins or glyco-nucleoproteins. While bacteria are morphologically simple in structure and without differentiation in parts, chemically they are complicated in structure, quite as much so as many of the tissues of the higher plants and animals. The

demonstration that bacteria are not only proteins, but relatively complex proteins, is a matter of marked importance. It shows that in many of their life processes they must bear a close resemblance to the cells of the higher animals; that they require the same kind of food, which they select, assimilate, and excrete in much the same way; that the conditions of life are much the same; what is favorable to one bearing a like relation to the other, and what proves injurious to one having a like effect upon the other.

2. *All true proteins contain a poisonous group.* At first we found that the cellular substance of certain pathogenic bacteria could be split up with the liberation of a poisonous substance, then we tested non-pathogenic bacteria, then animal and vegetable proteins, and all with the same result. Not only do all these contain a poison, but so far as its gross effects on the higher animals have been studied, the same poison. We have held that when we know more about these poisonous bodies obtained from all proteins, it will be found that chemically they are not identical, but physiologically they are so closely similar that up to the present time we have not been able to distinguish one from the other by the symptoms induced. The poison obtained from the typhoid bacillus, that from egg-white, and that from edestin of hemp-seed kill animals in the same doses, with the same symptoms and with the same lesions. This is striking evidence of the similarity in the structure of the protein molecule, whether it be of bacterial, animal, or vegetable origin. One cannot resist the temptation to formulate a theory to fit these facts. Indeed, the theory unfolds itself and may be briefly expressed as follows: All proteins are constructed on the same model and contain a chemical nucleus, archon, or key-stone. This is the poisonous group and is practically the same in all proteins. One protein differs from all others in its secondary and possibly its tertiary groups. In these lies the specificity of proteins. Living proteins function through their secondary and tertiary groups. When the primary group is detached from its own subsidiary and specific groups it manifests

its poisonous action through the avidity which it has for the secondary groups of other proteins. These are thus detached from their normal positions and consequently the living protein is deprived of its capability of functioning normally. This is only a theory, but it is one which naturally suggests itself.

3. *The chemical nucleus does not become a poison until stripped in part at least of its secondary groups, and the intensity of its poisonous action is determined by the thoroughness with which the secondary groups have been removed.* The protein molecule may be regarded as a highly complex neutral salt, made up of many basic and acid groups. One of these components, it may be either a basic or an acid group (or it may have within itself both a basic and an acid group), is the chemical nucleus of the molecule. In its natural condition its chemism is satisfied by nicely adjusted combination. When this combination is disrupted, which may be accomplished either by chemical agents or by enzymes, the chemical nucleus is set free, more or less completely, and to the extent that it is released from combination, it becomes, in the presence of living proteins, a poison because it disrupts the same. We have shown by direct experiment that the protein poison may be at least partly neutralized by being kept for some days in the presence of an alkaline carbonate at 37° C.

4. *When proteins are submitted to the action of disrupting agents there is the possibility of the chemical nucleus being set free more or less completely, and to the extent that it is detached it becomes a poison.* We have found that this occurs when proteins are carefully disrupted by either dilute acid or dilute alkali. So far as our work has gone the best agent with which to disrupt the protein molecule and obtain the largest yield of poison is a 2 per cent. solution of caustic soda in absolute alcohol. This is a crude procedure and much of the poison is destroyed in the process. The disruption easily extends beyond the point where the poison is set free and much of the product sought is destroyed. In peptic digestion the poison becomes active

at about the stage of the formation of peptone, and it has long been known that peptones are quite highly poisonous when administered parenterally. This also is a crude method of obtaining the poison, and with all the work that has been done along this line, we do not know whether the peptone is itself poisonous or whether its poisonous action is due to admixture with some other digestive product. We do know that as alimentary digestion proceeds the protein poison itself is destroyed. Indeed, we had no conception of the small amount of protein necessary to furnish a lethal dose of the poison, until we submitted proteins to the blood sera and organ extracts of sensitized animals. Then we found that 1 mg. of protein may supply enough poison to kill a guinea-pig when injected intravenously. But to produce the poison in this way necessitates a delicate adjustment between substrate and ferment which is imperfectly understood, and consequently inadequately controlled, and we can know that we have produced the poison in any given experiment only by its effect on an animal. Thus it happens that after years of study we are still quite ignorant of the true nature and chemical composition and structure of the protein poison.

5. *The pathogenicity of a bacterium is not determined by its capability of forming a poison.* Non-pathogenic bacteria yield just as much of the protein poison as the most highly pathogenic, and the proteins of our food contain the same poison that is found in pathogenic bacteria.

6. *The pathogenicity of a bacterium is dependent upon its ability to grow and multiply in the animal body.* Any micro-organism which can grow and multiply in an animal body is pathogenic to that animal. Growing and multiplying in the animal body means that the invader converts the proteins of the animal into its own proteins, transforms native into foreign proteins, and the accumulation of foreign proteins can result only from the destruction of the native. There are two conditions which determine whether or not a foreign protein can grow and multiply in the animal body: One is the capability of the invader of digesting and utilizing

the proteins of the body. All living cells grow by means of their own digestive ferments, and these must act upon the pabulum within their reach. If the ferment of the bacterial cell cannot digest and prepare food for the bacterium from the body proteins, then the invading bacterial cell dies. The second factor in determining whether a given bacterial cell will grow in the animal body is the effect of the ferments of the body cells on the invader. If these are rapidly and thoroughly destructive there is no bacterial development, and the organism is innocuous. The prodigiousus is not pathogenic, but the cellular substance of this bacillus obtained by growth on artificial culture media is highly poisonous to animals. This is true, with modification as to degree, of the cellular substance of all non-pathogenic bacteria. It is not the lack of poison in the substance when placed under conditions favorable to its growth, but it is its inability to grow under unfavorable conditions. The smallpox virus is pathogenic to the unvaccinated but non-pathogenic to the vaccinated, because by vaccination there has been developed in the body a ferment which destroys the smallpox virus before it can develop.

7. *Any foreign protein which can grow and multiply in the body of a given animal may prove pathogenic to that animal.* Our idea of the development of an infectious disease may be stated as follows: An infective agent is any protein which possesses the capability of growth in the animal body. What these essentials are have been stated under 6. We will take as illustration typhoid fever. The infective agent is the typhoid bacillus, a specific, particulate protein. It is infective because by means of its digestive ferment it can feed upon the proteins of man's body. This means that it can convert man's proteins into typhoid proteins and thus multiply its kind. Moreover, it is not, immediately on its entrance in man's body, destroyed by the ferments of the body cells. Having found admission to the body it proceeds to grow and multiply. This continues through the period of incubation, which in this disease is somewhere about ten days. During this period of incuba-

tion there is no effective resistance on the part of the body cells to the growth and multiplication of the foreign protein. During this time the man is not sick, and we conclude therefore that it is not the growth of the foreign protein which *per se* gives rise to the symptoms of typhoid fever. However, during this time the body cells are being prepared for their combat with the foreign protein. This preparation consists in the development in certain of the body cells of a new function, that of elaborating a new and specific ferment which will digest and destroy the foreign protein. When this new ferment begins its action the first symptoms of the disease appear. The active stage of the disease, with its symptoms and the lesions induced, marks the period over which the parenteral digestion of the foreign protein extends. Death may come from the too rapid breaking up of the foreign protein and the consequent liberation of a fatal dose of the protein poison, which is always formed on the disruption of the protein molecule, or it may result from some lesion induced by the products of this disruption, such as perforation and hemorrhage, or it may follow from chronic intoxication and consequent exhaustion. In case of recovery the individual is for a time at least immune to the typhoid bacillus because his body cells are now able to elaborate and make immediately effective the specific ferment which destroys the typhoid protein.

8. *The infectious diseases result from parenteral protein digestion.* Parenteral digestion, like all fermentative processes, is influenced in its rate of progress by many conditions, among which may be mentioned the relation between amount of ferment and substrate, the physical condition of the substrate, and the presence of the fermentation products. These influences upon parenteral digestion are not easily ascertained, and consequently not as yet measurable or controllable. The liberation of heat as measured by body temperature has recently received attention, and we can say in a general way that fever is one of the most easily recognizable effects of the process. While natural infec-

tion is due to living proteins, we have recently learned that experimental fever can be induced by repeated injections of foreign proteins and by changes in size of dose and in intervals between doses, fever of any desired type can be induced.

9. *Natural immunity to any infection is due to inability of the infecting agent to grow in the animal body.* This, of course, does not include toxin immunity, which is due to the presence in the body of an antitoxin or of something which destroys or neutralizes the toxin. The inability of an infection to multiply in the animal body has been explained under 6.

10. *The immunity which is due to recovery from an infection is the result of the development in the body during the course of the infection of a specific ferment which immediately destroys the infection on renewed exposure.* As has been stated, the cells of the body acquire the function of developing the specific ferment, and this function is awakened and made immediately effective on subsequent exposure. This new function developed in the body cells by disease may continue throughout life, or it may be lost after a period which is variable in different diseases. The immunity induced by one attack of yellow fever is believed to continue through life; that from smallpox generally holds through life, and but few have typhoid fever more than once. Some infectious diseases, such as pneumonia, are apparently not followed by immunity. In most instances it seems that the immunity induced by one attack of an infectious disease is not absolute, but only relative, and may be overcome by severe or prolonged exposure to a virulent form of the infection.

11. *Immunity established by vaccination is similar to that induced by an attack of the disease.* The vaccine is the same protein that causes the disease. It must be so modified that it will not induce the disease, but yet so little altered that it will stimulate the body cells to form a specific ferment which will promptly and quickly destroy the infecting agent on exposure. The smallpox virus is modified by

passage through the cow. The anthrax bacillus is converted into a vaccine by growth at high temperature. The typhoid bacillus is killed by heat. In all these instances the protein is so little changed in being converted from an active, infecting agent into a vaccine that it still sensitizes the animal to itself in the unmodified state. It seems reasonable to conclude that the protein retains its capability of sensitizing so long as there is no radical alteration in its chemical structure. The results secured by vaccination with killed typhoid bacilli prove that in this instance at least a vaccine is not necessarily a living organism. The possibility of obtaining vaccines from the split products of pathogenic proteins has led to some of the investigations detailed in this volume, and while we do not claim success in this particular, we think that continued efforts in this direction are justified.

12. *Protein sensitization and bacterial immunity, apparently antipodal, are in reality identical.* This statement first made by us in 1907 has since met with wide acceptance, and will be discussed in detail in later chapters.

13. *Protein sensitization consists in developing in the animal body a specific proteolytic ferment which digests the same protein on reinjection.* Protein sensitizers may be living or dead, particulate or in solution. Soluble proteins sensitize more readily and more fully than those not in solution. The development of the specific proteolytic ferment in sensitization is due to the action of the foreign protein upon the body cells. There is developed in certain body cells a new function, that of elaborating this new ferment. In order for a given body cell to be thus influenced by a foreign protein, the latter must come in contact with the former. Cell permeation by the foreign protein is probably essential to the perfect elaboration of this process.

14. *When a foreign protein is introduced into the blood of an animal it soon leaves the circulating fluid and is distributed throughout the tissues.* The truth of this has been demonstrated by the researches of independent investigators, and will be detailed later. This is true of both particulate and

soluble proteins, but the distribution is more prompt and effective with soluble than with particulate proteins. This explains why the former are more efficient sensitizers than the latter. It will be understood that a protein relatively insoluble *in vitro* may become more readily soluble *in vivo*.

15. *Vaccines are protein sensitizers.* One of the most important problems in scientific medicine now awaiting solution is that of the preparation and employment of vaccines. The term vaccine—from *vacca*, a cow—was first used when Jenner employed the infection of cowpox to induce immunity to smallpox. Since that time the use of the word “vaccine” has been extended to include every form of preventive inoculation. Through the researches of Wright vaccine therapy has been developed, and now vaccines are employed not only in the prevention but in the treatment of disease. Under 11 we have spoken of the employment of vaccines in inducing immunity, and now we wish to speak briefly of vaccine therapy. As we understand it, these two uses of vaccines depend upon the same principle. The action of the vaccine is the same in both instances. The protein of the organism responsible for the diseased condition and that of the vaccine must be identical or closely related bodies. Both must be protein sensitizers. In most, if not in all, of the systemic infectious diseases the infecting organism sensitizes the body either throughout or over large areas. It seems to us to treat such diseases with vaccines is irrational, and we believe that much harm has been done by such attempts. There are, however, local infections in which the area of sensitization is limited and circumscribed. Such diseases may be treated rationally with vaccines, provided such agents can be obtained in such forms that they will act both effectively and harmlessly. The future of vaccine therapy, in our opinion, depends upon our ability to secure such vaccines. That we have not yet fully established our ability to obtain vaccines that are both harmless and effective we are ready to admit. This does not mean, however, that all efforts to accomplish this should be dis-

continued. In our own attempts in this direction we have met with enough encouragement to lead us to be hopeful of ultimate success, while admitting present failure. In our opinion, it is not only unwise, but unjustifiable to treat advanced cases of tuberculosis with tuberculin or other tuberculo-sensitizer. So long as the disease is strictly localized and the body in general is not sensitized, such treatment can find reasonable justification. Our theory is that in strictly localized infections the proper use of a specific sensitizer may cause the more general and abundant formation of a specific proteolytic ferment which may aid in the destruction of the infecting organism. In our opinion sensitization consists in the development of a new function in the body cells. In strictly local infections this new function has been developed only in the infected area, and to establish a like function in more distant cells may be beneficial. The present tendency on the part of the profession to employ all kinds of bacterial proteins as vaccines is, in our opinion, not only unscientific, but wholly without justification. It should be clearly understood that with every protein injected into the body a most potent poison is introduced, and caution in the use of vaccines is not out of place.

16. *Toxin immunity and bacterial immunity are radically different.* This is a point upon which we shall frequently touch, and we hold that attempts to describe one of these forms of immunity in terms of the other have not only been unwarranted by the facts, but have led to unnecessary confusion.

17. *The protein poison is not a toxin.* It elaborates no antibody, and while its repeated use in non-fatal doses may establish a certain tolerance, it gives no immunity comparable in either nature or degree with that obtained by like employment of toxins.

18. *The protein poison is not specific.*

19. *The tolerance which may be secured by the protein poison is not specific.*

20. *The sensitization developed by a protein is specific, but is not due to the poisonous group in the protein.* As we have stated, the specificity of a protein is not due to its poisonous group, which is much the same in all proteins, but to its secondary groups, for it is in these that one protein differs from all others.

21. *Different proteins find in the body certain predilection places in which they are most prone to accumulate.* The pneumococcus accumulates in the lungs, the smallpox virus in the skin, the typhoid bacillus in the spleen, and mesenteric glands; the tubercle bacillus finds its most frequent location in the lungs, but it has been a parasite so long that it may grow on any human tissue.

22. *The symptoms of a given disease are largely determined by the location of the foreign protein.* The most skilful physician may not be able to tell what organism is responsible for a meningitis. The symptoms of acute miliary tuberculosis and those of typhoid fever are much alike. It is the location of the infection rather than the exact nature of the infecting agent which gives rise to the more or less characteristic symptoms of the several infectious diseases.

23. *The poison elaborated in all the infectious diseases is the same.* It is the protein poison, and it is physiologically the same whatever its source, whether it comes from coccus, bacterium, spirillum, or protozoan. The specificity which characterizes the infectious diseases is not due to the poison formed, but to the protein cause and the specific ferment produced.

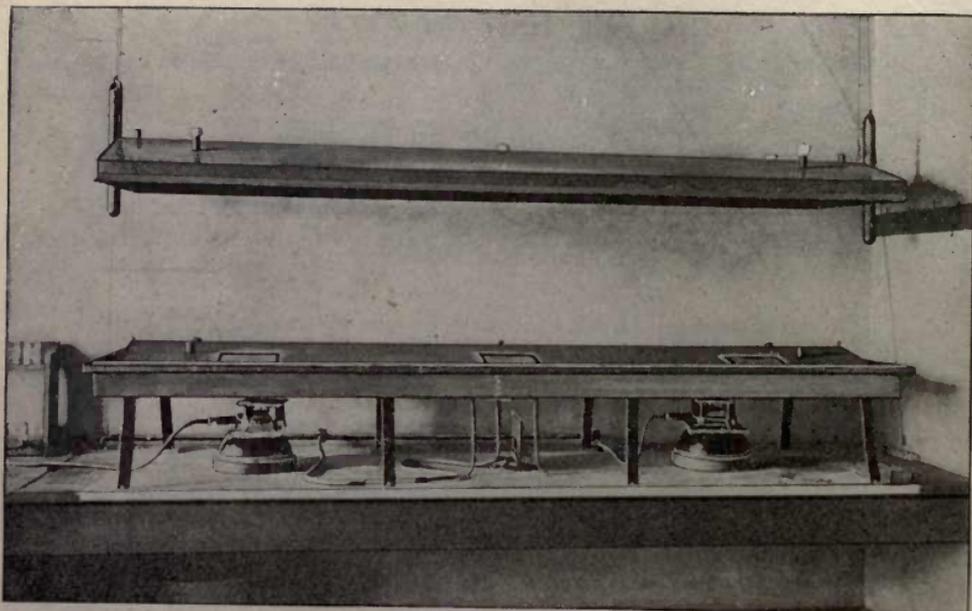
24. *When a cell in the animal body is permeated by a foreign protein, the former strives to elaborate a ferment by which the latter is destroyed.* We believe this to be a biological law, and we think that it lies at the foundation of a correct understanding of many of the problems of immunity and disease.

## CHAPTER II

### THE GROWTH OF MASSIVE CULTURES OF BACTERIA

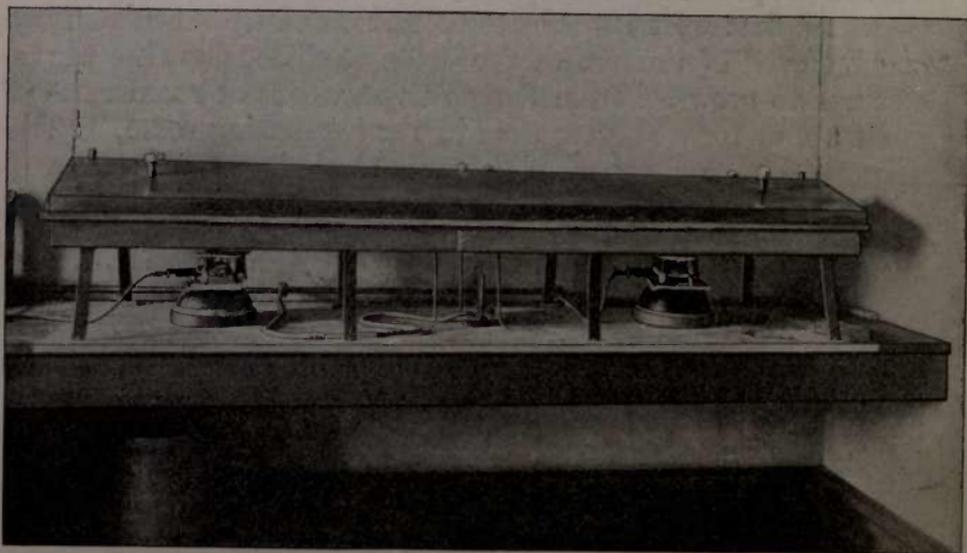
HAVING decided to study the chemistry of the bacterial cell, it soon became evident that we must devise some way of obtaining this substance in large quantity and fairly free from admixture with foreign material. Bacteria had been grown only in test-tubes, Petri dishes, and Roux flasks, and none of these methods of growth gave the amount of material necessary to promise any satisfactory investigation. As a medium, agar suits the purpose admirably, because the bacterial growth can be detached and washed from the surface of this medium quite free from admixture, but it remained to devise some means of obtaining a large surface so protected as to be guarded against contamination. At first we tried the Roux flasks, and by inoculating one hundred of them with the colon bacillus, allowing the cultures to grow for from two to three weeks at room temperature, or for a shorter time in the incubating room, and then washing off the growth with alcohol, we secured a somewhat bulky and promising volume of bacterial, cellular substance; but when this had been thoroughly washed with sterile salt solution, extracted with alcohol and ether, dried and weighed, we found the total yield, under the most favorable circumstances, was not more than three grams. This enabled us to make some preliminary experiments and to demonstrate that the dead bacterial cells, thus prepared, gave all the general color reactions for proteins and were highly poisonous to animals, but the possibility of making any satisfactory chemical study was not promising. Moreover, the labor and care necessary to

FIG. 1



Tank with raised lids.

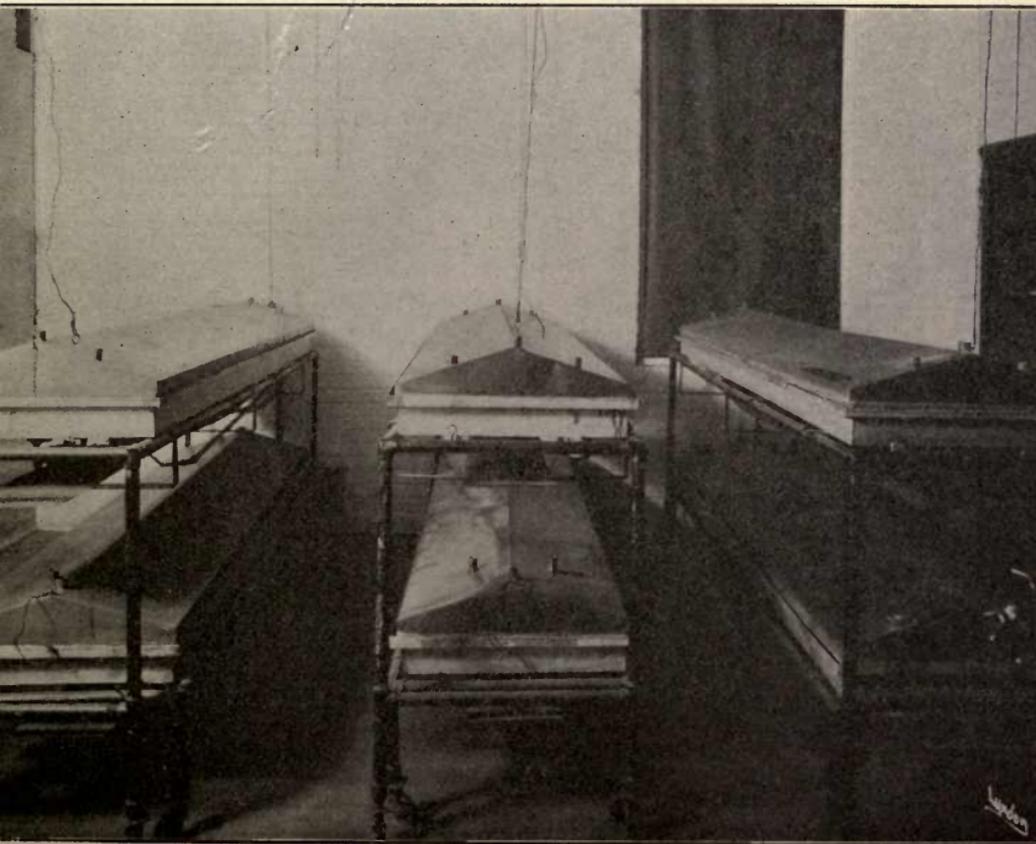
FIG. 2



Tank with lids lowered.

remove with anything like completeness the bacterial growth from one hundred Roux flasks were greater than we could afford to exert more than a few times; therefore, attempts to secure the desired quantity of bacterial cellular substance by this method were abandoned. We then tried

FIG. 3

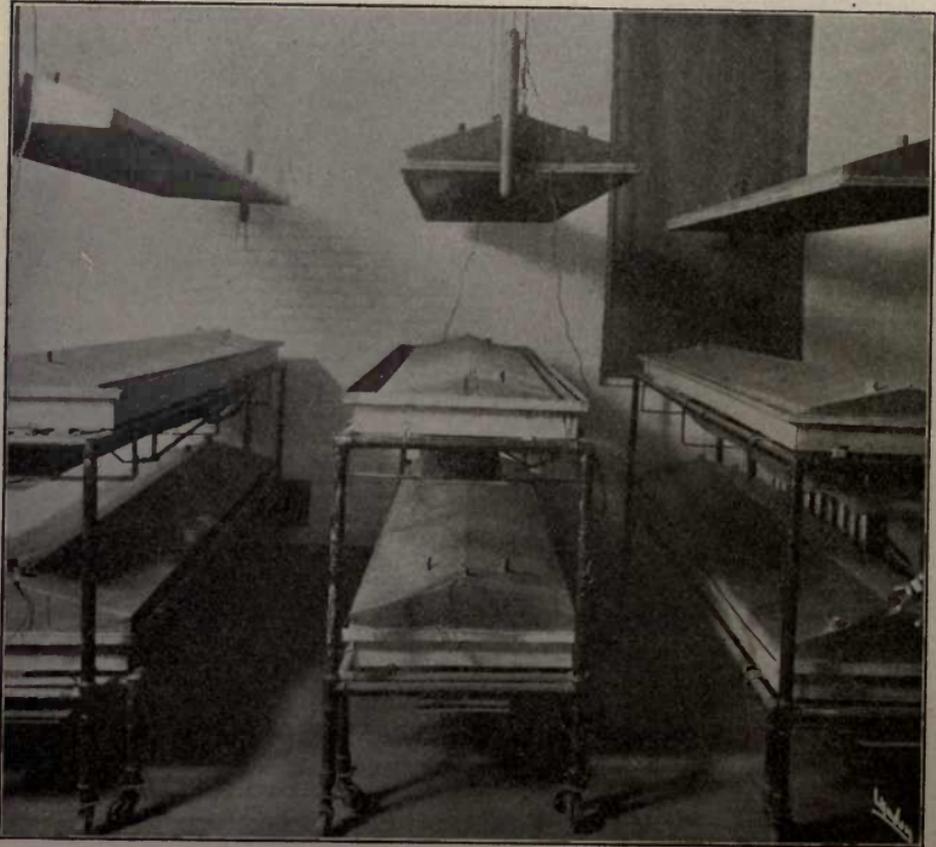


The incubating room, lids lowered.

growing the colon bacillus in ordinary moist chambers, used as Petri dishes, but with the greatest care many of these cultures became contaminated. Finally, we devised the large copper double tanks which have proved wholly satisfactory and have supplied abundant growths, easily obtain-

able and free from contamination. This tank, photographs of which are here given, was first put into operation in 1900, and was described in the following year<sup>1</sup> (Figs. 1, 2, 3, 4). A copper tank ten feet long, two feet wide, and four

FIG. 4



The incubating room, lids raised.

inches deep, with a trough around the edge one inch deep, has a cover which, when lowered into place, rests in the trough. This tank is supported by an iron frame of gas piping, the legs of which rest on rollers, so that the whole

may be easily moved about the room. An inner tank, two inches shorter and two inches narrower, also provided with a trough that runs around the edge, sits in the large one, and is supported two inches from the bottom of the larger one by iron cross-bars. The bottom of the outer tank and the seal trough on its edges are filled with water. The seal trough of the inner tank is filled with glycerin. Both lids are raised and lowered by wire ropes passed through pulleys fixed in the ceiling. The iron frame supporting the tanks may be of any desired height. In our incubating room we have a nest of six tanks, three of which are on frames four feet high and three on frames two feet high. This economizes space, as the lower ones can be rolled under the higher ones. Both lids are supplied with vent tubes which are plugged with cotton in sterilization. Twenty liters of 3 per cent. agar is placed in the inner tank; both lids are lowered into their respective troughs, and with large gas burners at full blast underneath the apparatus is a sterilizer. After three sterilizations on successive days the medium is inoculated by pouring a liquid culture through the vent tubes in the lid of the inner tank. Then with upper lid lowered into the water trough and gentle heat, which may be controlled by a thermoregulator, it becomes an incubator. With a number of tanks in a small room it is better to heat the room to the desired temperature, thus regulating the heat, than it is to heat each tank separately.

When the growth has reached its maximum, the time necessary for this varying with the organism grown and the temperature maintained, both lids are raised, the growth is detached from the subjacent agar with sterilized bent glass rods, sterile salt solution added if necessary, and the bacterial mass is drawn by means of a water pump into a sterilized receiver.

The tanks are inoculated from special glass bulbs in which the organism has been grown for some days. With the colon bacillus we have usually employed Uschinsky's solution, or some modification of it, in the inoculating bulbs, in order that there may be no trace of foreign protein in

the bacterial growth. While it was highly desirable that this should be done at least once in order to demonstrate that the protein reaction given by the bacterial substance was not due to some constituent of the culture medium, ordinarily beef-tea cultures may be employed. As will be seen later, we did grow the colon bacillus once in liquid Uschinsky medium for the purpose of fully satisfying ourselves that the protein material obtained did not come from the culture medium.

After removal from the tanks the bacterial cellular substance may be washed with various fluids. As a rule, we have washed once or twice with sterile salt solution by decantation and then repeatedly with alcohol, beginning with 50 per cent. and increasing to 95 per cent. The substance is then placed in large soxhlets and extracted first for one or two days with absolute alcohol, and then for three or four days with ether. These extractions with alcohol and ether should be thorough in order to remove all traces of fats and waxes.

After extraction, the cellular substance is ground, first in porcelain, then in agate mortars, and passed through the finest meshed sieves. If there be bits of agar in the bacterial cellular substance, which is seldom the case, it is separated by the sieve and discarded. The one who grinds the cellular substance should wear a mask in order to protect himself; notwithstanding this precaution, several workers have been acutely poisoned, especially with the typhoid bacillus. Of course, there is no danger of infection, as the material, after the treatment already described, contains no living bacilli. The finely ground cellular substance in the form of an impalpable powder may be kept in wide-mouthed bottles in a dark place, and if so kept it retains its toxicity for years, but when long exposed to the light, even if kept perfectly dry, it becomes less poisonous.

The yield from the tanks varies with the organism, but generally amounts to from 60 to 80 grams of the purified cellular substance for each tank, and with six tanks in

operation, and with a crop every three weeks, one may obtain several kilograms within a few months.

Three successive crops of the colon bacillus have been grown on the same agar, reesterilizing and reinoculating after each harvest, but the third crop is not abundant. It has been found to be well to follow the example of the scientific agriculturalist and rotate the crops. Colon grows well after typhoid, but typhoid does not grow well after colon. Five crops have been obtained in the following order: (1) pneumococcus, (2) typhoid, and (3) three successive colon growths, or better, non-pathogenic bacteria following one colon growth.

Many non-pathogenic organisms, the colon, typhoid, pneumococcus, and diphtheria organisms, have been grown on the tanks, and so simple is this method of obtaining bacterial cellular substance in large amount that any intelligent person, after some experience, may repeatedly go through the whole manipulation, producing growth after growth, without contamination. In the laboratory it is best to have one man make a specialty of producing these growths.

The anthrax and tuberculosis cellular substances with which we have worked have not been produced in the tanks. The former has been grown in Roux flasks and the latter in glycerin beef-tea cultures. After these growths have been obtained, their further preparation has been the same as that already outlined.

Prepared, as described, the bacterial cellular substances form fine, white, or yellowish-white powders. This is true even of the chromogenic bacteria, such as *b. violaceus* and *b. prodigiosus*, the pigment being removed from the cells by its solubility in alcohol. One of our former students, Detweiler,<sup>1</sup> studied some of these pigments, but these do not concern us at present, because they constitute no part of the cellular protein. The same is true of the other bodies soluble in alcohol and ether. The extracts made with these

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902, xvii, 246.

solvents contain fats, waxes, pigments, and possibly other substances, but in this review we are interested solely in the cellular proteins.

Microscopic examination of the powdered bacterial substances show the bacilli, mostly intact, though many are more or less broken by the attrition to which they have been subjected. The cells still take the ordinary stains, but the tubercle bacillus and others of this group are no longer acid-fast, showing that the property of retaining the stain when washed with mineral acid is due to some constituent removed by the alcohol and ether.

## CHAPTER III

### PRELIMINARY EXPERIMENTS WITH BACTERIAL CELLULAR SUBSTANCE

*BACILLUS coli communis* was selected in the earlier experiments for the following reasons: (1) It is easily obtained at any time from the normal feces of man. (2) It is quite stable in artificial cultures, varying but little, if transplanted from day to day, in its effects upon experimental animals. (3) It elaborates no extracellular poison, at least under ordinary conditions and in beef-tea cultures.

Our early findings were reported in 1901,<sup>1</sup> and these, confirmed and enlarged by subsequent work, will be briefly reported as follows:

1. The poison is contained within the bacterial cell from which it does not, at least under ordinary conditions, diffuse into the culture medium.

This was demonstrated by the following experiment, which was repeatedly made, and always with the same result: Beef-tea cultures of the colon bacillus, grown for three weeks or longer, in the incubator, were filtered through porcelain. From 8 to 10 c.c. of the clear, sterile filtrate was injected intra-abdominally in guinea-pigs. The animals thus treated were restless and evidently in pain for some minutes after the injection, probably due to the volume of the fluid and its slightly irritating character, but gave no other evidence of any effect of the injection.

As controls to the above, other guinea-pigs received intra-abdominally 0.25 c.c. of the same culture unfiltered, and all died within twelve hours. It will be understood

<sup>1</sup> Trans. Assoc. Amer. Phys., xvi, 201.

that these animals died from infection, and the object in inoculating them was to show that the culture contained living, virulent bacteria, while its filtrate was without effect on animals. However, it might be claimed that the poison, although in solution in the beef-tea, will not pass through porcelain. This suggestion is reasonable, and calls for further experimentation; consequently an unweighed portion of the dead cellular substance of the colon bacillus was suspended in water, heated in the autoclave at 154° under 2 kilos of pressure, and filtered through porcelain. Four cubic centimeters of this clear, sterile filtrate injected intra-abdominally into a guinea-pig caused death within thirty-six hours, and section showed the same lesions that are found after death from either the living bacillus or the dead cellular substance. This demonstrates that when the bacterial cells have been disrupted by superheated steam, their poisonous constituent becomes to some extent soluble in water, and may be passed through porcelain. Furthermore, experiment showed that colon cultures when boiled in open dishes and filtered through porcelain supplied inert filtrates. This indicates that the disrupting effect of a high temperature is necessary to the extraction of the poison from the cell.

Filtrates from living cultures of the diphtheria bacillus contain a toxin which is a secretion of the living micro-organism. The colon bacillus produces no such active toxin. Old, dead cultures of the colon or typhoid bacillus may contain soluble poisons, but these are not secretions of the living cells. They come from the autolysis of the dead cells, and, as we shall see later, they are not properly toxins, capable of producing antibodies, but are chemical poisons. Moreover, the toxin of the diphtheria bacillus is specific, while the cellular poison is not.

2. The poison is not extracted from the bacterial cell by dilute saline solution, alcohol, or ether, either at ordinary temperature or at the boiling-point of these fluids. Extracts of the cellular substance of the colon bacillus with these agents were repeatedly made, filtered, evaporated

*in vacuo*, taken up in a small volume of water, and injected into animals without effect. That the extraction of the cellular substance of the colon bacillus with alcohol and ether has no destructive action on the intracellular poison is shown by the fact that this material after extraction with these agents does not lose any of its toxicity. Furthermore, it may be stated that prolonged boiling of the cellular substance of the colon bacillus with alcohol or ether, or with the two successively, neither sets free nor destroys the intracellular poison. These agents dissolve the fats, waxes, and coloring matter from bacterial cells, but do not remove or destroy the intracellular poison. As will be seen later, this is also true of the intracellular poison of those bacteria that produce a soluble toxin in their cultures. Moreover, it will later appear that the poisonous group not only in bacterial but in vegetable and animal proteins is soluble in absolute alcohol after it has been well detached from the other groups in the protein molecule, but it is not removed from its place in the complex molecule by either alcohol or ether. Indeed, the poisonous group in the protein molecule is not removed from its attachment to other groups by any purely physical solvents, and the molecule must be disrupted by high temperature, chemical agents, or enzymes before its poisonous constituent can be extracted by physical solvents.

3. The cellular substance of the colon bacillus may be heated with water without destruction of its poisonous group.

Two hundred milligrams of the cellular substance of the colon bacillus was suspended in 10 c.c. of water in a tube, which was then sealed and heated at 184° for thirty minutes. On opening the tube, the milky content was found on microscopic examination to contain granular debris with a few unbroken cells. Portions of this heated substance injected into guinea-pigs caused death, and autopsy revealed the same lesions that are seen after death from either the living bacillus or the unbroken cellular substance.

Another portion of the content of this heated tube was placed in a centrifuge and separated into a deposit and a

supernatant fluid, the latter being somewhat opalescent. Guinea-pigs were treated with both portions, and all died. This demonstrates that superheated steam disrupts the bacterial cells, but does not destroy the intracellular poison.

Heating the cellular substance of the colon bacillus in physiological salt solution to  $140^{\circ}$  in the autoclave does not destroy or even weaken the poison. The cell substance used in this experiment had been prepared six years previously. One gram was thoroughly mixed with 100 c.c. of salt solution. Two cubic centimeters of this mixture, containing 20 mg. of the cell substance, killed guinea-pigs of 300 grams' weight when injected intra-abdominally, and 0.5 c.c. or 5 mg. made the animals very sick. The emulsion was then heated in the autoclave to  $140^{\circ}$  and held at this temperature for ten minutes. One cubic centimeter of this heated emulsion, containing 10 mg. of the cell substance, killed guinea-pigs of 300 grams' weight, and 0.1 c.c. or 1 mg. made the animals sick. Animals treated with the heated emulsion died more promptly and from smaller doses than those treated with the unheated preparation. Evidently the heating prepared the cell substance so that it was more promptly split up by the ferments of the body. When heated to this temperature a part of the poison passes into solution. This was shown by filtering the heated emulsion through hard paper. The filtrate was clear, slightly acid to litmus, gave the biuret, Millon, and  $\alpha$ -naphthol tests, and killed guinea-pigs of 300 grams' weight. The animals that had the unheated suspension showed marked peritoneal inflammation, with bloody exudate. In those having the larger doses (40 mg. of the cell substance) the inflammatory condition extended to the muscular walls of the abdomen. In those that had the heated suspension the inflammatory condition was much less marked, there being only a slight serous exudate, less and less stained with blood as the amount of the cell substance injected decreased. In those killed with the filtrate there was no evidence of peritoneal inflammation. This furnishes a beautiful illustration of the nature of inflam-

mation as caused by bacterial cells. When such cells are disrupted wholly by the body cells, and in a restricted locality, there is marked destruction of the local body cells, and this is the condition which we designate as "local inflammation." On the other hand, when the bacterial cells are disrupted and the cellular poison made soluble before being introduced into the body of the animal, there is no local reaction, or no special local reaction, and consequently no recognizable inflammatory conditions.

While heating suspensions of the cellular substance in water and salt solution does not lessen its toxicity, the poison passes into solution only partially. The heated suspension is more than twice as poisonous as the filtrate from the same. Some of the poison actually goes into solution and the filtrate from hardened paper may be perfectly clear, provided the first portion be returned to the filter, but the greater part of the poison is removed by filtration through paper. It is worthy of note that heated suspensions of the colon cellular substance, filtered or unfiltered, easily become contaminated, and apparently furnish acceptable culture media.

4. Dilute (0.5 per cent.) solutions of the caustic alkalis disrupt the cellular substance of the colon bacillus slowly and imperfectly.

This is shown by the following: 100 mg. of the cellular substance was boiled for five minutes in an open dish with a 0.5 per cent. solution of sodium hydroxide. The fluid was centrifuged and the deposit found to be still poisonous, while the supernatant fluid was without effect. However, stronger solutions (2 per cent.) of alkali completely disrupt the bacterial cell and dissolve the poison after prolonged heating. As will appear later, the method finally selected for splitting off the poisonous group consists in heating the cellular substance with a 2 per cent. solution of sodium hydroxide in absolute alcohol.

5. Boiling with a 0.2 per cent. dilution of hydrochloric acid has but little effect upon the bacterial cell or its contained poison.

One hundred milligrams of the cellular substance was boiled in an open test-tube with 10 c.c. of a 0.2 per cent. dilution of hydrochloric acid. This induced no visible alteration in the bacterial cells, as seen under the microscope, and the injection of this material into animals caused death in the usual time and with the usual findings.

6. Heating the cellular substance for an hour in an open dish on the water-bath (about 80°), with from 1 to 5 per cent. solutions of hydrochloric acid, breaks up the cells but does not wholly destroy the toxicity of the cell content; however, prolonged boiling with 1 per cent. or stronger dilutions of hydrochloric acid does destroy the poison.

Five hundred milligrams of the colon cellular substance was heated on the water-bath for one hour with 500 c.c. of a 5 per cent. solution of hydrochloric acid and then decanted through a hard filter. The filtrate was clear and colorless and gave no appreciable precipitate when dropped into absolute alcohol, but that the acid had dissolved some part of the cellular substance was shown by the response of the filtrate to the biuret test.

The undissolved material was suspended in a dilute solution of sodium bicarbonate, sufficient to neutralize the acid, and injected into guinea-pigs which died in the characteristic way, and showed the usual lesions.

7. The bacterial cellular proteins are, so far as their toxicity is concerned, quite resistant to the action of pepsin and trypsin.

A given sample of the cellular substance of the colon bacillus was tested upon a large number of guinea-pigs, in order to determine the minimum lethal dose, which was found to be for half-grown animals 0.5 mg. given intra-abdominally, and 1 mg. subcutaneously. This material was then subjected for three days to an artificial gastric juice, the efficiency of which was demonstrated simultaneously by its action on coagulated egg-white. The soluble and insoluble parts were separated and their toxicity tested. One-half milligram of the undigested part given intra-abdominally did not kill but 1 mg. did; while 1 mg.

given subcutaneously no longer killed but 2 mg. did. Of the part that was dissolved in the acid pepsin solution, doses up to 100 mg. had no effect. A like result was obtained with the cellular substance of the typhoid bacillus. The amount of cellular substance left undigested after three days' exposure to the acid-pepsin was about 10 per cent. of that originally taken. The conclusion is that the gastric juice slowly digests the bacterial cellular proteins, and in so doing destroys the poison.

With trypsin the effect is somewhat different. The cellular protein goes into solution more rapidly, and at least a part of the poison goes into solution without complete loss of its properties. The parts of both the colon and typhoid cellular substance that passed into solution after three exposures to trypsin killed in doses of from 35 to 40 mg. given intra-abdominally, while the undigested portion killed in doses of from 4 to 7.5 mg.

One gram of the cellular substance given to a rabbit through a stomach-tube had no recognizable effect on the animal.

At one time early in these investigations we had an idea that the poison in the colon cell resisted peptic digestion, and we therefore quite naturally suspected that it might be a nuclein. This belief was founded upon the following: The growth on fifty Roux flasks was removed, extracted with 96 per cent. alcohol so long as the alcohol took up coloring matter, then dried, placed in a beaker, and stirred with 1 liter of 0.2 per cent. hydrochloric acid dilution in which 0.5 grams of active pepsin had been dissolved. The beaker with content was kept in the incubator for two days, with occasional stirring. The undigested portion on microscopic examination was found to be amorphous, but still easily stained with methylene blue. It was collected on a filter, washed thoroughly with 96 per cent. alcohol, dried at 100°, and pulverized. One hundred milligrams of this powder was shaken with 50 c.c. of water, forming an acid, colloidal mixture. On adding sodium bicarbonate to a faintly alkaline reaction, the substance

dissolved to an opalescent fluid. This was heated in order to insure sterilization, and injected into guinea-pigs, which it killed within from six to twenty-four hours. One milligram and even less of this undigested portion killed the animals thus treated, but subsequent investigation showed that the poisonous portion, when fully detached from the other constituents of the protein molecule, kills in a few minutes, and we concluded that the undigested part consisted of several groups still attached, or, in other words, of a larger and more complex group of which the poison is only a part. The action of the proteolytic enzymes on bacterial cells deserves a more thorough study than we have given it.

These preliminary studies quite convinced us so long ago as 1901 that a typical colon bacillus, obtained from normal human feces, does not elaborate in its cultures a soluble poison, but that its cells do contain a highly active body. Moreover, these studies indicate that the poison of the colon bacillus exists in the essential proteins of the bacterial cell, and that it cannot be isolated until these proteins are broken up into their constituent parts. In other words, the poison consists of one or more groups in the protein molecule. Since the colon bacillus may grow in a medium consisting solely of inorganic matter and a small amount of some organic compound, as asparagin, its protein must be formed synthetically, and its poison, as a constituent of its protein, must be developed in the same way. One is forced to the conclusion that the poison of this bacillus, at least, does not result from the cleavage action of the bacterial cell or its soluble ferments on the constituents of the medium in which it grows, but that it is built up synthetically, and is set free only when the cellular protein is disrupted. In other words, the harmful action of bacillus coli communis upon animals is not due directly to the growth and multiplication of the organism in the animal body, but to the breaking up of the bacterial protein and the consequent liberation of its poisonous group.

Subsequent and more extended research has shown that

the stability of the protein molecule varies within wide limits, and that the facts learned in the study of the special strain of the colon bacillus do not hold strictly true in every particular with all proteins. This was expected, and we hoped to obtain from these preliminary studies nothing more than certain standards by which our findings in more extended studies might be measured. With some strains of the colon, and in many more of the typhoid bacillus, we have obtained evidence of the presence of soluble poisons in old cultures. For the most part at least these come from autolysis of the bacterial cell. This is a subject to which we shall return in recording the development of these researches.

The findings in our early studies quite naturally developed several inquiries, some of which may be formulated as follows: If the protein of the colon bacillus contains a poisonous group, may not the proteins of other pathogenic bacteria contain similar groups, and if the proteins of pathogenic bacteria contain poisonous groups, why should not the proteins of non-pathogenic bacteria possess like constituents. If bacterial proteins contain poisonous groups, why should not other proteins, such as those of vegetable and animal origin, contain like groups, and if all proteins possess in their structure, poisonous bodies, how is it that the animal world, including man, lives so largely on proteins? Attempts to solve these questions have taken our time and energy, and given us much pleasure. The results of these labors constitute the principal record of this volume.

Marshall and Gelston<sup>1</sup> made an exhaustive study of the toxicity of the cellular substance of the colon bacillus. At first they employed material coarsely ground in a porcelain mortar. This was suspended in water, boiled to insure complete sterilization, and then injected intra-abdominally in guinea-pigs. Up to 1 part of poison to 40,000 parts of body weight all animals treated in this way died. When the proportion was reduced to 1 to 50,000 and less, none of

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902, xvii, 298.

the animals died. The same powder more finely ground in an agate mortar killed 15 out of 16 animals up to 1 to 75,000; out of 28 pigs it killed 9 at 1 to 100,000; out of 8 it killed 5 at 1 to 200,000; out of 34 it killed 4 at 1 to 2,000,000. Heating the cellular protein for fifteen minutes under 2 kilos of pressure at 134° did not appreciably lessen its toxicity. The finely ground powdered substance killed rabbits when injected intra-abdominally: 2 out of 4 at 1 to 75,000; 4 out of 7 at 1 to 100,000; 4 out of 12 at 1 to 200,000; and 2 out of 18 at 1 to 2,000,000.

One gram of the cellular protein was incinerated and the whole ash injected intraperitoneally in a guinea-pig without effect. The toxic effect when subcutaneously injected is practically the same as when employed intraperitoneally, but death is longer delayed.

The intracellular poison of the diphtheria bacillus was studied by Gelston,<sup>1</sup> using different strains, among which was the well-known extracellular toxin producer designated as Park No. 8. These were grown in Roux flasks, as it was found that this organism does not grow well in the tanks, supposedly on account of limited aëration. The growth scraped from the surface of the agar was placed in physiological salt solution and heated for two and one-half hours at 50° to secure sterilization. This suspension was then poured onto hard filters and placed in an ice-box until filtration was complete. The mass on the filter was washed with sterile physiological salt solution, dried on porous plates over sulphuric acid *in vacuo*, and reduced to a fine powder in agate mortars. It is worthy of note that the physiological salt solution contained small amounts of the extracellular toxin, and that still larger quantities could be obtained by macerating the agar, on which the bacillus had grown, with salt solution. It will be observed that this cellular material was not extracted with alcohol and ether. It killed guinea-pigs when injected subcutaneously or intraperitoneally up to 1 to 33,000. On post-

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902, xvii, 308.

mortem examination all animals dying from intraperitoneal injection showed marked congestion of the mesentery, omentum, peritoneum, and adrenals; also numerous ecchymoses and hemorrhagic effusions in the serous coat of the stomach, intestine, and peritoneum. The spleen, liver, and kidneys were slightly enlarged and dark. In the kidneys a dark line sharply divided the cortex from the medulla. The heart was in diastole. When mixtures of diphtheria antitoxin and suspensions of the cell substance in salt solution were injected into guinea-pigs, death followed as promptly as when the dead germ only was given. One thousand immunity units failed to protect against 10 minimum fatal doses of the cell substance as shown by the following:

TABLE I

| No. | Weight. | Amount injected. | Antitoxin. <sup>1</sup> | Antitoxin. <sup>2</sup> | Immunity units. | Result. |
|-----|---------|------------------|-------------------------|-------------------------|-----------------|---------|
| 1   | 155     | 47.1 mg.         | 0.0005 c.c.             | 0.00031 c.c.            | 0.118           | +       |
| 2   | 135     | 40.9 mg.         | 0.0050 c.c.             | 0.00290 c.c.            | 1.175           | +       |
| 3   | 200     | 60.6 mg.         | 0.0242 c.c.             | 0.01940 c.c.            | 5.700           | +       |
| 4   | 150     | 46.0 mg.         | 0.0500 c.c.             | 0.03000 c.c.            | 11.750          | +       |
| 5   | 135     | 40.9 mg.         | 0.2000 c.c.             | 0.10900 c.c.            | 47.000          | +       |
| 6   | 175     | 53.0 mg.         | 0.5000 c.c.             | 0.35000 c.c.            | 117.500         | +       |
| 7   | 120     | 37.0 mg.         | 1.0000 c.c.             | 0.48000 c.c.            | 235.000         | +       |
| 8   | 120     | 37.0 mg.         | 2.0000 c.c.             | 0.96000 c.c.            | 470.000         | +       |

In these experiments the minimum lethal dose of the cellular substance was 1 to 33,000, and 1 c.c. of antitoxin was equivalent to 235 immunity units.

With another sample of cell substance and of antitoxin, the following figures were obtained:

TABLE II

| No. | Weight. | Amount injected. | Antitoxin.  | Toxin cor. | Anti-toxin cor. | Immunity units. | Result. |
|-----|---------|------------------|-------------|------------|-----------------|-----------------|---------|
| 1   | 257     | 55.39 mg.        | 2.5000 c.c. | ....       | 2.5680          | 1000            | +       |
| 2   | 280     | 0.05 c.c.        | 0.0025 c.c. | 0.056      | 0.0028          | 1               | -       |
| 3   | 245     | 52.80 mg.        | control     |            |                 |                 | +       |

In this series No. 2 received 10 minimum lethal doses of a filtered bouillon culture of the same bacillus from which

<sup>1</sup> Antitoxin for 250 grams body weight.

<sup>2</sup> Antitoxin for actual body weight.

the cellular substance had been obtained, and 1 immunity unit of antitoxin. These experiments show that while diphtheria antitoxin protects against the extracellular toxin it fails to protect against the intracellular poison.

Suspensions of the diphtheria cellular substance when exposed to a temperature of 50° or higher, for fifteen minutes or longer, gradually decrease in toxicity, but this is not wholly lost after exposure to 122° in the autoclave for thirty minutes. The minimum lethal dose of our preparation having been found to be 1 to 33,000, it was heated to 122° for 20 minutes, and then proved to be 1 to 6400. It is worthy of note that the several strains of the diphtheria bacillus employed yielded cellular substances that varied widely in toxicity. As a rule, one that supplied a potent extracellular poison yielded relatively an indifferent intracellular poison. Possibly this is due to the greater lability of the molecular structure of the former, which leads to the partial breaking down of the protein molecule in the heating resorted to in order to sterilize the growth.

The cellular substance of the anthrax bacillus was prepared and studied by J. Walter Vaughan.<sup>1</sup> This kills guinea-pigs in only relatively large doses, and this fact indicates that the intensity of the infectious properties of a microorganism is not, always at least, measured by the potency of its intracellular poison. The bacillus prodigiosus is non-pathogenic to the higher animals, not from its inability to furnish a poison, but because it cannot grow and multiply in the animal body; while, on the other hand, the anthrax bacillus is highly infectious to some of the higher animals, not from the intensity of the poison which it elaborates, but rather from the fact that in these animals this bacillus finds conditions favorable to its growth and multiplication.

Detweiler<sup>2</sup> prepared and demonstrated the poisonous action of the cellular substances of *b. prodigiosus*, *b. violaceus*, *sarcina aurantiaca*, and *s. lutea*.

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902, xvii, 313.

<sup>2</sup> Ibid., 257.

The relative toxicity of the finely powdered cellular substances of certain bacteria in proportion to body weight of animal, is shown in the following figures:

|                                  |             |
|----------------------------------|-------------|
| Bacillus anthracis . . . . .     | 1 to 1,700  |
| Sarcina lutea . . . . .          | 1 to 2,050  |
| Micrococcus pneumoniae . . . . . | 1 to 10,000 |
| Sarcina aurantiaca . . . . .     | 1 to 25,500 |
| Bacillus violaceus . . . . .     | 1 to 26,500 |
| Bacillus diphtheriae . . . . .   | 1 to 33,000 |
| Bacillus typhosus . . . . .      | 1 to 40,000 |
| Bacillus pyocyaneus . . . . .    | 1 to 50,000 |
| Bacillus coli . . . . .          | 1 to 75,000 |
| Bacillus prodigiosus . . . . .   | 1 to 90,000 |

Numerous and varied attempts to immunize animals with the bacterial substances were made. Guinea-pigs, rabbits, and goats were used in these experiments. The following quotation is taken from the report Marshall and Gelston<sup>1</sup> made in 1902:

“1. Although guinea-pigs and rabbits acquire immunity to the germ substance but slowly, yet if sufficient time be given, and the animal be allowed to recover before a second dose is administered, a fair degree of immunity may be obtained after many months of treatment. Unless the animal be allowed to recover completely before a second injection is given, death generally results. In these experiments for the purpose of inducing immunity, we employed the finely divided germ substance which had been used in determining the toxicity. Our previous experiments had shown that one part of the finely divided powder to 75,000 parts of body weight in a guinea-pig was surely fatal. Guinea-pig No. 93 received in the fifteenth injection a dose of 28 mg. (1 to 17,321), and recovered from the same. Guinea-pig No. 109 received on the ninth injection 25.5 mg. (1 to 22,745), and recovered. Rabbit No. 17 received on the fifteenth injection 372.3 mg. (1 to 4780), and recovered. Rabbit No. 29 received on the thirteenth injection 293.3 mg. (1 to 6409), and recovered.

<sup>1</sup> Loc. cit.

"2. Study of the blood serum from animals partially immunized to the germ substance led to the following results:

"(a) The blood serum of rabbit No. 2, which had received on the twelfth injection 562.3 mg. (1 to 3503), obtained twenty days after the last injection, had no bacteriolytic action on the organisms contained in a beef-tea culture of the colon bacillus of the same strain as that which had been used in immunizing the animals.

"(b) Tubes of immune serum and others of normal serum were inoculated with virulent cultures of the colon bacillus, and placed in the incubator at 37.5°. Both tubes showed good and equal growth within seven hours.

"(c) Tubes of immune and normal serum were inoculated with virulent cultures of colon and typhoid bacillus, and, from these gelatin plates were made at the end of one minute, thirty minutes, one hour, and forty-eight hours. The number of colonies on the colon plates did not diminish while those on the typhoid plates did with both sera, thus showing that the immune serum had no specific action.

"(d) Immune serum, when mixed with filtered cultures of the colon bacillus, gives a slight precipitate, which, however, is also given by normal serum.

"(e) The immune serum gives very positive agglutinating reactions with suspensions of the dead colon germ used in immunizing the animals. Suspensions of from 1 to 50 mg. of the germ substance in 1 c.c. of physiological salt solution are completely precipitated in from three to five minutes on the addition of an equal quantity of immune serum. Control tubes to which normal serum had been added gave negative results, inasmuch as complete subsidence did not occur in these within fourteen days.

"(f) The immune serum exerts, when mixed with suspensions of the germ substance and injected into the abdominal cavity of rabbits, a slight protective action, as is shown by the following:

Rabbit No. 1, 850 grams, had 17.0 mg. (1 to 50,000), 2 c.c., I. S., -.

Rabbit No. 2, 700 grams, had 14.0 mg. (1 to 50,000), 2 c.c., N. S., +.

Rabbit No. 3, 840 grams, had 16.8 gm. (1 to 50,000), 2 c.c., beef-tea, +.

“(g) The immune serum of the rabbit exerts no protective action for the guinea-pig against the germ substance.

“3. The immunity obtained by treating animals with the germ substance is apparently of short duration, and if the interval between the administration of doses be prolonged, death is likely to follow even when there is no increase in the dose. It will thus be seen that the attempt to immunize animals to the germ substance of the colon bacillus is beset with difficulties. If the intervals be too short, and the animal has not fully recovered, death is likely to result, and the same will also probably happen when the interval is unduly long. Even after a marked degree of immunity has been obtained, this is apparently lost within a few weeks, and a repetition of a dose of the same size causes death. The following table, showing next to the last, and the last injections given to certain animals, will illustrate our meaning:

January 19, 1902, rabbit No. 17, 1780 grams, had 372.3 mg. (1 to 4780), and recovered.

March 29, 1902, rabbit No. 17, 1880 grams, had 273.5 mg. (1 to 5048), and died March 31.

January 18, 1902, rabbit No. 29, 2000 grams, had 293.3 mg. (1 to 6819), and recovered.

March 29, 1902, rabbit No. 29, 1880 grams, had 293.3 mg. (1 to 6409), and died March 31.

January 6, 1902, pig No. 93, 485 grams, had 28 mg. (1 to 17,329), and recovered.

March 29, 1902, pig No. 93, 645 grams, had 28 mg. (1 to 19,464), and died March 31.”

Later these experiments were repeated and extended to goats by V. C. Vaughan, Jr., and Cumming, with practically the same results. We concluded that the capability of the animal to bear increased doses of the cellular substance was not sufficiently marked to be designated by the term immunity, and it was decided to recognize it as increased tolerance. This opinion we still hold.<sup>1</sup>

<sup>1</sup> It will be seen from this work as here recorded that we met with the phenomena of protein sensitization or so-called anaphylaxis as early as 1902 in our studies on the bacterial cellular substances, but that we failed to follow it up and indeed did not attach much importance to it.

## CHAPTER IV

### CHEMICAL STUDIES OF BACTERIAL CELLULAR SUBSTANCE

**Proteins.**—Nencki and Schäffer<sup>1</sup> obtained the cellular substance from a mixed culture of putrefying bacteria, dried it to a constant weight, first on the water-bath and then at 110°, pulverized, and extracted with alcohol and ether. The residue thus obtained was extracted on the water-bath with 0.5 per cent. potassium hydroxide. From the alkaline extract a protein, designated as mykroprotein, was precipitated by neutralization and saturation with sodium chloride. Mykroprotein when freshly precipitated was found to consist of amorphous flakes soluble in water, but losing in solubility when dried at 110°. It contains 52.32 per cent. of C, 7.55 per cent. of H, 14.75 per cent. of N, and neither sulphur nor phosphorus. In aqueous solution it gives an acid reaction, and is not precipitated by alcohol, but is precipitated by picric acid and other alkaloidal reagents. It gives the biuret and Millon reactions, but not the xanthoproteic. On being fused with potash it furnishes ammonia, amylamin, phenol, valerianic acid, leucin, and traces of indol and skatol.<sup>2</sup> Later, Nencki<sup>3</sup> attempted to prepare mykroprotein from anthrax. He obtained from anthrax spores a substance which he designated as anthrax protein, closely related to plant casein and animal mucin, soluble in alkalies, but insoluble in water, acetic, and dilute mineral acids. Like mykroprotein, it contains no sulphur. Dyrmont<sup>4</sup> made an analysis of

<sup>1</sup> Jour. f. prakt. Chem., 1879, xx, 443.

<sup>2</sup> Ibid., 1881, xxiii, 302.

<sup>3</sup> Berichte, 1884, xxvii, 2605.

<sup>4</sup> Arch. f. exper. Path. u. Pharm., 1886, xxi, 309.

anthrax protein, with the following results: C, 52.1 per cent.; H, 6.82 per cent.; N, 16.2 per cent., and a trace of ash. We now know that both mykroprotein and anthrax protein are cleavage products obtained from bacterial cellular substances through the action of alkali.

Brieger<sup>1</sup> found in Friedländer's pneumococcus a protein, partially soluble in water and precipitated on boiling, containing less nitrogen than is found in mykroprotein. Lewith,<sup>2</sup> reporting work done by Hellmich on a hay bacillus grown on synthetic medium, stated that a globulin was extracted from the cellular substance by neutral salts at ordinary temperature. This probably was in fact no part of the cellular protein. Subsequent extraction with dilute alkali gave a protein body described as an albuminate and said to resemble casein.

Buchner<sup>3</sup> demonstrated that certain bacterial cells contain pyrogenetic bodies. These are extracted from the cellular substances with dilute alkalies from which they are precipitated with dilute acids. The amount of protein obtained in this way varied greatly with the species of bacteria. *Bacillus pyocyaneus* gave the most abundant yield, supplying 19.3 per cent. of the dried cells. By heating on the sand-bath under a reflux condenser, or in an autoclave at 120°, filtering through sand, and precipitating with absolute alcohol, he obtained a more soluble protein.

Brieger and Fränkel,<sup>4</sup> Proskauer and Wassermann,<sup>5</sup> and Dzierzowski and Rekowski<sup>6</sup> prepared so-called toxalbumins from diphtheria cultures, and made ultimate analyses of the same, but, as we now know, these were mixtures and gave us no information concerning the composition of the bacterial cell.

<sup>1</sup> Zeitsch. f. physiol. Chem., 1885, ix, 1.

<sup>2</sup> Arch. f. exper. Path. u. Pharm., 1890, xxvi, 341.

<sup>3</sup> Berl. klin. Woch., 1890, xxvii, 673; *ibid*, 1084; Münch. med. Woch., 1891, xxxviii, 841.

<sup>4</sup> Berl. klin. Woch., 1890, xxvii, 241, 268.

<sup>5</sup> Deutsch. med. Woch., 1891, xvii, 585.

<sup>6</sup> Arch. d. Sci. biol., 1892, i, 167.

Hammerschlag<sup>1</sup> extracted tubercle bacilli with dilute alkali and precipitated a protein with ammonium sulphate. Buchner<sup>2</sup> extracted tubercle bacilli with from 40 to 50 per cent. glycerin, and obtained what he believed to be the active principle of tuberculin, but which in reality consisted of a mixture of the autolytic products of this bacillus. Hoffman<sup>3</sup> reported the isolation of six proteins from the tubercle bacillus, but these were mixtures. Weyl<sup>4</sup> believed that he had succeeded in separating the membrane from the protoplasmic content of the bacterial cell, and from the latter he obtained a body which he designated as a toxomucin, but there is no proof that the bacterial cell has any such structure as he supposed. Ruppel<sup>5</sup> prepared from the tubercle bacillus a body that he named tuberculosamin.

Vandervelde<sup>6</sup> reported the presence of nuclein in bacillus subtilis, and Dreyfuss,<sup>7</sup> basing his opinion on the behavior of bacteria toward the basic aniline dyes, concluded that nuclein is a constituent of all bacteria. Gottstein,<sup>8</sup> finding that various bacteria decompose hydrogen peroxide, both during life and after death, concludes from this, from the presence of phosphorus, and from the affinity of bacteria for basic aniline dyes, that they contain nuclein. Nishimura<sup>9</sup> reported the finding of nuclein in a water bacillus grown on potato. The bacterial cells were removed from the potatoes, extracted with alcohol and ether, heated under a reflux condenser with 0.15 per cent. sulphuric acid, and then heated in an autoclave at 105°. From this acid extract he obtained 0.17 per cent. xanthin, 0.08 per cent. adenin, and 0.14 per cent. guanin. Lustig and Galeotti<sup>10</sup> prepared

<sup>1</sup> Monats. f. Chem., 1899, x, 9; Centralbl. f. klin. Med., 1891, xii, 9.

<sup>2</sup> Münch. med. Woch., 1891, xxxviii, 45.

<sup>3</sup> Wien. klin. Woch., 1894, 712.

<sup>4</sup> Deutsch. med. Woch., 1891, xvii, 256.

<sup>5</sup> Zeitsch. f. Physiol. Chem., 1898, xxvi, 218.

<sup>6</sup> Ibid., 1884, viii, 367.

<sup>7</sup> Ibid., 1893, xviii, 358.

<sup>8</sup> Virchow's Archiv, 1893, cxxxiii, 302.

<sup>9</sup> Arch. f. Hygiene, 1893, xviii, 318.

<sup>10</sup> Deutsch. med. Woch., 1897, xxiii, 228.

a nucleoprotein from the pest bacillus. From the effect of methylene blue on this bacillus and its microscopic appearance after extraction, they conclude that the alkali removes the nuclein without destruction of the cell membrane. Our studies do not indicate the existence of a membrane in any bacterial cells. Galeotti<sup>1</sup> extracted an organism similar to bacillus ranicidus with 1 per cent. potassium hydroxide, and obtained a protein body containing from 11.99 to 12.21 per cent. of nitrogen and from 0.94 to 1.16 per cent. of phosphorus, and which he believed to be a nucleoprotein. The percentage of phosphorus increased after several reprecipitations.

Aronson<sup>2</sup> extracted the diphtheria bacillus with from one-tenth to one-fifth normal alkali in the cold, at 100° and at 130°, precipitated these extracts, first with acetic acid, then with acidulated alcohol, sometimes with alcohol to which ether and a little acetic acid had been added. The precipitate with acid furnished a white powder, giving the biuret, xanthoproteic, and Adamkiewicz reactions. Xanthin bases, a pentose, and an albumin were found among its decomposition products, thus proving the presence of a nucleoprotein. On the addition of alcohol to this acid filtrate, a new precipitate was formed, and this on purification yielded nucleic acid, from which xanthin bases, a pentose, and a phosphate were obtained. Blandin<sup>3</sup> obtained a nuclein and a nucleo-albumin from typhoid cultures, but these may have come from constituents of the medium or from the bacterial cells. Klebs<sup>4</sup> concluded that nuclein makes up a large part of the tubercle bacillus. He extracted the bacilli with ether and benzol, digested with hydrochloric acid and pepsin and dissolved the residue in alkali. By precipitating the alkaline extract with alcohol, he obtained a nuclein containing from 8 to 9 per cent. of phosphorus. Hahn<sup>5</sup> rubbed moist tubercle bacilli with

<sup>1</sup> Zeitsch. f. physiol. Chem., 1898, xxv, 48.

<sup>2</sup> Arch. f. Kinderheilkunde, 1900, xxx, 23.

<sup>3</sup> La Riforma Medica, April 17, 1901.

<sup>4</sup> Centralbl. f. Bakteriol., 1896, xx, 488.

<sup>5</sup> Münch. med. Woch., 1897, xlv, 1344.

sand, mixed with water, 20 per cent. glycerin, or physiological salt solution, to a dough consistency, and subjected the mass to a gradually increased pressure of from 400 to 500 atmospheres. The clear, expressed fluid contained a large quantity of coagulable protein, which decomposed hydrogen peroxide, but lost this property on being heated. It gave the protein tests and behaved like a nucleoprotein. Ruppel<sup>1</sup> obtained a nuclein containing 9.42 per cent. of phosphorus from the residue left after the preparation of his tuberculosamin. This he called tuberculinic acid, and he believed that it exists in the cell partly combined with the tuberculosamin and partly free. Levene<sup>2</sup> prepared three proteins from tubercle bacilli grown on protein-free medium. The dried bacilli were ground for two or three days in a porcelain mill, then extracted repeatedly for two days with an 8 per cent. solution of ammonium chloride. These proteins coagulated at from 50° to 64°, 72° to 75°, and 94° to 95° respectively. Ammonium sulphate precipitated all of them; sodium chloride only the first; 50 per cent. magnesium sulphate the first; magnesium sulphate to saturation the second, but not the third. It required less acid to precipitate the first, but 0.2 per cent. hydrochloric acid precipitated all three. The third was richer in phosphorus than the others, and Levene concluded that the tubercle bacillus consists principally of nucleoproteins, one of which differs from the others in that it is not precipitated by magnesium sulphate and does not give the biuret reaction. He called attention to the coincidence between the coagulation temperature of the first protein and that necessary for the sterilization of the bacillus. He believed that tuberculin is a specific substance having the constitution of a nucleoprotein. He also made a study of tuberculinic acid, finding but little of this free in mannite synthetic cultures, but considerable in beef-broth cultures. Samples differ in composition, and experiments suggest that tuberculinic acid is less stable than

<sup>1</sup> Loc. cit.

<sup>2</sup> Jour. Med. Research, July, 1901, 135; Medical Record, 1898, liv, 873.

any other known nucleic acid. De Schweinitz<sup>1</sup> thought that a nucleo-albumin is the fever-producing agent in the tubercle bacillus. Maragliano<sup>2</sup> made an aqueous extract of the tubercle bacillus by digesting it on the water-bath and obtained a poisonous substance. This comes from the autolytic cleavage of the bacillus.

**Carbohydrates.**—One of the earliest studies of the chemistry of bacteria was made by Scheibler<sup>3</sup> upon leuconostoc mesenteroides. The viscous growth of this germ in beet juice, after extraction with alcohol was boiled with milk of lime. The filtrate furnished a gum which was regarded as an anhydride of dextrose, since by slow hydrolysis it is converted into the latter. This substance, to which the name of dextran was given, is a white, amorphous powder, soluble in water and dextrorotatory, having three times the rotary power of cane sugar. Scheibler stated that this germ contains ash, fat, water, dextra, and a substance containing nitrogen, believed to be protagon or some closely related body. Kramer<sup>4</sup> separated from the slime of bacillus viscosus sacchari two modifications of a carbohydrate of the formula  $C_6H_{10}O_5$ . Both were optically active, and on being boiled with acid reduced Fehling's solution. Ward and Green<sup>5</sup> found that a species of bacterium from Madagascar sugar-cane secretes invertose. In sugar solutions it produces a viscous growth that gives an opalescent solution in water, which, when treated with alcohol, yields a bulky flocculent precipitate, found to contain two carbohydrates, one of which gives an osazone, and is optically active, while the other is inactive. They regard these bodies as related to, but not identical with, Scheibler's dextran, and are not certain whether they are products of the vital processes of the organism or are cleavage products. Vincenzi<sup>6</sup> could

<sup>1</sup> Bulletin No. 7, Bureau of Animal Industry; Jour. Amer. Chem. Society, 1897, xix, 782.

<sup>2</sup> Berl. klin. Woch., 1899, xxxvi, 385.

<sup>3</sup> Zeitsch. f. Rubenzuckerindustrie, 1874, xxiv, 309.

<sup>4</sup> Monats. f. Chem., 1889, x, 467.

<sup>5</sup> Proc. Roy. Soc., 1899, lxxv, 65.

<sup>6</sup> Zeitsch. f. physiol. Chem., 1887, ix, 181.

find no evidence of cellulose in bacillus subtilis, but Dreyfuss<sup>1</sup> heated masses of this organism to 180° with concentrated alkali and from this extract obtained a substance that reduced Fehling's solution and from which crystals of glucosazone were prepared. From this Dreyfuss concluded that cellulose is present. However, this conclusion is hardly justifiable. Like results were obtained from pyogenic bacilli. Hammerschlag<sup>2</sup> concluded that the tubercle bacillus contains cellulose. The cell substance, previously extracted with alcohol, ether, and 1 per cent. potassium hydroxide, was dissolved in concentrated sulphuric acid, diluted, and boiled, after which it reduced Fehling's solution. A second portion was treated with potassium chlorate and nitric acid, but most of the substance remained undissolved. A third portion was partly dissolved in ammoniacal copper solution. Hammerschlag stated that if one assumes that the nitrogenous material in the tubercle bacillus is all protein and that the protein contains 16 per cent. of nitrogen, this bacillus contains 36.9 per cent. of protein, 28.1 per cent. of cellulose, 27 per cent. of substance soluble in alcohol, and 8 per cent. of ash. Nishimura<sup>3</sup> thought that he found hemicellulose in a water bacillus, in prodigiosus, and in staphylococcus pyogenes. De Schweinitz and Dorset<sup>4</sup> extracted dried tubercle bacilli with alcohol, digested the residue with 1.25 per cent. sodium hydroxide for from forty to sixty minutes, washed the residue, then digested with 1.25 per cent. sulphuric acid, washed, dried, and ignited. The loss by ignition they calculated should give the cellulose. Accordingly, they reported 6.95 per cent. cellulose in the tubercle bacillus. However, this conclusion is hardly accepted by these authors themselves, since in the same paper they state that cellulose is probably present in small amount in the tubercle bacillus, and not present in the bacillus of glanders. Brown<sup>5</sup> boiled "the membrane" of bacterium xylinum twenty

<sup>1</sup> Loc. cit.

<sup>2</sup> Loc. cit.

<sup>3</sup> Loc. cit.

<sup>4</sup> Jour. Amer. Chem. Soc., 1895, xvii, 605; *ibid.*, 1896, xviii, 449; *ibid.*, 1897, xix, 782; *ibid.*, 1898, xx, 618.

<sup>5</sup> Jour. Chem. Soc., 1886, xlix, 432; *ibid.*, 1887, li, 643.

minutes with 10 per cent. potassium hydroxide and found that this digested the bacteria but left "the film" apparently unchanged. The residue was washed with dilute hydrochloric acid, then with water, and treated with bromine according to Müller's method for obtaining cellulose. The product seemed to be identical with that from cotton, dissolving in ammoniacal copper solution and in strong sulphuric acid. It gave a reducing sugar, dextrorotatory, even when grown on media containing only levorotatory substances. Analysis showed a close agreement with cellulose, and Brown regarded this as a cellulose proper, differing from the metacellulose usually found in yeast and the fungi. No trace of dextran was found. Brown regarded the formation of cellulose as a process of assimilation and not of fermentation. Bendix<sup>1</sup> extracted dried bacilli with 5 per cent. hydrochloric acid over the free flame, cooled, made alkaline, then acidulated with acetic acid in order to precipitate the protein. The filtrate gave with phenylhydrazin an osazone which when purified melted at 153° to-155°, thus showing it to be pentosazone. It gave the orcin and optical tests for pentose. He obtained pentose from diphtheria and tubercle bacilli, also from mixed fecal bacteria, but not from the typhoid bacillus. The pentose exists in the nucleoprotein. Aronson<sup>2</sup> found a nucleoprotein containing pentose in alkaline extracts of diphtheria bacilli. This author stated that the residue after complete extraction with alkali contains carbohydrate which is dextrorotatory and yields an osazone; it is neither cellulose nor chitin.

Meyer<sup>3</sup> came to the conclusion that in some species of bacteria fat is stored up, while in others complex carbohydrates take their place. One species grown on barley gave a substance, colored blue by iodine, and easily soluble in malt diastase and in saliva. *Bacillus subtilis* gave a body that is colored red by iodine and is dissolved by saliva, and on boiling with dilute sulphuric acid. Meyer

<sup>1</sup> Deutsch. med. Woch., 1901, xxvii, 18.

<sup>2</sup> Loc. cit.

<sup>3</sup> Flora, 1889, 432.

thought that this might be either glycogen or amyloextrin. He also obtained a substance that he considered a mixture of much amyloextrin and a little  $\beta$ -amylose. It is easily extracted from the cell with water. It may be remarked here that our researches have shown that substances removable from the cell by physical solvents constitute no part of the actual cell protein. The carbohydrate in the essential part of the cell is a constituent of the protein molecule. All carbohydrates, fats, waxes, and inorganic salts that may be washed out of the cell substance are either no part of the cell proper or result from autolytic changes in the cell molecules.

Levene<sup>1</sup> obtained a glycogen-like body from the tubercle bacillus. The cell substance was extracted with salts, or better, with alkali, the albumins removed from the extract with picric and acetic acids, the nuclein and carbohydrate carried down together with alcohol, and then separated by means of copper chloride. The glycogen thus obtained is soluble in water, gives the iodine color test, and reduces Fehling's solution after being boiled with dilute mineral acid. Emmerling<sup>2</sup> prepared chitin or a closely related body from the zoöglea of bacterium xylinum. From 110 grams of moist, impure material he secured 0.2 gram of crystalline glucosamine hydrochloride. Helbing<sup>3</sup> concluded that chitin makes up a large part of the tubercle bacillus, and to this constituent he attributed the peculiar staining properties of this organism. He was clearly wrong in this inference. All the early work on the carbohydrate constituent of the bacterial cell, when the material was grown on media containing carbohydrate, must be regarded as not possessed of practical value.

*Fat, Wax, etc.*—In the earlier studies of the chemistry of bacterial cells it was assumed that the alcoholic and ethereal extracts consisted of fats exclusively. Kramer<sup>4</sup>

<sup>1</sup> Loc. cit.

<sup>2</sup> Berichte, 1899, xxxii, 541.

<sup>3</sup> Deutsch. med. Woch., xxvi, Vereinsbeilage, 1900, 133.

<sup>4</sup> Arch. f. Hygiene, 1891, xiii, 71; *ibid.*, 1893, xvi, 151; *ibid.*, 1895, xxii, 167; *ibid.*, 1897, xxviii, 1.

noted that such an extract had the appearance of fat and melted not much over 40°. Hammerschlag<sup>1</sup> obtained from the tubercle bacillus free fatty acids that melt at 63° and concluded that the fat of this organism consists mainly of tripalmitin and tristearin, and that it contains little or no triolein. Nishimura<sup>2</sup> obtained from the alcoholic and ethereal extracts of his water bacillus a putty-like mass with the properties of lecithin. Meyer<sup>3</sup> found that the fat in bacillus tumescens gradually increases until spore formation occurs, when it disappears, the spores also being free from fat. Klebs<sup>4</sup> found in the tubercle bacillus 20.5 per cent. of a red fat, melting at 42°, and 1.14 per cent. of a white fat melting above 50°; the latter being insoluble in ether, but soluble in benzol. De Schweinitz and Dorset<sup>5</sup> saponified fats from the tubercle bacillus and from the melting-points of the acids concluded that the fat of this organism contains palmitic and arachidic acids, while that of the glanders bacillus contains oleic and palmitic. They also found a crystalline acid, for which they suggested the name tuberculinic acid, though this is quite different from Ruppel's nucleic acid. This new fatty acid was obtained mainly from the culture medium, only in small amounts from the bacilli. The crystals are prismatic or needles, melting at 161° to 164°, readily soluble in water, alcohol, and ether, and not responsive to the biuret test. Analysis showed close correspondence to the formula,  $C_7H_{10}O_4$ . The authors called attention to the similarity in composition and properties of this body to teraconic acid, and suggested that this may be the substance which is responsible for the coagulation necrosis and that it is the temperature reducing substance. In a later paper they described a crude fat extracted from the tubercle bacillus and from which they obtained an acid melting at 62°, unchanged by recrystallization. In concluding they decided that the fat of the tubercle bacillus consists principally of a glyceride of palmitic acid with a minute amount of the

<sup>1</sup> Loc. cit.<sup>2</sup> Loc. cit.<sup>3</sup> Loc. cit.<sup>4</sup> Loc. cit.<sup>5</sup> Loc. cit.

glyceride of a volatile fatty acid to which cultures of this bacillus owe their characteristic odor, also a very small amount of an acid (probably lauric) melting at  $42^{\circ}$  to  $43^{\circ}$ , and an unusually high melting acid, one apparently with a larger carbon content than any before noted in plants. Ruppel<sup>1</sup> obtained three extracts from the tubercle bacillus by using successively cold alcohol, hot alcohol, and ether. The first contains free fatty acids and a fat melting between  $60^{\circ}$  and  $65^{\circ}$ , easily saponified and decomposed into a free acid and a higher alcohol. The second contained a waxy mass, saponified with difficulty, and which seemed to be the ester of a fatty acid and a high alcohol. The third melted at  $65^{\circ}$  to  $67^{\circ}$ , and had an odor resembling that of beeswax. Aronson<sup>2</sup> obtained from tubercle bacilli, by means of a mixture of five parts of ether and one of absolute alcohol, a yellowish-brown tenacious mass, constituting from 20 to 25 per cent. of the dried bacillus. From the growth of several hundred liters of culture 70 grams were secured. This contained 17 per cent. of free fatty acids. The remainder was wax, not acid and glycerin, but esters of acid and alcohol insoluble in water. Most of this wax is not in the cells, but lies around and between them. Levene<sup>3</sup> found almost 30 per cent. of fat or wax in tubercle bacilli. Kresslig<sup>4</sup> extracted tubercle bacilli successively with ether, chloroform, benzol, and alcohol, and obtained 38.95 per cent. of fatty and waxy substances. Repeated extraction with chloroform gave a dark brown mass of the consistency and color of beeswax and melting at  $46^{\circ}$ . He found 14.38 per cent. of free fatty acid, 77.25 per cent. of neutral fat and esters of fatty acids, and some volatile fatty acid, probably butyric. He decided that the fat of the tubercle bacillus is quite different from that obtained from any other source.

**Reducing Action of Bacteria.**—Although the general subject of the reducing action of bacteria scarcely falls

<sup>1</sup> Loc. cit.

<sup>2</sup> Berl. klin. Woch., 1896, xxxv, 484.

<sup>3</sup> Loc. cit.

<sup>4</sup> Centralbl. f. Bakteriol., 1901, xxx, 897.

within the domain of this work, it may be well to mention the results of a few investigations. W. Smith<sup>1</sup> found that many bacilli, including the colon, decolorize methylene blue, sodium indigo sulphate, litmus, etc. He concluded that this reducing action is common to all bacteria, both aërobic and anaërobic; that the velocity of reduction depends upon the number of bacteria and the temperature; that it is a function of the bacterial plasma, and that the reducing substance does not diffuse into the culture medium, but that the cell retains this property for a time after death. Klett,<sup>2</sup> testing this reducing action of bacteria on sodium silicate, tellurate, and some other salts, also concluded that the reducing agent exists in the cell, and is not found among the cleavage products. Jegunow<sup>3</sup> showed that hydrogen sulphide is formed by the reducing action of bacteria on sulphates and on organic bodies containing sulphur. Sulphur bacteria oxidize hydrogen sulphide and store sulphur in the form of oily spheres, which may constitute as much as 90 per cent. of the cell substance. This sulphur is oxidized to sulphuric acid, thus serving as a source of energy in the vital processes of the bacterium. The sulphuric acid is neutralized by carbonates and separated as a sulphate; then by bacterial activity the sulphate is reduced, thus forming a complete cycle. If the bacteria can obtain no sulphur they use that stored up in their cells and die in from one to two days.

The above is a *resume* of the work done on the chemistry of bacterial cells up to the time when our work was begun. It should be clearly understood that we are not now concerned with the cleavage products of bacteria produced in the media in which they grow. This subject is discussed in *Cellular Toxins* by Vaughan and Novy (fourth edition, 1902).

**Moisture, Ash, and Nitrogen.**—Leach,<sup>4</sup> in studying the chemistry of the cellular substance of the colon bacillus

<sup>1</sup> Centralbl. f. Bakteriolog., 1896, xix, 181.

<sup>2</sup> Zeitsch. f. Hygiene, 1900, xxxiii, 137.

<sup>3</sup> Annuaire géologique et mineralogique de la Russie, 1900, ii, 157.

<sup>4</sup> Jour. Biol. Chem., 1906, i, 463.

prepared in our laboratory, makes substantially the following statement. The cell substance, prepared by the method already described, takes up moisture readily and holds it tenaciously, but may be dried to constant weight by heating small amounts in a steam-drying oven for many days at from 85° to 95°. If the temperature falls to 60°, it may absorb moisture even in the oven. One sample was heated to 105° during working hours for three days and kept in a desiccator during the intervals; it increased in weight. Drying *in vacuo* over sulphuric acid is, on the whole, the most satisfactory method, although it may require days and even weeks.

The dried cellular substance burns with a flame, forming volatile and liquid products, giving off odors characteristic of nitrogen compounds, and finally leaving a greenish ash. Two determinations gave the following results:

0.346 gram gave 0.0296 gram of ash, or 8.55 per cent.

0.496 gram gave 0.0431 gram of ash, or 8.68 per cent.

Values reported for other bacteria vary from 3 per cent. in putrefactive organisms to 13 per cent. in prodigiousus, or, by using special media, to nearly 30 per cent. in the cholera bacillus; while in the tubercle bacillus the ash has been found to vary from 1.77 to 5.92 per cent. according to conditions. The ash from the colon bacillus, as we have prepared it, contains sodium, potassium, small amounts of calcium, aluminum, copper, and phosphates. A slight residue insoluble in acid is probably silica. Sulphate is present in so small an amount that it may escape detection, and chloride has not been found. In comparison with the data obtained with other bacteria, these findings are noteworthy only in the absence of magnesium, and in the presence of copper and aluminum. Presumably the former comes from the tanks and the latter from the agar.

Phosphorus was the only constituent of the ash quantitatively determined. The ash was dissolved in nitric acid, the phosphate precipitated with ammonium molybdate, dissolved in ammonia, precipitated with magnesia mixture,

and weighed as pyrophosphate. The following results were obtained:

| Weight of sample. | Weight of phosphate. | Weight of P. | Per cent. of P. |
|-------------------|----------------------|--------------|-----------------|
| 0.496 gram        | 0.0475 gram          | 0.01323 gram | 2.68            |
| 0.346 gram        | 0.0380 gram          | 0.01059 gram | 3.06            |

The mean of these determinations, 2.87 per cent., agrees quite closely with Levene's finding, 2.67 per cent. of phosphorus in the tubercle bacillus from mannite cultures. Most observers report smaller results, but it should be noted that our samples are free from fat and wax, and therefore the percentage is higher than if calculated for the cells not previously extracted with alcohol and ether.

In view of the fact that the cellular substance, notwithstanding the washings to which it has been subjected, cannot be regarded as chemically pure, we have not wasted time in making elementary analyses. Wheeler has collected the nitrogen and ash determinations made in bacterial cellular substance in this laboratory and has arranged them in the following table:

| Substance.                   | Per cent. of nitrogen. | Per cent. of ash. |
|------------------------------|------------------------|-------------------|
| Typhoid . . . . .            | 11.55                  | 5.70              |
| Colon . . . . .              | 10.65                  | 8.615             |
| Colon . . . . .              | .....                  | 8.38              |
|                              |                        | 7.20 (air-dried)  |
| Tuberculosis . . . . .       | 10.55                  | 11.47             |
|                              | 9.27 (air-dried)       | 9.98 (air-dried)  |
| Anthrax . . . . .            | 10.285                 | 7.76              |
| Subtilis . . . . .           | 5.964                  | 10.83             |
| Proteus vulgaris . . . . .   | 6.791                  | 10.88             |
| Ruber of Kiel . . . . .      | 10.655                 | 6.71              |
| Megaterium . . . . .         | 8.349                  | 10.18             |
| Pyocyaneus . . . . .         | 10.843                 | 9.04              |
| Violaceus . . . . .          | 11.765                 | 6.90              |
| Sarcina aurantiaca . . . . . | 11.460                 | 6.40              |

As will be seen, the nitrogen varies from 5.964 per cent. in subtilis to 11.765 per cent. in violaceus, and the ash from 5.7 per cent. in the typhoid bacillus to 11.47 per cent. in the bacillus tuberculosis.

Nicolle and Alilaire<sup>1</sup> give the following table, showing the percentage of water, nitrogen, substance soluble in acetone, and phosphorus in the bacterial cells named:

| Organism.                     | Per cent. of water. | Per cent. of nitrogen. | Per cent. of sub. sol. in acetone. | Per cent. of acetone sub. sol. in chloroform. | Per cent. of acetone sub. sol. insol. in chloroform. | Per cent. of phosphorus in fats. | Per cent. of phosphorus in fats as H <sub>4</sub> PO <sub>4</sub> . |
|-------------------------------|---------------------|------------------------|------------------------------------|---|--|----------------------------------|---|
| B. glanders . . . .           | 76.49               | 10.47                  | 11.69                              | 8.59  | 3.10   | 2.530                            | 8.0   |
| B. chicken cholera . . . .    | 79.35               | 10.79                  | 7.54                               | 6.30  | 1.24   | 2.370                            | 7.5   |
| B. cholera . . . .            | 73.38               | 9.79                   | 8.70                               | 6.77  | 1.93   | 2.370                            | 7.5   |
| B. of Shiga . . . .           | 78.21               | 8.89                   | 12.80                              | 10.57   | 2.23   | 1.570                            | 5.0   |
| B. proteus . . . .            | 79.99               | 10.73                  | 10.87                              | 7.10  | 3.77   | 1.580                            | 5.0   |
| B. typhoid . . . .            | 78.93               | 8.28                   | 15.44                              | 10.64   | 4.80   | 1.160                            | 3.5   |
| B. anthrax . . . .            | 81.74               | 9.22                   | 6.31                               | 1.48  | 4.83   | 0.948                            | 3.0   |
| B. pseudotuberculosis . . . . | 78.83               | 10.36                  | 15.63                              | 10.31   | 5.32   | 0.793                            | 2.5   |
| B. pneumonia . . . .          | 85.55               | 8.33                   | 15.45                              | 7.36  | 8.06   | 0.790                            | 2.5   |
| B. coli . . . .               | 73.35               | 10.32                  | 15.25                              | 11.77   | 3.48   | 0.790                            | 2.5   |
| B. prodigiosus . . . .        | 78.00               | 10.55                  | 9.00                               | 6.60  | 2.40   | 0.474                            | 1.5   |
| B. psittacosis . . . .        | 78.05               | 9.55                   | 11.08                              | 7.03  | 4.05   | 0.474                            | 1.5   |
| B. diphtheria . . . .         | 84.50               | .....                  | 7.04                               | 5.23  | 1.81   | 0.158                            | 0.5   |
| B. pyocyaneus . . . .         | 74.99               | 9.79                   | 15.77                              | 10.67   | 5.10   | 0.157                            | 0.5   |
| B. lymphangitis . . . .       | 77.90               | 9.17                   | 6.83                               | 2.53  | 4.30   | 0.157                            | 0.5   |
| Froberg's yeast . . . .       | 69.25               | 10.00                  | 4.22                               | 2.92  | 1.30   | 0.000                            | 0.0   |
| Chlorella vulgaris . . . .    | 63.06               | 3.96                   | 21.10                              | 12.81   | 8.29   | 0.000                            | 0.0   |

**Carbohydrates.**—In no case have we been able to detect cellulose in the bacterial cell substance. Wheeler made special search for it in *sarcina lutea*. Twenty grams of substance was autoclaved with 25 parts (500 c.c.) of 10 per cent. potassium hydroxide at 120°, first for thirty minutes and then for an hour. There remained a considerable residue which no longer gave the protein reactions, but did respond to the carbohydrate test with  $\alpha$ -naphthol, although it did not reduce Fehling's solution even after prolonged boiling with dilute hydrochloric acid. Cellulose could not be detected by any of the tests employed. Schweitzer's reagent failed to dissolve it, and it gave no

<sup>1</sup> *Annales de l'Institut Pasteur*, 1909, xxiii, 547.

color with iodine even after treatment with sulphuric acid. A portion was dried, and heated with soda lime, when it evolved a gas which turned red litmus paper blue, thus indicating nitrogen which had been reduced to ammonia. The odor of burning feathers also indicated the presence of nitrogen. From these results it was concluded that the residue left after extraction of the cellular substance with 10 per cent. potassium hydroxide at 120° contains a carbohydrate, but there is nothing to indicate that it is cellulose. Leach made a search for cellulose in the cells of the colon bacillus, with like negative results.

There are two carbohydrate bodies in bacterial cellular substances. One of these furnishes a reducing sugar after being boiled with dilute mineral acid, while the other does not. The former may be extracted from the cells with either alkali or acid, better with the former. In Wheeler's studies of *sarcina lutea* the portion soluble in 10 per cent. potassium hydroxide was filtered through paper, acidified with hydrochloric acid, and treated with three volumes of 95 per cent. alcohol, which produced an abundant white, curdy, sticky precipitate. Precipitation by means of alcohol with acetic and picric acids was also tried, but did not prove satisfactory. Purification was attempted by repeated solution and precipitation with acidified alcohol, but the quantity was diminished each time on account of its relative solubility in dilute alcohol. After filtering, washing, and drying in an atmosphere of carbon dioxide, the powder obtained weighed only 0.7872 grams. It contained phosphorus, responded to the carbohydrate test, and reduced Fehling's solution after prolonged boiling with dilute mineral acid. Its phosphorus content was determined and found to be 0.861 per cent., which is too low to indicate the presence of nucleic acid or nuclein in anything like a pure condition. The same investigator at one time took 300 grams of the colon germ substance and heated on the water-bath with six liters of 2 per cent. potassium hydroxide. The extract was filtered through paper and acidified with acetic acid. The precipitate produced,

presumably protein, was filtered out after standing twenty-four hours, and was so small that it was lost on the filter paper.

The filtrate was then poured into three volumes of 95 per cent. alcohol acidified to the extent of 0.5 per cent. with hydrochloric acid. This formed a heavy, white, curdy, fibrous precipitate, which was filtered, washed acid-free with alcohol and then with ether. It was purified by repeated solution in 0.5 per cent. alkali and precipitation with alcohol. Finally there was obtained a fine white powder amounting to something less than 10 per cent. of the original cellular substance, but much had been lost by its partial solubility in dilute alcohol. This powder consists almost wholly of a carbohydrate which is converted into a reducing sugar after prolonged boiling with dilute mineral acid. However, it contained 5.9 per cent. of ash and 0.194 per cent. of phosphorus. Solutions of this powder give none of the protein reactions, with the exception of the xanthoproteic, to which they responded imperfectly.

Leach prepared the same body, but with a higher phosphorus content, from the colon bacillus. The cellular substance, after repeated extraction with dilute (1 to 5 per cent.) sulphuric acid, was extracted upon the water-bath or over a free flame with from 2 to 4 per cent. of sodium hydroxide. The alkaline extract, after filtration, was neutralized with hydrochloric acid and poured into 95 per cent. alcohol. A light colored, flocculent precipitate was obtained. This turned dark on the exposure to air incident to filtration. It was twice dissolved in 0.5 per cent. potassium hydroxide and reprecipitated with acidified alcohol. Each time the fresh precipitate was white or nearly so, but the utmost care in filtering, even in an atmosphere of carbon dioxide, did not prevent its turning dark. The solution in alkali gave the xanthoproteic and furfurol tests, but neither the biuret nor Millon test. Copper chloride gave a precipitate, but picric acid and platinum chloride did not. The solution was accordingly acidified with picric and acetic acids, copper chloride

added, and the mixture poured into three volumes of alcohol. An aqueous solution of the precipitate thus obtained did not reduce Fehling's solution, but after boiling with hydrochloric acid it reduced both Fehling's and Nylander's solutions, and also gave the furfural test, thus showing the presence of a carbohydrate. The original powder burned readily, puffing up and glowing as does nucleic acid, then fusing and leaving a dark ash. Two determinations of phosphorus gave the following:

| Weight of sample. | Weight of pyrophosphate. | Weight of P. | Per cent. of P. |
|-------------------|--------------------------|--------------|-----------------|
| 0.4723 gram       | 0.0524 gram              | 0.01450 gram | 3.09            |
| 0.6469 gram       | 0.0685 gram              | 0.01895 gram | 2.93            |

In our attempts to extract the poisonous groups from bacterial proteins this carbohydrate gave us great trouble. It is readily soluble in water, whether acid or alkaline, and more or less soluble in alcohol, the degree of solubility depending upon the strength of the alcohol. In absolute alcohol it is insoluble, but it cannot be precipitated completely from aqueous solution by the addition of alcohol. Concentrated solutions and residues obtained by evaporation *in vacuo* are sticky and unsatisfactory in all attempts at purification. As we ascertained after much experimentation, the poisonous group in the protein molecule is freely soluble in absolute alcohol, and finally when we disrupted the protein molecule with a dilute solution of alkali in absolute alcohol we secured a complete separation of the poisonous group from both carbohydrates. Therefore the best material in which the bacterial or other protein carbohydrates can be studied is the non-poisonous portion after complete removal of the poisonous group by heating the protein repeatedly with a 2 per cent. solution of sodium hydroxide in absolute alcohol. This method will be discussed in detail later, but it needs to be stated here that when this is done the poisonous group, free from any trace of either carbohydrate, goes into solution in the alkaline alcohol, while the non-poisonous part, or the haptophor, as

we have designated it, containing all the carbohydrates, remains insoluble in this menstruum.

Leach<sup>1</sup> has studied the carbohydrate in the haptophor portion of the cellular substance of the colon bacillus. Gram samples of the haptophor portion were dissolved in water containing a little alkali, neutralized with hydrochloric acid to definite strength and heated on a water-bath in a flask with a reflux condenser. The hydrolyzed solution was neutralized and titrated with Fehling's solution. Although there is undoubtedly some pentose present, there is no proof that the reducing substance is all carbohydrate. However, for purposes of comparison the reducing substance was calculated as xylose. In order to find conditions giving the maximum yield, amount and strength of acid as well as time of boiling were varied as shown in the following table:

REDUCING POWER OF COLON HAPTOPHOR

| No. of sample. | Amount of HCl. | Per cent. of HCl. | Hours boiled. | Per cent. calculated as xylose. |
|----------------|----------------|-------------------|---------------|---------------------------------|
| 1              | 26.0 c.c.      | 1.0               | 1             | 7.05                            |
| 2              | 38.8 c.c.      | 2.5               | 1             | 16.45                           |
| 3              | 38.5 c.c.      | 2.5               | 2             | 21.56                           |
| 4              | 38.5 c.c.      | 2.5               | 4             | 23.12                           |
| 5              | 72.0 c.c.      | 2.5               | 3             | 23.93                           |
| 6              | 72.0 c.c.      | 2.5               | 9             | 23.53                           |

As shown by these figures the maximum amount of reducing substance was obtained by using 2.5 per cent. acid, and boiling for three hours. Longer heating changes the result very little.

Attempts were made to separate this carbohydrate from the other constituents of the haptophor of the colon bacillus. A 5 per cent. aqueous solution of the haptophor was poured into four volumes of absolute alcohol, containing 10 c.c. of hydrochloric acid and 100 c.c. of ether per liter. After settling, the supernatant liquid was siphoned off and the precipitate (known as G) collected with suction, washed

<sup>1</sup> Jour. Biol. Chem., 1907, iii, 443.

with alcohol containing ether, then with ether, dried, and pulverized. The yield from 50 grams of haptophor was 19 grams. This was twice dissolved in water made faintly alkaline with sodium acid carbonate, and reprecipitated by alcohol containing hydrochloric acid and ether. The final precipitate G weighed 16 grams. With water, G forms an emulsion, acid in reaction and cleared by the addition of alkali. The biuret test is negative, Millon doubtful, xanthoproteic, Adamkiewicz,  $\alpha$ -naphthol, and orcin tests are all positive, the carbohydrate tests being very marked. After boiling with acid there is copious reduction of Fehling's solution. G was tested for glycogen, with negative results. One gram of G boiled two and one-half hours with 72 c.c. of 2.5 per cent. hydrochloric acid, gave 38.63 per cent. of reducing substance calculated as xylose. A second gram boiled for five hours yielded 43.77 per cent.

The second carbohydrate, or, more properly, the second substance giving the  $\alpha$ -naphthol test in bacterial proteins, is not converted into a reducing sugar on being boiled with dilute mineral acid. In Wheeler's work with *sarcina lutea* it remained as a residue after extracting the cell material with 10 per cent. potassium hydroxide at 120°. This residue responded to the  $\alpha$ -naphthol test, did not give the protein reactions, and did contain nitrogen. The same investigator also found this body in alkaline extracts of the residue left after extraction with dilute acid. In her work with the cell substance of the colon bacillus, Leach makes the following statement touching this body: "The residue, after repeated extraction with dilute sulphuric acid (from 1 to 5 per cent.), was treated with 2 to 4 per cent. sodium hydrate either upon the water-bath or over a free flame. In every case the substance went into solution readily, leaving only a slight coating on the filter. The slight residue gave no protein test, contained no nitrogen, but gave test for carbohydrate. In one case it was removed from the filter, and the organic matter approximately determined. The total residue was about 0.4 gram, equivalent to 0.8 per cent. of the cell substance used. The organic

matter was only 0.15 gram, equivalent to 0.3 per cent. of the original." This body is also found in the dilute acid extracts of cellular proteins, as is shown by the following additional quotation from Leach: "Some earlier investigations in this laboratory upon the toxicity of the colon germ showed the desirability of studying the action of dilute acid upon the cell substance. Accordingly, samples were treated with 1 per cent. sulphuric acid under varying conditions. On filtering, a light brown or straw-colored fluid was obtained. This readily reduced nitric acid and gave the typical xanthoproteic color on the addition of ammonia. In no case was there more than a slight biuret test, and there was too much sulphate present for a satisfactory Millon test. The  $\alpha$ -naphthol test for furfural was positive. Alcohol gave a voluminous precipitate, A, which will be described more fully under another heading. The alcoholic filtrate, B, was neutralized with sodium hydroxide, the sodium sulphate filtered out, together with some organic matter mechanically carried down, and the liquid distilled under diminished pressure at 30° to 38°. The liquid residue, C, left after distillation, turned yellow on heating with potassium hydroxide, but gave neither the biuret nor Millon test. Again, the xanthoproteic and  $\alpha$ -naphthol tests were positive, but it failed to reduce either Fehling's or Nylander's solution (after boiling with dilute mineral acid). It yielded precipitates with ammonium molybdate, phosphomolybdic acid, ammoniacal silver nitrate, and picric acid. A guinea-pig was injected with 5 c.c. of C with no apparent effect."

We are inclined to attribute the sticky, mucilaginous properties of both acid and alkaline extracts of bacterial cellular substances to these bodies giving the furfural reaction, and here regarded as carbohydrates. Furthermore, we are of the opinion, though this cannot be considered as conclusive, that the one yielding a reducing sugar after boiling with dilute mineral acid exists in the cellular molecule as a constituent of the nucleic acid group, while the other is a part of the protein component.

The reducing carbohydrate is present in the bacterial cellular substance examined in this laboratory in a minimum of something over 10 per cent.; in the colon haptophor in about 24 per cent.; and in precipitate G from the haptophor in about 44 per cent. So far, we have not obtained it free from phosphorus. The percentage of the other furfural giving body we have no means of determining even approximately, although we can safely say that the amount is much smaller. The one yielding a reducing sugar probably exists in the nucleic acid group as a pentose.

**Nuclein Bodies.**—In her work on *sarcina lutea*, Wheeler<sup>1</sup> makes the following statement: “So far as the xanthin bases are concerned, Nishimura<sup>2</sup> found 0.17 per cent. of xanthin, 0.08 per cent. of adenin, and 0.14 per cent. of guanin in his water bacillus. It has been suggested that in Nishimura’s experiments these bases might have come from the potato upon which his organism was grown, but inasmuch as the potato contains only a very small percentage of protein, this is not likely. Lustig and Galleotti<sup>3</sup> report guanin obtained from the pest bacillus, and Galleotti<sup>4</sup> says that a nucleoprotein separated from the bacillus *ranicidus* yielded xanthin bases, although the percentage of nitrogen was low.

“I have gone through the process of testing for xanthin bases four times. Three times the acid extracts were carefully precipitated with powdered silver nitrate crystals until a drop of the solution gave a yellow instead of a white precipitate with barium hydrate. The precipitate was filtered out, washed, dried, and then worked up for xanthin bases. The fourth time the process was almost the same, the difference being that 33 $\frac{1}{3}$  per cent. acid extract had been made. This was first almost neutralized with barium hydrate, the barium sulphate filtered out, carefully washed out and boiled with water, and then the slightly acid extract was precipitated with silver sulphate instead of silver nitrate. The first silver nitrate precipitate was

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902, xxvii, 265.

<sup>2</sup> Loc. cit.

<sup>3</sup> Loc. cit.

<sup>4</sup> Loc. cit.

investigated according to the method given by Kruger and Solomon;<sup>1</sup> as no satisfactory separation was thereby obtained, the last three precipitates were separated according to the method of Kossel and his pupils, as outlined by Hammersten,<sup>2</sup> for the separation of the four bases, xanthin, hypoxanthin, guanin, and adenin. The precipitate was dissolved as completely as possible in boiling nitric acid (specific gravity, 1.1), a little urea having been added to prevent nitrification, filtered hot, concentrated somewhat, and allowed to cool. On cooling, only a slight residue of the guanin-hypoxanthin-adenin portion separated out. On decomposing this precipitate, treating with ammonia and evaporating, the amount obtained was so small that it was not possible to make separation of the bases, but ammoniacal solution produced a comparatively heavy flocculent organic precipitate. The nitric acid filtrate containing the xanthin portion was precipitated with ammonia. A heavy, reddish-brown, mucilaginous precipitate came down, but was not sufficient in quantity or sufficiently free from impurities to justify an ultimate analysis."

Leach<sup>3</sup> obtained from 1 per cent. sulphuric acid extracts of the colon cellular substance a body containing 7.33 per cent. of phosphorus. It gave none of the protein color reactions except the ubiquitous xanthoproteic. It could hardly be anything else than a nucleic acid. However, the percentage of nitrogen was only 8.98, and no known nucleic acid contains so small an amount of nitrogen. In the same extracts she obtained indications of the presence of two xanthin bases, xanthin and guanin. The evidence of the existence of nuclein bodies in the haptophor of the colon cellular substance will be given later.

**Diamino-acids.**—Wheeler failed to obtain any evidence of the presence of arginin or histidin in *sarcina lutea*, but in each of five attempts she secured quite convincing proof

<sup>1</sup> Zeitsch. f. Physiol. Chem., 1898, xxvi, 373.

<sup>2</sup> Physiol. Chem., p. 120, as translated by Mendel.

<sup>3</sup> Jour. Biol. Chem., 1906, i, 463.

of the existence of lysin. She says: "As to lysin, I have obtained in every instance a yellow, thick, oily body, where lysin picrate should be formed, which, however, could not be crystallized. This oily body was shaken up with ether to remove excess of picric acid, but when I attempted to purify it by redissolving it in alcohol, it was no longer completely soluble, inasmuch as a part of it hardened into a solid which seemed somewhat crystalline, and the remainder was precipitated by alcohol. However, it was found to be readily soluble in water, especially in hot water, and although no crystals were obtained on concentration of the aqueous solution, the same heavy oil separated. In order to obtain the hydrochloride, if possible, the oily substance was treated with hydrochloric acid in a little water, and the picric acid shaken out with ether. From the solution on concentration an imperfectly crystalline mass was obtained. It may be that I have not lysin, but there is undoubtedly present some organic body which, in its chemical behavior at least, is very similar to lysin."

In searching for the hexon bases in the cellular substance, Leach proceeded as follows: "The cell substance was stirred with nine times its weight of 33.33 per cent. sulphuric acid, allowed to stand overnight, then heated in an evaporating dish on the water-bath. When all danger of frothing was over, the mixture was transferred to a flask fitted with a reflux condenser, and boiled on a sand-bath for eight hours one day and six the next. After cooling and filtering, some water was added to the filtrate, and it was neutralized by the addition of the calculated amount of barium hydrate. When the barium sulphate had completely settled, the supernatant liquid was siphoned off, the precipitate stirred up with boiling water, heated to boiling, settled overnight, and again siphoned. This was repeated until the wash water was nearly colorless. The extract and wash water were united, acidified with acetic acid,<sup>1</sup> concentrated on

<sup>1</sup> If there is a large excess of barium present, it is well to remove it by carbon dioxide.

the water-bath, cooled, and filtered to remove any tyrosin and leucin that may crystallize out. The filtrate was diluted to about one and one-half liters for each 100 grams of cell substance, made decidedly acid with nitric acid, and 20 per cent. silver nitrate added as long as it gave a precipitate. It was left overnight to settle, and the silver precipitate of xanthin bases filtered out. To this filtrate excess of silver nitrate and barium hydrate were added to remove arginin and histidin. After their removal, silver and barium were precipitated by hydrochloric and sulphuric acids, these inorganic precipitates boiled out with water several times, the filtrate and wash water united, and concentrated. The solution, which should contain some 5 per cent. of acid, was treated with a 50 per cent. solution of phosphotungstic acid as long as it gave an immediate precipitate. The precipitate was rubbed up with 5 per cent. sulphuric acid, carefully washed with the same solution, and filtered with suction. The heavy white precipitate was again rubbed up with water, hot saturated solutions of barium hydrate added, until the mixture was no longer acid, settled overnight, and the supernatant liquid siphoned off. The precipitate, consisting of barium phosphate, tungstate, etc., was washed several times with hot barium hydrate solution, decanted, and finally filtered by suction. The filtrate and wash water were united, and barium was removed as carefully as possible, first by running in carbon dioxide, and then by adding ammonium carbonate to the solution. This precipitate, like all the other inorganic ones, was boiled out several times with water, and the washings added to the original filtrate. The resulting liquid was concentrated nearly to dryness on the water-bath, the residue taken up with water, filtered to remove barium carbonate, and again concentrated to a thick syrup.

“The alkaline syrup was vigorously stirred with alcohol, and then with an alcoholic solution of picric acid. Sometimes a crystalline precipitate came down at once, sometimes there was a viscous mass like molasses candy, which became

granular or crystalline after long kneading and stirring, as Fischer and Weigert suggest. When picric acid would no longer give a precipitate even on standing, the crystals were filtered out by suction, washed with alcohol, and dried on a porous plate. On concentration the alcoholic mother liquid became gummy and viscous, but no more crystals were obtained. The crude picrate was recrystallized from hot water several times. On dissolving there was much sediment which mainly filtered out, but on concentration more appeared upon the sides of the vessel. The loss of substance by the first crystallization was very large; as it became pure, however, it crystallized like an inorganic salt. All mother liquors were treated with hydrochloric acid to remove picric acid, reprecipitated with phosphotungstic, the precipitate worked up as before, and a further crop of crystals obtained. The crystals are slender, yellow, silky, felted needles or prisms. On heating in a melting-point tube the substance begins to change color at  $216^{\circ}$ , and is very dark at  $230^{\circ}$ . Heated side by side with lysin picrate from fibrin and from gelatin, they agree within a degree. The authorities all agree that lysin picrate turns black at  $230^{\circ}$  to  $232^{\circ}$ , while Kutscher and Lohmann also say that it begins to change color at  $215^{\circ}$ .

"To change the picrate into the chloride, 2 grams were dissolved in 33 c.c. of hot water, 5 c.c. of concentrated hydrochloric acid added, cooled, the picric acid filtered out and washed with water containing hydrochloric acid. The filtrates were shaken out with ether as long as there was any yellow color. The solution should be colorless or nearly so; if it is not, it can be decolorized by treatment with animal charcoal. The solution was evaporated nearly to dryness, first on the water-bath, and finally in a desiccator. When down to a thick syrup, stirring gave crystals. These were recrystallized out of hot water containing hydrochloric acid, giving long prisms, which melt at  $192^{\circ}$ , again agreeing with the corresponding salt from gelatin and fibrin. Henze says that lysin chloride becomes soft at  $193^{\circ}$  and melts at  $195^{\circ}$ ; Lawrow says that it has no sharp

melting-point, but begins to melt at 194° to 195°. Henderson collected samples melting from 190° to 200°, prepared by different individuals from widely different sources. By careful purification he obtained from each sample a product melting at 192° or 193°. Thus it would appear that the apparent discrepancy in the melting-point is due to impurities. Reactions, crystalline form, properties, and melting (or decomposing) show that the picrate and chloride from the germ are identical with lysin picrate and chloride from gelatin and from fibrin. Thus the presence of one of the hexon bases in the bacterial cell has been demonstrated, and another point of resemblance between bacterial and other proteins has been established."

**Mono-amino-acids.**—The phosphotungstic acid filtrate obtained by Leach in her work on the hexon bases was turned over to Wheeler, who has made the following report on it: "From the solution phosphotungstic acid was removed with barium hydrate and carbon dioxide used to remove excess of barium. By concentration and crystallization bodies were obtained resembling tyrosin and leucin under the microscope. These were purified by repeated recrystallization from water or from ammoniacal water, the tyrosin being so much less soluble than the leucin that they could be separated by difference of solubility. It was necessary to boil the leucin fraction with animal charcoal to remove the coloring matter. The tyrosin formed the characteristic, colorless, silky needles, many grouped in the characteristic sheaves. As it became more pure the needles were longer and longer, and grouped in the sheaves less positively. After many purifications the crystals melted at a constant temperature, though this was difficult to determine, since tyrosin melts with decomposition. The melting-point maintained after each of two or three recrystallizations was 288°, uncorrected. The correction was 8.13°, which made the corrected point 296.13°. A Kahlbaum preparation of tyrosin in the laboratory melted within a degree of the same point, and agreed in the chemical tests, to be mentioned presently. Richter gives the melting-point of

tyrosin as 235°; Cohn as 295°; while Fischer says that with rapid heating the corrected point is 314° to 318°.

"The tyrosin obtained gives a Hofmann test with Millon's reagent. It gives Scherer's test with nitric acid and sodium hydrate on platinum foil, and also a beautiful Piria test with sulphuric acid, then barium carbonate and ferric chloride, which test is characteristic for tyrosin.

"The leucin crystallized in the characteristic knobs or balls. As it became purer it crystallized more and more in shining, white, very thin plates, sometimes in radial groups, sometimes not. The crystals were finally obtained with a practically constant melting-point, 262° to 263° or corrected, 268.5° to 269.6°. The pure laboratory leucin (Kahlbaum) melted at the same point. Schwanert, Hammersten, and others give the melting-point for active leucin as 170°; that for the inactive form is given as 270°. Fischer says the melting-point is 293° to 295° (corrected) if heated quickly in a closed tube. Cohn gives 275° to 276°. The leucin obtained melted with darkening and decomposition. With careful heating in an open tube it sublimed with the characteristic white, woolly deposit. It also responded to Scherer's test on platinum foil with nitric acid and sodium hydrate, which test Hammersten says is characteristic for leucin."

Agnew<sup>1</sup> has made the following contribution to the mono-amino-acid content of the cellular proteins of the colon and tubercle bacilli.

The material used in this research consisted of the cellular substance of the bacteria. Growths from massive cultures were placed in large Soxhlets and extracted for three days with absolute alcohol and for the same time with ether. The protein bacterial substance thus freed from everything soluble in alcohol and ether was ground into a powder and passed through a fine-meshed sieve. For the preparation of the amino-acids Fischer's method slightly modified was employed. The cellular protein was boiled with three and

<sup>1</sup> Unpublished research.

one-half times its weight of strong hydrochloric acid under a reflux condenser until it failed to respond to the biuret test. The humus substance was filtered out, repeatedly extracted with hydrochloric acid, and the concentrated extracts added to the filtrate from the humus. From this filtrate glutamic acid was isolated as a hydrochloride by saturation with hydrochloric acid gas and standing for some days in the ice-box. The deposited glutamic acid was collected on a filter with the aid of a pump. The filtrate was concentrated *in vacuo* to a syrup, diluted with an equal volume of alcohol, and esterified by saturating with hydrochloric acid gas, the solution being warmed to complete the esterification. The alcohol was distilled off at a temperature under  $40^{\circ}$ , more alcohol added, and removed by distillation, this being repeated three times. At this point glycocoll, as the hydrochloride of the ethyl ester, crystallized out on account of difficult solubility in alcohol. For convenience, the thick syrup containing the esters of the hydrochlorides of the amino-acids was divided into portions and each treated separately but in the same way. The syrup was diluted with half its volume of water, cooled in a freezing mixture, and made slightly alkaline with sodium hydrate. This alkaline solution was extracted with ether, the liquid being kept alkaline by the addition of a few drops of a saturated solution of sodium hydrate. The liquid was then converted into a paste by the addition of solid potassium carbonate and repeatedly shaken with ether. The combined ethereal extract was dried by shaking with solid potassium carbonate for a short time, and by being left for twelve hours over fused sodium sulphate. The ether was then distilled off and the free esters subjected to fractional distillation *in vacuo*. The highest vacuum that I was able to obtain varied from 20 to 30 mm. The pasty mass left after the distillation of the esters, according to Abderhalden's recommendation, was made acid with hydrochloric acid, concentrated, and the inorganic salts allowed to crystallize. The filtrate freed from these crystals was again esterified, furnishing a further but smaller amount of esters.

*Hydrolysis of the Cellular Protein of Bacillus Coli Communis.*—Three hundred and fifty grams of this cellular protein was thoroughly mixed with 1100 c.c. of hydrochloric acid, of specific gravity 1.19, and allowed to stand overnight. The next morning it was found to be a frothy mixture of deep purple color (Liebermann's reaction). It was placed in a 2-liter flask connected with a long reflux condenser, and boiled on the sand-bath for six hours, when it no longer gave the biuret reaction. It was then filtered through heavy paper, giving a clear brown filtrate and leaving a heavy deposit of humus on the paper. The latter was repeatedly extracted with dilute hydrochloric acid, the extracts concentrated, and added to the filtrate. The humus, air-dried, weighed 77 grams, making 22 per cent. of the cellular protein.

The filtrate was concentrated *in vacuo* to half its volume, placed in a freezing mixture, saturated with hydrochloric acid gas and left in the ice-box for two days. By this time the hydrochloride of glutamic acid had crystallized out. An equal volume of ice-cold alcohol, previously saturated with hydrochloric acid gas, was added, and the glutamic acid collected, dried over solid sodium hydrate, and weighed. It yielded 10.5 grams of 3 per cent. of the cellular protein.

The filtrate from the glutamic acid was esterified with hydrochloric acid gas, followed by gentle heat. The alcohol was distilled off *in vacuo*, an equal volume added, again saturated with the gaseous acid, heated, and removed by distillation, this being repeated several times. The fluid was left in the ice-box for forty-eight hours, when crystals were deposited. These were purified by reprecipitating them from alcoholic solution with hydrochloric acid gas. The melting-point was  $144^{\circ}$  and the crystals were identified as the ethyl ester of glycocoll. The yield of glycocoll was 1 gram.

The filtrate from the glycocoll containing the hydrochlorides of the esters was treated as already stated and fractionated with the following yields:

|                        | Bath temperature. | Yield.     |
|------------------------|-------------------|------------|
| Fraction I . . . . .   | 40° to 60°        | 16.5 grams |
| Fraction II . . . . .  | 60° to 80°        | 8.5 grams  |
| Fraction III . . . . . | 80° to 100°       | 22.5 grams |
| Fraction IV . . . . .  | 100° to 130°      | 20.0 grams |
| Fraction V . . . . .   | 130° to 160°      | 16.5 grams |

The dark red residue left in the distillation flask was dissolved in hot alcohol, decolorized with animal charcoal, filtered, and from the filtrate 0.5 gram of leucinimid was obtained.

*Fraction I.*—This was saponified by boiling with five times its weight of water for five hours with a reflux condenser. On concentration two crops of crystals were obtained. The first was dissolved in the smallest possible amount of hot water, then treated with an equal volume of alcohol, and left in the ice-box for twenty-four hours. The white crystalline mass, tasting sweet and closely resembling alanin, weighed 1.75 grams.

The second crop was obtained by evaporation to dryness, and weighed 1 gram. It was dissolved in 3 c.c. of hot water; this was poured into an equal volume of hot absolute alcohol, and set in the ice-box for twenty-four hours. White needle-like crystals, sweet to the taste and arranged in bundles, formed. These were recrystallized, washed with absolute alcohol, dried, and weighed. The yield was 0.5 gram. These crystals sublimed on heating and were probably valin, although it will be seen that neither of these bodies has been positively identified.

*Fraction II.*—This on being saponified by boiling with five times its weight of water for five hours under a reflux condenser and concentrated, yielded a crystalline mass that contained no prolin, since no part of it was soluble in hot absolute alcohol. The crystalline mass was dissolved in 700 c.c. of water and boiled for an hour with an excess of freshly prepared copper oxide. On concentration of the blue filtrate, a difficultly soluble salt was obtained. This was crystallized and the percentage of copper in it found to be 20.35, thus identifying it as the double salt of copper with leucin and valin.

|   |       |
|---|-------|
| Calculated per cent. of Cu in Cu-leucin . . . . .       | 19.66 |
| Calculated per cent. of Cu in Cu-valin . . . . .        | 25.52 |
| Calculated per cent. of Cu in Cu-leucin-valin . . . . . | 20.48 |

The more soluble copper salt was freed from its copper with hydrogen sulphide. The filtrate furnished, on treating it with 80 per cent. alcohol and allowing it to stand in the ice-box, 0.5 gram of alanin.

*Fraction III.*—After saponification by boiling for five hours under reflux condenser with five times its weight of water, this fraction yielded 7.3 grams of solid. The absence of prolin was demonstrated by the fact that hot absolute alcohol dissolved nothing. The solution was boiled with copper oxide which took up all the substance. The percentage of copper was found to be 20.38, thus showing the compound to be the double salt of copper with leucin and valin.

*Fractions IV and V.*—When these were mixed with water a brownish oil separated. This was filtered off and saponified by heating for several hours on the water-bath with an excess of baryta. When the barium had been removed there was obtained 0.5 gram of phenylalanin. It was found difficult to purify this, but finally we did so, and found the melting-point to be 262.5°. The filtrate from the oily ester of phenylalanin was also saponified by heating on the water-bath with an excess of baryta. The absence of aspartic acid was shown by failure to obtain an insoluble barium asparaginate. When the barium was removed a small amount of glutamic acid was found in the filtrate.

The pasty mass left after the extraction of the esters was neutralized with hydrochloric acid and prepared for a second esterification, but owing to an accident this was not completed. No ultimate analyses were made in this or the subsequent hydrolyses, and we have relied for the recognition of the amino-acids on (1) the boiling-point of the esters, (2) crystalline form, (3) melting-point, and (4) the percentage of copper in the copper compounds.

In this hydrolysis we have accounted for only a little more than 10 per cent. of the nitrogen, distributed as

follows: glutamic acid, 3 per cent.; glycocoll, 0.33 per cent.; alanin, 1 per cent.; valin, 1.6 per cent.; leucin, 2 per cent.; and phenylalanin, 0.2 per cent.

The cellular protein of the bacillus coli communis with which we did this work contained 13.74 per cent. of moisture and 7.2 per cent. of ash, or 8.38 per cent. of ash in the moisture-free substance.

*Hydrolysis of the Cellular Protein of the Bacillus Tuberculosis.*—Five hundred grams of this substance was hydrolyzed after the manner already given. The air-dried humus from this substance weighed 120 grams or 24 per cent. of the cellular substance, or 27 per cent. of the moisture-free substance. The humus was found to contain 14.34 per cent. of moisture, 0.15 per cent. of ash, and 1.69 per cent. of nitrogen.

Before hydrolyzing this substance samples were taken, and the following determinations made.

|  |       |
|--|-------|
| Percentage of moisture . . . . .                       | 12.07 |
| Percentage of ash . . . . .                            | 7.08  |
| Percentage of ash in moisture-free substance . . . . . | 8.05  |

Nitrogen was found distributed as follows:

|   |               |
|---|---------------|
| Percentage of nitrogen in cell substance . . . . .                        | 9.27          |
| Percentage of nitrogen in moisture-free cell substance . . . . .          | 10.55         |
| Percentage of nitrogen in ash-free cell substance . . . . .               | 9.98          |
| Percentage of nitrogen in ash- and moisture-free cell substance . . . . . | 11.47         |
|   |               |
| Total amount of nitrogen in 500 grams of cell substance . . . . .         | 46.3500 grams |
| Total amount of nitrogen in the hydrolyzed fluid . . . . .                | 36.3075 grams |
| Total amount of nitrogen in the extract of humus . . . . .                | 4.0530 grams  |
| Total amount of nitrogen in the extracted humus . . . . .                 | 2.0388 grams  |
| Total amount of nitrogen in 500 grams of cell substance . . . . .         | 46.3500 grams |
| Total amount of nitrogen in the hydrolytic product . . . . .              | 42.3993 grams |
| Total amount of nitrogen lost in hydrolysis . . . . .                     | 3.9507 grams  |
| Amount of nitrogen in hydrolyzed fluid . . . . .                          | 36.3075 grams |
| Amount of nitrogen in extract of humus . . . . .                          | 4.0530 grams  |
| Amount of nitrogen in fluid to be esterified . . . . .                    | 40.3605 grams |

It is thus seen that there is in the fluid to be esterified, 40.3605 grams of nitrogen, and it is supposed that this exists in the form of mono- and diamino-acids.

Glutamic acid was separated as the hydrochloride. Chlorides of ammonium and sodium were present in large amount, but were easily separated on account of their greater solubility in water. We obtained 1 gram of glutamic acid, equivalent to 0.2 per cent. of the cellular substance or 0.23 per cent. of the moisture-free substance.

The filtrate from the glutamic acid on being esterified, the esters extracted, dried, and distilled, yielded the following:

|                    | Temperature<br>of bath. | Temperature<br>of vapor. | Weight of<br>distillate. |
|--------------------|-------------------------|--------------------------|--------------------------|
| Fraction I . . .   | 50° to 85°              | 35° to 60°               | 14 grams                 |
| Fraction II . . .  | 85° to 100°             | 60° to 85°               | 33 grams                 |
| Fraction III . . . | 100° to 140°            | 85° to 105°              | 34 grams                 |
| Fraction IV . . .  | 140° to 180°            | 105° to 130°             | 12 grams                 |
| Fraction V . . .   | 180° to 210°            | 130° to 160°             | 12 grams                 |

The pasty mass left after extraction of the esters was acidified with hydrochloric acid, evaporated, the salt filtered out from time to time, and when brought to a thick syrup it was diluted with an equal volume of absolute alcohol and the esterification repeated. However, the yield from this esterification was exceedingly small, not more than a few drops for each fraction.

From the residue left after distillation, we obtained 2 grams of leucinimide, equivalent to 0.4 per cent. of the cellular protein or 0.45 per cent. of the moisture-free substance.

*Fraction I.*—This was saponified by being boiled with five times its weight of water for five hours under a reflux condenser. It was evaporated to dryness and the white crystalline mass was dissolved in 25 c.c. of hot water, treated with an equal volume of hot absolute alcohol and left in the ice-box for two days when a white crystalline mass separated. This was purified and yielded 7 grams of alanin, equivalent to 1.4 per cent. of the cellular substance or 1.57 per cent. of the moisture-free substance.

*Fraction II.*—From this there was obtained by the method already described a copper-leucin-valin compound repre-

senting 3.7 grams of leucin and 12.4 grams of valin. Prolin was not present.

*Fraction III.*—From this there was secured by the formation of the copper salt 5.4 grams of leucin and 10.6 grams of valin.

Fractions II and III gave a combined yield of 9.1 grams of leucin and 23 grams of valin, equivalent to 1.82 per cent. of leucin and 4.6 per cent. of valin in the cellular substance or 2.04 per cent. and 5.17 per cent. respectively in the moisture-free substance.

*Fractions IV and V.*—Each was shaken with three times its weight of cold water and filtered through a damp paper, leaving a brown oil. The oil, after being washed twice with cold water, was saponified with excess of baryta. From this there was obtained 2.5 grams of crude phenylalanin. This was purified and the melting-point found to be 263°. The yield of phenylalanin amounted to 0.5 per cent. of the cellular substance or 0.56 per cent. of the moisture-free substance. From the filtrate from the oil a few crystals which were probably glutamic acid, were obtained, but the amount was too small for identification.

From 500 grams of the cellular protein of the bacillus tuberculosis the following substances in the amounts and percentages given were obtained.

| Substance.              | Amount.     | Per cent. of cell substance. | Per cent. of dry cell substance. |
|-------------------------|-------------|------------------------------|----------------------------------|
| Humus . . . . .         | 120.0 grams | 24.00                        | 27.00                            |
| Glutamic acid . . . . . | 1.0 gram    | 0.20                         | 0.225                            |
| Alanin . . . . .        | 7.0 grams   | 1.40                         | 1.57                             |
| Leucin . . . . .        | 9.1 grams   | 1.82                         | 2.04                             |
| Valin . . . . .         | 23.0 grams  | 4.60                         | 5.17                             |
| Phenylalanin . . . . .  | 2.5 grams   | 0.50                         | 0.56                             |
| Leucinimide . . . . .   | 2.0 grams   | 0.40                         | 0.45                             |

It will be seen that of the total of 40.3605 grams of nitrogen in the fluid esterified there has been recovered in the form of mono-amino-acids only 5.2 grams of nitrogen or 12.88 per cent. However, under the best conditions one cannot hope to obtain more than a part of the mono-amino-acids present and the diamino-acids probably take up a considerable part of the nitrogen.

*Hydrolysis of the Non-poisonous Portion of the Cellular Protein of the Bacillus Tuberculosis.*—Five hundred grams of this non-poisonous bacterial split product, known in this laboratory as “residue” or haptophor, was hydrolyzed. The humus was found to constitute 29 per cent. of the air-dried, or 40 per cent. of the moisture-free substance, the percentage of moisture being 27.5.

The distribution of nitrogen was studied with the following results:

|  |              |
|--|--------------|
| Percentage of nitrogen in residue . . . . .                        | 4.59         |
| Percentage of nitrogen in moisture-free residue . . . . .          | 6.34         |
| Percentage of nitrogen in ash-free residue . . . . .               | 5.54         |
| Percentage of nitrogen in ash- and moisture-free residue . . . . . | 8.29         |
| Amount of nitrogen in the hydrolyzed fluid . . . . .               | 18.980 grams |
| Amount of nitrogen in the extract of humus . . . . .               | 1.888 grams  |
| Amount of nitrogen in the extracted humus . . . . .                | 2.030 grams  |
| <hr/>  |              |
| Total . . . . .  | 22.898 grams |
| Total amount of nitrogen in the 500 grams of residue . . . . .     | 22.990 grams |
| Total amount of nitrogen in the products of hydrolysis . . . . .   | 22.898 grams |
| <hr/>  |              |
| Loss during hydrolysis . . . . .                                   | 0.092 gram   |
| Amount of nitrogen in the hydrolyzed fluid . . . . .               | 18.980 grams |
| Amount of nitrogen in the extract of humus . . . . .               | 1.888 grams  |
| <hr/>  |              |
| Amount of nitrogen in the fluid to be esterified . . . . .         | 20.868 grams |

No glutamic acid could be obtained from the residue.

The result of the fractional distillation is shown as follows:

|                        | Temperature of bath. | Yield.     |
|------------------------|----------------------|------------|
| Fraction I . . . . .   | 40° to 60°           | 21.5 grams |
| Fraction II . . . . .  | 60° to 80°           | 5.0 grams  |
| Fraction III . . . . . | 80° to 100°          | 8.0 grams  |
| Fraction IV . . . . .  | 100° to 130°         | 9.0 grams  |
| Fraction V . . . . .   | 130° to 170°         | 10.0 grams |

By the hydrolysis of 500 grams of tubercle residue the following substances were obtained in the amounts and percentages given:

| Substance.             | Amount.      | Per cent. of residue. | Per cent. of dry residue. |
|------------------------|--------------|-----------------------|---------------------------|
| Humus . . . . .        | 145.00 grams | 29.00                 | 40.00                     |
| Alanin . . . . .       | 4.00 grams   | 0.80                  | 1.10                      |
| Leucin . . . . .       | 6.40 grams   | 1.28                  | 1.76                      |
| Valin . . . . .        | 2.30 grams   | 0.46                  | 0.63                      |
| Phenylalanin . . . . . | 1.70 grams   | 0.34                  | 0.46                      |

It has been possible in this hydrolysis to recover only 8.19 per cent. of the nitrogen in the fluid esterified. Doubtless we should have obtained a greater percentage had we been able to secure a higher vacuum. Fischer does his work with a vacuum of 1 to 2 mm. of mercury, while the best we could obtain with the facilities at our command varied from 20 to 30 mm.

While the results of this work are not so satisfactory as one might wish it does indicate that the proteins of the two bacilli studied are different in their chemical composition. This is shown by the distribution of the amino-acids as is indicated by the following figures:

|                         | Colon.         | Tuberculosis.  |
|-------------------------|----------------|----------------|
| Glutamic acid . . . . . | 3.00 per cent. | 0.20 per cent. |
| Glycocoll . . . . .     | 0.33 per cent. | 0.00 per cent. |
| Alanin . . . . .        | 1.00 per cent. | 1.40 per cent. |
| Valin . . . . .         | 1.60 per cent. | 4.60 per cent. |
| Leucin . . . . .        | 2.00 per cent. | 1.82 per cent. |
| Phenylalanin . . . . .  | 0.20 per cent. | 0.50 per cent. |

Wheeler has reported as follows upon the mono-amino acids of the toxophor group:

The poisons selected for this work were those from tuberculosis, typhoid, and colon germ substances, and for comparison that from egg albumin. For the tuberculosis, the poison from 900 grams germ substance was used—296 grams; for the typhoid, 100 grams of the poison; for the colon, that from 300 grams of germ substance, estimated as 61.5 grams; while for the albumin, that from 200 grams of the protein was employed, yielding, it was estimated, 93 grams of crude poison. These poisons were hydrolyzed by boiling under a reflux condenser for fourteen hours with concentrated hydrochloric acid. The nitrogen of each extract was determined by the Kjeldahl method, using an aliquot part of each as a sample, and giving the following results:

| NITROGEN OF ACID EXTRACT OF POISONS |                 |
|-------------------------------------|-----------------|
| Source of poison                    | Per cent. of N. |
| Tuberculosis . . . . .              | 9.895           |
| Typhoid . . . . .                   | 10.380          |
| Colon . . . . .                     | 10.185          |
| Egg albumen . . . . .               | 11.477          |

From this point Fischer's ester method for obtaining the individual mono-amino-acids was carried out.

This method is so well known that it is not necessary to outline it here other than to say that after the amino-acids have been produced by cleavage of the protein with concentrated acid, the hydrochloride of their ethyl esters is formed, and later the free esters are separated by distillation in the highest possible vacuum. These are then saponified and the amino-acids crystallized and purified.

The efficiency of this method depending in large measure upon the vacuum secured, the yields here presented for cleavage of the poisons might have been materially increased with a better vacuum, the highest one possible with the apparatus at hand varying from 20 to 30 mm. The following table shows the results of the distillations of the free esters, both the bath and vapor temperatures being given, the amount of the distillates, and the yield of crude crystals after saponification.

DISTILLATION OF THE ESTERS OF THE MONO-AMINO-ACIDS FROM PROTEIN POISONS

*Tuberculosis Poison*

| Fraction. | Temperature of oil bath. | Temperature of vapor. | Amount of distillate. | Weight of crude crystals. |
|-----------|--------------------------|-----------------------|-----------------------|---------------------------|
| 1         | 40° to 60°               | 20° to 40°            | 5.0 c.c.              | 2.0 grams                 |
| 2         | 60° to 80°               | 40° to 60°            | 5.0 c.c.              | 2.0 grams                 |
| 3         | 80° to 100°              | 60° to 80°            | 25.0 c.c.             | 16.0 grams                |
| 4         | 100° to 130°             | 80° to 100°           | 25.0 c.c.             | 16.0 grams                |
| 5         | 130° to 160°             | .....                 | 25.0 c.c.             | 7.0 grams                 |

*Typhoid Poison*

| Fraction. | Temperature of bath. | Temperature of vapor. | Amount of distillate. | Weight of crude crystals. |
|-----------|----------------------|-----------------------|-----------------------|---------------------------|
| 1         | 25° to 60°           | 10° to 20°            | 12.0 c.c.             | 0.3158 gram               |
| 2         | 60° to 80°           | 20°                   | 8.0 c.c.              | 1.2558 grams              |
| 3         | 80° to 110°          | 20°                   | 2.0 c.c.              |                           |
| 4         | 110° to 130°         | 95° to 108°           | 7.0 c.c.              | 6.0000 grams              |
| 5         | 130° to 145°         | 108° to 110°          | 4.0 c.c.              |                           |
| 6         | 145° to 200°         | 138° to 185°          | 4.0 c.c.              |                           |

No distillate passed over between 20° and 95°, inside temperature, or between 110° and 138°.

*Colon Poison*

| Fraction. | Temperature of bath. | Temperature of vapor. | Amount of distillate. | Weight of crude crystals. |
|-----------|----------------------|-----------------------|-----------------------|---------------------------|
| 1         | 40° to 60°           | 28° to 41°            | 1.5 c.c.              | 0.07 gram                 |
| 2         | 60° to 80°           | 41° to 56°            | 1.0 c.c.              | 0.06 gram                 |
| 3         | 80° to 104°          | 56° to 84°            | 4.0 c.c.              | 2.00 grams                |
| 4         | 104° to 120°         | 84° to 88°            | 2.0 c.c.              | 0.63 gram                 |
| 5         | 120° to 160°         | 88° to 139            | 5.5 c.c.              |                           |

The yield from the colon poison was exceedingly small, due to the fact that at one stage of the process part of the solution was lost.

*Albumin Poison.*

| Fraction. | Temperature of bath. | Amount of distillate. | Weight of crude crystals. |
|-----------|----------------------|-----------------------|---------------------------|
| 1         | 40° to 60°           | 5 c.c.                | 0.2638 gram               |
| 2         | 60° to 80°           | 3 c.c.                | 0.3338 gram               |
| 3         | 80° to 100°          | 8 c.c.                | 4.0000 grams              |
| 4         | 100° to 130°         | 10 c.c.               | 6.0000 grams              |
| 5         | 130° to 160°         | 7 c.c.                | 7.0000 grams              |

After repeated recrystallizations these crude products were obtained in a state of chemical purity. From the tuberculosis poison, fractions 1 and 2 yielded needle-shaped crystals, soluble in water and alcohol, sweet to the taste, and containing 15.773 per cent. of nitrogen, the average of four determinations by the Kjeldahl method. Alanin,  $C_3H_7NO_2$ , has all these properties and contains 15.73 per cent. of nitrogen, thus identifying the crystals as alanin. Fisher, Fraenkel, and others do not give a melting point for d-alanin, saying that it is not sharp, due to the presence of a mixture of the optically active and racemic forms. The melting-point of the crystals from the tubercle poison varied from 268° to 280° corrected, showing no constant temperature. In fractions 3 and 4 the crystals were beautiful, shiny, satiny plates, sweet to the taste, soluble in water, but almost insoluble in alcohol. These sublimed readily, melted with decomposition, and contained 11.976 per cent. of nitrogen (average of eight determinations). These properties and the nitrogen correspond with valin,  $\alpha$ -aminoisovaleric acid,  $C_5H_{11}NO_2$ , which contains 11.965 per cent. nitrogen. Fränkel gives the melting-point of

valin as  $298^{\circ}$ , corrected, when heated in a closed tube, decomposition taking place at the same time. The valin from the tubercle poison melted as high as  $296.28^{\circ}$ , corrected, but after continued recrystallizations the melting-point dropped as low as  $285^{\circ}$ , and was never reliable. Whether this was due to a partial racemization on repeated heating is not known. Heated in a closed tube the melting-point of the final product was  $275.8^{\circ}$  to  $278.2^{\circ}$ . As is well known, valin closely resembles leucin in its properties, so that it is very difficult to demonstrate the existence of one in the presence of the other. On page 78, by another method, the presence of leucin in the poison has been shown, but by the Fischer method of ester distillation valin seems to be the one obtained. The presence of leucin was further demonstrated by the fact that from the final residue left after the esters had been distilled, crystals of its decomposition product, leucinimide were obtained. This crystallized from dilute alcohol in the form of needles and melted at  $295.4^{\circ}$ . Cohn gives the melting-point of leucinimide as  $295^{\circ}$ , Fränkel as  $262^{\circ}$ . From fraction 5 of the tubercle poison a qualitative test only was obtained for phenylalanin, the quantity obtained being too small for complete purification. After evaporation of the ethereal solution of the thick, oily ester, according to the method, the ester is saponified by twice evaporating with hydrochloric acid. It is then evaporated with ammonia, dissolved in a small amount of water, and poured into a large volume of absolute alcohol, which precipitates the phenylalanin. From this precipitate the qualitative test was obtained, according to Fränkel, by dissolving in dilute sulphuric acid and adding an excess of potassium dichromate, producing the characteristic odor of phenylacetaldehyde and showing thus the presence of phenylalanin. From fraction 5, after removal of the phenylalanin, upon saponification with barium hydrate, there was obtained, after the barium had been removed, rhombic hemihedral crystals which had a distinctly sour taste. These, after purification, showed 9.54 per cent. of nitrogen, the average of two Kjeldahl determinations,

identifying them as glutamic acid,  $C_5H_9NO_4$ , which has 9.52 per cent. of nitrogen. Fränkel gives the melting-point of glutamic acid as  $202^\circ$  to  $202.5^\circ$ , or quickly heated,  $213^\circ$ , with decomposition. The product above obtained melted in an open tube at  $242^\circ$  to  $245^\circ$ , in a closed tube at  $236^\circ$  to  $238^\circ$ . From fraction 1 of the typhoid poison was obtained alanin, with characteristic properties, as described above. These crystals showed 15.633 per cent. of nitrogen and melted at  $267^\circ$  to  $271^\circ$ . Fractions 2, 3, 4, and 5 contained only valin, which showed 11.932 per cent. of nitrogen, the average of four determinations. This melted at  $287^\circ$  to  $290.6^\circ$  in an open tube,  $278^\circ$  to  $280^\circ$  in a closed one. From fraction 6 the qualitative test for phenylalanin was obtained as from the tubercle poison.

Owing to the small yield of esters and crystals, fractions 1 and 2 from the colon poison could only be determined qualitatively. Both fractions, however, showed needle-shaped crystals and a sweet taste, which in conjunction with the temperature at which their esters distilled indicated alanin. Fractions 3 and 4 gave characteristic valin crystals containing 11.942 per cent. of nitrogen and melting at  $283.4^\circ$  to  $285^\circ$  in an open tube, or  $274^\circ$  to  $277^\circ$  in a closed tube. Phenylalanin was obtained qualitatively from this as from the two preceding poisons. After its extraction from fraction 5, and after saponification with barium hydrate and its removal, crystals in the form of rhombic plates and prisms, insoluble in alcohol, were obtained. These corresponded with those of aspartic acid, and as the quantity was not sufficient for purification by recrystallization, the copper salt was formed with copper acetate. This was obtained in the form of needles, very difficultly soluble in cold water, difficultly in hot, which again corresponded with the properties of aspartic acid.

When the crystals from the fractions obtained from albumin poison were examined the result was not different. Fractions 1 and 2 produced characteristic alanin crystals with 11.75 per cent. of nitrogen, the average of four determinations. The melting-point was  $277^\circ$  to  $279.6^\circ$ . Valin,

with form and properties as already given, was obtained from both fraction 3 and 4, containing 11.935 per cent. of nitrogen, and showing a melting-point of 282° to 286.4° in an open tube, and of 279° to 283° in a closed tube. Likewise from fraction 5 the heavy oil of phenylalanin ethyl ester was obtained, and from this as in the other cases the qualitative test for phenylalanin by the production of phenylacetaldehyde. The remaining portion of fraction 5 yielded the same rhombic plates and prisms as described under the colon fractions, and which are like those of aspartic acid properly obtained at this point if present. The copper salt was again formed, the same needles, very difficultly soluble in cold water, difficultly in hot, being obtained. The amount of crystals was too small for further identification.

From this it will be seen that mono-amino-acids are obtained from the protein poisons after hydrolysis with strong acid. It is not claimed that these are the only mono-amino-acids present, or that all of these have been sufficiently identified, but in consideration of the fact that those discussed were found in the proper fraction according to Fischer's separation and according to the boiling-points of their esters, that the crystalline form and qualitative properties corresponded, and that, when it could be determined, the percentage of nitrogen was close to the theoretical, it seems fair to conclude that the following tabulation is not far from correct:

## MONO-AMINO-ACIDS OF THE PROTEIN POISONS

| Tuberculosis<br>poison. | Typhoid<br>poison. | Colon<br>poison. | Albumin<br>poison. |
|-------------------------|--------------------|------------------|--------------------|
| Alanin                  | alanin             | alanin           | alanin             |
| Valin                   | valin              | valin            | valin              |
| Phenylalanin            | phenylalanin       | phenylalanin     | phenylalanin       |
| Glutanic acid           | —                  | —                | —                  |
| —                       | —                  | Aspartic acid    | aspartic acid      |
| Leucinimide             | —                  | —                | —                  |

This is sufficient to establish the point for the proof of which the method was employed, that is, the protein nature

of the poisonous group of the protein molecule. Attention is called also to the comparative simplicity of the group and to the great similarity of acids obtained from the different poisons. This accords well with the great similarity and non-specificity of their physiological action.

It is interesting that the final residue left after distillation of the esters gives still a very intense Millon reaction, which cannot be ascribed to the presence of tyrosin.

It will be clearly understood that this work does not show that the active agent or agents in the "crude soluble poison" is or are protein in nature.

## CHAPTER V

### THE CLEAVAGE OF PROTEINS WITH DILUTE ALKALI IN SOLUTION IN ABSOLUTE ALCOHOL

THE researches detailed in the preceding pages seem to establish the following propositions:

1. The cellular substances of bacteria consist largely of proteins that yield split products identical with those obtained by the hydrolysis of vegetable and animal proteins.

It has been shown that the bacterial cellular substances, when broken up with mineral acids or alkalies, furnish ammonia, mono-amino and diamino nitrogen, one or more carbohydrate groups, and humin substances. It seemed therefore logical to conclude that the bacterial cell consists largely of proteins.

2. The proteins of the bacterial cell contain at least one group which when injected intra-abdominally, subcutaneously, or intravenously in animals, has a markedly poisonous effect.

3. This poisonous group may be detached from the cell protein by hydrolysis with either dilute acids or alkalies.

4. The dilute alkali furnishes the better means of extracting the poisonous group.

5. When the bacterial protein is broken up with alkali in dilute aqueous solution, at least two groups are split off and pass into solution. These are the carbohydrate and the poisonous groups. Both are soluble in water and in dilute alcohol, and their separation, when the cell protein is disrupted by alkali in aqueous solution, is difficult and unsatisfactory.

6. Since the carbohydrate group is insoluble in absolute alcohol, while the poisonous group is more readily soluble

in this menstruum than in water, it was decided to attempt to disrupt the cell protein with a solution of alkali in absolute alcohol. Another idea also acted as a determining factor in attempting this method of hydrolysis, and in fact it was at that time the dominating factor. The effect of the poisonous group on animals so closely resembles that of neurin that it was thought that the two might be identical, or at least that the poisonous body might contain neurin. Knowing that neurin can be heated without decomposition in alkaline alcohol was, therefore, a reason for trying this method.

7. Previous experiments had demonstrated the advantage of extracting the cell substance thoroughly with alcohol and ether before submitting it to hydrolysis. This frees the material from fat, wax, and other substances soluble in alcohol or ether, and since it had been shown that these are no part of the cell protein it is beneficial to get rid of them *in toto* before hydrolysis is attempted.

The following preliminary trials were made by Vaughan and Wheeler (in the fall of 1903) in order to compare hydrolysis with aqueous and alcoholic solutions of alkali.

Two samples, of 10 grams each, of the cellular substance of the colon bacillus were taken. This material had previously been thoroughly extracted with alcohol and ether. One sample was mixed with 250 c.c. of a 1 per cent. aqueous solution of sodium hydroxide and the other with the same volume of an absolute alcohol solution of the same substance in the same strength. These mixtures were heated in flasks, fitted with reflux condensers, for one hour on the water-bath. Ten cubic centimeters of the clear filtrate from each was evaporated, the aqueous solution to 5 c.c. and the alcoholic to dryness, and then taken up in 5 c.c. of water. Each was carefully neutralized with dilute hydrochloric acid and injected into the abdominal cavity of a guinea-pig. Both animals developed in a characteristic manner the first and second stages of poisoning with the split product, but neither died. This experiment showed that the poison was present in both extracts, and, so far

as we could judge by the development and intensity of the symptoms, in similar amounts. That the poison could be extracted by alkaline alcohol was proved. However, the yield was not satisfactory, and a second test was made, and in this the strength of the alkali was doubled. These were treated as before, and the pigs that received the injections developed the characteristic symptoms and died. The one that had the alcoholic extract died within six, and the other within eight minutes. This confirmed the hope that the alcoholic alkali was quite as efficient as the aqueous in the extraction of the poisonous group. While the aqueous extract contained a large amount of the carbohydrate group, it was found that the alcoholic extract, after evaporation to dryness and solution in water, gave the biuret, Millon, and xanthoproteic tests, but failed wholly to give the Molisch reaction. The carbohydrate group had been split off in both samples, but being insoluble in absolute alcohol, it remained with the insoluble portion of the cellular substance.

The above and many other experiments have demonstrated that the best method, so far devised, for extracting the poisonous group from the cell protein, or, as subsequent work has shown, from any protein, is by means of a 2 per cent. solution of sodium hydroxide in absolute alcohol. If satisfactory results are obtained, the alcohol used in the extraction must be absolute. If it is not, more or less of the carbohydrate will be mixed with the poison; a sticky mass will be obtained, and the patience of the experimenter will be taxed severely. Previous thorough extraction of the protein with alcohol and ether for the removal of fats, waxes, and other substances soluble in these agents, is also essential to satisfactory work.

The method for preparing the bacterial cellular substance has been given, but it may be well to give here some details for the preparation of egg-white before splitting it up into poisonous and non-poisonous proteins.

Fresh eggs (we have usually taken twenty dozen at a time) are broken and the whites dropped into a beaker or

precipitating jar, then poured with constant stirring into four volumes of 95 per cent. alcohol. This stands with frequent stirring for two days, then the alcohol is decanted, and replaced with the same volume of absolute alcohol. This is allowed to stand for from one to two days, when the coagulated albumin is collected on a filter, allowed to drain, then placed in large Soxhlets and extracted with ether for from one to two days. It is then ground in porcelain mortars and passed through fine meshed sieves. This gives a beautifully white powder which may be kept in bottles in stock from which portions are taken for the purpose of hydrolyzing it.

Twenty dozen eggs yield about 735 grams of this powder, a little more than 3 grams per egg.

A weighed portion of the protein, prepared as above, is placed in a flask, covered with from fifteen to twenty-five times its weight of absolute alcohol in which 2 per cent. of sodium hydroxide has been dissolved. The flask, fitted with a reflux condenser, is heated on the water-bath for one hour, when it is allowed to cool and the insoluble portion collected on a filter. After thorough draining the insoluble part is returned to the flask and the extraction repeated. It has been found that three extractions are necessary in order to split off all the poisonous group. The temperature of these extractions is  $78^{\circ}$ , the temperature of boiling absolute alcohol. By this method the protein is split into two portions, one of which is soluble in absolute alcohol and is poisonous, while the other is insoluble in absolute alcohol and is not poisonous.

A large number of protein bodies, bacterial, vegetable, and animal, have been split up in this way and no true protein has failed to yield a poisonous portion. Among the proteins with which we have worked the following may be mentioned: egg-white, casein, serum albumin, edestin, zein, Witte's peptone, Macquaire's peptone, deChapoteaut's peptone, the tissue of cancers, and the cellular substance of bacillus coli communis, b. typhosus, b. anthracis, b. tuberculosis, b. Moelleri (timothy), sarcina lutea, b. ruber

of Kiel, *b. proteus*, *b. subtilis*, *b. megaterium*, *b. pyocyaneus*, *b. pneumoniae*, and *b. diphtheriae*. Gelatin contains no poison, but gelatin is an albuminoid and gives the Millon test imperfectly, if at all. Nicolle and Abt<sup>1</sup> found that Defresne's peptone does not yield a poison when treated by our method, and we have confirmed this finding. It would be interesting to know whether this peptone is made from gelatin or from a true protein. The probabilities are that in peptic digestion a point is reached when the poisonous group in proteins is disrupted. In fact, as has been stated (page 42), we have shown that the poison in the cellular substance of the colon bacillus is slowly digested and destroyed by digestion with pepsin-hydrochloric acid. Therefore, it is not strange that certain peptones fail to yield a poisonous body when disrupted with dilute alkali in absolute alcohol. Witte's peptone, so-called, as is well known, is not a peptone, but an albumose.

This poison, like the whole protein of which it is a part, is formed synthetically by the living cell. In case of the colon poison we demonstrated this by growing the bacillus in Fraenkel's modification of Uschinsky's medium, which has the following composition:

|  |              |
|--|--------------|
| Water . . . . .                        | 10,000 parts |
| Sodium chloride . . . . .              | 50 parts     |
| Asparagin . . . . .                    | 34 parts     |
| Ammonium lactate . . . . .             | 63 parts     |
| Di-sodium hydrogen phosphate . . . . . | 20 parts     |

After a week's development the contents of these flasks were poured into from two to three volumes of 95 per cent. alcohol. The precipitate was filtered out and put into absolute alcohol; next it was extracted in Soxhlets with ether, dried, and powdered. This powdered cellular substance, when split up with 2 per cent. sodium hydroxide in absolute alcohol, furnished the poison, the action of which was demonstrated on guinea-pigs. Moreover, the poison obtained in this way gave all the protein reactions

<sup>1</sup> Annales de l'Institut Pasteur, February, 1908.

hereafter described as being obtained from the poison from agar-grown cultures. This demonstrates that the poison is an integral part of the cellular substance, and it is evident that the bacterial cell must synthetically produce this protein body during its growth from the chemical constituents of the medium.

When the protein is split up by dilute alkali in absolute alcohol according to the method described, the poison is in solution in the alkaline alcohol. The preparation is filtered and the filtrate neutralized with hydrochloric acid, avoiding an excess of acid. This throws down the greater part of both base and acid as sodium chloride, which is removed by filtration. In this way a solution of the poison in absolute alcohol is obtained. This is evaporated *in vacuo* at 40°, redissolved in absolute alcohol to remove traces of sodium chloride, and again evaporated *in vacuo* at 40° or less. Evaporation may be done in an open dish, but the toxicity of the substances is somewhat decreased when this is done. The poisonous part of the protein molecule when obtained in this way and powdered, when there is no water present, forms a dark brown scale which pulverizes into a lighter brown powder.

It should be clearly understood that we regard this method of extracting the poisonous group from the protein molecule as by no means ideal. We know that it is crude and that much of the poison is destroyed in the process. In disrupting a protein by our method with dilute alkali in absolute alcohol, ammonia is given off and the odor of this gas is apparent even at the end of the third extraction. An effort was made to discover how much nitrogen was converted into ammonia in the process. A device was arranged for conducting the ammonia into standard acid, and four 10-gram samples of Witte's peptone were extracted with 2 per cent. sodium hydrate in absolute alcohol, one for three hours in a current of air, the others in a current of hydrogen for two and one-half, eight and one-half, and nineteen and one-half hours respectively. At the end of each operation the excess of acid was titrated with deci-

normal sodium hydrate, and the percentage of nitrogen calculated. The relative toxicity of the split products was determined. In every case ammonia was still being produced when the process was interrupted. Again, a 10-gram sample of the poison from egg albumen was boiled for fifty-four and one-half hours with 2 per cent. alcoholic alkali to ascertain if ammonia could be split from the poison itself. The results of this work are shown in the following table:

AMMONIA PRODUCED BY CLEAVAGE OF PROTEIN WITH DILUTE ALKALI IN ABSOLUTE ALCOHOL.

| Sample.       | Time in hours. | Atmosphere. | Per cent. of |                | Toxicity.                 |
|---------------|----------------|-------------|--------------|----------------|---------------------------|
|               |                |             | N given off. | Rate per hour. |                           |
| Witte peptone | 3.0            | air         | 0.4305       | 0.1435         | Diminished.               |
| Witte peptone | 2.5            | H           | 0.3956       | 0.1582         | Greater than that in air. |
| Witte peptone | 8.5            | H           | 0.7383       | 0.0868         | Diminished.               |
| Witte peptone | 19.5           | H           | 1.0517       | 0.0539         | Diminished.               |
| Poison        | 54.5           | H           | 1.4800       | 0.0270         | Diminished by half.       |

The albumin poison as ordinarily obtained contains 13.74 per cent. of nitrogen. By the fifty-four and one-half hours' heating with alcoholic alkali, 10.77 per cent. of its nitrogen was converted into ammonia. After this treatment the poison still gave a good Millon test, but no longer the biuret.

It is probable that by continued heating in the same manner quite all of the nitrogen could be separated, though it is noticeable that the rate was greatly diminished as the time lengthened. The decrease in toxicity with the evolution of ammonia suggests that this group is essential to the toxicity of the poison. This seems to be highly probable.

**Properties of the Crude Soluble Poison.** The poison split off from the protein molecule by the method above given is designated as "the crude soluble poison;" "crude" because it is undoubtedly a mixture of chemical bodies, and "soluble" in contradistinction to the bacterial cellular substance, from which it was first prepared, and which is poisonous, but not soluble.

The brownish toxic powder, varying in shade of color somewhat with the protein from which it has been obtained, has a peculiar odor. It is highly hygroscopic, and the poisonous portion is freely soluble in water. The solubility of the whole powder, however, varies with the protein from which it is obtained, and possibly with the length of time that it has been exposed to the alkali in the alcohol. Any portion insoluble in water should be removed by filtration, and in some instances we have found filtration through porcelain necessary. Generally the powder dissolves in water with a slight opalescence easily removed by filtration through paper. In all cases we have found the portion insoluble in water free from toxic effect. Aqueous solutions of the poison are decidedly acid to litmus, the acidity being due to some organic body and probably not to the poison itself. On neutralization with sodium bicarbonate a brownish, non-toxic precipitate is formed. Prolonged contact with alkali, as we shall see later, lessens the activity of the poison, and even neutralization has some effect, which is more marked the longer the preparation stands. We are inclined to attribute this to the formation of a salt with the acid poison and the alkali. The poison is freely soluble in alcohol, more readily than in water. Alcoholic solutions on long standing deposit small brownish sediments which we have always found to be inert. When an alcoholic solution is evaporated, there is a part of the residue that is insoluble in absolute alcohol. These portions also are devoid of toxic effect. Alcoholic solutions have been kept for five years without recognizable loss in toxicity, and even aqueous solutions decompose very slowly. The poison is soluble in methyl as well as in ethyl alcohol. It is insoluble in ether, chloroform, and petroleum ether. Each of these removes a small amount of fatty substance, which is non-toxic, but they do not dissolve an appreciable quantity of the poison. From its alcoholic solution the poison is precipitated by ether, but contact with ether decreases its toxicity to such an extent that this method is not applicable in attempts at purification.

The "crude soluble poison" is soluble in strong mineral acids, and such solutions remain clear on being boiled and on dilution with water. However, a few drops of mineral acid added to an aqueous solution cause a precipitate, which seems to indicate that the acidity of the aqueous solution is caused by the presence of some organic acid.

The poison diffuses slowly through collodion sacs both within the animal body and when suspended in distilled water. The following experiments bear on this point: Two hundred milligrams of the crude soluble poison from the cellular substance of the typhoid bacillus dissolved in 20 c.c. of water was placed in each of two collodion sacs which were then suspended in distilled water. At the end of twenty-four hours, the Millon reaction was given by the dialysate. This was replaced every twenty-four hours by fresh distilled water, and the dialysis continued for ninety-six hours. At the end of this time the combined dialysates were concentrated to dryness, the residues dissolved in absolute alcohol, filtered, and again evaporated. The brown, sticky residue, thus obtained, dissolved in water, was acid in reaction, had the characteristic odor, and when injected into a guinea-pig, killed in twenty minutes with typical symptoms, thus showing that the poison does diffuse through a collodion sac. So slowly, however, does it diffuse that at the end of ninety-six hours it was not wholly removed from the sac. In another experiment one gram of the same poison in 8 c.c. of water was put into a collodion sac which was introduced into the abdominal cavity of a medium-sized rabbit. After twelve days, the animal not being visibly affected, the sac was removed and found to contain 6 c.c. of a clear fluid which looked more like blood serum than anything else. Five cubic centimeters of this injected into the abdomen of a guinea-pig had no effect. We conclude from this that the poison had diffused from the sac, but so slowly that it was disposed of by the animal's body without recognizable discomfort.

Notwithstanding the ready solubility of the crude soluble poison in absolute alcohol, we must regard it as either

being a protein itself or as being mixed with one or more proteins. Its aqueous solutions give all the protein color reactions with the important exception of that of Molisch. It is worthy of note that the part that separates from alcoholic solution on long standing is inert and does not give the protein reactions, while the solution does not decrease in toxicity. This indicates that the protein is permanently soluble in absolute alcohol. The Millon reaction shows most perfectly and persistently whenever the poison is found. It is generally believed by physiological chemists that this reaction is given by all benzene derivatives in which one hydrogen atom has been replaced by a hydroxyl group, and it is also generally supposed that tyrosin is the only oxyphenyl compound in the protein molecule, therefore this reaction is presumed to show the presence of tyrosin. This is interesting in view of the fact already stated that gelatin, which contains no tyrosin, or but little, yields no poison. The fact that the poison contains no carbohydrate, as shown by its failure to respond to the Molisch test, an exceedingly delicate test, is, in our opinion, strong evidence that the cleavage in the protein molecule induced by dilute alkali in absolute alcohol at the temperature of  $78^{\circ}$  follows along structural lines. If the change were one of simple degradation without chemical cleavage it would be difficult to explain the absolute failure of the carbohydrate test in the crude soluble poison. It seems quite evident from our work that in the process the complex protein molecule is split into several groups, one of which is the poison and another is a carbohydrate, the former being freely soluble in absolute alcohol, while the latter is insoluble in this reagent. It should be stated that the crude, soluble poison not only fails to respond to the Molisch test, but it also fails to reduce Fehling's solution after prolonged boiling with dilute mineral acid.

The crude soluble poison gives the biuret test beautifully, therefore we must say that the poison either is itself a biuret body or is mixed with such a body. As is well known, the biuret test is regarded as the landmark between

proteins and their simpler non-protein disruption products, and, so long as a disrupted protein continues to give the biuret test it must still be classed among the proteins. It will certainly be understood that the pure poison may not be a protein, but until it is purified sufficiently to fail to give the biuret test it must be regarded as a protein.

The poison responds nicely to the Adamkiewicz or glyoxylic acid test. Hopkins and Cole have shown quite convincingly that this color test depends upon the presence of tryptophan or indol-amino-propionic acid; therefore, while we have made no direct search for tryptophan in our poison, we assume its presence on account of the unequivocal response to this test.

When the poison is boiled with concentrated hydrochloric acid to which a drop of concentrated sulphuric acid has been added, the powder passes into solution and a violet color results, thus giving Liebermann's test. At one time Hofmeister believed this to be a carbohydrate reaction in which furfural and the aromatic oxyphenyl radicals take part, but Cole has shown that this, like the Adamkiewicz test, also once regarded as a carbohydrate test, is due to the tryptophan group. We are quite convinced that our soluble poison contains no carbohydrate, and we regard the fact that it does respond to the Liebermann test as a strong confirmation of the error of Hofmeister's explanation of this test, and in favor of the explanation given by Cole.

When heated with strong nitric acid the powdered poison goes into solution, more or less yellow according to the amount used, and this becomes orange on the addition of ammonia, thus giving the xanthoproteic test and indicating the existence of aromatic radicals.

The ordinary test for sulphur in proteins, that of heating with excess of sodium hydrate in the presence of a small amount of acetate of lead, is not given by the portion of the protein split off by alkali in absolute alcohol. If, however, a portion of the substance in a test-tube is fused with metallic sodium and the cooled mass treated with water, a few drops of a freshly prepared solution of sodium

nitroprussiate added to a part of the clear filtrate, a beautiful violet color is produced, indicating the presence of sulphur. Also, if the other part of the clear filtrate be treated with a lead acetate solution, lead sulphide is precipitated. If the solution be acidified before lead acetate is added a faint but unmistakable odor of hydrogen sulphide is detected. It is known that sulphur may exist in the protein molecule in at least two forms, one part being readily split off with dilute alkali as a sulphide, the other being obtained only when the disruption of the protein molecule is carried much farther. It is still a question whether or not both of these sulphur groups come from cystin. Since the nitroprussiate reaction is very delicate, no conclusion as to the amount of sulphur can be drawn from this test, and although a good precipitate of lead sulphide is formed, the amount of sulphur in the poison is probably not large, since Leach failed entirely to find sulphur in the ash of the colon bacillus, though both the cellular substance and the non-poisonous portion, as well as the poison, respond to the nitroprussiate test for sulphur and also give the lead sulphide precipitate in the clear acidified filtrate from the fused mass.

A solution of this toxic substance is not coagulated by heat in acid, neutral, or alkaline solution, though, as already stated, a few drops of a mineral acid added to an aqueous solution causes the appearance of a considerable precipitate, which is not soluble on heating or on the further addition of acid. This precipitate is produced regardless of the previous removal of the opalescence from the aqueous solution.

Among the metallic salts, copper sulphate produces no precipitate and ferric chloride only on heating. Silver nitrate naturally precipitates any trace of chlorides present, but after the addition of an excess of ammonia there still remains a small precipitate. Potassium ferrocyanide gives a precipitate, also potassium bismuth iodide in acid solution. Lead acetate, mercuric chloride, and platinum chloride all produce heavy precipitates. With lead acetate and mercuric chloride, however, after removal of lead and

mercury with hydrogen sulphide from their respective precipitates and filtrates, the protein reactions are given by the filtrates, and here also is found the poison in each case. From 10 to 15 per cent. of the crude poison can be precipitated by the use of platinum chloride in either water or alcoholic solution. All attempts to crystallize this precipitate failed, as only a small part of it is dissolved by hot water, and the insoluble part is unaffected by any of the ordinary solvents. The protein reactions are given by the platinum precipitate, by both soluble and insoluble parts, but not by the filtrate. The poison is found in the insoluble part of the precipitate after removal of the platinum by hydrogen sulphide, its toxicity being markedly increased. The other parts, after removal of the platinum, are inert.

The most active products have been obtained by precipitation from solution in absolute alcohol with alcoholic solutions of the chlorides of platinum, mercury, and copper and removal of the base from the precipitate with hydrogen sulphide. By this method we have obtained a body which kills guinea-pigs of from 200 to 300 grams' weight in doses of 0.5 mg. given intravenously.

From a water solution of the poison, bodies giving protein reactions may be salted out by the addition of ammonium sulphate or sodium chloride to saturation, but in neither case is the separation complete, the filtrates still responding to the protein color tests after removal of the neutral salts. In case of salting out with ammonium sulphate, the solubility of both parts is thereby lessened and the toxicity diminished, possibly on account of decreased solubility, though both parts exhibit some poisonous action, and likewise both show the protein color tests.

Phosphotungstic, phosphomolybdic, and picric acids all give abundant precipitates. Since these reagents are also used in the precipitation of alkaloidal bodies, the precipitates with phosphomolybdic and phosphotungstic acids were further examined, the possibility suggesting itself that the toxic body might be alkaloidal in nature, and that the

protein part might be entirely separate from the poison. A sample was precipitated with phosphomolybdic acid in acid solution, the precipitate removed, washed, and dissolved in ammoniacal water. This solution was then shaken with amyl alcohol, but the alcohol was not colored and the residue obtained on concentration was so slight as to be practically nothing. Another sample was precipitated with phosphotungstic acid, the solution being acid in reaction. The precipitate was allowed to settle, removed by filtration, washed with acidulated water, decomposed with a saturated solution of barium hydrate, and the remaining insoluble part filtered out. So far as possible, the barium was removed from the filtrate with carbon dioxide, alternating with concentration, and further addition of carbon dioxide. The solution was then allowed to concentrate to dryness, when the residue was dissolved in absolute alcohol, leaving barium salts behind. On concentrating the slightly opalescent solution, more barium salts came down during the process and were filtered out. The dry residue was taken up in water and ammonium carbonate used to precipitate the barium that still remained. After removing the barium carbonate by evaporating on the water-bath, both carbon dioxide and ammonia were expelled, the solution again becoming acid. Dryness being reached, absolute alcohol was once more used, leaving undissolved a small amount of inorganic material. In this way the final residue after evaporation of the alcohol was practically freed from inorganic impurities. Sulphuric acid no longer gave a barium precipitate in water solution. The amount obtained by this method was very small and an exceedingly small part of the original toxic powder. Since the substance obtained in this way still gave good Millon's, biuret and xanthoproteic reactions, it is fair to say that it was not alkaloidal. The very small amount obtained by this method given to a guinea-pig intra-abdominally made the animal sick, but did not kill. Either phosphotungstic acid does not precipitate the toxic body or else the amount obtained was less than a fatal dose.

Should the poison consist of an alkaloidal body existing as a salt in the acid solution, the possibility of extracting the base with ether or chloroform, after the solution had been made alkaline with ammonia, is apparent. This was tried with negative results. To a water solution of colon poison, acid in reaction, ammonia was added, drop by drop, to a slightly alkaline reaction, the mixture shaken with ether, the ether separated and evaporated. The residue remaining was non-toxic. The ammoniacal water solution was next shaken with chloroform, the slightly colored chloroform drawn off and evaporated at low temperature, leaving a small amount of a dark, thick, semiliquid, which was not poisonous either as it was or after faintly acidifying with hydrochloric acid. The water solution remaining being still poisonous, it is evident that the toxic part is not an alkaloidal body capable of being extracted directly.

Potassium bismuth iodide in acid solution of the crude soluble poison produces an abundant precipitate, apparently more or less soluble in excess, and soluble in ammoniacal water.

Kowalewsky has shown that uranyl acetate will completely remove from various albuminous fluids every trace of protein giving a biuret reaction, while Jacoby and others have used this reagent for the removal of proteins from faintly alkaline solutions. Abel and Ford used it to remove protein from an extract of poisonous fungi. In a slightly alkaline solution of albumin poison, uranium acetate gave an abundant precipitate, but not a complete separation, as both precipitate and filtrate still gave the Millon and biuret tests, and the filtrate, after removal of excess of uranium with a solution of di-sodium hydrogen phosphate, filtration, evaporation, solution in alcohol, and reëvaporation, was still poisonous. In acid solution, the precipitation was complete, the filtrate no longer giving the protein reactions.

Freshly prepared metaphosphoric acid also produced an abundant precipitate, but not a complete separation, the filtrate showing both Millon and biuret reactions.

Likewise a heavy precipitate is produced by the use of a saturated solution of picric acid, but the poison is not in the precipitate, which gives only a very poor Millon test after removal of the picric acid, and no biuret. Hofmeister has given a method for introducing iodine into the molecule of egg albumen. This was tried with the poison split from egg albumen. The iodized compound no longer gave either the Millon or biuret reactions, and while it affected animals more or less, they did not die, and the symptoms were not those induced by toxin poisoning. The iodine seemed to have entered into chemical combination in the poison molecule, and to have thus changed its characteristics. The iodized body was freely soluble in absolute alcohol, and in alkaline water, not in water alone, and was precipitated by acid water from alcoholic solution, also on acidifying an alkaline water solution. Though it no longer responded to the Millon and biuret reactions, a good test for nitrogen was obtained after fusing with metallic sodium.

An attempt was made to benzoylate the poison by the Schotten-Baumann method, using albumin poison. Practically no precipitate was obtained. From the filtrate in a part soluble in hot alcohol there were obtained shiny, glistening plates or flat needles which matted together under suction, and had much the appearance of some of the fatty acids. These were insoluble in water or very difficultly so, if at all, difficultly soluble in cold alcohol, readily in hot. They gave no Millon test, no biuret, no Molisch, and contained no nitrogen. After recrystallization from alcohol they melted constantly at 62°. Palmitic acid melts at 62° and boils at 339° to 350° (Mulliken). A Merck preparation of palmitic acid melted at 62° and boiled at about 345° to 350°. Our crystals had not yet boiled at 360°, though above 300° there was some decomposition. From the remainder of the filtrate there was obtained from the part soluble in cold alcohol a non-crystallizable body, giving both Millon and biuret tests and containing 9.335 per cent. of nitrogen, and from the part soluble only in water, likewise a non-crystalline compound, with 9.66 per cent. nitrogen,

and showing both Millon and biuret tests, but not seriously affecting animals in usual doses.

The nitrogen in a number of the crude poisons has been determined by Gidley in this laboratory as follows:

PERCENTAGE OF NITROGEN IN PROTEIN POISONS.

| Source of poison.               | Per cent. of N. in crude poison. |
|---------------------------------|----------------------------------|
| Colon bacillus . . . . .        | 13.49                            |
| Typhoid bacillus . . . . .      | 11.52                            |
| Tubercle bacillus . . . . .     | 11.00                            |
| Pyocyaneus . . . . .            | 10.50                            |
| Ruber of Kiel . . . . .         | 10.495                           |
| Subtilis . . . . .              | 8.12                             |
| Megaterium . . . . .            | 8.595                            |
| Proteus vulgaris . . . . .      | 10.17                            |
| Yellow sarcine . . . . .        | 6.145                            |
| Egg albumen (Leach) . . . . .   | 13.74                            |
| Serum albumin . . . . .         | 10.48                            |
| Edestin . . . . .               | 12.78                            |
| Zein . . . . .                  | 10.69                            |
| Witte peptone . . . . .         | 11.14                            |
| De Chapoteaut peptone . . . . . | 12.735                           |

To study the distribution of the nitrogen, determinations were made in both the colon and albumin poisons, of the ammonia nitrogen, the mono-amino, and diamino nitrogen, by the method already described under cleavage with dilute mineral acids. The following are the results:

DISTRIBUTION OF NITROGEN IN PROTEIN POISONS.

| Source of poison.        | Total poison. | Total N of acid extract. | Ammonia N. | Mono-amino N. | Diamino N. |
|--------------------------|---------------|--------------------------|------------|---------------|------------|
| Colon bacillus . . . . . | 13.49%        | 10.185%                  | 1.525%     | 6.472%        | 1.753%     |
| Egg albumen . . . . .    | 13.74%        | 11.477%                  | 0.745%     | 7.999%        | 1.400%     |

It will be seen that the greater part of the nitrogen is to be found in mono-amino combination. From the phosphotungstic filtrates, from both the albumin and colon poisons, containing the mono-amino acids, crystalline bodies were obtained. Judged by the strong Millon test, tyrosin was

undoubtedly present, but the crystalline masses were largely leucin, and no tyrosin was obtained in purified form. From the crude crystals, after many and repeated crystallizations, what was thought to be leucin was obtained pure, melting at  $264^{\circ}$  to  $265^{\circ}$  uncorrected, or  $269.42^{\circ}$  to  $270.46^{\circ}$  corrected. The crystals were thin plates characteristically grouped, and sublimed readily. From another 5 per cent. sulphuric acid extract of albumin poison was obtained a large mass of crystals in characteristic tyrosin-like sheaves, and giving a deep Millon reaction. These were undoubtedly tyrosin, though at the time no melting-point was taken.

**Properties of the Haptophor or Non-poisonous Group.**—Leach<sup>1</sup> has investigated this split product with the following general results: After cleavage of the protein with alkaline alcohol, the haptophor remains undissolved. It is collected on a filter, then transferred to Soxhlets, and for some days extracted with 95 per cent. alcohol. This is for the purpose of removing as thoroughly as possible the alkali which it has absorbed from the alkaline alcohol. This cannot, however, be wholly washed out by this method, and it is possible that in part it is held chemically. After this extraction the substance is easily reduced to a fine brownish powder. On burning it puffs up, gives off the characteristic odor of nitrogenous compounds, and leaves a copious ash containing phosphate. The solubility of the haptophors from different proteins differs widely; that from egg-white is wholly soluble in water, while that from the cellular substance of the tubercle bacillus is only sparingly soluble. However, it is only the part soluble in water from any of these haptophors that is of special interest. The studies of Leach, referred to, were made with the non-poisonous portion of the colon bacillus. This is mainly soluble in water, giving an opalescent solution from which a light-colored sediment is deposited on standing, leaving a clear, golden brown solution. The sediment is not soluble in

<sup>1</sup> Jour. Biol. Chem., 1907, iii, 443.

either dilute alkali or acid in the cold, but is soluble in alkali on boiling. The clear, aqueous solution of the haptophor is alkaline from sodium hydrate held either mechanically or chemically; it is precipitated by mineral acids and by alcohol. It responds to the biuret, xanthoproteic, Millon, and Adamkiewicz tests. Millon's test is not very satisfactory, and in some samples has failed altogether, even after care has been exercised in neutralizing the alkali. It is quite evident that the substance or substances in the protein molecule to which the Millon test is due are for the most part found in the toxophor group. However, the readiness of response to this test varies greatly in the different haptophors. The haptophor substance does not reduce Fehling's solution directly, but does so readily and abundantly after prolonged boiling with dilute hydrochloric acid. The presence of carbohydrate in the haptophor has already been discussed (page 70). Tests with  $\alpha$ -naphthol, phloroglucin, and orcin give positive results. Ammonium molybdate gives an organic precipitate, but no evidence of free phosphoric acid. The preliminary tests show the presence of protein, nucleic, and carbohydrate groups. Comparing these results with those obtained in the study of the toxophor, the following statements may be formulated: (1) The toxophor is freely soluble in absolute alcohol, the haptophor is insoluble in this menstruum. (2) The toxophor contains no carbohydrate, all of which is found in the haptophor. (3) The toxophor freely responds to the Millon test, while the haptophor does so slightly and in some instances not at all. (4) The toxophor contains no phosphorus, or but little of this element, while the haptophor is rich in phosphorus. (5) The toxophor from different proteins seems to be the same, possibly with unrecognizable differences in chemical structure, while the haptophor of each protein differs from that from all other proteins.

Leach<sup>1</sup> gives the following table showing the percentages of ash, nitrogen, and phosphorus in the haptophor of the colon bacillus:

<sup>1</sup> Loc. cit.

|                            | Ash.  | Fixed ash. | Inorganic ash. | N.    | P.   | N ash free. | P ash free. | Ratio N:P. |
|----------------------------|-------|------------|----------------|-------|------|-------------|-------------|------------|
| Cell substance . . .       | ...   | 8.61       | ...            | 10.65 | 2.87 |             |             |            |
| Haptophor . . .            | 33.25 | ..         | 26.08          | 5.56  | 2.34 | 7.52        | 3.99        | 2.38       |
| Prep. A. . . .             | 26.76 | 20.36      | 15.66          | 6.76  | 3.61 | 8.02        | 4.28        | 1.87       |
| Prep. B. . . .             | 35.34 | ..         | 30.74          | 4.87  | 1.50 | 7.03        | 2.16        | 3.25       |
| Prep. D. . . .             | 15.38 | 15.05      | 8.48           | 4.95  | 2.25 | 5.41        | 2.46        | 2.20       |
| Prep. G. . . .             | 6.99  | ..         | 1.66           | 4.65  | 1.74 | 4.73        | 1.77        | 2.67       |
| Prep. G. pur. . .          | 5.50  | 5.50       | 1.36           | 3.43  | 1.35 | 3.48        | 1.37        | 2.53       |
| Prep. H. . . .             | 35.91 | 14.00      | 27.67          | 5.98  | 2.68 | 8.27        | 3.71        | 2.23       |
| Prep. K <sub>2</sub> . . . | 7.57  | ..         | 2.08           | 5.50  | 1.79 | 5.62        | 1.83        | 3.07       |
| Prep. M. . . .             | 11.71 | 11.71      | 3.74           | 3.16  | 2.47 | 3.28        | 2.70        | 1.28       |
| Prep. M <sub>2</sub> . . . | 8.30  | ..         | 3.47           | 5.35  | 1.58 | 5.55        | 1.64        | 3.39       |

*Explanation of the Table.*—Ash, residue heating at low redness. Fixed ash, residue after heating to full heat of powerful burner. Inorganic ash, ash less calculated amount of PO<sub>4</sub>. N, nitrogen by Kjeldahl-Groening method. P, phosphorus by the Neumann method. N and P ash free, reckoned free from “inorganic ash.” N:P, quotient of column 4 divided by column 5. A, portion of haptophor dissolved by acid alcohol. B, portion of haptophor not dissolved by acid alcohol. D, substance precipitated by acid alcohol from solution of B in aqueous alkali. G, substance precipitated by acid alcohol from aqueous solution of haptophor. H, obtained by concentration of the alcoholic filtrate from G. K, substance precipitated by dilute acetic acid from aqueous solution of haptophor. K<sub>2</sub>, same as K, except that strong acid was used. M, precipitated by alcohol from filtrate from K. M<sub>2</sub>, precipitated from filtrate from K<sub>2</sub>.

Leach states: “As these preparations are all mixtures, the absolute values are worth nothing taken singly, but the comparative values, especially the ratio of N to P, as given in the last column, are of interest. The determinations were made for the sake of tracing the nucleo compounds. There are many indications of nucleic acid, but the amount of both nitrogen and phosphorus is much too small. The ratio between them is, however, quite within the range for nucleic acids from other sources, as may be seen by comparison in the following table. Moreover, the

nucleic acid and the nucleates are the only nucleo compounds in which the ratios are at all comparable with those given in the preceding table. Nuclein contains a little less phosphorus than any of these preparations from the germ, while other nucleo compounds are much richer in nitrogen and poorer in phosphorus. It is perhaps worthy of mention that contact with mineral acid apparently breaks up the nucleic acid, the phosphoric acid going into solution; thus, preparation A gives evidence of phosphorus in inorganic combination, while G does not."

| Substance.            | Source.          | Observer.             | N.    | P.   | N:P.  |
|-----------------------|------------------|-----------------------|-------|------|-------|
| Nucleic acid          | Salmon sperm     | Miescher              | 15.24 | 9.62 | 1.58  |
| Nucleic acid          | Sea urchin sperm | Mathews               | 15.34 | 9.59 | 1.60  |
| Nucleic acid          | Yeast            | Miescher              | 16.03 | 9.04 | 1.77  |
| Nucleic acid          | Pancreas         | Bang                  | 18.20 | 7.67 | 2.37  |
| Nucleic acid          | Thymus           | Kostytschew           | 15.55 | 9.25 | 1.69  |
| Nucleic acid          | Thymus           | Kostytschew           | 15.26 | 9.30 | 1.65  |
| Nucleic acid          | Wheat embryo     | Osborne and<br>Harris | 15.88 | 8.70 | 1.83  |
| Inosinic acid         | Muscle           | Haiser                | 16.00 | 8.60 | 1.86  |
| Clupein nucleate      | —                | Mathews               | 21.06 | 6.07 | 3.48  |
| Nucleohiston          | Thymus           | Huiskamp              | 18.37 | 3.70 | 4.97  |
| Nucleoprotein         | Thymus           | Huiskamp              | 16.42 | 0.95 | 17.30 |
| Nucleoprotein         | Pancreas         | Umber                 | 17.82 | 1.67 | 10.65 |
| Nuclein               | Pancreas         | Umber                 | 17.39 | 4.48 | 3.88  |
| Ba $\alpha$ -nucleate | Thymus           | Kostytschew           | 12.83 | 7.63 | 1.68  |
| Ba $\beta$ -nucleate  | Thymus           | Kostytschew           | 10.16 | 8.48 | 1.38  |

In a later paper, Leach<sup>1</sup> has made a study of the haptophor of egg-white. The percentages of ash, nitrogen, phosphorus, and sulphur in egg-white and its split products are given and compared with the cellular substance of the colon bacillus in the following table:

|                  | Inorganic |       |       |       |      | N ash<br>free. | P ash<br>free. | S ash<br>free. |
|------------------|-----------|-------|-------|-------|------|----------------|----------------|----------------|
|                  | Ash.      | ash.  | N.    | P.    | S.   |                |                |                |
| Egg-white . . .  | 2.48      | 2.066 | 14.48 | 0.135 | 2.66 | 14.70          | 0.138          | 2.73           |
| Toxophor . . .   | 1.14      | ..    | 13.74 | Trace | 2.19 | 13.90          | ..             | 2.22           |
| Haptophor . . .  | 13.57     | 12.80 | 12.67 | 0.253 | 2.79 | 14.53          | 0.290          | 3.20           |
| Cell substance . | 8.61      | ..    | 10.65 | 2.870 |      |                |                |                |
| Toxophor . . .   | 2.33      | ..    | 11.15 | Trace |      |                |                |                |
| Haptophor . . .  | 33.25     | 26.08 | 5.56  | 2.340 | ..   | 7.52           | 3.990          |                |

<sup>1</sup> Jour. Biol. Chem., 1908, v, 253.

Leach split up edestin, casein, egg-white, and colon cellular substance with alkaline alcohol. The insoluble part of each gave the various protein color tests, Millon's reaction less satisfactorily than the others. On stirring with water, the edestin preparation was entirely soluble, there was a slight flocculence with the casein preparation, the others were mainly but not wholly soluble. Addition of a little sodium hydroxide increases the solubility. Mineral acids give precipitates with the casein and egg preparations.

The most marked difference was found on testing for carbohydrates. As edestin contains no carbohydrate, its preparation showed no evidence of such a group. Although casein is said to contain no carbohydrate, it has been found to respond to the Molisch test, and so does its haptophor. As was to be expected, the egg preparation gives evidence of hexose and not pentose. The lead sulphide reaction shows the presence of loosely combined sulphur in the preparations from egg and edestin, not in the ones from casein and the colon bacillus.

Samples of the haptophor of egg-white were stirred with water, filtered, and attempts made to separate protein and carbohydrate in the filtrate by means of uranium acetate. The acetate was added both with and without sufficient alkali to keep the solution alkaline. A copious precipitate resulted in both cases and this was filtered out with some difficulty. The slight excess of uranium was removed from the filtrate by the addition of sodium phosphate. The filtrate gave evidence of carbohydrate, but the separation was not sufficiently sharp, and that method was abandoned. Acidifying until there was a slight permanent precipitate, the addition of either ethyl or methyl alcohol cleared the solution. Phosphotungstic acid precipitated both protein and carbohydrate. In short, no method was found that would remove the protein from the solution and leave the carbohydrate. It is perhaps a legitimate inference that the combination of the two is a chemical one.

Samples were subjected to hydrolysis and titrated with Fehling's solution. The proteins and possibly other bodies

present interfered with the reaction, but by adding the solution all or nearly all at once it was possible to obtain comparative results. Experiments with the haptophor of the colon bacillus had shown that the maximum reduction was obtained by boiling for two and one-half hours with 2.5 per cent. hydrochloric acid (see p. 70).

Three grams of the haptophor of egg-white was mixed with 200 c.c. of water, and 20 c.c. of 25 per cent. hydrochloric acid. A second sample was prepared in the same way except that it was filtered before adding the acid. Both were boiled with reflux condenser. After boiling half an hour and then at intervals of three hours, aliquot parts were removed, neutralized, titrated with Fehling's solution, and the amount of reducing substance calculated. Other samples were hydrolyzed with sulphuric acid, with less satisfactory results. These preliminary experiments indicated that the reducing substance is all present in the portion soluble in water, and that the maximum yield, which if calculated as dextrose, is about 9 per cent., is obtained by boiling from ten to twelve hours, and until the mixture no longer gives the biuret test.

Accordingly, 25 grams of the egg-white haptophor was shaken for two hours on a shaker with ten times its weight of water, filtered, 200 c.c. more of water added, the solution neutralized with hydrochloric acid, then 50 c.c. of 25 per cent. hydrochloric acid added, thus making approximately a 5 per cent. solution of material in 2.5 per cent. acid. This was boiled with a reflux condenser for ten or twelve hours, until the solution no longer gave the biuret test. It was then filtered, leaving very little on the filter. The clear, red-brown filtrate was cooled, neutralized with sodium hydroxide, and benzolated by the Schotten-Baumann method. The mixture became very warm, but was cooled by surrounding the flasks with pounded ice and salt. When the reaction ceased, the compound settled nicely, and was filtered by suction after standing two or three hours. The precipitate was washed with water containing a little ammonia, and treated with boiling water, in which a large

portion was freely soluble. On cooling and concentrating the alcoholic solution, a fine yield of crystals was obtained. The crystals from several samples were united and recrystallized from hot absolute alcohol until the solution was clear and colorless. Macroscopic bundles of needles were thus obtained, showing very characteristic grouping. They were washed in alcohol and in ether, dried upon porous plates, the operations being repeated until samples from two recrystallizations melted side by side within  $1^{\circ}$  or  $1.5^{\circ}$ . The crystals are pure white, readily soluble in benzol, chloroform, and in glacial acetic acid as well as in alcohol, and melt at  $203^{\circ}$ . When boiled with sodium hydroxide, ammonia is given off; after removing benzoic acid by boiling with hydrochloric acid, the resulting product reduces Fehling's solution.

0.4150 gram gave 0.00891 gram N, corresponding to 2.14 per cent. N.

0.4220 gram gave 0.00962 gram N, corresponding to 2.279 per cent. N.

Average is 2.213 per cent. N.

These characteristics suffice to identify the compound as glucosamin benzoate which Pumm reports as melting at  $203^{\circ}$ . Kueny prepared different benzoates of glucosamin by varying the conditions of the experiment. The one most readily formed was the tetrabenzoate, melting at  $199^{\circ}$  when recrystallized from alcohol, and at  $207^{\circ}$  when recrystallized from glacial acetic acid. He tried by various methods to prepare a pentabenzoate, but without success. Langstein prepared glucosamin benzoate from egg-white, which, after once recrystallizing from hot alcohol, melted at  $201^{\circ}$  to  $202^{\circ}$ , and gave 1.95 per cent. of nitrogen. The theoretical amount of nitrogen in the tetrabenzoate is 2.35 per cent. Thus, the benzoate prepared from the haptophor of egg-white agrees with glucosamin benzoate prepared from glucosamin and from egg-white, at least as well as those preparations do with each other. Numerous observers have found glucosamin in egg-white, and this work shows that it remains in the haptophor when egg-white is disrupted by alkaline alcohol.

## CHAPTER VI

### ACTION ON ANIMALS<sup>1</sup>

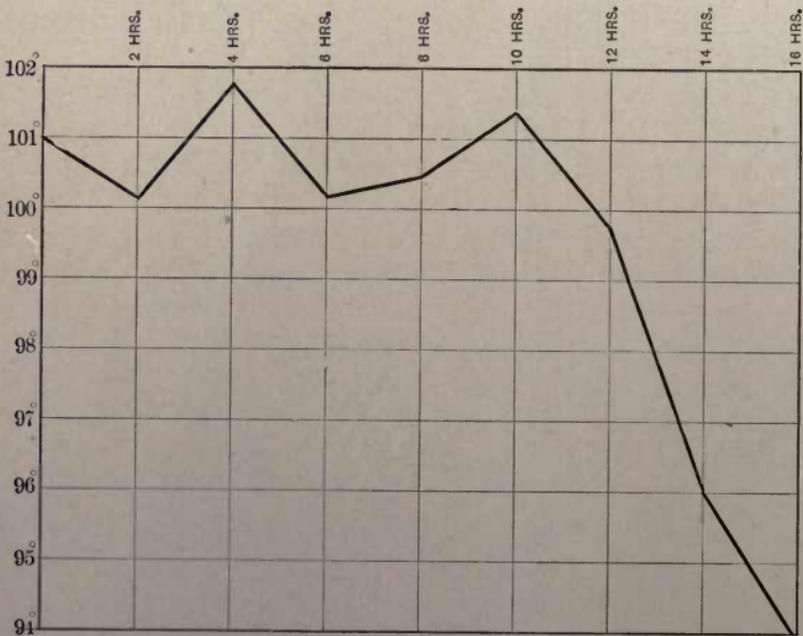
It will be interesting and instructive to compare the effects of the living bacillus, the dead cellular substance, and the soluble poison on animals.

**The Action of the Living Bacillus.**—When a guinea-pig is inoculated with a fatal dose of the living colon germ, practically no symptoms whatever are noticeable for a period varying from five to twelve hours, according to the size of the dose given. This may be considered as the period of incubation and is roughly proportional to the amount of living germ injected. We have always worked with a bacillus 1 c.c. of a twelve-hour or older, bouillon culture of which has invariably proved fatal to guinea-pigs within twenty-four hours. If 1 c.c. of such a culture is given, no effects will be seen for a period of from ten to twelve hours. If, on the other hand, 2 c.c. of the same culture be injected, the animal will begin to manifest symptoms of illness in from eight to ten hours, and if larger doses are given the symptoms will become apparent in a shorter time. This period of incubation undoubtedly represents the time taken for the bacillus to multiply and to be destroyed to such an extent that sufficient poison may be liberated through its disintegration to produce noticeable toxic effects in the animal. This period of incubation is, therefore, in reality the crisis of the disease and the outcome depends solely on whether all bacteria have been destroyed before a lethal dose of the poison has been set free or not. It is

<sup>1</sup> This chapter is a reproduction, without material change, of an article by Victor C. Vaughan, Jr., published in the Jour. Amer. Med. Assoc. in 1905.

during this period that individual resistance and acquired immunity are important factors acting by causing increased bacteriolysis and the destruction of all bacilli before a fatal dose of poison has been set free. During this time the temperature of the animal may rise to a greater or less extent or may remain stationary; the animal remains active, eats; its coat is not roughened and it appears in

FIG. 5



Temperature curve of guinea-pig after inoculation with 1 c.c., sixteen-hour bouillon culture of the colon bacillus. Death occurred twenty hours after inoculation.

all respects as well as a normal animal. At the end of this period, however, the appearance changes. The animal becomes less active. It remains in one corner of its cage; its coat becomes roughened; it hangs its head and apparently enters into a state of stupor. At the same time the rectal temperature begins to fall abruptly, as can be seen from a study of Fig. 5.

Indeed, this fall of body temperature is often the first marked symptom and, when occurring to a marked degree, it is invariably a bad omen. The body temperature will often fall from 101° to 94° F. or even lower within from two to four hours, and this fall is progressive and continuous until the animal's death, immediately preceding which a temperature as low as 87° or 86° F. is not uncommon. At the same time the animal shows signs of the most marked peritoneal inflammation, as is evidenced by rigidity and spasm of the abdominal muscles on pressure. At autopsy, the only gross lesion present is a marked hemorrhagic peritonitis with a large amount of bloody fluid containing intact red corpuscles and leukocytes in the peritoneal cavity. The parietal and visceral peritoneum are studded with minute punctiform hemorrhages. Hemorrhage is an especially prominent feature in the great omentum and is present to a less marked degree in the mesentery.

**The Action of the Cellular Substance.**—The dead bacterial substance used in the following work was obtained by growing a large amount of the colon germ on tanks filled with agar for a period of two weeks at room temperature. At the end of this time the growth was removed from the tanks and extracted with absolute alcohol and ether. The crude bacterial substance thus obtained was reduced to a fine powder by pulverization in an agate mortar, and was then ready for use.

It is interesting to note that the person who did the pulverizing was often quite seriously poisoned during the process unless he took the precaution of wearing a mask which hindered the inhalation of the powder. The symptoms of such poisoning were exceedingly interesting. The first thing noticed was a marked irritation of the nasal mucous membrane and a huskiness of the voice, due no doubt to the mechanical irritation of the inhaled powder. This was followed by a feeling of depression and malaise, and chilly sensations. Occasionally a decided chill would be experienced. It is unfortunate that no accurate observations of temperature were taken in these cases. Nausea

and even vomiting were occasionally noted. After a period of discomfort varying from six to ten hours, during which the patient often complained of dull pain in the various joints, recovery would rapidly and completely take place.

On examining the powder obtained in this manner microscopically we found that it consisted of colon bacilli which still retained their morphological characteristics and could still be stained by aniline dyes. On the other hand cultures made from this powder have, of course, never given a growth. In other words, the bacillus has not been broken up by this treatment, but simply has been deprived of life and of the power of reproduction. It is worthy of note that neither by the action of alcohol, ether, physiological salt solution, distilled water, nor any simple solvent have we been able to extract a poison from the colon bacillus. Nor, again, can a poison be split off by the action of heat even when the germ substance is heated to 184° C. in a sealed tube for thirty minutes. It is only when we make use of agents which will chemically break up the colon bacillus that we are enabled to obtain a poison apart from the rest of the cellular substance. The powdered bacterial substance is not soluble, but can be held in suspension in normal salt solution and, since it can be boiled without appreciably affecting its toxicity, suspensions were always heated to 100° for fifteen minutes before injection in order to insure sterility.

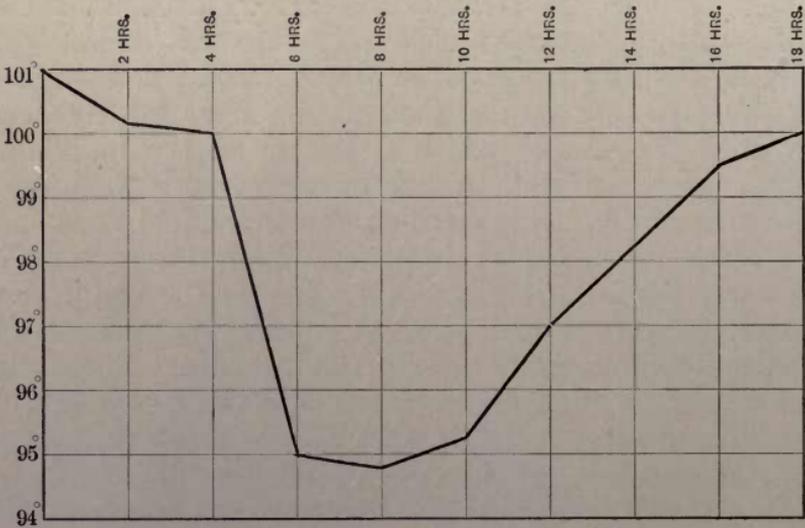
This coarsely powdered cellular substance killed guinea-pigs when injected intraperitoneally in doses of 1 to 40,000, body weight, and invariably proved fatal within twelve hours, usually causing death at the end of from six to eight hours. On the injection of a fatal dose of the cellular substance intraperitoneally, we noticed that the most marked change was in the length of the period of incubation. Thus, whereas in the case of the living germ from eight to twelve hours passed before noticeable symptoms appeared, in that of the dead germ substance the animal almost invariably showed symptoms of illness at the end of four hours. In regard to the character of these symptoms

it may be stated that they are similar in all respects to those induced by the living bacillus. The temperature remains the same or may rise slightly during the first two hours. At the end of the four hours it has begun to fall, and there is a decided drop from then on until the time of death, provided the dose given is a fatal one. If a non-fatal dose has been injected intraperitoneally the temperature, as will be seen from Fig. 6, has reached a minimum at the end of from six to eight hours and has returned to normal again in from twelve to twenty hours.

Moreover, as a general rule, it may be stated that the fall in non-fatal cases seems to be directly proportional to the amount of bacterial substance injected. That this should be the case seems to be only natural when we consider the fact that in this instance we have largely done away with that factor which is known as the individual resistance of the animal. As has been previously mentioned in the case of the living bacillus, the individual resistance plays an important part in determining the amount of poison which will ultimately be set free in the body. For example, whereas 1 c.c. of a twelve-hour culture of our colon bacillus invariably proved fatal, 0.25 c.c. never did. The explanation of this is to be found in the fact that with the smaller dose all animals were able to cause disintegration of all bacilli injected before a fatal dose of poison was set free. If now 0.5 c.c. be given some would recover, while others would die. In this case we would speak of the former as possessing a greater individual resistance than the latter. This simply means that, in the first instance the animal has possessed a sufficient quantity of bactericidal substance directly available to cause disintegration of all bacilli before the latter have multiplied to a sufficient extent to furnish enough poison to kill the animal on its liberation. On the other hand, those animals which succumbed did not possess quite enough of the bactericidal substance, or at least did not possess it in a form available for immediate use. When, however, the dead bacterial substance is given the dose of poison which the animal receives is a certain definite amount and is not capable of subsequent increase.

Accompanying the fall in temperature there is apparent lassitude, stupor, and roughening of the coat. In cases in which many times the fatal dose has been given, the animals occasionally die within from four to six hours with convulsions, a feature which can now and then be observed after the injection of large quantities of the living bacillus. At autopsy we find a picture similar in all respects to that following inoculation with the living colon bacillus. There is a marked hemorrhagic peritonitis, the peritoneal cavity

FIG. 6



Temperature curve of guinea-pig after intraperitoneal injection of non-fatal dose of crude bacterial cell substance.

containing bloody fluid, together with unabsorbed bacterial cell substance, and the omentum and mesentery showing numerous punctiform hemorrhages. It is needless to state that in all cases cultures were made from the peritoneal cavity and heart's blood immediately after death, and these proved to be sterile. From this we see that practically the sole difference between the effects following inoculation with the living bacillus and the injection of the dead bacterial substance is a shortening of the period of incubation

due, no doubt, to the fact that the intracellular poison is liberated much more rapidly and in greater concentration in the second case. As will be seen later, it is not so much the absolute quantity of the poison which is injected that determines the result, as the amount which is active at a given time.

**The Action of the Soluble Poison.**—When doses of this powder are given intraperitoneally in amounts varying from 8 to 60 milligrams, according as to whether we have been careful to remove most of the common salt or not, a fatal result follows in guinea-pigs in from thirty to sixty minutes. Within fifteen minutes after injection the temperature begins to fall and sometimes within half an hour has reached 94° F. or even lower. At first, after an interval of from five to ten minutes immediately following the injection, the animal appears restless, runs about the cage, and shows a great tendency to scratch itself, this undoubtedly being due to itching sensations in the skin caused by irritation of the peripheral nerves. The animal then begins to show evidence of lack of coördination, which is rapidly followed by partial paralysis, which is especially marked in the hind extremities. This stage lasts for from five to ten minutes, during the later part of which the animal usually lies quietly on one side. From this state the animal passes into what one might term the convulsive stage. These convulsions are usually clonic in nature and, as a rule, at first involve only the neck muscles, the head being momentarily drawn backward on the back. At first these convulsions are but slight in degree and are separated by considerable intervals of time. Soon, however, they become much more frequent and of much greater severity. Gradually they become more and more general in their extent, until all the muscles of the body become involved in violent clonic convulsions. This stage when present presages a fatal outcome; rarely an animal recovers after reaching the convulsive stage. During a convulsion, or occasionally in the interval of calm, respiration ceases. The heart, however, continues to beat, at first with perfect regularity

and no acceleration; indeed, the rate seems to be somewhat slower than normal. Gradually the beat becomes more and more feeble, the rate and regularity being preserved to the end. It is usually only after an interval of from three to four minutes after the cessation of respiration that the heart ceases to beat. As has been previously stated, a fatal issue, if it occurs at all, always results within one hour after injection and usually within from thirty to forty minutes. This is to a large extent independent of whether the dose is the minimum lethal one or two or three times that amount. It is certainly entirely independent of the size of the pig. Death, of course, results at slightly different times with different batches of the poison, but even in this case the interval of time between injection and a fatal issue does not vary to any great extent. A dose which has proved to be the minimum fatal dose for one pig will almost surely prove to be the same for another. In other words, we have done away practically entirely with the period of incubation, and the poison acts so rapidly that individual resistance plays no part; hence, the animal acts almost with the exactitude of a chemical compound into which for all practical purposes it has been converted. The period of incubation has ceased to exist since the poison is no longer contained within either the dead or the living bacillus, but is present in a free and uncombined form, capable of uniting immediately with those body cells for which it may possess a special affinity.

At autopsy no special gross lesions can be made out. The peritoneum is smooth and shiny throughout, and there is not the slightest evidence of either hemorrhage or even marked congestion in the omentum or mesentery. This is very important and in marked contrast to the hemorrhagic peritonitis found after injection of either the living or the dead colon bacillus. We are inclined to believe that it is the distinguishing feature between the injection of the poison in a comparatively free and in a combined state. At one time we attempted to obtain the poison by a simpler method, omitting the extraction of the crude substance

with ether. The result was that on evaporation of the alcoholic filtrate we obtained a sticky residue which it was utterly impossible to pulverize or to weigh. We were compelled, therefore, to content ourselves with evaporating it to a sticky mass, which was then immediately dissolved in water. The solution of the substance thus prepared was very poisonous, but, as a rule, took from one to two hours or even longer to bring about a fatal result. The animals showed the roughening of the coat and the stupor characteristic of the living and dead bacillus, but not as a rule seen in the case of the soluble poison. Furthermore, the majority of the animals showed during life unmistakable signs of peritoneal inflammation. They died in convulsions. At autopsy an intense hemorrhagic peritonitis was present, which was particularly prominent in the omentum and mesentery, and hemorrhage was often present in the capsules of the liver and the spleen. From the fact that death was slower in these cases and that the symptoms were more like those seen after inoculation with the living bacillus, we are inclined to believe that in this instance the poison, although split off from the bacillus itself, still exists in combination with some other cell group, and that it is essential that this combination be broken up before the poison can be set free and can act on the body cell.

Another interesting fact in this connection is furnished by the action of the poison in solutions which have been rendered strongly alkaline by the addition of sodium bicarbonate. As has been previously stated, the aqueous solutions of the poison are slightly acid in reaction, and in order to avoid the irritative effects which might follow their injection into the peritoneal cavity, they were neutralized or rendered slightly alkaline by the addition of sodium bicarbonate.<sup>1</sup> At first no attempt was made to secure perfect neutralization, with the result that sometimes we were making use of neutral, while again slightly or

<sup>1</sup> The precaution of neutralizing the soluble poison, when properly prepared, is unnecessary as it has no appreciable irritative action.

decidedly alkaline solutions were employed. It was soon noticed, however, that the results obtained in the three cases were very different. Thus, whereas in the neutral or faintly alkaline solution the injection of 60 mg. of the powder invariably killed, in the case of a stronger alkaline solution the same amount did not cause a fatal result, although the animals were very ill. From this fact it became evident that some change had taken place in the poison on standing in alkaline solution. In order to study this change more in detail, experiments were conducted with solutions of different degrees of alkalinity, with the results found in the following tables:

TABLE III.—RESULTS WITH SOLUTION OF POISON BARELY NEUTRALIZED WITH SODIUM BICARBONATE AND PLACED IN INCUBATOR

| No. of animal. | Dose of poison. | Solution kept in incubator. | Weight of pig. | Result of injection. | Time of death after injection. |
|----------------|-----------------|-----------------------------|----------------|----------------------|--------------------------------|
| 1              | 60 mg.          | Fresh                       | 325 gm.        | +                    | 30 minutes                     |
| 2              | 60 mg.          | 2 hours                     | 330 gm.        | +                    | 20 minutes                     |
| 3              | 60 mg.          | 20 hours                    | 370 gm.        | +                    | 20 minutes                     |
| 4              | 60 mg.          | 2 days                      | 320 gm.        | +                    | 15 minutes                     |
| 5              | 60 mg.          | 4 days                      | 350 gm.        | +                    | 20 minutes                     |
| 6              | 60 mg.          | 6 days                      | 350 gm.        | +                    | 45 minutes                     |
| 7              | 60 mg.          | 8 days                      | 320 gm.        | +                    | 20 minutes                     |

TABLE IV.—RESULTS WITH SOLUTION OF POISON RENDERED DECIDEDLY ALKALINE WITH SODIUM BICARBONATE AND PLACED IN INCUBATOR

| No of animal. | Dose of poison.          | Solution kept in incubator. | Weight of pig. | Result of injection.  | Time of death after injection. |
|---------------|--------------------------|-----------------------------|----------------|-----------------------|--------------------------------|
| 1             | 60 mg.<br>(barely neut.) | Fresh.                      | 350 gm.        | +                     | 30 minutes                     |
| 2             | 60 mg.<br>decidedly alk. | Fresh.                      | 370 gm.        | Very sick for 2 hours | Recovered                      |
| 3             | 60 mg.                   | 2 hours                     | 325 gm.        | Not very sick         | Recovered                      |
| 4             | 80 mg.                   | 4 hours                     | 310 gm.        | Sick                  | Recovered                      |
| 5             | 120 mg.                  | 24 hours                    | 280 gm.        | +                     | More than 5 hours.             |
| 6             | 160 mg.                  | 3 days                      | 350 gm.        | +                     | 7 hours.                       |

TABLE V.—RESULTS WITH SOLUTION OF POISON RENDERED DECIDEDLY ALKALINE WITH SODIUM BICARBONATE AND KEPT AT ROOM TEMPERATURE

| No. of animal. | Dose of poison. | Time at room temperature. | Weight of pig. | Result of injection.   | Time of death after injection. |
|----------------|-----------------|---------------------------|----------------|------------------------|--------------------------------|
| 1              | 60 mg.          | Fresh                     | 350 gm.        | +                      | 35 minutes                     |
| 2              | 60 mg.          | 12 hours                  | 265 gm.        | Sick                   | Recovered                      |
| 3              | 90 mg.          | 2 days                    | 280 gm.        | Sick                   | Recovered                      |
| 4              | 120 mg.         | 2 days                    | 460 gm.        | +                      | 20 minutes                     |
| 5              | 120 mg.         | 7 days                    | 405 gm.        | Sick for 5 hours       | Recovered                      |
| 6              | 160 mg.         | 7 days                    | 440 gm.        | Sick for several hours | Recovered                      |

From the above tables it will be seen that the degree of alkalinity of the solution, and especially the length of time that the poison has stood in alkaline solution are very important factors in determining its toxicity. Thus in Table III, in which the solution was barely neutralized, the poison seems to have retained its full potency after eight days in the incubator, whereas, in the case of the strongly alkaline solution, the potency has decreased markedly within from twenty-four to forty-eight hours. Again, there are great differences to be seen depending on whether the strongly alkaline solution has been kept at room temperature or at that of the incubator, the decrease in toxicity being much less rapid in the first instance.

A more detailed report of the effects on animals than it was possible to give in the above tables is not without interest. For example, in Table IV, No. 2, which received 60 milligrams immediately after the solution had been rendered decidedly alkaline, was very sick indeed, whereas No. 3, which received the same amount after two hours in the incubator, was only slightly affected. In the case of Nos. 5 and 6 the effects observed corresponded more closely to those obtained with the crude bacterial cell substance. It is unfortunate that the time of death was not ascertained in the case of No. 5. No. 6 did not succumb until seven hours after the injection. On autopsy

there was considerable fluid in the peritoneal cavity, and the vessels of the mesentery were markedly congested. The omentum was particularly injected and a few minute hemorrhages could be made out. The most plausible explanation of the above facts is found in the theory that the poison has not been destroyed in the alkaline solution, but rather has entered into chemical combination with the alkali and that we are again dealing with it in a combined instead of in a free state. The fact that the same amount will not cause a fatal result is thus readily explained, since the outcome depends largely on the rapidity with which the poison acts. If it is present in a state of combination which must be broken up before it can exert its deleterious action on the body, and if this combination is only slowly decomposed, the nerve cells, for which it apparently has a special affinity, are not subjected to an overwhelming dose at one time, as in the case of the intraperitoneal injection of the free poison.

The results obtained in animals Nos. 5 and 6, Table V, are very interesting. In these instances there were two distinct illnesses, the first becoming manifest within from twenty to thirty minutes after the injection and corresponding in all respects to that following a non-fatal dose of what we have for convenience termed the free poison. The animals were decidedly in better condition at the end of an hour; however, they then began to show symptoms similar to those noticed after the injection of the crude cell substance, *i. e.*, roughening of the coat, stupor, and slight convulsive movements. Recovery from this state did not occur until after the lapse of from five to six hours. It is evident that here the first signs of illness were due to some of the poison which had not as yet combined with the alkali, and hence still existed in the free state, whereas the later symptoms were due to the effects of the slow liberation of the same poison from its combination. In this connection it is interesting to note that the combination between the poison and the alkali which apparently takes place in decidedly alkaline solutions is not an immediate

one, but occurs gradually and reaches a maximum only after the lapse of a considerable interval of time. That the rapidity with which this combination is effected depends largely on temperature is shown by the fact that it occurs much more rapidly in a solution kept in the incubator than in one which is allowed to stand at room temperature.

The results which follow the injection of a fatal dose of the soluble poison intraperitoneally have already been described. When a non-fatal dose has been injected the symptoms first noticed are similar in all respects to those following a fatal dose. The animal becomes restless, shows signs of irritation of the peripheral nerves, incoördination, and partial paralysis. The convulsive stage is not present, as a rule, and when it is noticed is evidenced solely by slight movements separated by considerable intervals of time. We have never seen a case showing marked generalized convulsions which recovered.<sup>1</sup> Recovery is apparently rapid and complete, and within two hours after injection the animal which has been desperately ill appears as well as any untreated animal. The maximum effect is obtained within from forty-five to sixty minutes in every instance. The study of the changes in temperature in these animals is particularly interesting. Within fifteen minutes the rectal temperature has begun to fall and has reached a minimum within one hour.

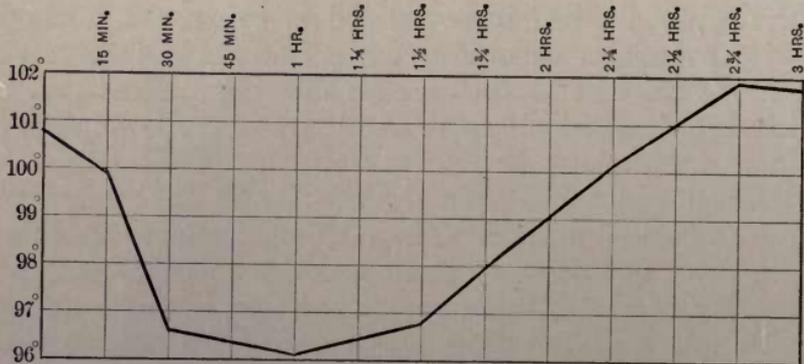
It remains stationary for a short time and then begins to rise again, and at the end of three hours after the injection has usually returned to normal or above.

On injecting the soluble poison subcutaneously, we find that animals are able to withstand a much larger dose than when the poison is given intraperitoneally. Thus, in the case of a poison, 60 mg. of which invariably killed when given intraperitoneally, it was found that 120 mg. could be given subcutaneously without causing a fatal result. However, the injection of a solution containing 180 mg. invariably caused death, the fatal issue occurring

<sup>1</sup> This does rarely occur.

in about the same length of time as in the case of animals treated intraperitoneally. Thus a dose of 180 mgs. always proved fatal in from one-half to three-quarters of an hour. The symptoms are practically identical with those following the intraperitoneal injection with the exception of the fact that the various stages are much more sharply defined. For example, the stage of peripheral irritation is much more marked. The animal soon after injection becomes very restless, runs around his cage, and scratches his body. This itching seems, however, to be general from the outset, and is not, apparently, more pronounced in the immediate

FIG. 7



Temperature curve of guinea-pig treated with 45 mgs. of the soluble poison intraperitoneally.

neighborhood of the site of injection. If the animal has been injected under the skin of the abdomen, its attention is not necessarily first attracted to this spot, but it may begin by scratching its nose or one of the extremities. Another peculiar symptom, which is probably due to peripheral irritation, and which is seldom seen in cases of intraperitoneal injection, is the tendency which the animals show to dig furiously in the shavings in the bottom of their cages. This feature is quite characteristic, and is seldom absent in pigs which have been treated subcutaneously. The later stages are similar in all respects to those seen following the intraperitoneal injection. The animal shows

symptoms of incoördination, lies on one side, and finally develops convulsions, with failure of respiration, the heart continuing to beat regularly for some time after the complete cessation of respiration. Here also the symptoms are accompanied by a decided fall in the body temperature.

The results following the intravenous injection of the soluble poison are given in the following table:

TABLE VI

| No. of animal. | Amount of poison injected intravenously. | Time of cessation of respiration after injection. | Time of cessation of heart-beat after injection. |
|----------------|--|---|--|
| 1              | 10 mg.                                   | 4 minutes   | 7 minutes  |
| 2              | 10 mg.                                   | Recovered   |  |
| 3              | 10 mg.                                   | 3 minutes   | 6 minutes  |
| 4              | 10 mg.                                   | Recovered   |  |
| 5              | 15 mg.                                   | 4 minutes   | 6 minutes  |
| 6              | 15 mg.                                   | 3 minutes   | 5 minutes  |
| 7              | 15 mg.                                   | 4 minutes   | 7 minutes  |
| 8              | 15 mg.                                   | 4 minutes   | 6 minutes  |
| 9              | 20 mg.                                   | 3 minutes   | 5 minutes  |
| 10             | 20 mg.                                   | 4 minutes   | 6 minutes  |
| 11             | 20 mg.                                   | 3 minutes   | 6 minutes  |
| 12             | 20 mg.                                   | 3 minutes   | 7 minutes  |

From the above table it will be seen that in all cases respiration ceased within four minutes after injection. Indeed, the respiratory embarrassment becomes pronounced immediately following the injection. The animal struggles for breath and there is violent retraction of the sternum. No convulsions are seen following the intravenous injection, this being probably due to the inhibitory influence of the anesthetic which has been used during the preparation of the animal for the operation. The failure of respiration in the absence of convulsions would seem to be conclusive evidence that the cessation of this function is due not to mechanical interference during a convulsive attack, but to a direct paralysis of the respiratory centre itself. Furthermore, the fact that the heart continues to beat in a perfectly normal manner for from two to four minutes after respiration has entirely ceased, would tend to show that the

immediate cause of death is asphyxia brought about by paralysis of respiration through the action of the poison.<sup>1</sup>

This action of the heart after the cessation of respiration is exceedingly interesting, and is entirely analogous to that mentioned as following the intraperitoneal injection. The rate of the beat is decidedly lessened and at first the individual beats are stronger. They gradually become more and more feeble, however, until finally the heart stops in diastole, the rate after the preliminary slowing remaining unchanged until the end. It is worthy of note that in the case of intravenous injections the fall of temperature, which is so marked a feature after the intraperitoneal and subcutaneous injections of the poison does not occur. The explanation of this fact is doubtless to be found in the very short interval of time which elapses between the injection and a fatal outcome. As regards the size of the lethal dose when given intravenously, we see that 10 mg. often, and 15 mg. invariably, proved fatal.<sup>2</sup>

For purposes of comparison, we have always made use of the poison obtained from the same extraction in our intravenous, subcutaneous, and intraperitoneal injections, and have therefore been able to ascertain with a fair degree of accuracy the differences in dose required to bring about a fatal result in the three cases. Thus, whereas 60 mg. represents the fatal dose when given intraperitoneally, it requires between 120 and 180 mgs. subcutaneously, and only from 10 to 15 mgs. of the same poison to cause death when given intravenously. These differences are undoubtedly due to the rapidity of absorption in the various cases, and a fatal issue depends entirely on whether sufficient poison reaches the sensitive area at one time to cause cessation of respiration or not.

It has now been shown that a very powerful intracellular poison can be obtained from the colon bacillus. As has been previously stated, the results given in the foregoing experiments are those obtained with the poison from one

<sup>1</sup> The physiological action of this protein poison is discussed on page 315.

<sup>2</sup> The fatal dose of the purest form of the poison which we have obtained is 0.5 mg. intracardially.

extraction only. It must be understood that the poison is not in a pure state and when it is stated that 60 mgs. causes death when injected intraperitoneally we refer simply to the powder obtained from a given extraction. We have been able to procure powders which kill in doses of 15 and even as low as 8 mgs. when given intraperitoneally. This difference is in large amount due to the presence of sodium chloride, since no attempt has been made to remove this salt in the case of the less toxic powders by redissolving in absolute alcohol.

There are several facts which lead us to believe that this poison is the one which causes the symptoms of illness and death in animals infected with the colon germ. Most of these facts have already been brought out, but it may not be out of place to briefly recapitulate at this point. As has been previously seen, the results obtained with the living germ, the dead bacterial substance, and the soluble poison can best be explained on the ground that the poisonous body in each case is the same. The differences in action are not differences in symptoms, but simply in the rapidity with which these symptoms become manifest. While it is undoubtedly true that in animals dying with the minimum fatal dose of the living germ, the convulsive stage is not present or is only slightly marked, it is rarely absent in cases where from three to four times the fatal amount has been given. The sole difference between the living germ and the soluble poison which would appear to demand an explanation is the lack of evidence of a peritonitis in the latter case. This, we think, is best explained by the fact that in the case of the soluble poison the poisonous substance exists in an uncombined form, which, of course, is not true in the case of either the living or the dead bacterial cell. The uncombined poison is rapidly absorbed from the peritoneal cavity, and hence the irritative effects which would result from its retention in this place are absent.

As has been stated, one of the first signs of the action of the poison is a lowering of the body temperature. This hypothermia is usually present to a marked degree, and is noticeable before any visible symptoms occur. It is,

therefore, the best index which we have as to the exact time at which the poison begins to exert its effect. In the same manner the rise of temperature after the development of hypothermia is the first indication of recovery. Moreover, if in an animal with a subnormal temperature a rise occurs, it is an infallible sign of ultimate recovery, no matter how grave the general condition may appear to be at the time. We have laid great stress, therefore, on the changes in body temperature as furnishing the most delicate test of the action of the poison. It may be here stated that the body temperature of guinea-pigs is ordinarily fairly constant within certain narrow limits, and they are much more satisfactory animals to work with in this respect than are rabbits. Moreover, their temperature does not seem to be materially altered by the injections of sterile salt solution or such inert substances as a suspension of pumice stone into the peritoneal cavity. As has been seen in the case of the living germ, it is only after the lapse of several hours that a fall in temperature occurs. This would indicate that it is not until this time that sufficient poison is liberated to cause notable toxic effects in the animal. That it takes an appreciable time for the poison to be liberated from the bodies of the bacilli is well illustrated in the instance of the dead bacterial substance. Here it is only a question of dissolution of the bacilli and the setting free of the contained poison, and yet it will be noticed that an interval of at least two hours and usually longer elapses before there is any noticeable fall in temperature. The maximum effect in this case is reached between four and six hours, and if the dose has been a non-fatal one, recovery begins at the end of from eight to ten hours, as is indicated by the upward trend of the temperature curve at this point. In the case of the soluble poison, the toxic effect begins at once. Within fifteen minutes the temperature has begun to drop and within an hour has reached a minimum. Recovery then begins and within three hours the effect of the poison has worn off, as is best evidenced by the return of the body temperature to normal or above by this time.

## CHAPTER VII

### THE PRODUCTION OF ACTIVE IMMUNITY WITH THE SPLIT PRODUCTS OF THE COLON BACILLUS<sup>1</sup>

It may be stated that the work which we have done with the colon bacillus up to the present time has in every instance upheld the belief that the substances which give rise to the phenomena occurring in animals infected with the living colon germ exist as essential groups within the bacterial cell and can be liberated from the latter only by its disruption. Moreover, until these substances have been separated from the other constituents of the bacterial cell with which they are normally combined they are unable to exert any deleterious action upon the body cells. If the belief that the phenomena which result from infection with the colon bacillus are due to the action of the intracellular constituents of this organism is correct, we would expect that it might be possible by chemical means to split up this bacillus into different groups, the injection of some of which into animals would be followed by some of the results which are seen after inoculation with the living germ. In a previous chapter we have shown that it is possible to split off a toxic group which causes death in animals with symptoms similar to those observed after the injection of a fatal dose of living bacilli. However, death is by no means the sole phenomenon which results from the inoculation of animals with the colon bacillus. The results which follow the injection of non-fatal doses of the living germ are of equal if not of greater importance.

<sup>1</sup> This chapter is taken, with but few changes, from an article by Victor C. Vaughan, Jr., in the *Journal of Medical Research*, 1905, xiv, 67.

It is a well-known fact that animals which have been treated with non-fatal doses of either the living or dead colon germ acquire a certain degree of immunity toward subsequent infection with this bacillus. If, now, our theory as regards the action of this bacillus is correct, one would suppose that among the groups which we have been able to split off there exist certain ones which possess the power of producing immunity when injected into susceptible animals. To ascertain whether such an active immunity can be established in animals through treatment with the split products of the colon bacillus is the aim of this chapter, and we shall find it convenient to take up (1) the active immunity obtained with the toxic portion, and (2) the immunity obtained with the residue which remains after the separation of the poisonous portion from the cellular substance.

**1. Immunization with the Poisonous Portion of the Cellular Substance of the Colon Bacillus.**—From the description of the action of the “crude soluble poison” of the colon bacillus given in the preceding chapter, it can be readily seen that the poison with which we are working is one which exerts its action with great rapidity. The difficulties of immunizing animals with such a poison can be readily appreciated, and it is inevitable that during the course of treatment a large number should be lost. Up to the present time our attempts to produce immunity with the toxic portion have been largely confined to intraperitoneal and subcutaneous injections with what we have termed the free or uncombined poison. However, as has been shown in a previous chapter, it is possible to make use of this poison in a combined state by rendering the solution of the toxic part decidedly alkaline with sodium bicarbonate and allowing it to stand for some time, preferably at incubator temperature. With this combined poison, one is able to give much larger doses without producing a fatal result, and, moreover, the effect of the poison is in this instance manifested over a much longer period of time. These two factors are, of course, of primary importance in the production of immunity and it

is quite possible that with the employment of the combined poison a higher degree of immunization may be obtained.

That it is possible by means of repeated doses to induce a certain amount of tolerance in animals for the poisonous portion is illustrated in the following tables:

TABLE VII

20 MG. OF THIS POISON INVARIABLY CAUSED DEATH IN UNTREATED PIGS WITHIN ONE HOUR

| Guinea-pig No. | Weight in grams. | Dose of poison in mg. and when given. |     |      |      |      |     |      |      |      | Total amount poison in mg. |
|----------------|------------------|---------------------------------------|-----|------|------|------|-----|------|------|------|----------------------------|
|                |                  | 3/2                                   | 3/7 | 3/15 | 3/28 | 3/28 | 4/1 | 4/7  | 4/14 | 4/22 |                            |
| 1              | 815              | 15.0                                  | 20  | 25.0 | 30.0 | 30   | 30  | 35.0 | 40.0 | 45   | 270                        |
| 2              | 705              | 12.5                                  | ..  | 15.0 | 17.5 | 20   | 25  | 30.0 | 35.0 | 40   | 195                        |
| 3              | 575              | 10.0                                  | ..  | 15.0 | 20.0 | 20   | 25  | 30.0 | 35.0 | 40   | 195                        |
| 4              | 700              | 10.0                                  | ..  | 15.0 | 20.0 | 20   | 25  | 30.0 | 35.0 | 40   | 195                        |
| 5              | 580              | 9.0                                   | ..  | 12.5 | 15.0 | 20   | 25  | 27.5 | 30.0 | 35   | 174                        |
| 6              | 790              | 10.0                                  | ..  | 15.0 | 20.0 | 25   | 25  | 30.0 | 35.0 | 35   | 195                        |
| 7              | 545              | 7.5                                   | ..  | 15.0 | 20.0 | 20   | 25  | 30.0 | 32.5 | 35   | 185                        |
| 8              | 655              | 10.0                                  | ..  | 15.0 | 20.0 | 20   | 25  | 30.0 | 35.0 | 40   | 195                        |
| 9              | 640              | 15.0                                  | ..  | 20.0 | 25.0 | 25   | 30  | 35.0 | 40.0 | 45   | 235                        |

The following table furnishes an index to the degree of tolerance established in rabbits through the administration of gradually increasing doses of the poison:

TABLE VIII

350 MG. OF THIS POISON CAUSED DEATH IN UNTREATED ANIMALS WITHIN ONE HOUR

| Rabbit No. | Weight in grams. | Dose of poison in mgs. and when given. |       |       |     |      |      |      |                                    |
|------------|------------------|--|-------|-------|-----|------|------|------|------------------------------------|
|            |                  | 12/12                                  | 12/19 | 12/28 | 1/9 | 1/14 | 1/19 | 1/26 |                                    |
| 1          | 1850             | 200                                    | 200   | 400   | 500 | 700  | 1000 | 1400 | Died 20 min. after last injection. |
| 2          | 2800             | 250                                    | 300   | 400   | 500 | 700  | 900  | 1000 |                                    |
| 3          | 1950             | 300                                    | 250   | 400   | 600 | 700  | .... | .... | Died 30 min. after last injection. |
| 4          | 2100             | 200                                    | 250   | 400   | 500 | 700  | 1000 | 1200 | Died 35 min. after last injection. |

From a study of the above tables it can be seen that in the case of both guinea-pigs and rabbits after the administration of several doses of gradually increasing strength, a point is reached at which the animal is able to withstand

the injection of from two to three times the amount which would surely have proved fatal for an untreated control. This would indicate that during the course of the treatment the animal had developed either a slight degree of immunity, or had established a certain amount of tolerance for the poison. Which of these explanations is the correct one can only be determined after a careful study of the subject of the possible production of passive immunity and the demonstration of a possible antibody in the blood of treated animals. At present, owing to the slight amount of increased resistance which the animals exhibit to the action of the poison, we are inclined to believe that the question is one of tolerance. Although the degree or tolerance thus far secured has been limited, we do not feel justified in concluding that greater resistance to the poison may not be obtained. There are many factors of primary importance in this work, all of which must be carefully studied before definite conclusions can be drawn. For example, the interval of time which is allowed to elapse between the injections is a matter of first importance. Since the length of time over which the poison acts is apparently so short, it seemed quite probable that any reaction which might occur on the part of the body would develop in a comparatively short time after the injection. With the object of ascertaining whether this was true or not, animals were treated daily with gradually increasing doses with the following results:

TABLE IX

60 MG. OF THIS POISON INVARIABLY CAUSED DEATH IN UNTREATED ANIMALS WITHIN ONE HOUR

| Day. | Pig No. 1.             |              | Pig No. 2.             |              | Pig No. 3.  |              | Pig No. 4.             |              | Pig No. 5.  |              |
|------|------------------------|--------------|------------------------|--------------|-------------|--------------|------------------------|--------------|-------------|--------------|
|      | Wt.,<br>gm.            | Dose,<br>mg. | Wt.,<br>gm.            | Dose,<br>mg. | Wt.,<br>gm. | Dose,<br>mg. | Wt.,<br>gm.            | Dose,<br>mg. | Wt.,<br>gm. | Dose,<br>mg. |
| 1    | 425                    | 45           | 385                    | 45           | 460         | 45           | 390                    | 45           | 405         | 45           |
| 2    | 380                    | 50           | 360                    | 50           | 415         | 50           | 355                    | 50           | 385         | 50           |
| 3    | 385                    | 60           | 375                    | 60           | 420         | 60           | 365                    | 60           | 385         | 60           |
| 4    | 385                    | 80           | 375                    | 80           | 450         | 80           | 370                    | 80           | 380         | 80           |
| 5    | 405                    | 100          | 370                    | 100          | 450         | 100          | 375                    | 100          | 390         | 100          |
| 6    | 405                    | 112          | 385                    | 112          | 460         | 112          | ...                    | ...          | 385         | 112          |
| 7    | 420                    | 125          | 400                    | 125          | 470         | 100          | ...                    | ...          | 410         | 112          |
|      | Died in 30<br>minutes. |              | Died in 30<br>minutes. |              |             |              | Died in 30<br>minutes. |              |             |              |

From this we see that it is possible to establish a certain amount of tolerance by means of daily injections of the poisonous portion. Here again we find that it is comparatively easy to reach a dose which corresponds to about twice the fatal amount, but above this the animal cannot be carried. When death does result from a dose of the poison which is too large to be borne by the treated pig, the symptoms are identical in all respects with those which occur in the case of an untreated animal, and a fatal result follows in the same length of time.

The question now arose as to whether these animals which had acquired a tolerance for the poisonous portion of the colon bacillus were more resistant to inoculation with the living germ than were untreated animals. In order to ascertain this point, guinea-pigs which had received from 174 to 235 mg. of the toxic portion were inoculated intraperitoneally with doses of the living germ with the following results:

TABLE X

1 C.C. OF A 16-HOUR CULTURE OF THE COLON BACILLUS USED IN THESE EXPERIMENTS INVARIABLY KILLED A CONTROL WITHIN TWENTY-FOUR HOURS.

| Guinea-pig No. | No. of injections of poison. | Total amount of poison received. | Interval between last injection and inoculation with germ. | Amount and age of culture. | Result.         |
|----------------|------------------------------|----------------------------------|--|----------------------------|-----------------|
| 1              | 9                            | 270 mg.                          | 11 days  | 1 c.c. 24-hour culture     | Recovery.       |
| 2              | 8                            | 195 mg.                          | 11 days  | 1 c.c. 24-hour culture     | Recovery.       |
| 3              | 8                            | 195 mg.                          | 11 days  | 1 c.c. 4-day culture       | Died in 22 hrs. |
| 4              | 8                            | 195 mg.                          | 11 days  | 2 c.c. 4-day culture       | Dead in 24 hrs. |
| 5              | 8                            | 174 mg.                          | 15 days  | 2 c.c. 24-hour culture     | Recovery        |
| 6              | 8                            | 195 mg.                          | 25 days  | 2 c.c. 24-hour culture     | Recovery        |
| 7              | 8                            | 185 mg.                          | 33 days  | 2 c.c. 24-hour culture     | Recovery        |
| 8              | 8                            | 195 mg.                          | 25 days  | 2 c.c. 24-hour culture     | Recovery        |
| 9              | 8                            | 235 mg.                          | 8 days   | 2 c.c. 24-hour culture     | Recovery        |

That an active immunity to the living colon bacillus is also developed in rabbits which have been treated with repeated injections of the toxic portion is illustrated by the following experiments:

Rabbit No. 1 received between May 25 and July 5 eight injections of the toxic part, the total amount of poison injected being 855 mg. On July 8 this animal received 5 c.c. of a twenty-four-hour culture of the living germ without apparent effect. The control inoculated at the same time was found dead in eight hours.

Rabbit No. 2 received between May 28 and July 18 eleven injections of the toxic portion, the total amount of poison given being 2475 mg. On July 27 this animal was inoculated with 5 c.c. of a sixteen-hour culture of the colon bacillus without effect. The control was found dead in ten hours.

Rabbit No. 3 received between June 27 and July 18 seven injections of the poison, the total amount given being 2100 mg. On July 28 this animal was inoculated with 6 c.c. of a twenty-four-hour colon culture. Recovered.

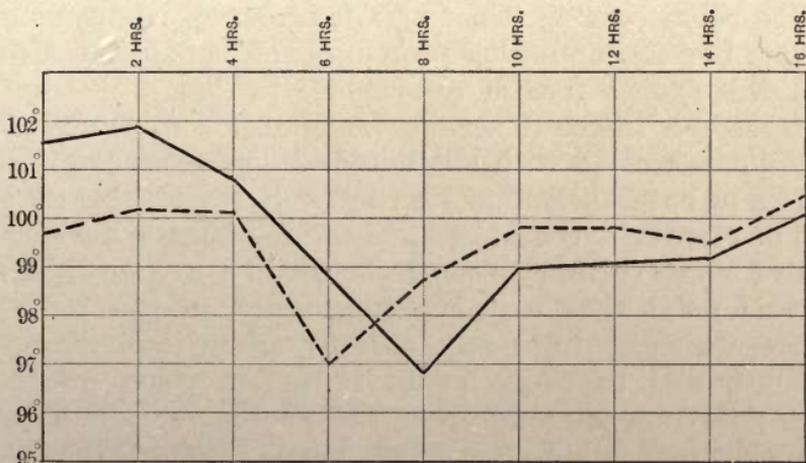
Rabbit No. 4 received the same amount of poison as the preceding one. Twelve days after his last treatment this animal was given 6 c.c. of a forty-hour colon culture and recovered. The control was found dead in eight hours.

From the foregoing experiments it becomes evident that animals which have been treated with the toxic portion of the colon bacillus acquire a certain degree of immunity to the living germ. We are as yet unable to state whether it is possible to obtain a high degree of immunity with the poisonous portion or not. Thus far we have had animals which have withstood inoculation with from two to four times the fatal dose of the living germ. It is worthy of note in this connection that animals which have received one injection of a non-fatal dose of the poison are able to withstand inoculation with twice the lethal dose of the living germ on the following day. The immunity which follows a single injection is, however, exceedingly transitory, and has usually disappeared on the second day following the treatment. This would seem to be the most marked difference between the immunity which results from a single injection of the toxic part and that which follows a series of injections extending over a considerable interval

of time. In the first instance the protection afforded is very temporary, while in the second it is still present even after the lapse of from twenty-five to thirty days. It may be possible that in the case of the injections extending over a long period of time the immunity obtained is of a higher degree. This is a point which will require further study.

In the case of an animal which has been treated with the poisonous portion and has subsequently received a dose of the living germ which would surely have proved fatal for a normal animal, the symptoms noticed are identical

FIG. 8



— Curve of normal animal inoculated with non-fatal dose of living bacillus.  
 - - - - - Curve of immune animal inoculated with living bacillus.

in every respect with those which follow the injection of a non-lethal dose in an untreated animal. This is a very important fact and one on which we have laid much stress. Moreover, as can be seen from Fig. 8, the temperature curve corresponds very closely with that obtained in the case of a normal animal inoculated with a non-fatal dose of the living colon bacillus.

We see that in both instances there is no appreciable fall in body temperature until from six to eight hours after inoculation. At this time the minimum temperature has

been reached in each case, and within from ten to twelve hours it has again returned to normal. The similarity of the symptoms in the two instances leads us to believe that in all probability we are here dealing with an immunity which is identical in character with that which is usually spoken of as natural immunity. This idea has been further upheld by the fact that we have been able to obtain from egg albumen and peptone poisonous substances which resemble the toxic portion of the colon bacillus in their action, and by the injection of single non-fatal doses of which it is possible to obtain the same transitory immunity to the living colon germ as is observed after the injection of the colon poison. That this toxic group is common to certain bacteria and other protein bodies is not improbable, and this would furnish an explanation not only of the increased resistance to certain bacterial infections occurring in animals treated with albumin and peptone, but of some phases of natural immunity as well. However, this subject will be more fully considered in a future paper on a comparison of these various poisons. It may be well to reiterate at this point that we have conclusively shown that the poison which we have been able to obtain from the colon bacillus, and to which death is due in colon infection, does not come directly from the peptone or albumen in the culture medium, since we have obtained the same poison from the bacillus when grown upon a protein-free medium.

2. **Immunization with the Residue Remaining after the Extraction of the Poison from the Colon Bacillus.**—The residue remaining after the extraction of the poison from the colon bacillus, which is insoluble in alkaline absolute alcohol, is soluble in water. The resulting solution is, however, quite decidedly alkaline in reaction, owing to the presence of free alkali. Since it is essential that we should avoid the irritative effects which would follow the injection of this free alkali into the peritoneal cavity, the solution is first rendered slightly acid with hydrochloric acid, and then neutralized with sodium bicarbonate before injection.

The solution of the residue thus obtained after sufficient extraction with alkaline alcohol is non-toxic in the ordinary sense of the term. However, the toxicity of a substance for the body as a whole depends largely upon whether the cells which it attacks are of fundamental importance in maintaining the life of the animal or not. Thus a poison which possesses a special affinity for the cells of the respiratory centre will inevitably lead to the production of marked symptoms of poisoning on the part of the animal, while one which exerts its effect upon the blood or connective-tissue cells would not necessarily do so. Of course, in the latter case, treatment over a prolonged period of time would undoubtedly result in symptoms of chronic poisoning. The residue is as potent a cell poison as is the toxic portion, but the cells which it poisons are not directly concerned in the carrying on of a function, the cessation of which would prove immediately fatal to the organism as a whole. That the residue is possessed of but slight toxicity is seen from the fact that the injection of from 300 to 400 mg. into the peritoneal cavity of guinea-pigs at a single dose has apparently no effect upon the animal. There is no fall of temperature such as is observed after the injection of the poisonous portion, nor, on the other hand, is there any appreciable rise. It may be well to emphasize at this point that in order to study this portion of the colon bacillus and its action it is absolutely essential that the toxic portion should have been completely removed. In order to accomplish this it is necessary to extract the bacterial cell substance at least three times with the alkaline alcohol, and frequently a fourth extraction is required. If all of the poisonous portion has not been removed, the treated animal begins to show evidences of poisoning, as lowering of temperature, stupor, and, provided the extraction has been very imperfect, death. These symptoms do not, however, become manifest to a marked degree until from two to four hours after the injection. This is in marked contrast to the rapidity with which the free poison acts, and would indicate that the poison in the imperfectly extracted residue

still exists in combination with other constituents of the bacterial cell.

The question now arose as to whether the animals treated with increasing doses of the residue had acquired any immunity to infection with the living colon bacillus. To ascertain this point, guinea-pigs were treated with this portion and subsequently inoculated with the living germ with the following results:

TABLE XI

NOS. 1, 2, 3, AND 4 RECEIVED A CULTURE, 1 C.C. OF A 12-HOUR CULTURE OF WHICH CAUSED DEATH IN UNTREATED PIGS WITHIN TWENTY-FOUR HOURS. THE REST RECEIVED A CULTURE,  $\frac{1}{2}$  C.C. OF WHICH INVARIABLY PROVED FATAL TO UNTREATED ANIMALS.

| Guinea-pig No. | No. of injections of residue. | Total amount received in gm. | Time between last injection and inoculation. | Amount of culture injection. | Result.  |
|----------------|-------------------------------|------------------------------|--|------------------------------|----------|
| 1              | . . 9                         | 0.29                         | 16 days                                      | 1 c.c. 16-hour culture       | Recovery |
| 2              | . . 9                         | 0.26                         | 15 days                                      | 1 c.c. 24-hour culture       | Recovery |
| 3              | . . 9                         | 0.3                          | 16 days                                      | 2 c.c. 16-hour culture       | Recovery |
| 4              | . . 2                         | 0.3                          | 4 days                                       | 2 c.c. 16-hour culture       | Death    |
| 5              | . . 4                         | 0.9                          | 3 days                                       | 2 c.c. 18-hour culture       | Recovery |
| 6              | . . 3                         | 1.0                          | 5 days                                       | 2 c.c. 24-hour culture       | Recovery |
| 7              | . . 3                         | 1.0                          | 5 days                                       | 3 c.c. 24-hour culture       | Recovery |
| 8              | . . 4                         | 0.8                          | 7 days                                       | 3 c.c. 20-hour culture       | Recovery |
| 9              | . . 4                         | 0.9                          | 14 days                                      | 3 c.c. 20-hour culture       | Recovery |
| 10             | . . 5                         | 1.1                          | 17 days                                      | 3 c.c. 16-hour culture       | Death    |
| 11             | . . 4                         | 0.8                          | 30 days                                      | 3 c.c. 16-hour culture       | Death    |
| 12             | . . 4                         | 0.9                          | 4 days                                       | 4 c.c. 16-hour culture       | Recovery |
| 13             | . . 4                         | 0.8                          | 7 days                                       | 4 c.c. 16-hour culture       | Recovery |
| 14             | . . 4                         | 0.9                          | 14 days                                      | 4 c.c. 18-hour culture       | Death    |
| 15             | . . 4                         | 0.9                          | 5 days                                       | 5 c.c. 16-hour culture       | Death    |
| 16             | . . 4                         | 0.8                          | 7 days                                       | 6 c.c. 16-hour culture       | Death    |

From the above table it will be seen that guinea-pigs which have been treated with that portion of the colon bacillus which is represented by the residue have acquired an active immunity to at least eight times the fatal dose of the living germ. The degree of immunity produced does not depend so much upon the amount of residue which has been injected as upon the number of treatments and the interval of time over which they have been continued.

For example, No. 3, which received a total amount of 0.3 gram in nine doses, was able to withstand 2 c.c. of a sixteen-hour culture after an interval of sixteen days, while No. 4, which received the same total amount in two doses, succumbed to the injection of 2 c.c. of a sixteen-hour culture given at an interval of four days after the last dose of residue. Again we notice that the length of time over which the immunity lasts is rather short in the case of animals which have received a large amount of the substance in a few doses continued over a short period. Thus the immunity to 3 c.c. of a culture, 0.5 c.c. of which proved fatal for untreated pigs, was lost between the fourteenth and the seventeenth day following the injection of the last dose of residue, and that to 4 c.c. of the same culture disappeared between the seventh and the fourteenth day.

That it is possible to secure active immunity to the living colon bacillus in rabbits by the injection of the colon residue is shown in the following experiments:

Rabbit No. 1 received on August 13 a solution which contained 1 gram of the residue. On August 20 a second injection of 2 grams was given. Eighteen days later this animal was inoculated with 5 c.c. of an eighteen-hour culture of the colon bacillus. From two to four hours after injection this rabbit was apparently very sick, the symptoms resembling those which are seen following the injection of the toxic portion. However, it then began to improve, and eventually completely recovered. A control inoculated at the same time with an equal amount of the same culture died in five hours.

Rabbit No. 2 received doses of 1 gram of the residue on October 5, 10, and 20. Four days after the last injection this animal was inoculated with 5 c.c. of an eighteen-hour culture from which he recovered. A control given the same dose at the same time died within five hours.

Rabbit No. 3 had the same treatment as did No. 2, and six days after the last dose of residue withstood 5 c.c. of an eighteen-hour culture. Eight days later this animal received 10 c.c. of a sixteen-hour culture and did not die.

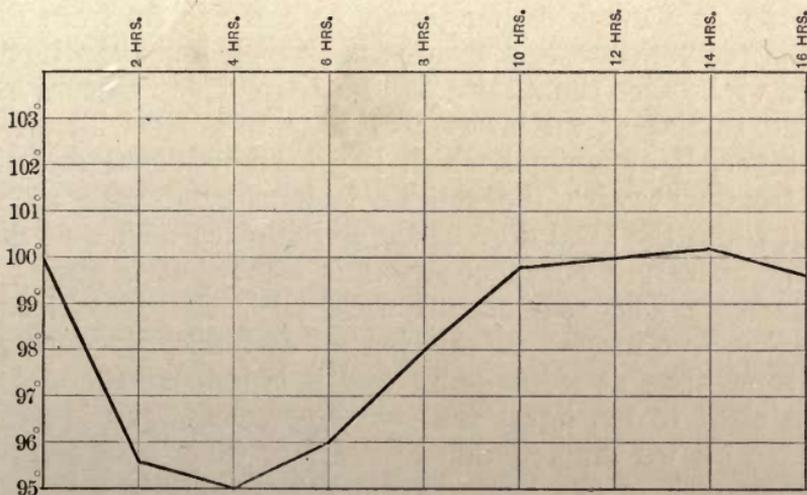
Rabbits No. 4 and 5 had the same treatment as did Nos. 2 and 3, and seven days after the last dose each received 5 c.c. of a twenty-four-hour culture of the living colon bacillus without a fatal result. A control which received the same amount of the same culture died within five hours.

When we turn our attention to the symptoms which follow the injections of living cultures of the colon bacillus into animals which have been actively immunized with the split products, we find that the clinical picture differs materially according as to whether they have been treated with the toxic portion or with the residue. As has been previously mentioned, the symptoms which are observed in a pig immunized with the toxic part are apparently identical with those which one sees in a normal animal after inoculation with a non-lethal dose of the living bacillus. The picture which is obtained on inoculation of animals rendered immune by previous treatment with the residue is, however, quite different. In this case the animals become apparently very ill within an hour after inoculation with the living germ. Indeed, so noticeable was this fact and the treated pigs appeared so much sicker than did the controls that our first thought was that we had in some manner increased their susceptibility to subsequent infection by treatment with the residue. However, after from six to eight hours the treated animals appeared in much better condition and eventually recovered, whereas the controls invariably died. The temperature of the treated animals runs a course entirely in accord with the symptoms. Thus in a pig which had received 290 mg. of the residue, and subsequently was inoculated with 1 c.c. of a twenty-four-hour culture, the temperature fell from 100° to 95° F. within four hours, and by six hours had once more begun to rise, as is illustrated in Fig. 9.

The difference between the behavior of animals treated with the toxic part and those which have been treated with the residue toward cultures of the living germ is easily explained if we consider the fact that in the first case we are dealing with an animal which has acquired a certain

amount of tolerance for the intracellular poison of the colon bacillus as represented by the toxic part. In the case of animals treated with the residue, however, no tolerance for the poison contained within the colon bacillus has been developed. If now the process which takes place in both instances is a bacteriolytic one, it results that in the case of the animal immunized with the toxic group the effects of the poison contained within the bacterial cell and liberated upon its disintegration will not become manifest

FIG. 9



The temperature curve of an animal immunized with the colon residue and afterward inoculated with twice the lethal dose of the living culture.

until a sufficient amount of poison has been set free to overcome the tolerance which the animal has attained during the process of immunization. In the case of the animal immunized with the residue there is no tolerance to be overcome other than that which is present in all animals, and the effects of the poison liberated through bacteriolysis become apparent sooner and to a more marked extent. Again, the fact that bacteriolysis may occur more rapidly in the case of residue pigs than in those immunized with the toxic group might explain in part the difference

in behavior in the two cases. This is a point on which we are as yet unable to give any definite results.

In order to study the differences in reaction to the living germ in animals treated with the toxic part and those immunized with the residue it is not only essential that they should receive the same amount of the same culture, but the dose given should not exceed twice that which would prove fatal for a control. When a larger amount of the living culture is given the differences are by no means so clearly defined, although even in this case the animal which has been treated with the residue shows symptoms of severity at a much earlier time. As can be seen from Fig. 9, the temperature of a residue pig which had been inoculated with twice the fatal dose of a living colon culture had begun to rise at an interval of six hours after injection. However, if an animal which has been rendered immune by treatment with the residue is inoculated with six to eight times the fatal dose of the living culture we find that the temperature curve obtained is somewhat different in character. The temperature falls with the same initial rapidity, but instead of showing an early rise it continues for some time at a low point and it is only at the end of from eight to ten hours that any appreciable rise is manifest. This we think is due to the fact that there has not been enough of the bacteriolytic substance directly available to destroy all the bacilli contained in the large amount of culture injected. The remainder of the germs are destroyed by the same factors which are operative in normal animals after the injection of a non-fatal dose of the living bacillus. As can be seen from Fig. 8, it is only after an interval of six to eight hours that there is any appreciable fall in temperature in the case of a normal animal inoculated with a non-fatal dose of the living culture. This, we think, indicates that it is not until this time that any appreciable amount of poison is liberated by bacteriolysis since, as we have seen in a previous chapter, one of the first signs of the action of the intracellular poison is a fall in body temperature. In a pig which has been

immunized with the residue and subsequently inoculated with a large amount of the living germ, we obtain evidence of hypothermia at a much earlier period, owing to the fact that bacteriolysis takes place very rapidly since the bacteriolytic substance is present in a form available for immediate use. If, however, the amount of this substance directly available is not sufficient to cause death and bacteriolysis of all germs present, those bacilli which remain are still capable of further reproduction. The same mechanism which causes destruction of the bacteria in normal animals, and which is probably connected with the phenomenon of phagocytosis is, however, still operative in the immune animal. Thus we shall have two influences at work in the immune animal to cause bacteriolysis, one acting rapidly, and the other manifesting its action only after a considerable interval of time. We should therefore expect theoretically that we would find in the immunized animal a marked fall in temperature at an early time, due to the setting free of the poison from the bodies of the bacteria disintegrated by the directly available bacteriolytic substance followed by a secondary rise, and a succeeding fall due to the liberation of the poison by means of the factors present in the normal animal. However, this is not actually the case, since the effect of the poison liberated at first has not worn off before the second period of bacteriolysis becomes well established. Consequently, the intermediate rise of temperature is absent.

The results which follow the injection of the dead bacterial substance into animals immunized with the residue are very interesting. As has been previously mentioned, whereas animals treated with the residue develop an active immunity to colon infection, they do not possess any greater degree of tolerance for the colon poison than do untreated animals. This is shown by the fact that the fatal dose of the soluble poison is the same for the treated pig as for the untreated control. This would lead to the belief that the immunity obtained to the living colon bacillus is, in the case of the residue animals, purely a bacteriolytic one. If

this is true, one would suppose that on the injection of a fatal amount of the dead bacterial substance death would occur more rapidly in the immunized than in a normal pig, provided the immune animal possesses a sufficient amount of bacteriolytic substance directly available to cause disintegration of all bacteria present. If, now, a pig which has been immunized with the residue receives 5 c.c. of a twenty-four-hour culture of the colon bacillus which has been deprived of life by means of heat, the animal is very sick within from fifteen to twenty minutes. The symptoms noted are similar in all respects to those which are observed after the injection of the soluble poison. The pig runs about the cage, scratches itself, and shows the same evidence of lack of coördination and partial paralysis of the hind extremities. This behavior is in marked contrast to that seen in the case of a normal animal which has received an injection of 5 c.c. of a twenty-four-hour culture which has been rendered sterile by means of heat. In this instance the animal appears perfectly well until after the lapse of about an hour, when it begins to show signs of illness such as roughening of the coat, stupor, and indications of a beginning peritonitis. The latter symptoms are those which we have described as being due to the slow liberation of the poison from a combined state. The same symptoms are observed in the case of the immune pig, and are noticed at the same length of time after the injection. The difference in the behavior of the immune and the normal pig is seen to consist in the fact that in the first instance we have symptoms of the action of the free poison shortly after the injection of the dead culture, which are entirely lacking in the second case. This shows beyond doubt that in the immune pig there is marked bacteriolysis of the dead bacilli and a consequent liberation of the contained poison shortly after the injection of the dead bacterial cell into the peritoneal cavity. Although we have as yet been unable to actually cause death in an immune pig at an early period, the animals are in every instance very ill within thirty minutes after

the injection of the dead culture. In fact, several of them have shown signs of the commencement of the convulsive stage as evidenced by slight convulsive movements of the head separated by considerable intervals of time. We have been unable to secure a fatal result in these animals up to the present time simply because we have worked with pigs which did not possess a sufficient amount of bacteriolytic substance directly available to cause disintegration of enough bacilli to liberate a fatal amount of poison at one time. It is worthy of note that this behavior of animals immunized with the residue toward the dead bacterial substance furnishes additional proof of the fact that the poison of the colon bacillus is an intracellular one. If the poison existed free in the culture medium we should expect that the control would show evidences of its action at as early a period as does the treated animal. However, as has been stated above, this is not the case. The fact that the treated animal shows symptoms of poisoning to a much greater degree and at an earlier time than does the control can be explained only on the ground that the poison with which we are dealing is an intracellular one and is set free only after the disintegration of the bacillus by bacteriolysis.

The question now arose as to whether the immunity induced through the residue is specific for the colon bacillus or not. In order to test this point, animals which had been treated with this portion were inoculated with living cultures of the typhoid bacillus with the following results:

TABLE XII

1 C.C. OF A 16-HOUR CULTURE OF THIS TYPHOID BACILLUS KILLED CONTROLS WITHIN TWENTY-FOUR HOURS.  $\frac{1}{2}$  C.C. DID NOT CAUSE DEATH

| Guinea-pig No. | No. of injections of residue. | Total amount received in gm. | Time between last injection and inoculation. | Amount of typhoid culture injection. | Result. |
|----------------|-------------------------------|------------------------------|--|--------------------------------------|---------|
| 1              | 4                             | 0.8                          | 6 days                                       | 1 c.c. 16-hour culture               | Death   |
| 2              | 4                             | 0.8                          | 6 days                                       | 2 c.c. 16-hour culture               | Death   |
| 3              | 4                             | 0.8                          | 2 days                                       | 2 c.c. 16-hour culture               | Death   |
| 4              | 4                             | 0.8                          | 6 days                                       | 3 c.c. 16-hour culture               | Death   |
| 5              | 4                             | 0.8                          | 5 days                                       | 4 c.c. 16-hour culture               | Death   |

Although these experiments are by no means sufficient in extent to warrant the conclusion that the injection of the residue obtained from the colon bacillus furnishes no increased resistance to typhoid infection, it can be seen that the degree of immunity established must be very slight. As far as the typhoid bacillus is concerned, the immunity produced by the colon residue would appear to be specific. If the immunity induced by the colon bacillus is indeed specific, one would suppose that the immunizing group is one which is found only in the residue obtained from the colon germ.

As has been previously mentioned, we have found it possible by treatment similar to that which we have used in splitting up the colon bacillus to secure toxic substances from egg albumen and peptone, which possess a similar action when injected into the animal body, to that observed after the injection of the colon poison. We have also stated that the same transitory immunity to colon infection followed the injection of the albumin and peptone poison as was obtained with the colon poison itself. The albumen and peptone bear a further resemblance to the bacterial cell substance in that the residue which remains after alcoholic extraction is non-toxic. The question now arose as to whether the injection of the albumen and peptone residue afford any immunity to the living colon germ or not. In order to ascertain this point animals were treated with gradually increasing doses of these residues, and subsequently inoculated with the colon bacillus with the following results:

TABLE XIII

1 C.C. OF A 16-HOUR CULTURE OF COLON BACILLUS KILLED CONTROLS IN TWENTY-FOUR HOURS.  $\frac{1}{2}$  C.C. DID NOT KILL

| Guinea-pig No. | No. injections of residue. | Total amount received in gm. | Time between last injection and inoculation. | Peptone Residue                    |  | Result.  |
|----------------|----------------------------|------------------------------|--|------------------------------------|--|----------|
|                |                            |                              |  | Amount of colon culture injection. |  |          |
| 1              | 4                          | 0.9                          | 3 days                                       | 2 c.c. 16-hour culture             |  | Death    |
| 2              | 4                          | 0.9                          | 4 days                                       | 2 c.c. 16-hour culture             |  | Death    |
| 3              | 4                          | 0.9                          | 6 days                                       | 2 c.c. 16-hour culture             |  | Death    |
| 4              | 4                          | 0.9                          | 3 days                                       | 2 c.c. 18-hour culture             |  | Recovery |
| 5              | 4                          | 0.9                          | 4 days                                       | 2 c.c. 18-hour culture             |  | Recovery |
| 6              | 4                          | 0.9                          | 5 days                                       | 2 c.c. 18-hour culture             |  | Death    |
| 7              | 4                          | 0.9                          | 4 days                                       | 2 c.c. 16-hour culture             |  | Death    |
| 8              | 4                          | 0.9                          | 6 days                                       | 3 c.c. 16-hour culture             |  | Death    |

TABLE XIV

| Guinea-pig No. | No. injections of residue. | Total amount received in gm. | Time between last injection and inoculation. | Albumin Residue                    |  | Result. |
|----------------|----------------------------|------------------------------|--|------------------------------------|--|---------|
|                |                            |                              |  | Amount of colon culture injection. |  |         |
| 1              | 4                          | 0.9                          | 3 days                                       | 1 c.c. 16-hour culture             |  | Death   |
| 2              | 4                          | 0.9                          | 5 days                                       | 1 c.c. 16-hour culture             |  | Death   |
| 3              | 4                          | 0.9                          | 3 days                                       | 2 c.c. 16-hour culture             |  | Death   |
| 4              | 4                          | 0.9                          | 4 days                                       | 2 c.c. 18-hour culture             |  | Death   |
| 5              | 4                          | 0.9                          | 5 days                                       | 2 c.c. 16-hour culture             |  | Death   |
| 6              | 4                          | 0.9                          | 5 days                                       | 3 c.c. 16-hour culture             |  | Death   |
| 7              | 4                          | 0.9                          | 4 days                                       | 4 c.c. 16-hour culture             |  | Death   |

From the above tables it can be readily seen that the residues obtained from the peptone and albumin possess little if any immunizing properties against infection with the colon bacillus. In the case of the animals treated with the peptone residue, the first three pigs received a different residue from that given to the remainder. This residue had been thoroughly extracted with alkaline alcohol, and was evidently possessed of no immunizing properties whatever. On the other hand, the residue which was received by pigs No. 4 to No. 8 inclusive had not been subjected to so thorough an extraction. It is therefore highly probable that the slight degree of immunity apparently obtained in some of the latter animals was due to the presence of

some of the toxic portion which had been left in the residue as the result of incomplete extraction. In the animals treated with the albumen residue we were unable to obtain any evidence whatever of increased resistance to colon infection.

It has now been shown that active immunity to the colon germ can be produced in animals by treatment with the split products obtained from this bacillus. This would seem to furnish conclusive evidence that there exist within the colon bacillus certain immunizing groups which are capable of being separated more or less completely from the other constituents of the bacterial cell by means which bring about a chemical cleavage of the latter. Furthermore, it has been seen that the colon bacillus contains at least two different groups, each of which when injected into the animal body is capable of establishing a certain degree of immunity toward subsequent infection with the living germ. One of these groups is contained within the toxic portion, and probably represents a group which is common to many protein bodies, since, as has been shown, it is contained in the poisons secured through the chemical cleavage of egg albumen and peptone, as well as from the colon bacillus. The degree of immunity thus far obtained through the agency of this group is not great. The fact that this group is apparently not specific to the colon bacillus, but can also be obtained from other protein bodies, furnishes an explanation of the increased resistance to infection observed in animals previously treated with solutions of egg albumen and peptone. Again it has been shown that the residue, which, as has been previously stated, is for all practical purposes non-toxic, also contains an immunizing group. The immunizing group contained in the residue differs from that found in the toxic portion in one very important respect. It represents a group which up to the present time we have been able to find only in the colon bacillus, and which when injected into animals affords protection against this bacillus alone. In other words, the immunity produced with the residue is strictly specific in character. Moreover, the degree of

immunity to the living germ obtained through the employment of the residue is apparently much higher than that which follows treatment with the toxic portion. It does not seem improbable that the specific immunizing group which is contained in the residue represents that group of the colon bacillus which is of primary importance in the development of specific acquired immunity to this germ.

The work just detailed may be summed up as follows:

1. Guinea-pigs treated at intervals of from three to four days with intra-abdominal injections of the colon residue acquire an active immunity to at least eight times the ordinary fatal dose of the living bacterium.

2. The degree of immunity secured does not depend so much upon the amount of the residue or non-poisonous portion that has been injected as upon the number of treatments and the interval of time over which they have been continued.

3. The length of time over which the immunity continues is rather short in the case of animals that have received a large amount of the residue in a few doses continued over a short period.

4. Rabbits that received from two to three injections of 0.5 gram each of the residue acquire an immunity to quantities of the living bacillus that kill the controls within five hours.

5. The immunity induced by the colon residue or non-poisonous part is specific, and previous treatments of animals with the residues of egg-white, peptone, and the typhoid bacillus give no immunity to the colon bacillus.

In continuing this work Vaughan and Wheeler<sup>1</sup> decided in the first place to ascertain whether or not a single dose of the residue gives any immunity; if so, what degree of immunity does it afford and how long does it continue?

The number of immunizing doses given in the work already reported ran from three to nine, and the special object of the work here reported is to ascertain the effects of a smaller number of immunizing doses.

<sup>1</sup> New York Med. Jour. June, 29, 1907.

TABLE XV

These animals had one dose of 50 mg. of the residue, as the non-poisonous portion is designated. The protocol number, the weight of the animal, the interval in days between the administration of the residue and the inoculation with the twenty-four hours beef-tea culture of the bacillus, the amount of the culture and the result are shown:

| Protocol,<br>No. | Weight,<br>gm. | Interval,<br>days. | Amount given,<br>c.c. | Result.  |
|------------------|----------------|--------------------|-----------------------|----------|
| 324              | 400            | 1                  | 5                     | Recovery |
| 325              | 420            | 3                  | 5                     | Recovery |
| 269              | 505            | 4                  | 3                     | Recovery |
| 275              | 300            | 4                  | 5                     | Recovery |
| 326              | 315            | 7                  | 5                     | Death    |
| 270              | 600            | 9                  | 3                     | Recovery |
| 328              | 345            | 11                 | 2                     | Recovery |
| 207              | 455            | 21                 | 2                     | Recovery |
| 209              | 495            | 27                 | 4                     | Death    |
| 210              | 625            | 27                 | 4                     | Death    |
| 211              | 485            | 28                 | 3                     | Death    |
| 139              | 240            | 36                 | 2                     | Recovery |
| 140              | 250            | 40                 | 3                     | Death    |
| 141              | 250            | 42                 | 2                     | Death    |
| 142              | 255            | 42                 | 2                     | Death    |

In studying these results it will be well to consider the minimum fatal dose as the unit, which in case of the cultures used in these experiments is 1 c.c. of the twenty-four hour beef-tea growth, and we will regard the animal that succumbs to 2 c.c. as having practically lost its immunity. With this measure it will be seen that a single dose of 50 mg. of the colon residue gives to the animal a temporary immunity of at least 5 units, which is in force twenty-four hours after the treatment, and continues for at least four days, but has begun to disappear by the seventh day. However, some slight degree of immunity continues up to the thirty-sixth day, but practically all is lost by the fortieth day.

TABLE XVI

These animals had a single dose of 25 mg. of the residue. The data are the same as given in the preceding table.

| Protocol,<br>No. | Weight,<br>gm. | Interval,<br>days. | Amount given,<br>c.c. | Result.  |
|------------------|----------------|--------------------|-----------------------|----------|
| 330              | 460            | 1                  | 5.0                   | Recovery |
| 331              | 300            | 3                  | 5.0                   | Death    |
| 332              | 225            | 7                  | 5.0                   | Death    |
| 333              | 290            | 9                  | 2.5                   | Recovery |
| 334              | 235            | 11                 | 2.0                   | Death    |
| 335              | 360            | 14                 | 1.0                   | Recovery |

Comparing Tables XV and XVI, it will be seen that the immunity given by 25 mg. of the residue, although it may be as great as that given by 50 mg. at the end of the first twenty-four hours, declines more rapidly and is less at the end of three days, and continues to be less at eleven days. The only element of doubt that we can see in these conclusions lies in the small size of all the animals, save No. 1 used in Table XVI. However, we have not found that size or weight of guinea-pigs are important factors, provided, of course, that the animals are in good condition, in influencing the result after inoculation with the colon or the typhoid bacillus.

TABLE XVII

These animals had from two to three treatments, receiving each time 50 mg. of the residue. These treatments were at intervals of three days. The protocol number, the weight of the animals, the number of treatments, the total amount of residue given, the interval in days between the last treatment and the inoculation, the amount of the culture twenty-four hours old, and the result are given:

| Protocol,<br>No. | Weight,<br>gm. | No. of<br>treatments. | Amount of<br>residue,<br>mg. | Interval,<br>days. | Amount of<br>culture,<br>c.c. | Result.  |
|------------------|----------------|-----------------------|------------------------------|--------------------|-------------------------------|----------|
| 270              | 600            | 2                     | 100                          | 3                  | 3                             | Recovery |
| 271              | 600            | 2                     | 100                          | 3                  | 4                             | Recovery |
| 212              | 520            | 2                     | 100                          | 3                  | 3                             | Recovery |
| 213              | 530            | 2                     | 100                          | 3                  | 4                             | Recovery |
| 272              | 530            | 3                     | 150                          | 5                  | 4                             | Recovery |
| 273              | 540            | 3                     | 150                          | 5                  | 5                             | Recovery |
| 274              | 475            | 3                     | 150                          | 7                  | 6                             | Recovery |
| 278              | 580            | 3                     | 150                          | 7                  | 6                             | Recovery |
| 280              | 615            | 3                     | 150                          | 7                  | 6                             | Recovery |
| 214              | 585            | 3                     | 150                          | 12                 | 5                             | Recovery |
| 215              | 600            | 3                     | 150                          | 12                 | 6                             | Recovery |
| 216              | 485            | 3                     | 150                          | 12                 | 6                             | Recovery |
| 217              | 530            | 3                     | 150                          | 12                 | 6                             | Recovery |
| 218              | 475            | 3                     | 150                          | 12                 | 7                             | Recovery |

Comparing Table XVII with Tables XV and XVI, it is plainly evident that this immunity induced by two and three doses at intervals of three to four days is greater in degree and more lasting in its effects than that produced by a single injection. This confirms the conclusion already stated, but at the same time this additional work shows that a single dose may serve to furnish protection against at least five times the ordinary fatal dose for a few days and against twice the fatal dose for one month.

TABLE XVIII

These animals received a single dose of 100 mg. of the typhoid residue. The protocol number, weight of animal, interval between treatment and inoculation, amount of twenty-four-hour culture given, and the result are shown:

| Protocol,<br>No. | Weight,<br>gm. | Interval,<br>days. | Amount of<br>culture,<br>c. c. | Result.             |
|------------------|----------------|--------------------|--------------------------------|---------------------|
| 354              | 250            | 1                  | 3                              | Recovery            |
| 355              | 320            | 1                  | 4                              | Death on second day |
| 356              | 280            | 3                  | 3                              | Death               |
| 357              | 265            | 3                  | 4                              | Death               |
| 358              | 270            | 6                  | 2                              | Death               |
| 359              | 230            | 6                  | 1                              | Recovery            |
| 219              | 570            | 28                 | 2                              | Death               |

TABLE XIX

These animals received a single dose of 50 mg. of the typhoid residue.

| Protocol,<br>No. | Weight,<br>gm. | Interval,<br>days. | Amount of<br>culture,<br>c. c. | Result.             |
|------------------|----------------|--------------------|--------------------------------|---------------------|
| 336              | 360            | 1                  | 3                              | Recovery            |
| 337              | 285            | 3                  | 3                              | Death               |
| 338              | 245            | 7                  | 3                              | Death               |
| 339              | 350            | 9                  | 2                              | Death on second day |
| 340              | 290            | 11                 | 1                              | Recovery            |
| 341              | 255            | 14                 | 1                              | Recovery            |

TABLE XX

These animals received two and three immunizing doses of the typhoid residue. The protocol number, the weight, the number of immunizing doses, the interval in days between the last treatment and the inoculation, the amount of culture twenty-four hours old, and the result are given:

| Protocol,<br>No. | Weight,<br>gm. | No. of<br>treatments. | Amount of<br>residue,<br>mg. | Interval,<br>days. | Amount of<br>culture,<br>c.c. | Result.  |
|------------------|----------------|-----------------------|------------------------------|--------------------|-------------------------------|----------|
| 220              | 375            | 2                     | 100                          | 3                  | 2                             | Recovery |
| 221              | 495            | 2                     | 100                          | 3                  | 3                             | Death    |
| 222              | 650            | 3                     | 150                          | 5                  | 2                             | Recovery |
| 223              | 480            | 3                     | 150                          | 5                  | 3                             | Recovery |
| 224              | 435            | 3                     | 150                          | 5                  | 3                             | Recovery |
| 144              | 605            | 3                     | 150                          | 7                  | 4                             | Recovery |
| 145              | 665            | 3                     | 150                          | 13                 | 4                             | Recovery |

The minimum fatal dose of the twenty-four-hour culture of the typhoid bacillus employed in this case is 0.5 c.c. It will be seen from Table XVIII that a single dose of 100 mg. of the residue gives the animal an immunity of six units at the end of twenty-four hours, and that the immunity was less than eight units at that time. On the third day the immunity was found to be diminishing, but on the sixth day the animal bore twice the fatal dose. The animal which received eight units at the end of the first day evidently was close to the borderline, because it did not die until the second day, and the normal time for an untreated guinea-pig to live after receiving the minimum fatal dose is less than twelve hours. Table XIX shows that a single dose of 50 mg. is quite as efficient as one of 100 mg. Table XX indicates that multiple immunizing doses give a higher degree and a more lasting immunity than that secured by a single dose.

**Theoretical Considerations and Conclusions.**—We wish to offer certain theories that we have reached after making these experiments. In order to save space we will condense our views as follows:

1. All the proteins with which we have worked contain a poisonous group, and the probabilities are that this is true of all proteins, be they bacterial, vegetable, or animal.

2. Proteins may be split into poisonous and non-poisonous groups, either artificially in the retort or in the animal body.

3. The splitting up of the protein in the animal body is due to a proteolytic ferment which is the product of certain cells.

4. This ferment is specific for the protein which calls it into existence.

5. Our conception of the origin and nature of these specific ferments is as follows: The cell is made up of molecules; the molecules consist of atoms, and the atoms of electrons. The molecule may be likened to the universe, composed of suns, planets, and satellites. These are in harmonious and rhythmic motion. The molecule of the foreign protein introduced into the body has a structure similar to that of the cell molecule, and when one is brought within the attractive range of the other, one or the other, or both, must undergo certain disturbances. Suppose that an atomic group is split off from the animal cell and enters the attraction sphere of the molecule of the foreign protein, then the harmonious arrangement of the atoms and electrons of the latter will be affected; indeed, the molecule may be disrupted as completely as it is in the retort under the influence of dilute alkali.

Our residues evidently have the same effect on the cells of the body that the proteins from which they come do, and in this way we may explain the specific action of the residues. The special group broken off from the cell molecule depends upon the composition of the protein which comes within its attractive sphere, and it seems that our residues contain that portion of the molecule of the foreign protein which possesses this property of bringing into existence, or rather of activating, its own specific ferment. The ferment is, according to our conception, a portion of the animal cell, an atomic group within the cell molecule,

and does not become a real active ferment, or it is not activated until the foreign protein comes within its sphere of attraction. It occurred to us that if this theory has much of truth in it we might test it. We thought that the introduction of a small portion of the residue might give some immunity immediately. We therefore injected doses of 25 mg. of the colon and 12.5 mg. of the typhoid residue into the abdominal cavity of guinea-pigs, and thirty minutes later inoculated these animals intra-abdominally with living cultures, and found that the colon animals had in that short time acquired an immunity of five units and the typhoid one of six units. However, we found that larger immunizing doses did not give us results so good, and this is easily explained by supposing that this ferment set free or activated by the residue is in part used up in its reaction with the residue itself. The reason multiple doses repeated at intervals give us a higher degree of immunity than single doses may be due to more cells being acted upon or to the accumulation of the ferment in the blood. The theory of the *modus operandi* of the residue which we have offered is tentative, and we hope to be able to investigate it further.

It will undoubtedly occur to the reader, as it has to us, to ask the question how it is that the residue sensitizes or activates, while the bacillus itself, living or dead, has no such effect or at least is not nearly so effective. The only answer that we can suggest to this question is that in order to be effective in its action the sensitizer must be in solution, and that being in this state it reaches every part of the circulatory system in a few seconds. Possibly cell permeation may be necessary to the most perfect sensitization.

## CHAPTER VIII

### THE SPLIT PRODUCTS OF THE TUBERCLE BACILLUS AND THEIR EFFECTS UPON ANIMALS<sup>1</sup>

**The Organism.**—The tubercle bacillus employed in the experimental work herewith reported is one which, after having been grown for many years on artificial culture-media, has lost its virulence for rabbits and guinea-pigs. We have repeatedly demonstrated this fact during the past six years, but in order to have renewed evidence we have, at the beginning of this research, inoculated 4 rabbits and 5 guinea-pigs intra-abdominally with loops of the glycerin beef-tea culture, and these animals having been killed from three to six months after inoculation, have in no instance showed any evidence of infection. On artificial culture-media this bacillus grows abundantly, and in this respect it has served our purpose in furnishing a large amount of cellular substance. It has been grown in the ordinary glycerin beef-tea and has been harvested after periods of from one to six months. Growths obtained after from one to two months at 37° have given us the most satisfactory material.

**The Cellular Substance.**—The bacterial substance is collected on hard filters, dried between folded filters, and thoroughly extracted, first with alcohol and then with ether, in large Soxhlets. In this paper we will say nothing concerning the fats and waxes extracted with alcohol and ether. The cellular substance is next rubbed up in a mortar and passed through a fine-meshed sieve. As thus prepared,

<sup>1</sup> The first part of this chapter is taken from a paper read by Vaughan and Wheeler before the International Congress on Tuberculosis in 1908.

the powder shows the bacilli more or less broken into a cellular debris when examined microscopically. The individual bacilli take the carbohc stain, but this is now washed out with dilute nitric acid.

**The Cleavage of the Cell.**—The cellular substance, prepared as stated above, is placed in large flasks, fitted with reflux condensers, covered with from fifteen to twenty times its weight of absolute alcohol, in which 2 per cent. of sodium hydroxide has been dissolved, and heated for one hour at 78°, the boiling-point of absolute alcohol. Three successive extractions are made, using a new portion of alkaline alcohol each time. This treatment splits the cellular substance into two portions—one soluble and the other insoluble in absolute alcohol. These portions we will designate as the “cell poison” and the “cell residue.”

**The Cell Poison.**—This is soluble in the alcohol, which is carefully neutralized with hydrochloric acid. The precipitated sodium chloride is removed by filtration and the filtrate containing the poison is evaporated *in vacuo* at or below 40°. This leaves the cell poison as a brownish mass containing a small amount of sodium chloride, which can be removed by repeated solutions in absolute alcohol and evaporation. The poison resembles that obtained from other protein bodies. It is freely soluble in absolute alcohol; less freely in water. Its aqueous solutions give all the color protein reactions, including that of Molisch, which is not given by the poisonous groups that we have obtained from other proteins. In powder form it is deliquescent and becomes darker as it absorbs water. The tubercle protein apparently contains much less poison than the cellular proteins of the colon and typhoid bacilli. The latter are split up by our method into about one-third poison and two-thirds residue, while 25 grams of the cellular substance of the tubercle bacillus yielded less than 3 grams of poison.

**The Cell Residue.**—This is the portion insoluble in the alkaline alcohol. It is placed in Soxhlets and extracted for many hours with absolute alcohol in order to remove traces of the cell poison and free alkali. After this it is

dried and powdered. It is partially soluble in water, and the soluble part constitutes that which we have used in our experiments.

**The Bacterial Filtrate.**—After the bacilli have been removed, the culture medium is concentrated on a steam-bath to one-sixth its volume. This concentrated fluid is poured into five times its volume of absolute alcohol, which throws down a heavy, sticky precipitate. This precipitate is placed in Soxhlets and extracted, first with alcohol and then with ether. Next, it is powdered and split up with alkaline alcohol after the method used with the cellular substance. This breaks it up into poisonous and non-poisonous groups, which we distinguish from the corresponding bodies obtained from the cellular substance by designating them as “the precipitate poison” and “the precipitate residue.”

**The Precipitate Poison.**—This differs in none of its physical or chemical properties, so far as we have investigated, from the cell poison.

**The Precipitate Residue.**—This is freely and wholly soluble in water. It gives all the protein reactions and is precipitated by uranyl acetate and metaphosphoric acid.

**The Final Filtrate.**—In this manner we have designated that portion of the culture-medium that remains after the concentrated medium has been precipitated by five times its volume of absolute alcohol. The alcoholic filtrate gives a voluminous precipitate with an alcoholic solution of mercuric chloride, showing that all the protein material has not been precipitated by the alcohol. This filtrate is freed from alcohol by distillation and has been used in some of the animal experiments described later.

It will be seen that we have split up the tubercle cell into two portions: the cell poison and the cell residue. The culture-medium has been concentrated and then precipitated with five times its volume of absolute alcohol, and this precipitate has been broken up into two portions: the precipitate poison and the precipitate residue, and the portion of the culture-medium left after the removal of

the alcoholic precipitate we have designated as the final filtrate.

**The Effect of the Cellular Substance on Animals.**—It must be borne in mind that the cellular substance with which we are now dealing is that of a tubercle bacillus that is avirulent to rabbits and guinea-pigs and that it has been thoroughly extracted with alcohol and ether. There remains, as it were, only the protein skeleton of the bacillus.

We have injected into the abdominal cavities of twenty-four guinea-pigs single doses, varying in amount from 5 to 200 mg. of the cellular substance, and from these experiments we make the following statements:

1. In no case was death caused directly by the injection. One pig that received 20 mg. was found dead six days later. There were several caseous nodules in the omentum and one on the under surface of the liver. Microscopic examination showed that these consisted of masses of leukocytes and the debris of the injected bacilli. Another that received 5 mg. was found dead nine days later, but careful search failed to reveal any traces or effect of the injection. Animals that received from 100 to 200 mg. remain apparently well four months after the injection.

2. It gives in guinea-pigs no immunity to a subsequent inoculation with a virulent bacillus. Six pigs that had received single intra-abdominal injections of the cellular substance in amounts varying from 15 to 200 mg. were inoculated one month later with a loop of a virulent culture of bacillus tuberculosis and all developed tuberculosis and died from it within from nineteen to one hundred days.

3. It does, for a short time at least, sensitize guinea-pigs to the tuberculosis bacillus. This is an interesting and, in our opinion, a hopeful point. The following are illustrations of this action: Pig No. 159, weight 530 grams, received, December 18, 25 mg. of the cellular substance. Thirteen days later it was given intra-abdominally a large loop of the avirulent culture suspended in salt solution. The animal was sick within a few minutes. Within half an hour it developed the first and second stages of anaphyl-

axis. Within forty-five minutes its rectal temperature had fallen to 96° F. and it was found dead the next morning. Postmortem showed a hemorrhagic peritonitis. Pig No. 163, weight 535 grams, received 45 mg. of the cellular substance December 18. Its subsequent treatment and its results were the same as recorded of the preceding animal. Pig. No. 151, weight 555 grams, received, December 18, 125 mg. of the cellular substance. Twenty-three days later it had intra-abdominally a large loop of the avirulent culture. It died within sixteen hours and showed a hemorrhagic peritonitis.

If we interpret these results correctly we infer that the cellular substance had sensitized these animals and that the bacilli of the second dose were broken up so rapidly and their poisonous constituents set free so speedily that the animals died. If this interpretation be correct, there remains at least the possibility that there may be found in the bacillary substance some constituent that may stimulate the cells of the animal body to split up and destroy tubercle bacilli. We will return to this before we close.

**The Effect of the Cell Poison on Animals.**—This body, obtained by splitting up the cellular substance with alkali in absolute alcohol, is, like all similar bodies that we have obtained from bacterial, vegetable, and animal proteins, a poison. It develops the three stages of peripheral irritation, partial paralysis, and terminal convulsions. When given in sufficient quantity it kills within an hour both healthy and tuberculous animals. When given to healthy animals in very small repeated doses it has no visible effect. In larger repeated doses it causes in healthy animals a condition of chronic intoxication characterized by loss of flesh and general marasmus. When given even in very small repeated doses to tuberculous animals it intensifies the tuberculous process, and in all cases the treated animals die before the controls. There is no evidence that it elaborates any antitoxin, and it is harmful, so far as our experiments show, and we have made many with

this body upon both normal and tuberculous animals; it has nothing to recommend it. What is true of the cell poison is equally true of the precipitate poison and the final filtrate. The effects of these poisons on animals are harmful—only harmful.

**The Effects of the Cell Residue on Animals.**—This is the non-poisonous group obtained by splitting up the cellular substance with alkali in absolute alcohol. On healthy animals it has no recognizable ill effect, either in single or repeated doses, either large or small. In this product we see the one small ray of hope of finding, among the split products, a body that may possibly be of service in the treatment of incipient and localized tuberculosis. Before giving the basis of this slight hope we will tell what we have done with this product.

In the first place, it sensitizes guinea-pigs to the tubercle bacillus. The following are illustrations: Guinea-pig No. 199, weight 530 grams, received, December 20, 50 mg. of the residue. Thirteen days later it had intra-abdominally a large loop of the avirulent culture (the same amount given to pigs 159, 163, and 151, see p. 167). This injection was made at 11.30 A.M. At 12 M. the temperature had fallen to 96° F. At 2.30 P.M. it was 97.1°, and at 5 P.M. it was 99°, and the animal was apparently well.

Pig No. 200, weight 530 grams, received 50 mg. of the residue intra-abdominally. Thirteen days later it had intra-abdominally one large loop of the avirulent culture. The rectal temperature before the injection was 101° F. Within half an hour it had fallen one degree, but went no lower and the animal seemed to be but little disturbed. We have similar records of other animals.

We compare these results with those recorded of animals 159, 163, and 151, and tentatively conclude that the sensitizing agent in the cellular substance is the portion that we have designated as the residue. But the avirulent bacilli killed the animals sensitized with the cellular substance because when the bacteriolytic ferment was set free or activated by the second injection, it split up not

only the bacilli introduced by the second injection, but also those remaining in the body from the first injection and these together supplied enough free poison to kill.

It will require much experimentation to show what degree of sensitization can be secured by the residue, what size doses should be used, and how long the condition of sensitization continues. If men, as well as guinea-pigs, can be sensitized with the residue, there is the possibility that it may be of service in the treatment of initial and localized tuberculosis, because it may be used to bring into existence and activate a specific bacteriolytic ferment which will split up and destroy the few bacilli that are in the body, but we can readily see that this might be harmful rather than beneficial when the number of bacilli in the body is large enough to furnish a dangerous amount of the poison when set free. In this case the old adage that it is not wise to disturb sleeping dogs might be remembered.

**The Effect of the Precipitate Residue on Animals.**—This is the most interesting of the split products of the tubercle bacillus, and it deserves much more study than we have as yet been able to give it. On healthy animals it has no recognizable ill effects either in single or repeated doses, large or small. We took 6 half-grown pigs and injected into the abdominal cavity every third or fourth day 50 mg. of this residue. These injections were begun April 20 and continued until June 11. During this time each animal received sixteen injections, a total of 800 mg. each. All increased normally in weight, and five days after the last injection all were killed and carefully examined and found to be perfectly normal. We were led to do this because of the following experience: Three sets of pigs were inoculated intra-abdominally with the living avirulent culture. These inoculations were made December 14, 1905. The animals of the first set had no treatment, and when killed April 11, 1906, were found to be perfectly normal. Those of the second set had, December 18, 50 mg., December 22, 60 mg., and December 26, 70 mg. of the cell residue. All in this set were killed April 11, and were also found to be

wholly free from infection. Those of the third set had, December 18, 30 mg., December 22, 75 mg., and December 26, 100 mg. of the precipitate residue. Half of this set died before April 11 of tuberculosis, and the other half were found to be tuberculous when killed on that date. To us this indicates that the precipitate residue has some specific effect upon tuberculous animals. We suspect that the ill effect in these instances was due to the size of the doses, because in reality the doses of the precipitate residue were much larger than those of the cell residue—much larger, indeed, than the figures indicate, because, as we have stated, the cell residue is not freely soluble in water, while the precipitate residue is wholly soluble. In making up our solutions we weighed out each residue and, in fact, the animals received only the soluble parts of the amounts stated in the figures. We can easily understand how excessive doses given soon after inoculation with the avirulent culture might induce such a result. This culture is avirulent because it makes only an ineffectual attempt to grow in the animal body. The feeble effort is resisted and overcome by the natural defences of the healthy body. Now, if these natural defences were wholly occupied in disposing of the material injected, which should have been only sufficient to awaken these defences, then the bacilli would meet with no resistance and would multiply.

The precipitate residue sensitizes guinea-pigs to the tubercle bacillus just as the cell residue does. Evidently our so-called residues are much alike, and it is more than probable that they contain the same active constituent. In cultures from three to six months old many of the bacilli have undergone autolytic changes and the cellular substance has in part passed into solution. This is true of both the poisonous and the non-poisonous groups of the protein that makes up the cell substance. Of one thing we have satisfied ourselves at least, and that is that no preparation from the tubercle bacillus should be used in the treatment of tuberculosis until the poisonous group of the tuberculous protein and other proteins in the culture-

medium be removed. This is too powerful a poison to be injected repeatedly even in small doses into the animal body.

One of us has for the past two years used solutions of the cell residue in the treatment of tuberculosis in man. The most suitable preparation is a 1 per cent. solution filtered through porcelain. The cell residue in weighed quantity is placed in a bottle with the proper volume of a 0.5 per cent. solution of carbolic acid, and the bottle is carried on a mechanical shaker for twenty-four hours, after which the content is passed through a porcelain filter. Such a solution will keep indefinitely. We have used this solution sufficiently to justify the following statements: (1) It is of no value in advanced cases of pulmonary tuberculosis. (2) It may prove harmful even in initial cases if the dose be too large or if small doses be too frequently repeated. (3) When properly used in initial cases or in localized tuberculosis, its action is apparently prompt and specific. If the tubercle bacilli wholly disappear from the sputum, as they may, the injections should be repeated at intervals of from two to four weeks for some months. We wish it clearly understood that in well-established cases of pulmonary tuberculosis no benefit from this treatment can be expected. We believe that in initial cases this preparation is preferable to any form of tuberculin.

**Toxophor Group.**—White and Avery<sup>1</sup> have reported an interesting research on the split products of the cellular substance of the tubercle bacillus, especially of the toxophor group. They used a strain virulent to guinea-pigs. This was grown on glycerin broth cultures for six weeks, and the cellular substance was washed with alcohol and ether, ground in a ball mill and split up by our method. The toxophor obtained by them agreed with that which we have prepared. It is a yellowish-brown powder of characteristic pungent odor, readily soluble in alcohol. Its aqueous solutions are faintly turbid, and give the biuret,

<sup>1</sup> Jour. Med. Research, 1912, xxvi, 317.

xanthoproteic, Adamkiewicz, Liebermann, Millon, and Molisch tests; the last two faintly. Bromine water produces a white flocculent precipitate, but no color, showing the absence of tryptophan. Injections were made into the right external jugular vein of guinea-pigs of about 200 grams weight. The poison, as prepared, killed in doses of 1 to 15,000 body weight. White and Avery give such an excellent statement of the symptoms and gross pathology that we are induced to make the following quotation: "When a quantity approaching the minimum fatal dose is given, the first symptoms appear immediately, or, at most, within thirty seconds. The animal becomes restless, scratches its nose, and frequently utters a sharp hiccough. The movements become incoördinate, the gait is unsteady. The eyes are fixed, and stare. Respiratory embarrassment, with diaphragmatic spasm sets in and increases to a degree which causes the animal to spring from its feet, to buck, and finally to fall on its side with convulsive twitching of its legs, intermittent, and both clonic and tonic in character. Involuntary micturition and defecation frequently take place. The dyspnea becomes more marked, and then ensue successive periods of apnea, lasting as long as twenty to thirty seconds. These are followed by violent inspiratory efforts, during which the chest wall becomes fixed in maximum inspiration. Cyanosis is noticeable in the lips and ears, and becomes more marked. The convulsive gasps increase in frequency and decrease in depth, until finally only the lips move, the feeble and rapid dilatations of the *alæ nasi* marking the onset of death. This sequence of symptoms is accompanied by a rapid and progressive fall of the body temperature. Death takes place in from one and one-half to six or seven minutes. Immediate autopsy reveals first a cyanotic hue of the subcutaneous and muscular tissues. The blood is dark in color and does not clot readily. Beyond an exaggerated peristaltic movement of the intestines, the abdominal viscera appear to be normal. On opening the chest the lungs are found to be in a state of maximum inflation, overlapping the peri-

cardium, and forming a cast of the thoracic cavity. They are pale and often slightly bluish in color, and frequently exhibit punctate hemorrhages on the surface. The heart still beats. Not infrequently there is definite heart block, with an auriculoventricular arrhythmia of three to one. Often there are petechial hemorrhages in the epicardium, Greater extravasations are also seen, and in two cases actual rupture of the ventricle had apparently taken place. On section the lungs do not collapse, and on pressure only a little frothy serum exudes. They are not edematous. They float on water. The excised heart continues to beat for several minutes. The gross appearance of the brain is normal. A study of the pathological changes in the histology of the lungs, heart, and brain has been undertaken, but has not yet progressed sufficiently to warrant any conclusions. When the dose is larger the acute symptoms appear instantaneously, and their sequence is more rapid. With a sublethal dose the onset is slower and the manifestations are less violent. The animal shows evidence of weakness, drops its hind legs, and frequently lies on its side in collapse. The apneic stage is never reached, its appearance therefore signifies inevitable death. Recovery from a non-fatal dose is comparatively prompt even when near the lethal borderline. Recovered animals exhibit no visible sequelæ of the intoxication."

These investigators have compared the acute intoxication produced in animals by the tuberculoprotein with anaphylactic shock, and conclude that there are no appreciable points of difference in the symptomatology and gross pathology of the two conditions. "They would therefore appear to be identical." The tuberculoprotein, like that obtained from other proteins, is thermostabile. It also agrees with the like protein obtained by the cleavage of other proteins in the following particulars: (1) It lowers the temperature when given in doses sufficient to produce recognizable effects. (2) It does not sensitize animals to the unbroken tuberculoprotein, while the haptophor group is not poisonous and does sensitize to the whole

protein. (3) The injection of non-fatal doses of the poison renders animals at least temporarily refractory to subsequent injections of what would normally be fatal doses. We have always held that this is due to the establishment of a tolerance. This is important and we will refer to it again when we discuss the action of tuberculin. (4) The poison is not absorbed *in vitro* by brain, lung, or liver tissue. "These experiments seem to emphasize the absence of any possible identity of this protein fragment with the true toxins. The results, however, are in accord with the symptoms produced by the cell poison. Recovery from a sub-lethal dose is rapid and complete, and this would imply that the contact between the body cells and the poison is transitory, and non-destructive. It appears to be more like a fulminating irritation, and may result in an arrest of function due to a disturbance in the physical equilibrium of the cells affected." (5) The serum of normal guinea-pigs incubated with the poison does not materially, at least, decrease its action. (6) The poison does not induce any local reaction when introduced intradermally in guinea-pigs sensitized to tuberculo-protein. Four animals sensitized nineteen days previously with the cell residue, and which had been found to be sensitive to an extract emulsion of tubercle bacilli, and four animals rendered and proved sensitive by a watery extract of tubercle bacilli received intradermal injections of the poison, and under close observation showed no reaction. This is as should be expected. The skin reaction, like other tuberculin reactions, results from the cleavage of the tuberculo-protein. The cleavage products produce no such reactions. (7) Auer and Lewis<sup>1</sup> showed that prophylactic treatments with atropine save a large percentage of animals from death by anaphylactic shock on the reinjection of the homologous protein. We claim that our protein poison is the active agent in anaphylaxis. Now, White and Avery show that atropine protects 75 per cent. of guinea-pigs from death after the

<sup>1</sup> Amer. Jour. Physiology, 1910, xxvi, 439.

administration of lethal and slightly supralethal doses of the poison. (8) Morphine sulphate has been shown by White and Avery to antagonize the action of the poison." Of the animals tested (19) only 3 showed typical symptoms, and with two of these death was slightly delayed. The three autopsies revealed typical inflation of the lungs, with epicardial hemorrhages in two. Nine of the pigs had only slight symptoms, and although the issue was fatal, death was delayed from forty-two minutes to over six hours. On section, however, six of the animals showed inflated lungs with epicardial hemorrhages. Two animals recovered. It will be noted that in five cases a dose of 1 to 12,000 failed to produce typical immediate symptoms. Further investigations of the effects of morphine might lead to a better knowledge of the factors concerned in the sequelæ of parenteral administration. (9) Banzhaf and Steinhardt<sup>1</sup> studied the effects of chloral hydrate upon the action of our poison prepared from egg-white, and came to the following conclusions: "Normal guinea-pigs under the influence of chloral (by intracardiac and intramuscular injections) were completely protected against one and one-fourth fatal doses of the poison (given intracardiacy). If two or more fatal doses were given death resulted. Chloral mixed with the poison and then given caused irregular results which were interpreted as meaning that there is no chemical union of the chloral and poison *in vitro*. We assume that the chloral protected by union with certain vital cells." White and Avery used a 2.5 per cent. solution of chloral in normal salt. The injections were made intravenously. "With the exception of 2 animals displaying typical symptoms, both of which received amounts of the poison considerably in excess of that required to kill, 6 of the 13 survived the injection with slight or no symptoms, while 5 succumbed in from two to thirteen hours without exhibiting the classic respiratory spasms. Autopsy showed the typical findings in 2, while in 2 others there was a partial inflation

<sup>1</sup> Jour. Med. Research, 1910, xxiii, 1.

of the lungs with punctate hemorrhages beneath the pericardium. The results, although not so strikingly positive as those of Banzhaf and Steinhardt, at least tend to confirm their conclusion." (10) Banzhaf and Steinhardt found that lecithin given intraperitoneally in doses of from 250 to 500 mg. or more to serum-sensitized guinea-pigs protected them from a second injection of 5 c.c. of horse serum given twenty-four hours later. When lecithin was emulsified with the Vaughan poison or given twenty-four hours before the poison was injected, no protection was afforded. From this Banzhaf and Steinhardt conclude that lecithin prevents the cleavage of the protein in a sensitized animal on reinjection and that it does not neutralize or modify the action of the preformed poison. White and Avery, from their experiments, come to the following conclusion: "Lecithin emulsion injected simultaneously with the poison seems to possess a slight and irregular prophylactic action. Incubation of the poison with lecithin emulsion for an hour at  $37.5^{\circ}$  increases this neutralizing property. A dose of 1 to 12,000 was not affected. The preliminary administration of lecithin protected some of the animals, delayed death in others, and was without effect in the remainder. The results were too inconstant to warrant definite conclusions."

White and Avery are inclined to the opinion that our crude protein poison contains a plurality of active substances, and in this they are probably right. They say: "The effects provoked by the parenteral administration of the artificially obtained poisonous substance in non-fatal doses, and as modified by atropine, morphine, chloral, and other drugs, seem to suggest the plurality of its action. It is conceivable that the poisonous fraction obtained by Vaughan's method contains either an essential component which is several in its physiological action, and which in sufficient doses exerts its primary and dominating effect on the respiratory mechanism, or that it contains groups or individual constituents of different selective vital affinities, the most eminent of which is for the peripheral or central

cells functioning in respiration. It is not unreasonable to hope that a further separation of the poisonous fraction into its components and a more intimate study of their various actions on the animal economy may furnish valuable clues not only to the relation of these chemical substances to true anaphylactic processes, but also to the physiological nature of the varied phenomena of hypersensitiveness." It seems to us, theoretically, that there must be a whole spectrum of poisons in the protein molecule. We have shown that at least one group in this molecule is poisonous. The poisonous action of the protein molecule becomes more marked as we proceed in stripping off certain side chains. Peptone is more poisonous than the native protein from which it is obtained. Our product is more active than peptone. Between the two there must be a group of bodies, each of which is more active than the peptone and less active than our split product. Indeed, we are confident that we have discovered some of these intermediate bodies. As has been stated, when the alcohol employed in the cleavage of the protein molecule is not absolute we obtain products that are quite unlike our poison in physical, chemical, and physiological properties. They are sticky and gummy. They contain some carbohydrate, responding to the Molisch test, and yielding a reducing substance after prolonged boiling with dilute mineral acid; while our final product is not gummy and fails to show any evidence of carbohydrate content, except in that from tubercle bacilli. These other bodies kill much less promptly. The paralytic symptoms are more marked, and the convulsive stage is either only slightly in evidence or wholly wanting.

Interesting experiments on sensitization to tuberculo-protein have been made by Baldwin<sup>1</sup> and Krause.<sup>2</sup> We make the following extracts from this work: Animals may be sensitized by any of the ordinary products of the tubercle bacillus. Sensitization may be secured by introducing the protein by any parenteral route, by the peritoneal

<sup>1</sup> Jour. Med. Research, 1910, xxii, 189.

<sup>2</sup> Ibid., xxii, 275; xxiv, 361.

cavity, subcutaneously, subdurally, intracerebrally, by post-orbital injection, and probably by intravenous injection, though the last-mentioned method was not tried. Sensitization may be obtained by the injection of only 0.05 mg. of the protein. The best preparations for sensitization are those in which the protein is in solution. The shortest period of incubation found was six days. This was when the sensitizing dose was given postorbitally. Before the twenty-first day sensitization is uneven and inconstant. After this period it proceeds with great regularity, and the longest duration noted was two hundred and eighty-six days. It is likely that it continues in the guinea-pig throughout life. The size of the sensitizing dose bears no relation to the period of incubation. Acute anaphylactic shock follows when the reinjection is given intravenously or postorbitally. The minimum toxic dose on reinjection was found to be 0.99 mg. of the dry protein, and the minimum fatal dose on reinjection 1.6 mg. Attempts to establish passive anaphylaxis have been uniformly unsuccessful. Infected animals become autosensitized and are killed by injections of large amounts of the tuberculoprotein. This protein does not act like a toxin, and when injected into animals does not lead to the elaboration of an antitoxin. "If an animal be infected experimentally it begins to react to tuberculin about the fifteenth day; in like manner, the non-tuberculous but protein-treated animal will react to a second injection about two weeks after the first. Again, both the tuberculous and the sensitized non-tuberculous animals react to exceedingly small doses of the protein; indeed, a certain proportion of the tuberculous will undergo an intoxication that is identical with acute anaphylaxis, provided the toxic dose is applied postorbitally, while if the sensitized animal receives its toxic injection by a route that renders absorption less rapid—*e. g.*, an intraperitoneal injection—the resulting intoxication will tend to approximate what is generally observed as the tuberculin reaction in the infected guinea-pig (without, of course, any focal reaction). Therefore, while the facts will not at present

warrant the flat declaration that the two phenomena result from the same fundamental causes, there are enough data at hand to justify the elaboration of a working hypothesis that such is the case." The important question of the relation between sensitization and immunity to infection has been tested by Baldwin and Krause. Series of guinea-pigs were sensitized to tuberculo-protein. The fact that they were in full sensitization was demonstrated by testing some of each set. Those that recovered from anaphylactic shock and known as "refractory" were inoculated an hour after the reinjection. Lot A had received a total of 25 c.c. of the watery extract in seventeen doses over a period of thirty-nine days. Lot B had received a total of 13 c.c. of the watery extract in ten doses over a period of thirty-nine days. Lot C had received a total of 8 c.c. of the watery extract in six doses over a period of thirty-nine days. Lot D had not been sensitized. The last sensitizing doses were given June 14, 1910. All of these animals were inoculated with the same amount of a virulent culture of the tubercle bacillus July 1, 1910. Sixty-two days after inoculation all the animals were killed and examined. A summary of the findings is stated as follows: "The refractory animals suffered most. The disease was pretty well disseminated in all of them, and they exhibited far more tuberculosis than any of the animals that had not been intoxicated, and than any of the controls. . . . The animals that were sensitized in various ways all became diseased. As a general thing, we may say that the more protein the animal received during preliminary treatment, the less was the resultant infection. So far as one could tell from the toxic symptoms of the test animals there was very little difference in the average degree of sensitization in the several sets of guinea-pigs. The results of inoculation were, however, different. It is most likely that the differences were altogether independent of any degree of raised or lowered resistance conferred by the sensitive state, but that they were due to the heightened immunity that followed the protein injections."

Krause concludes the paper from which the above was taken as follows:

"1. Sensitization of non-tuberculous guinea-pigs with tuberculo-protein does not alter their resistance to experimental tuberculous infection.

"2. Sensitization to tuberculo-protein and relative immunity (increased resistance) to infection can occur coincidentally in the same animals.

"3. Resistance to infection is markedly lowered during the period that a sensitized animal is suffering from symptoms of anaphylactic shock."

The third conclusion is certainly justified from the results of the research, and is what might have been predicted at the start. Whether the first conclusion is in any way contradictory to the previous statement "that the more protein the animal received during preliminary treatment, the less was the resultant infection," we leave the reader to determine for himself. This line of experimentation should be continued with all the tuberculo-protein preparations and with variations in size and frequency of dosage.

Thiele and Embleton<sup>1</sup> have reviewed the literature of sensitization in tuberculosis, and have experimented with reference to both active and passive hypersensitiveness to tubercle bacilli, and the relation to the tuberculin reaction in man. The conclusions reached are stated as follows: (1) Guinea-pigs may be typically sensitized with powdered tubercle bacilli. (2) Guinea-pigs may be passively sensitized with the blood or tissues of animals actively sensitized. (3) Guinea-pigs may be sensitized to tuberculin with the blood of tuberculous patients who are highly sensitive to tuberculin. (4) Likewise, guinea-pigs may be sensitized with tuberculous tissue from man, or with that from tuberculous guinea-pigs. (5) By regulating the dose one can induce fever or cause the temperature to fall below the normal in actively sensitized guinea-pigs with tuberculin. (6) The same results can be obtained in

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1913, xvi, 411.

animals sensitized with heterologous or homologous tissue. (7) A cutaneous reaction has not been obtained.

This work is confirmed and supplemented by that of Sata,<sup>1</sup> who sensitizes guinea-pigs with a single injection of tuberculous serum, in doses of 0.1, 0.5, or 1.0 c.c. subcutaneously, intraperitoneally, or intravenously, and uses a reinjection of old-tuberculin intravenously. When the dose of the reinjection is as much as 0.5 c.c. acute anaphylactic death results. With smaller doses there is elevation of temperature.

Many investigators have failed to sensitize animals with tuberculin, while most have succeeded with dead bacilli and with aqueous extracts. This is not surprising; indeed it is what should have been expected. Tuberculin consists of digested, denatured proteins of relatively simple composition. It is well known that peptones and polypeptids do not sensitize. The protein poison when detached from other groups in the protein molecule sensitizes neither to itself, nor to the unbroken protein. The fact that tuberculin does not sensitize or does so imperfectly raises a serious question as to its employment as a therapeutic agent. It is undoubtedly an excellent diagnostic agent because its relatively simple structure may favor its prompt cleavage when injected into an animal already sensitized by the disease. But if it is not a sensitizer its therapeutic good effect, if it has any such effect, must be confined to the possible establishment of a tolerance to the tuberculo-protein. Sensitization to tuberculo-protein can be induced by bacillary emulsions, with watery extracts, and with the non-poisonous residue. If the sensitization secured by the last-mentioned agent is as good as that produced by the others, it has the advantage of not containing any poison. On the other hand, if the therapeutic effect desired consists in the development of a tolerance to the poison, tuberculin must be preferred unless we should use the more completely isolated poison.

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1913, xvii, 62.

There are those who, while admitting that animals can be sensitized to tuberculo-protein, hold that the tuberculin reaction is not an anaphylactic one. We think that it is, and that the fact that tuberculin does not sensitize or does so imperfectly does not contradict this. It is probable that when tuberculin does sensitize at all it is due to the fact that it contains traces of but little altered or unaltered tuberculo-protein. The tuberculin reaction should be regarded as a phenomenon resulting from a reinjection. The animal is already sensitized by the disease.

Koch in his early work pointed out two facts, which in a way seemed to be contradictory, but which have been found to be true. First, he showed that a tuberculous animal behaves toward a second infection differently from a normal animal, the former resisting the second infection by forming an inflammatory area about the point of the second inoculation, this leading to necrosis, and recovery without extension of the infection. Second, Koch stated that the injection of dead tubercle bacilli into tuberculous guinea-pigs killed them within from six to forty-eight hours, while like injections into normal guinea-pigs had no such effect. These apparently contradictory statements have not only been confirmed, but have been found not in any way in conflict. The studies of Römer, Hamburger, and others have shown that the following conditions must prevail in order to fully demonstrate the first statement of Koch: (a) The first injection must be a weak one, permitting the disease to run a chronic course. (b) The time interval between the first and second inoculations must be relatively long, the resistance to the second infection increasing with time. (c) The dose of the second injection must not exceed a certain limit. What happens to the bacilli of the second inoculation? Why should these fail to develop, while those of the first inoculation continue to grow? Kraus and Hofer<sup>1</sup> have found that tubercle bacilli injected into the peritoneum of a tuberculous animal are

<sup>1</sup> Deutsch. med. Woch., 1912.

destroyed by lysis within an hour. There is some lytic destruction of tubercle bacilli in the peritoneum of a healthy guinea-pig, but this does not compare in rapidity and completeness with that occurring in the tuberculous animal. But why is the action of this lytic agent manifested so effectively against the bacilli of the second inoculation while those of the first apparently proceed in uninterrupted growth? One of Hamburger's experiments<sup>1</sup> may throw some light on this question. He made his reinoculation subcutaneously on each side. On the left he injected a small dose, on the right a large one. On the left there was no development; on the right a tuberculous nodule developed. We infer from this and similar observations made by others that the lytic agent which destroys the tubercle bacillus and which is produced in larger amount in tuberculous than in normal animals, because the cells of the former have been sensitized, is stored in the cells as a zymogen, and is activated only when tuberculoprotein is brought into contact with the cell, and possibly is active only, or is most active, in *statu nascendi*. The ferment is capable of destroying only a given amount of bacilli or is wholly inactive in the presence of a great excess of substrate, or its action is soon interrupted by the accumulation of fermentative products. However, it is quite certain that all the bacilli of the second inoculation are not always killed because it may happen according to observations of Hamburger that some months after the second inoculation, during which time there may have been no evidence of infection, tubercular processes appear and develop like a primary infection. Whatever the true explanation, it is a fact that the tuberculous animal is more resistant to additional infection than the normal animal is to primary infection. This led Löwenstein<sup>2</sup> to say: "Only the tuberculous organism is tuberculosis-immune." Ham-

<sup>1</sup> Beiträge z. klin. d. Tuberculose, xii.

<sup>2</sup> Handbuch d. path. Mikroorganismen, Kolle u. Wassermann, zweite Auflage.

burger and Toyosuku<sup>1</sup> infected guinea-pigs subcutaneously, and after the disease had become chronic, they submitted these animals along with normal ones to a dust rich in tubercle bacilli. The normal animals developed pulmonary tuberculosis, while the tuberculous ones failed to do so. Römer<sup>2</sup> developed chronic subcutaneous tuberculosis, and then inoculated intracutaneously and intravenously, and in this way demonstrated the immunity of the tissues of the tuberculous animal to infection with tuberculosis. The submental and cervical glands of normal guinea-pigs become tuberculous on feeding with as small an amount as 0.1 mg. of living bacilli, but these glands are not affected when tuberculous guinea-pigs are fed with living bacilli. Many other investigators have experimented along the same line, with like results. That the unaffected tissues and organs of tuberculous men are largely immune to infection with the tubercle bacillus is a matter of every-day observation. In pulmonary tuberculosis the sputum laden with bacilli passes through the upper air passages without, as a rule, infecting them. Besides, there are cases of healed tuberculosis with virulent tubercle bacilli in their expectoration. There are tuberculosis carriers just as there are typhoid carriers.

Koch tried various methods in his attempts to immunize animals to tuberculosis. Early in his investigations he tried feeding animals with both living and dead cultures. For two months he fed rats exclusively on the bodies of animals dead from tuberculosis. From time to time a rat was killed, and most of them were found normal. In a few, small nodules were detected in the lungs. But these animals after feeding for weeks upon tubercular tissue, developed tuberculosis promptly when inoculated intraperitoneally. Later, Koch made the following statement: "All attempts to cause absorption of living or dead bacilli, by administration subcutaneously, intraperitoneally, or intravenously, have failed me and also other investigators. When dead bacilli are injected subcutaneously they

<sup>1</sup> Beiträge z. klin. d. Tuberkulose, xviii

<sup>2</sup> Ibid., xiii.

uniformly cause suppuration, and they can be easily stained and detected in large numbers in the abscesses thus formed after months. When injected into the peritoneal cavity they are better absorbed, and I have obtained some immunity in this way, but they generally cause local inflammations, which lead to adhesions with stenosis and occlusion of the intestine, so that a large percentage of the animals is lost. When injected intravenously into rabbits, dead bacilli cause tubercular nodules, similar to those observed after infection, in the lungs, and in these nodules the unaltered bacilli can be found after a long time. By this method absorption does not proceed in the desired way. Having been convinced that the unaltered bacilli could not be used, I attempted to render them absorbable through the action of chemical agents on them. The only method of this kind which I have found effective consists in boiling the bacilli with dilute mineral acid or with strong alkali. In this way tubercle bacilli may be so changed that they are absorbed *in toto*, and in large amount, though slowly, when administered subcutaneously. But marked immunity has not been reached in this way, and it seems that these chemical agents cause such thorough alteration in the bacillary substance that its immunizing property is destroyed."

This conclusion reached by Koch has been justified by all subsequent investigators. Levy<sup>1</sup> has tried to prepare a vaccine by such chemically indifferent substances as glycerin, 25 per cent. solution of milk sugar, and 10 to 25 per cent. solutions of urea. The object in these experiments was to kill the bacilli by the withdrawal of water and without changing their immunizing properties. Levy stated that these vaccines contain no living bacilli, and with them he apparently increased the resistance of guinea-pigs to infection with tubercle bacilli, but Römer doubts the complete killing of the bacilli by these agents. Heating the bacilli to 70° or 80° has failed to furnish an effective vaccine. Löwenstein<sup>2</sup> tried to prepare a vaccine by exposing tubercle

<sup>1</sup> Med. Klinik, 1905, 1906; Centralbl. f. Bak., xlii, xlvi, and xlvii.

<sup>2</sup> Zeitsch. f. Tuberkulose, 1905, vii.

cultures to daylight for a year, but this failed. The same investigator tried formalin, with a like result. Bartel<sup>1</sup> made a brie of tubercle bacilli and lymph glands, and Schröder tried a like experiment with spleen pulp, but neither of these preparations proved effective. Calmette and his students have tried preparations obtained by the action of iodine and its salts upon tubercle bacilli, but without success. Similar preparations with chlorine have been tried by Mossu and Goupil.<sup>2</sup> Noguchi<sup>3</sup> stated: "The inoculation of guinea-pigs with tubercle bacilli, which have been killed by soaps (sodium oleate), develops in these animals a complete or partial resistance to subsequent inoculation with a virulent culture of the same strains of bacilli. In short, a condition of immunity to tuberculosis can be induced in guinea-pigs by the injection of an emulsion of tubercle bacilli in oleic soaps." Zeuner,<sup>4</sup> following the work of Noguchi, has used an extract of tubercle bacilli in solution of sodium oleate in the treatment of tuberculosis. Broll<sup>5</sup> found that guinea-pigs treated with this preparation survived only a few weeks. Marxner<sup>6</sup> tried it on goats, and found no evidence of infection in two cases on postmortem, but Löwenstein<sup>7</sup> states that this is explained by the fact that the animals were sectioned too soon after inoculation, and adds that he sectioned goats three years after this treatment, followed by inoculation, and found tubercular cavities, the size of a man's head, in the lungs. Deycke and Much<sup>8</sup> found that, "One part of tubercle bacilli is dissolved in two parts of a 25 per cent. solution of neurin when kept at 52° for twenty-four hours. This forms a perfectly clear syrup which becomes cloudy on cooling. We attribute this phenomenon to the presence in the bacilli of fatty bodies with high melting-points." Later it was

<sup>1</sup> Wien. klin. Wochenschrift, 1905.

<sup>2</sup> Compt. Rend. de l'Acad., 1907.

<sup>3</sup> Centralbl. f. Bak., 1909, lii, 85.

<sup>4</sup> Zeitschf. f. Tuberkulose, xv.

<sup>5</sup> Berlin tierärztliche Wochenschrift., No. 47.

<sup>6</sup> Zeitsch. f. Immunitätsforschung, x, xi, xii.

<sup>8</sup> Beiträge z. klinik d. Tuberkulose, xv, Heft 2.

<sup>7</sup> Loc. cit.

found that solutions of cholin have a similar, but less marked, solvent action on tubercle bacilli; also that these solutions injected into men are without harmful effect. Attempts to immunize animals with these solutions have been made, without success. Aronson<sup>1</sup> has extracted fat from tubercle bacilli with trichlorethylen, and has attempted to immunize with the residue, but has not reported any success. Calmette and Guerin<sup>2</sup> have tested the protective action of tubercle bacilli grown on media containing bile acids. They stated in 1910 that by the tenth generation, these cultures of bovine tubercle bacilli become so attenuated that they can be used as a vaccine. Since these investigators have made no later report it is fair to assume that their expectations have not been realized. Attempts have been made to immunize animals to tuberculosis with the virulent bacillus by beginning with so small an amount as one bacillus (Webb and Williams) and increasing the dose; with cultures attenuated in varying degrees; with living and dead cultures of various varieties of the tubercle bacillus, such as human, bovine, avian, chicken, from cold-blooded animals, etc.; with strains of other acid-fast bacilli, as those of timothy, butter, manure, etc.; with normal and specific sera; but up to the present time no satisfactory results have been obtained. This is an interesting subject, and we would like to go into some detail, but it lies outside the scope of this book.

Löwenstein<sup>3</sup> has shown that the tubercle bacillus will grow, though not abundantly, on a medium of the following simple composition:

|                              |            |
|------------------------------|------------|
| Ammonium phosphate . . . . . | 6 parts    |
| Glycerin . . . . .           | 40 parts   |
| Distilled water . . . . .    | 1000 parts |

Although the growth is slow in developing and sparse, it elaborates an active tuberculin. This is additional evidence that growth of bacteria consists essentially of synthetical processes.

<sup>1</sup> Berl. klin. Woch., 1910 No. 35.

<sup>2</sup> Comp. rend. de l'Acad., cli, 1.

<sup>3</sup> Centralbl. f. Bak., 1913, lxxviii, 591.

## CHAPTER IX

### THE ANTHRAX PROTEIN<sup>1</sup>

**Literature.**—Since anthrax is the most typically infectious of all diseases, and since so many theories have been evolved concerning it, we may be pardoned for briefly reviewing the literature. As early as 1805 Kausch<sup>2</sup> wrote a monograph on this disease in which he held that it is due to paralysis of the nerves of respiration; but he offered no explanation of the paralysis. Delafond<sup>3</sup> held that anthrax has its origin in the influence of the chemical composition of the soil on the food, thus inducing pathological changes from malnutrition. The contagious nature of the disease was clearly established in 1845 by Gerlach.<sup>4</sup> This was confirmed by the studies of Heuzinger,<sup>5</sup> and was endorsed by Virchow in 1855, since which time it has never been questioned. However, as early as 1849 the bacilli had been seen by Pollender.<sup>6</sup> Pollender did not publish his observations until 1855, but he states that they were made in the fall of 1849. First, he examined the blood of five cows dead from anthrax, and compared this with material taken from the spleen of a healthy animal. The examinations were not made until from eighteen to twenty-four hours after death, and he states that the blood was stinking, thus indicating that it had become contaminated with putrefactive organisms, but the description which he gives

<sup>1</sup> The first part of this chapter is abstracted from a paper by J. Walter Vaughan, published in the *Trans. Assoc. Amer. Phys.*, 1902, xvii, 313.

<sup>2</sup> *Ueber der Milzbrand des Rindviehes.*

<sup>3</sup> *Traité sur la Maladie du Sang des Bêtes a laine*, 1843.

<sup>4</sup> *Magazin f. Thierheilkunde.*

<sup>5</sup> *Die Milzbrandkrankheiten der Thieren und der Menschen.*

<sup>6</sup> *Vierteljahresschrift f. gerichtliche Medicin*, 1855, viii, 103.

shows that he actually saw anthrax bacilli. He used a crude compound microscope made by Plössl, and he gave his attention to the blood corpuscles, chyle globules, and the bacilli. His description of the microorganisms may be condensed as follows: The third and most interesting microscopic bodies seen in anthrax blood are innumerable masses of rod-like, solid, opaque bodies, the length of which varies from  $\frac{1}{400}$  to  $\frac{1}{200}$  of a line, and the breadth averages  $\frac{1}{3000}$  of a line. They resemble the "vibrio bacillus" or "vibrio ambiguosus." They are non-motile and neither water nor dilute acids, nor strong alkalies have any effect upon them, and for this reason he concluded that they must be regarded as vegetable organisms. He questioned whether they existed in the blood of the living animal or resulted from putrefaction, but was inclined to believe the former, and thought they might represent the infecting organism, or at least the bearer of the infection. It will be seen that Polender presented no positive proof that these rod-like bodies had any causal relation to the disease. In 1856 Brauell<sup>1</sup> inoculated sheep, horses, and dogs with blood taken from animals sick with anthrax, and in this way demonstrated that the disease could be transmitted to sheep and horses, but not to dogs. He found sheep highly susceptible, horses less so, and dogs quite immune. He also demonstrated the presence of the bacilli in the blood of sick animals before death. It is interesting to note that he fell into an error concerning the motility of the bacilli. He states that when seen in fresh blood they are non-motile, but later they become highly motile. This was, of course, due to contamination. It should be noted that Brauell also made examinations of the blood of various domestic animals suffering from other diseases, and demonstrated the absence of the bacillus in these. In 1863 Davaine<sup>2</sup> published three valuable papers on anthrax. In the first he states that in 1850 Rayer inoculated sheep with the blood of others dead from anthrax, and in this way trans-

<sup>1</sup> Virchow's Archiv, 1857, xi, 132.

<sup>2</sup> Compt. Rend. de l'Academie des Sciences, lvii, 220, 351, 386.

mitted the disease. It appears that Rayer published a short note of this work in the *Bull. de la Soc. de Biologie* in 1850, but we have not had access to this publication. Davaine's own work was of the greatest value and shows great skill for that time. Probably the most important experiments that he made were those in which he demonstrated that the blood of an animal sick with anthrax is not capable of transmitting the disease to others unless it contains the bacillus. It may be of interest to describe briefly the experiments which led to the establishment of this fact. Rabbit A was inoculated with anthrax blood. Forty-six hours later, examination showed no bacilli in the blood of A. At that time twelve or fifteen drops of blood were taken from the ear of this animal and injected into rabbit B. Nine hours later the blood of A was reëxamined and found to contain a large number of bacilli. This blood was injected subcutaneously into rabbit C. One hour later rabbit A died, and twenty hours later C died, while B remained free from infection. Space will not permit us to follow the literature of anthrax further, save those parts that bear on the presence of a chemical poison. Pasteur, DeBarry, Koch, and others studied the morphology, life history, and cultural characteristics of the bacillus, and in this way founded the science of bacteriology. The reader is referred for the theories of the action of this bacillus to the works of Bollinger,<sup>1</sup> Szpilman,<sup>2</sup> Joffroy,<sup>3</sup> Touissant,<sup>4</sup> and Nencki.<sup>5</sup>

**Investigations.**—In 1877 Pasteur and Joubert<sup>6</sup> filtered anthrax cultures and the blood of animals sick of this disease through porcelain, and injected the germ-free filtrate into animals without inducing the disease, and concluded, quite properly, that this bacillus does not

<sup>1</sup> Zur Path. des Milzbrandes, 1872.

<sup>2</sup> Zeitsch. f. phys. Chem., 1880, iv, 350.

<sup>3</sup> Compt. Rend. Soc. de Biol., 1873 and 1874.

<sup>4</sup> Comp. Rend. de l'Acad., 1879, xci, 195; xciii, 163.

<sup>5</sup> Berichte d. deutsch. Chem. Gesellschaft, 1884, 2605.

<sup>6</sup> Comp. Rend. de l'Acad., lxxxiv, 905.

produce a soluble poison. Subsequent investigations, in our opinion, have established the correctness of this conclusion. However, there have been several claims to the discovery of soluble poisons in cultures of the anthrax bacillus, and in the bodies of animals dead with this disease, and we will now briefly review some of these claims which are of historical interest.

Hoffa<sup>1</sup> obtained from pure cultures of the anthrax bacillus small quantities of a substance which he believed to be a ptomain, and the specific poison of this disease. When injected under the skin of certain animals it at first increased the respiration and the action of the heart. After a short period the respirations became deep, slow, and irregular. Later the temperature fell below the normal, the pupils were dilated, and a bloody diarrhea set in. Autopsy showed the heart to be in systole, the blood dark, and ecchymoses were found on the pericardium and peritoneum. Subsequently, Hoffa believed that he had succeeded in isolating this substance from the bodies of animals dead of anthrax. He named it anthracin, and undoubtedly convinced himself for the time at least that he had discovered the specific poison of this disease. No subsequent investigator—and several have repeated the experiments—has been able to confirm Hoffa's results, and it is now more than probable that his "anthracin" resulted from the action of the agents used in its detection and separation upon the constituents of the fluids with which he worked.

In 1889 Hankin<sup>2</sup> grew the anthrax bacillus in Liebig's meat extract, to which fibrin had been added, and from this filtered culture he precipitated with ammonium sulphate an albumose, which, while not directly poisonous to animals when injected simultaneously with an inoculation of the anthrax bacillus, caused more speedy death than when the bacillus only was used. He concluded that the albumose destroys or lessens the natural resistance of the

<sup>1</sup> Ueber die Natur des Milzbrandgiftes, 1886.

<sup>2</sup> British Med. Jour., 1890, ii, 65; Proc. Royal Soc., xlviii, 93.

animal to the disease, after which the bacillus is able to continue the elaboration of its poison in the body.

Petermann<sup>1</sup> repeated Hankin's experiments, and obtained an albumose which elevates the temperature from one to two degrees, but is otherwise without poisonous effects and without protective influence against the anthrax bacillus. Hankin and Westbrook<sup>2</sup> repeated and modified the experiments of the former, and reached the following conclusions: (1) The anthrax bacillus elaborates a proteolytic ferment by means of which albumose may be formed from proteins, but these have no immunizing action. (2) The bacillus produces another albumose which is not due to the soluble ferment, but to an intracellular ferment. (3) This albumose was obtained in a relatively pure condition. This was done by growing the bacillus in a solution of pure peptone. It confers partial immunity against anthrax, when given in small doses to mice. (4) To animals susceptible to anthrax this albumose, in ordinary doses at least, is not poisonous. (5) Those animals which are relatively immune to anthrax, such as the rat and frog, are easily poisoned by this albumose. (6) On the contrary, young rats which are susceptible to anthrax are not poisoned by this substance.

Klemperer<sup>3</sup> obtained from cultures of the anthrax bacillus a substance which caused elevation of temperature when injected subcutaneously, but which was not submitted to further investigation. Brieger and Fränkel<sup>4</sup> endeavored to prepare a tox-albumin from the organs of animals dead of anthrax. They cut the tissue into fine pieces, rubbed up with water, allowed to stand for twelve hours in an ice-box, and filtered through porcelain. The filtrate was concentrated *in vacuo* at 30° to one-third its volume, and after being acidified with a few drops of acetic acid, was treated with ten times its volume of absolute alcohol. The mixture was then allowed to stand twelve hours longer in an ice-box, after which the precipitate was collected on a

<sup>1</sup> Ann. de l'Institut Pasteur, 1892, vi, 32.

<sup>2</sup> Ibid., vi, 633.

<sup>3</sup> Zeitsch. f. klin. Med., 1892, xx, 165.

<sup>4</sup> Berl. klin. Woch., 1890, xxvii, 241, 268, 1133.

filter, dissolved in a small volume of water, refiltered, reprecipitated with alcohol, this being repeated until a perfectly clear aqueous solution was obtained. The albumose was further purified by dialysis, and as thus obtained, it was found to be freely soluble in water and to give the ordinary reactions for proteins. These investigators failed to make any satisfactory study of this product, and the repetition of their work by others has led to negative results.

Marmier<sup>1</sup> attempted to isolate a poison from cultures grown in a medium of the following composition:

|                               |        |
|-------------------------------|--------|
| Water . . . . .               | 1000.0 |
| Peptone . . . . .             | 40.0   |
| Sodium chloride . . . . .     | 15.0   |
| Sodium phosphate . . . . .    | 0.5    |
| Potassium phosphate . . . . . | 0.2    |
| Glycerin . . . . .            | 40.0   |

Before inoculation this fluid was filtered through porcelain, and then sterilized at 110°. The peptone used was obtained from the commercial preparation by the precipitation of the albumoses with ammonium sulphate, and the salts were removed by dialysis. In this medium the anthrax bacillus, especially the sporeless form, grew abundantly. In order to obtain the poison the culture was filtered and saturated at room temperature with ammonium sulphate, which produced a more or less abundant precipitate. This was allowed to stand for some hours and filtered, after which the precipitate was washed with a saturated solution of ammonium sulphate. Subsequently the precipitate was dissolved in water, freed from salts by dialysis, concentrated, feebly acidified with sulphuric acid, and precipitated with alcohol. The substance thus obtained was found to be soluble in water and in a 1 per cent. solution of phenol. It was said not to give any of the reactions for albumoses or alkaloids, but this can scarcely be true. This work has had no confirmation, and is mentioned here simply because of its historical interest.

<sup>1</sup> Ann. de l'Institut Pasteur, 1895, ix, 533.

Heim and Geiger<sup>1</sup> grew anthrax bacilli in eggs after the method of Hueppe, extracted with alcohol, precipitated the extract with mercuric chloride, filtered, treated the filtrate with platinum chloride, decomposed the precipitate thus formed with hydrogen sulphide, filtered, rendered alkaline with potassium hydrate, and divided into two portions, one of which was extracted with ether and the other with benzol. The amount of material removed with ether was small, but that obtained in the benzol extract was large. When either of these residues was taken up in a few cubic centimeters of feebly acidified water and injected intra-abdominally into mice, it caused salivation and lacrymation, followed by muscular convulsions and death. The smallest dose of the benzol extract which proved to be fatal was 0.5 c.c., while a similar amount of the ether extract caused only transient symptoms. Apparently no controls were employed by these investigators, and the evidence that they obtained any poison from the anthrax bacillus is too slight to deserve serious attention.

Ivanow<sup>2</sup> has demonstrated the presence of certain volatile acids, formic, acetic, and caproic, in anthrax cultures, but there is no proof that the bacilli had anything to do with the production of these bodies, or that they are concerned in any way in the symptomatology or pathology of the disease; certainly these same volatile acids are found in the cultures of many bacteria, both pathogenic and non-pathogenic.

Petri and Massen<sup>3</sup> detected hydrogen sulphide in anthrax cultures, but inasmuch as at the same time they found this gas in every one of the thirty-six other bacteria examined, it cannot be said to be of any specific importance. Moreover, spectroscopic examination of anthrax blood fails to show the presence of hydrogen sulphide or any of its compounds, and there is no evidence that this gas has any connection with the disease.

<sup>1</sup> Lehrbuch der Bakteriolog. Untersuchungen u. Diagnostik, 1894, 229.

<sup>2</sup> Ann. de l'Institut Pasteur, 1892, vi, 131.

<sup>3</sup> Arbeiten aus d. kaiserlich. Gesundheitsamte, 1893, viii, 318.

Fermi<sup>1</sup> has shown the presence of both diastatic and proteolytic ferments in anthrax cultures, but as all living cells, including bacteria, elaborate such ferments, this discovery fails to make us acquainted with the poison of the anthrax protein. Maumus<sup>2</sup> found that by its growth on potato the anthrax bacillus converts some starch into sugar, and Reyer<sup>3</sup> showed the presence in anthrax cultures of a ferment which coagulates casein.

Klein<sup>4</sup> removed anthrax bacilli from agar cultures of forty-eight hours' growth, placed them in 5 c.c. of bouillon, and after the tube had been held for five minutes in boiling water, injected the contents into the peritoneal cavity of a guinea-pig, without results. After a few days the injection was repeated, and four or five days later these animals were inoculated subcutaneously and intra-abdominally, with small doses of a living culture. All died within forty-eight hours, of typical anthrax. From these experiments Klein concluded that the anthrax bacillus contains no intracellular poison, and that treatment with the cellular substance confers no immunity on guinea-pigs.

Conradi<sup>5</sup> attempted to solve the question of the existence of an anthrax poison by the following methods:

1. Guinea-pigs were inoculated intraperitoneally with anthrax, and immediately after death the peritoneal fluid, which varied in amount in different individuals, but averaged from 10 to 15 c.c., and contained in each field from ten to twenty microorganisms, was filtered through porcelain. In some of the experiments the filter of Chamberland was employed while in others that of Kitasato was used. The filtered peritoneal exudate was injected into mice, rats, and guinea-pigs subcutaneously, intravenously, and intraperitoneally, and always without effect. The amounts of the filtered exudate injected into mice varied from 2 to 4 c.c.;

<sup>1</sup> Arch. f. Hygiene, 1890, x, 1.

<sup>2</sup> Compt. Rend. de la Soc. de Biologie, 1893, v, 1071.

<sup>3</sup> Ibid., 309.

<sup>4</sup> Centralbl. f. Bakteriologie, 1894, xv, 598.

<sup>5</sup> Zeitsch. f. Hygiene, 1899, xxxi, 287.

that into rats from 5 to 12 c.c.; in guinea-pigs from 4 to 15 c.c.; in rabbits from 10 to 20 c.c.; and in one dog, 25 c.c. was injected subcutaneously. In proportion to body weight the mice received by far the larger injections. The experiments indicate that in the peritoneal exudate of guinea-pigs inoculated with anthrax there is no appreciable amount of soluble toxin.

2. Many guinea-pigs were inoculated with anthrax, and directly after death their livers and spleens were removed under aseptic conditions and rubbed up in a sterilized mortar with sterile sand to which a little physiological salt solution had been added. After thorough rubbing the mixture was diluted with physiological salt solution and filtered through a Chamberland tube under four atmospheres of pressure. The filtrate was injected subcutaneously, intravenously, and intraperitoneally into mice, rats, guinea-pigs, and rabbits, and in every case without effect.

3. Conradi, finding the preparation of collodion sacs difficult, substituted for them the vegetable membranes from phragmites communis, first used by Podbelsky.<sup>1</sup> These sacs, after sterilization, were filled with bouillon cultures of the anthrax bacillus, and after laparotomy under ether were placed in the abdominal cavities of animals. The animals used were guinea-pigs, rabbits, and dogs, all of which remained well, notwithstanding the presence of these tubes containing virulent cultures of the anthrax bacillus in their abdominal cavities. These experiments satisfied Conradi that the anthrax bacillus does not produce any soluble toxin, and he next turned his attention to determining whether or not this organism possesses any intracellular poison.

4. The anthrax exudates obtained as described in the first series, in quantities of from 5 to 6 c.c., were placed in test-tubes, 0.5 c.c. of toluol added to each, the tube closed with a sterilized cork, thoroughly shaken, and then allowed to

<sup>1</sup> To one experienced in the preparation of collodion sacs this must be regarded as a clumsy substitution.

stand for ten days in the dark at room temperature. At the expiration of this time the contents of many tubes were placed in a separator and the toluol removed. Having shown by inoculation that the germ contained in these tubes was dead, the exudate thus sterilized was injected into susceptible animals subcutaneously, without effect.

5. Asporogenous cultures were sterilized by exposure for one hundred and ten hours to  $-16^{\circ}$ . After exposure to this temperature, the tubes were kept in an incubator at  $20^{\circ}$ , long enough to see that they remained sterile, after which they were injected subcutaneously into susceptible animals, and always without effect.

6. A number of rabbits and guinea-pigs were simultaneously infected with anthrax, and after death the livers and spleens were subjected to a hydraulic pressure of 500 atmospheres. The fluid thus obtained, which on microscopic examination showed the presence of bacteria, was passed through a Chamberland filter, and then injected subcutaneously, intraperitoneally, and intravenously into mice, rats, guinea-pigs, and rabbits, and always without effect.

7. The experiment of Brieger and Fränkel in which they prepared their anthrax toxalbumin was repeated with negative results.

From these experiments Conradi reaches the following conclusions: "By no method known at present can it be shown that the anthrax bacillus forms either an extracellular or an intracellular poison within the bodies of either susceptible or insusceptible animals. Indeed, these experiments increase the probability that the anthrax bacillus does not form any poisonous substance, therefore the solution of the manner in which anthrax infection results remains unknown. Whether improved chemical methods will lead to its detection or not cannot be determined, but for the present the anthrax bacillus at least must be regarded as a purely infectious microorganism." If this conclusion reached by Conradi be true, the mechanical interference theory is the best that can at present be offered so far as anthrax is concerned.

In all our work the anthrax bacillus has been grown in Roux flasks, as we have not dared try it in the large tanks, consequently the amount of cellular substance obtained has been small. This work was begun in 1900 and carried on intermittently. We have felt it desirable to exercise great care in handling quantities of the anthrax bacillus. We have always opened the flasks and removed the growth over a shallow tray containing some powerful germicide. We will make a few extracts from our protocols. In May, 1900, the growth was removed from twelve Roux flasks, placed in one liter of 1 per cent. sulphuric acid, and kept in the incubator at 37° for twenty-four hours. At the expiration of this time cultures showed the bacillus still alive. The suspension was then placed in the autoclave and heated at 100° for thirty minutes. Cultures now showed that the bacillus had been killed. The suspension was passed through a Chamberland filter with the aid of a pump. The clear filtrate was poured drop by drop into twice its volume of absolute alcohol. A finely flocculent, white precipitate formed, was collected on a hard paper, washed with alcohol until the filtrate was no longer acid, and dried *in vacuo* over potash. This substance is not colored with nitric acid and heat, but on the addition of ammonia the characteristic orange of the xanthoproteic test is developed beautifully. It does not give the biuret and other protein tests. Later an unweighed portion of this substance was dissolved in water and injected intra-abdominally in a guinea-pig. Twelve hours later the animal was found dead. The heart was in partial diastole with clots in both auricles and ventricles. The peritoneal cavity contained a few cubic centimeters of a clear fluid.

Two days later a guinea-pig received intra-abdominally 60 mg. of the alcoholic precipitate. When last seen that night, five hours after the injection, the animal was dying. The next morning it was posted and the condition described above was found. The heart's blood was found to be sterile. This experiment was repeated a number of times, with similar results.

This work was not resumed until 1902, and on repeating the work the alcoholic precipitate failed to manifest any poisonous action, but in this instance we heated the acid extract for two hours. Inasmuch as the prolonged heating seemed to be the only difference in the methods of procedure, another trial was made in which the acid extract was heated in the autoclave at  $110^{\circ}$  for exactly ten minutes. The alcoholic precipitate freed from acid as before was ground to a fine powder in an agate mortar. An unweighed portion of this powder was dissolved in 5 c.c. of water and injected intra-abdominally. When last seen that night, nine hours after the injection, the breathing was difficult and irregular. The animal was found dead the next morning. Autopsy showed extreme subcutaneous edema over the abdomen. The peritoneal cavity contained a few cubic centimeters of a clear fluid, and a smaller amount of bloody exudate was found in the pleural cavity. The heart was in diastole and the most marked changes were found in the lungs. These were greatly congested and the left upper lobe seemed to be consolidated. Closer examination showed portions of the lungs to be completely hepatized. Many of the air cells were filled with exudate and blood corpuscles. The kidneys were highly congested and the liver seemed pale and friable. Further experiments with weighed portions of the powder showed the minimum fatal dose for a guinea-pig when given intra-abdominally to be about 50 mg. Smaller doses down to 15 mg. made the animals very sick, but failed to kill.

The poisonous group obtained from the anthrax protein by cleavage with 1 per cent. sulphuric acid is destroyed, at least greatly weakened, by prolonged boiling in aqueous solution.

The cellular substance of the anthrax bacillus, prepared by our method, is the least toxic of all the bacterial proteins with which we have worked. This is true whether the cell protein is derived from a pathogenic or a non-pathogenic organism. It requires not less than 50 mg. of the cell substance, after extraction with alcohol and ether, to

kill a guinea-pig after intra-abdominal injection. Smaller doses make the animals sick, but do not kill, and confer no marked immunity to inoculation with living cultures.

The cell substance has been split up by our method into poisonous and non-poisonous portions. The former differs in no recognizable way from the poisonous group obtained from other proteins, and treatment of animals with the latter fails to establish any noticeable immunity to inoculation with the bacillus.

It has been shown that the poisonous group in the cellular protein of a non-pathogenic bacillus may be more effective than that in the anthrax bacillus. The minimum lethal dose of the air-dried cell of the prodigiosus for a guinea-pig of from 200 to 300 grams body weight, when injected intra-abdominally, is less than 3 mg., while that of the anthrax bacillus for the same animal is about 200 mg. Even the lemon sarcine, the least toxic of the non-pathogenic organisms examined, surpasses the anthrax bacillus in toxic action. These facts convince us that the pathogenicity of a bacterium is not measured by its capability of furnishing a poisonous group, but by its ability to grow and multiply in the animal body. The high degree of infectivity shown by the anthrax bacillus in some animals is due to the fact that it grows without hindrance on the part of the secretions of those animals. On the other hand, its failure to infect other species is due to the inhibiting action of certain secretions of these animals.

Like other proteins, that of the anthrax bacillus contains a poisonous group. The chief constituent of the anthrax bacillus is a glyconucleoprotein, and by this we do not mean a physical mixture of carbohydrate, nuclein, and protein, but a molecule containing these constituents as atomic groups. The intracellular poisons contained in bacterial cells are not preformed toxins, as supposed by Pfeiffer, but are atomic groups in a complex molecule. The poison can be obtained from the protein only by processes which disrupt the molecule. Mere solvents, such as water, alcohol, ether, saline solution, and glycerin,

do not detach the poisonous group. We have obtained poisonous substances from the anthrax bacillus by two methods. The substances thus obtained differ physically, chemically, and physiologically. The one obtained by the action of 1 per cent. sulphuric acid is insoluble in alcohol and does not give the distinctive protein reactions. The one obtained by cleavage of the bacterial cell with a 2 per cent. solution of sodium hydroxide in absolute alcohol is soluble in alcohol and does give the biuret and Millon reactions. The former kills only after some hours, and leaves marked pathological changes. The other kills in a few minutes and leaves no gross alterations. This, however, does not prove that the poisonous group in the two preparations is not the same. It may be that in the one the poisonous group is still closely attached to other groups, and energetic measures may be necessary to tear it off, and as a result of this the injury done to the body cells and recognized at autopsy may be due. In the other preparation the poisonous group is already detached, and consequently its effects are manifest immediately. On the other hand, our work does not show that the poisonous group in the two preparations is the same. It leaves this question quite undetermined. As we have stated elsewhere, there is probably in the protein molecule a whole spectrum of poisons, one derivable from the other, a chain of poisonous groups, one differing from the one next it by having one more or one less link. There is at present no more important and no more difficult subject than that of the chemistry of the protein molecule. The researches of Fischer have done much to show some features of the structure of the protein molecule. We know, as a result of Fischer's work, that proteins are to be regarded as polymers or condensation products of the amino acids, but between the native protein and the amino acids into which it may be split, there is a long list of intermediary products about which we know practically nothing. The ordinary, native proteins are not primarily poisons. The amino-acids which result from their ultimate cleavage are not poisonous, but between

the two there are many split products, of varied sizes, which are poisonous. Besides, some of the amino-acids may be converted into highly poisonous substances. Whether a given protein molecule, on being disrupted, supplies an active poison or not is determined by the lines of cleavage and these are dependent upon the cleavage agent and the conditions under which it acts. The work of Fischer, as valuable as it is, has been and is of little or no service in elucidating the processes of parenteral digestion, which must be better understood before we can read the first line in the true history of disease, either exogenous or endogenous.

Rosenau and Anderson<sup>1</sup> sensitized animals by subcutaneous injections of extracts of the anthrax bacillus. Sobernheim<sup>2</sup> was not able to confirm this work, and made some statements that deserve attention. He said that the cell substance of the anthrax bacillus is quite different chemically and biologically from that of other bacteria, and that it is wholly devoid of poisonous properties, whatever the amount and method of administration may be. Our own work, as already stated, shows that this is not true. Busson<sup>3</sup> has reinvestigated this question of sensitization with anthrax protein. Preisz<sup>4</sup> has shown that when the anthrax bacillus is grown at a high temperature (42.5° C.) after the manner used by Pasteur in preparing his vaccine, the membrane becomes mucilaginous and more permeable. With bacilli thus prepared Busson succeeded in inducing a mild form of sensitization by intraperitoneal injections. The sensitized state was recognized by a more marked elevation of temperature over the controls on reinjection. It is undoubtedly true that the anthrax bacillus is protected by its capsule against the action of ferments produced in the bodies of infected animals, but that anthrax protein is so radically different from other

<sup>1</sup> Hygienic Lab. Bull., 1907, No. 36.

<sup>2</sup> Kraus und Levaditi, Handbuch d. Technik u. Methodik d. Immunitätsforschung, ii.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, 1912, xii, 671

<sup>4</sup> Centralbl. f. Bak., 1911, lviii.

bacterial proteins is an unwarranted assumption, and that it is not poisonous in any dose we have shown not to be true. When the anthrax protein is obtained in solution without alteration of its constitution, and when this solution is properly administered we dare say that it will be found to sensitize animals as well as any other protein. As we have had occasion to point out more than once, it is necessary to have a protein in solution in order to develop exquisite sensitization, and it must be in solution on reinjection in order to induce the most striking form of anaphylactic shock. Permeation of the body cells seems to be essential to the most complete sensitization, also to its demonstration on reinjection.

Roos<sup>1</sup> has shown that salvarsan is an efficient germicide for the anthrax bacillus, both *in vitro* and *in vivo*, and Becker<sup>2</sup> and Bettman<sup>3</sup> have successfully treated anthrax in man with this preparation.

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1912, xv, 487.

<sup>2</sup> Deutsch. med. Woch., 1911.

<sup>3</sup> Ibid., 1912.

## CHAPTER X

### THE CELLULAR SUBSTANCE OF THE PNEUMOCOCCUS<sup>1</sup>

**The Strain.**—The strain of the pneumococcus with which this work was done was presented us by Dr. J. J. Kinyoun, of Washington. When the culture was received, a mouse, guinea-pig, and rabbit received intraperitoneal inoculations. The mouse died in twenty-three hours, the guinea-pig in twenty-four, and the rabbit in twenty-seven. Cultures were made from the heart blood of each of these animals, and all found to be pure. Our growths were made in 5 per cent. glycerin bouillon, and with these, Roux flasks and the tanks containing a medium made of 3 per cent. of agar and 1 per cent. of ox serum in the 5 per cent. glycerin bouillon were inoculated. The flasks and tanks were kept at 38° for four days, when the growth was removed. The growth seemed to reach maturity in this time at the temperature mentioned. When kept longer it began to dry and contract. In some instances the growth was not harvested until the sixth day. The cellular substance thus obtained was handled in the usual manner, *i. e.*, it was thoroughly extracted with alcohol and ether.

The strain was found to be highly virulent and remained so throughout the year of work with it. Fig. 10 shows the effects of the living organism on guinea-pigs after intra-abdominal inoculation of 0.00001, 0.000001, and 0.0000001 c.c. of a twenty-four-hour bouillon culture.

Fig. 11 shows the relative effects of the living organism and 5 mg. of the cellular substance. It will be seen

<sup>1</sup> The first part of this chapter is founded on work done in the Hygienic Laboratory of the University of Michigan in 1905-06 by Dr. J. F. Munson.



good with some variations in the exact time when the fall began in all our experiments with the living organism when twenty-four-hour cultures were employed. During the time before the fall begins, the organism is growing and multiplying. The fall indicates dissolution of the cell and the liberation of its poisonous constituents. When older cultures are used, especially when the amount is large, the fall in temperature may appear much earlier, and is due to the presence of autolyzed cells in the cultures. This is true not only of the pneumococcus, but of the cholera, typhoid, and other bacteria.

The cellular substance of the pneumococcus, prepared by our method, is a white powder, in which the individual cells readily take the stains, and it is found to be quite free from debris. We administered it in suspension in sterile salt solution, and generally intraperitoneally. It seems to be more irritating than other bacterial cellular substances with which we have worked, and even 5 mg. suspended in 5 c.c. of salt solution and injected into the peritoneal cavity seems to cause pain. The animal soon becomes quite normal in appearance, and remains so for an hour or two, when the fur behind the ears begins to roughen and gradually the whole coat takes on this state. The posterior extremities become weak and the animal is unable to maintain the erect posture. The weakness intensifies into a paralytic state, and finally the animal lies stretched out on its side, and seems quite unable to make a struggle. Rarely there are convulsions, but, as a rule, respiration slowly and quietly fails, and it is often difficult to tell just when it stops. Following these symptoms, the temperature which at first may be slightly elevated gradually falls, and has been frequently at 85°, rarely at 75°, before respiration wholly ceases. When the dose is a non-fatal one the lowest point is usually reached about the seventh hour, when the temperature rises as gradually as it fell. The cellular substance of the pneumococcus is not highly poisonous compared with similar preparations from other bacteria. It is rather interesting

to make some comparisons here. As has been stated, the strain with which our work was done was highly virulent, killing half-grown guinea-pigs in doses of 0.0000001 c.c. of a twenty-four-hour culture given intraperitoneally. At the same time our old stock culture of the pneumococcus did not kill in doses of less than 1 c.c., and yet the cellular substances of the two, measured by toxicity, were practically the same. This and similar observations with other bacteria lead us to conclude that virulence is measured by rate of multiplication and not by chemical differences in cellular poison content. Moreover, when two animals were killed with the two strains the cells seemed to be as abundant in one as in the other. The more virulent strain multiplies the faster. Virulence may depend upon several factors, but rate of multiplication is certainly one of them, and on a common medium as the animal body this must depend upon the effectiveness of the ferments whose function it is to prepare and utilize the pabulum on which the organism feeds. Our highly virulent strain furnished a cellular substance which killed guinea-pigs in doses of 1 to 10,000. Occasionally smaller doses killed. The smallest fatal dose on first injection of which we have a record was 1 to 19,000, but the surely fatal minimum was 1 to 10,000, and as we have stated, the less virulent strain of pneumococcus furnished cellular substance of the same degree of toxicity. A comparison of the virulent strain of the pneumococcus with our strain of colon is also of interest. With our colon bacillus the minimum constantly fatal dose was 1 c.c. of a twenty-four-hour bouillon culture. Sometimes a dose of 0.5 c.c. killed, and the smallest fatal dose, as we found it, was 0.25 c.c. This organism yielded a cellular substance, which as a coarsely ground powder always killed 1 to 50,000; when finely ground it killed 1 to 75,000, and sometimes as high as 1 to 2,000,000. Our virulent pneumococcus killed in 0.0000001 c.c. doses, and yielded a cellular substance which, when ground to the finest possible powder, killed only 1 to 10,000. Surely these are strong arguments for our belief that the pathogenicity of a microorganism is

not measured by its poisonous cell content, but by the rate with which it multiplies in the animal body or the intensity and rapidity with which it converts body proteins into its own proteins.

It must be borne in mind in considering what we are about to say in this paragraph that at the time these experiments were conducted we knew but little about protein sensitization, and they were not conducted with the phenomena of sensitization in view. Had we known then what we now know the lines of investigation would have been drawn somewhat differently. However, this makes a review of our old protocols all the more interesting and valuable. We tried to immunize animals with the cellular substance. It will be worth while to follow one set of these experiments through. We take the three tables on p. 210 just as they stand in the protocol.

It will be observed that in the second and third injections made at intervals of five and six days we killed one-third of our animals. Now we know that this was due to the fact that we partially sensitized the animals.

Failing absolutely to even render our animals tolerant to the dead germ substance, we tried to weaken it by heat, but in this we were equally unsuccessful. However, we did prove that heating the cellular substance of the pneumococcus to 144° for five minutes in the autoclave does not destroy its intracellular poison. We also found that by heating the cells some of the poison passes into solution, and may be filtered through porcelain.

We split up the cellular substance with a 2 per cent. solution of sodium hydroxide in absolute alcohol, and obtained a non-poisonous and a poisonous portion. In both small and large doses the former had no visible effect on animals, but it gave no immunity to subsequent inoculations.

The poisonous fraction kills animals in about the same doses as are required by similar preparations from other proteins. The symptoms are not wholly identical with those induced by poisons obtained from other proteins.

TABLE XXI.—MARCH 9, 1905. CAGE VI. PNEUMOCOCCUS GERM SUBSTANCE;  
DOSES SUSPENDED IN 5 C.C. SALT SOLUTION.

| Dose given at | Pigs.               | Weight. | Dose, mg. | 1/ B. W. | Normal temp | 1 hr. temp. | 2 hr. temp. | 3 hr. temp. | 4 hr. temp. | 5 hr. temp. | 7 hr. temp. | 10 hr. temp. | 21 hr. temp. | 26 hr. temp. |
|---------------|---------------------|---------|-----------|----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|--------------|--------------|
| 1 12.20       | Black               | 340     | 2.5       | 136,000  | 100.0       | 102.9       | 104.3       | 99.0        | 98.9        | 98.0        | 96.7        | 99.0         | 99.7         | 99.5         |
| 2 12.18       | Gray                | 350     | 5.0       | 70,000   | 100.2       | 101.5       | 103.4       | 100.4       | 97.1 +      | 96.6        | 94.2        | 95.0         | 94.8         | 98.2         |
| 3 12.17       | Gray curly          | 370     | 10.0      | 37,000   | 100.3       | 100.1       | 99.9        | 97.6        | 97.6        | 98.8        | 97.4        | 94.2         | 99.2         | 100.8        |
| 4 12.15       | Wh., yel.,<br>black | 390     | 15.0      | 26,000   | 100.8       | 101.3       | 101.2       | 96.4        | 98.1        | 98.6        | 95.8        | 94.2         | 97.6         | 101.4        |
| 5 12.10       | Yel., white         | 395     | 20.0      | 19,750   | 101.0       | 101.7       | 102.4       | 99.2        | 98.9        | 97.6        | 94.6        | 96.8         | 96.6         | 100.9 +      |
| 6 12.05       | Yellow              | 625     | 25.0      | 25,000   | 101.2       | 102.0       | 102.7       | 101.0       | 98.1        | 97.3        | 97.6        | 97.4         | 99.3         | 102.4        |

TABLE XXII.—MARCH 14, 1905. THIS IS, SO FAR AS POSSIBLE, AN EXACT  
REPETITION OF THE WORK OF MARCH 9, 1905, USING THE SAME PIGS. DOSES  
GIVEN AT 11.45 A.M.

| Pigs.                   | Weight. | Dose, mg. | 1/ B. W. | Normal temp | 1 hr. temp. | 2 hr. temp. | 3 hr. temp. | 4 hr. temp. | 5 hr. temp. | 7 hr. temp. | 8½ hr. temp. | 10 hr. temp. | 12 hr. temp. |      |
|-------------------------|---------|-----------|----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|--------------|--------------|--------------|------|
| 1 Black                 | 521     | 2.5       | 208,400  | 100.9       | 102.6       | 105.0       | 103.0       | 98.4        | 98.8        | 96.4        | 102.3        | 101.0        | 101.9        |      |
| 2 Gray                  | 508     | 5.0       | 101,600  | 100.2       | 105.4       | 103.0       | 103.8       | 100.2       | 100.1       | 98.7        | 101.2        | 100.6        | 100.8        |      |
| 3 Gray, curly           | 545     | 10.0      | 54,550   | 101.7       | 103.3       | 104.1       | 102.4       | 99.3        | 98.4        | 95.3        | 100.2        | 101.0        | 101.8        |      |
| 4 Yel., white,<br>black | 542     | 15.0      | 36,133   | 101.2       | 102.7       | 100.5       | 100.0       | 99.8        | 98.1        | 98.1        | 90.7         | 91.4         | 95.4         | Died |
| 5 Yel., white           | 545     | 20.0      | 27,250   | 100.4       | 100.6       | 101.2       | 97.9        | 96.6        | 95.2        | 87.8        | 86.7         | 88.7         | 90.3         | Died |
| 6 Yellow                | 601     | 25.0      | 24,040   | 102.0       | 103.0       | 103.7       | 100.1       | 100.0       | 97.0        | 91.4        | 92.5         | 97.0         | 97.3         |      |

TABLE XXIII.—MARCH 20, 1905. CAGE VI. THE USUAL DOSES WERE GIVEN  
(THIRD TIME). ANIMALS REACTED MORE SEVERELY THAN BEFORE. AN IMPRESSION  
DRAWN FROM THEIR BEHAVIOR. NOS. 3 AND 6 DIED. PLACING IN INCUBATOR DID  
NOT SAVE THEM

| Pigs.         | Weight. | Dose, mg. | 1/ B. W. | Normal temp | 1 hr. temp. | 2 hr. temp. | 3 hr. temp. | 4½ hr. temp. | 6 hr. temp. | 7½ hr. temp. | 9½ hr. temp. | 10½ hr. temp. | 15 hr. temp. |      |
|---------------|---------|-----------|----------|-------------|-------------|-------------|-------------|--------------|-------------|--------------|--------------|---------------|--------------|------|
| 1 Black       | 550     | 2.5       | ....     | 99.6        | 103.4       | 104.3       | 102.6       | 96.4         | 90.5        | 91.4         | 91.4         | 90.5          | 96.7         |      |
| 2 Gray        | 542     | 5.0       | ....     | 101.0       | 103.6       | 103.2       | 101.7       | 97.1         | 92.3        | 92.7         | 95.0         | 93.2          | 98.2         |      |
| 3 Gray, curly | 552     | 10.0      | ....     | 100.6       | 100.0       | 100.3       | 96.0        | 86.9         | 86.9        | 84.2         | 96.8         | ....          | ....         | Dead |
| 6 Yellow      | 609     | 25.0      | ....     | 101.0       | 98.2        | 97.8        | 93.2        | 90.5         | 86.0        | 86.0         | 95.9         | ....          | ....         | Dead |

The temperatures of Nos. 3 and 6 at nine and one-half hours rose because they were placed in the incubator.

The difference lies in the less marked convulsive character of the third stage. When injected intraperitoneally in guinea-pigs, the coat soon roughens, and for some minutes the animal seems quiet. Weakness of the hind limbs develops and the upper part of the body is shaken by spasms resembling severe hiccough. The paralysis rapidly develops and spreads, and the animal lies on one side. It dies in most instances without general convulsions. The respiration becomes slower and the heart continues to pulsate for some minutes after respiration has stopped. Death occurs in from twenty minutes to an hour. The temperature curve begins to fall soon after the injection, and continues until death. In cases of recovery the first sign of improvement is a rise in temperature, and it comes up more slowly than it went down.

It is worthy of note that in case of inoculation with the living organism there is an incubation period of about ten hours. This is followed by the complete triumph of the infection, and is shown by the even and constant fall in temperature. With the dead cellular substance the incubation period is shortened, but the character of the fall is the same. With the free poison there is no period of incubation, and the temperature begins to fall in a few minutes. In all, the temperature curve is the same in general character. Certainly, it must be true that the poison which affects and kills the animal must be the same in the living and dead cell, and in the split product.

We demonstrated that the poisonous portion, like that obtained from other proteins, establishes on repeated injections of non-fatal doses a certain degree of tolerance, but gives no immunity against infection.

The recent work of Rosenow<sup>1</sup> on the autolytic cleavage products of the pneumococcus, and certain other bacteria is of great interest and value. The pneumococcus readily undergoes autolysis, and Rosenow has studied the products resulting in this way. The following are some of the more

<sup>1</sup> Jour. Infect. Dis., ix, 190; x, 113; xi, 286, 480.

important facts demonstrated by this investigator: (1) Animals may be sensitized with dead pneumococci or with extracts from the same. The sensitizing dose may be given subcutaneously, intraperitoneally, intravenously, or intrapleurally. In order to induce anaphylactic shock the reinjection must be made intravenously or intracardiacly. In the sensitized animal both dead and living pneumococci are dissolved more rapidly than in normal animals. This explains the slight but definite immunity to virulent cultures manifested by sensitized animals. (2) Fresh pneumococci suspended in salt solution and kept at 37° for forty-eight hours, under ether or over chloroform, undergo autolysis by which a poison is liberated. This poison injected intravenously or intracardiacly in normal animals causes anaphylactic shock. In guinea-pigs this poison induces death by spasm of the bronchioles and consequent arrest of respiration. In dogs it causes marked fall in blood-pressure and delays the coagulation of the blood. This poison is split off from the pneumococcus protein not only in autolysis, but also by normal and immune sera and by leukocytic extracts. (3) The cleavage of pneumococcus cell substance by autolysis or the other agents mentioned, is accomplished by proteolytic ferments, as is shown by increased production of amino bodies as the poison is set free. Finally, the digestive process reaches a point when the poison itself is digested and rendered inert. "The fact that virulent pneumococci have within themselves a proteolytic enzyme which splits their protein into a highly toxic substance, is strong indication that certain strains of pneumococci may cause infection forthwith without first rendering the host allergic. This is quite in keeping with the fact that in pneumococcus infections an incubation period is not an invariable rule. On the other hand, in certain instances, a previous sensitization before symptoms set in, probably occurs. This might well be the case in lobar pneumonia when the chill occurs a week or ten days after the patient contracted a severe cold or bronchitis. The distribution by lobes in typical cases may be related to

the bronchial spasm which this toxic substance produces. That early dyspnea and increased respiration before consolidation is demonstrable is in keeping with this idea. (4) Morphine, ether, urethane, atropine, and adrenalin, protect normal guinea-pigs against the toxic material obtained *in vitro* from pneumococci, and also sensitized guinea-pigs on reinjection."

Recently (December, 1912) we found a small bottle of the powdered pneumococcus cellular substance prepared by Munson nearly seven years before (March, 1906). It is a fine, yellowish-white powder, looking very much like wheat flour. It has stood during these years in a cupboard, kept closed except when momentarily opened to put something in or take something out. Microscopic examination showed the pneumococci as clearly and in as perfect form as in a fresh preparation. It kills guinea-pigs on intra-abdominal injection in the same doses (1 to 10,000 of body weight), and just as promptly as it did more than six years ago. Five hundred milligrams of this was weighed, suspended in 500 c.c. of salt solution, 10 c.c. of chloroform added, and the whole allowed to stand at 37°. After twenty-four hours, 10 c.c. of the opalescent supernatant fluid was injected into the external jugular vein of a guinea-pig. Within two hours the rectal temperature had fallen below 94°, and the animal remained sick for some hours, but gradually recovered. The same experiment repeated at the end of forty-eight and seventy-two hours killed the guinea-pigs within two hours. These animals died with the symptoms of a subacute anaphylactic shock. We conclude from this that the intracellular autolytic ferment had remained intact during the years that had elapsed since the preparation of the cellular protein. Six days after the suspension had been prepared and placed in the incubator a like injection killed the guinea-pig within three minutes. This animal died with the symptoms of acute anaphylactic shock, and autopsy showed the lungs distended and minute petechial hemorrhages in the pericardium.

## CHAPTER XI

### PROTEIN SENSITIZATION OR ANAPHYLAXIS

**Introduction.**—The older medical literature occasionally records facts which in the light of more recent and extended knowledge are known as the phenomena of protein sensitization. Such were some of the experiences recorded in the early attempts at the transfusion of blood. Many of the untoward results reached in this procedure and beyond the ken of that time are now fully explained. Behring and Kitashima<sup>1</sup> found on immunizing an animal to tetanus toxin that it died in convulsions notwithstanding the fact that the blood serum was richly charged with antitoxin. They explained this by assuming the existence of a condition of “hypersensitiveness” to the toxin. With our present knowledge we see no reason for ascribing this to the toxin. There is, so far as we know, no evidence that animals can be rendered hypersensitive to either toxin or antitoxin. Neither has ever been obtained free from proteins, and since all true proteins, so far as we know, sensitize, there seems no sufficient justification in ascribing a sensitization induced by a protein solution containing a toxin to the latter. Buchner<sup>2</sup> repeatedly injected bacterial proteins into men and noticed that the cardinal indications of local inflammation, tumor, rubor, dolor, and calor resulted. Furthermore, he noted that fever increased with repeated injections. Krehl and Matthes<sup>3</sup> induced fever in animals by repeated injections of albumose and peptone. Weichardt<sup>4</sup> made an advanced study in the domain which

<sup>1</sup> Berl. klin. Woch., 1901, No. 6.

<sup>2</sup> Berl. klin. Woch., 1890, 216; Münch. med. Woch., 1891, No. 3.

<sup>3</sup> Arch. f. exper. Path. u. Pharm., 1895, xxxv, 232; *ibid.*, 1896, xxxvi, 437.

<sup>4</sup> Berl. klin. Woch., 1903, No. 1.

we now designate as anaphylaxis. He repeatedly treated rabbits with protein expressed from placental cells, and found that some of these died promptly on subsequent injections. Furthermore, he mixed the serum of animals thus treated with placental cells and obtained a soluble poison which he named synzytiotoxin. Later, he showed that hay fever results from the parenteral digestion of the proteins of pollen. Both of these points will be discussed in more detail later. Wolff-Eisner<sup>1</sup> discussed the theory of endotoxins and their application to various diseased conditions, in a very suggestive manner, but added little to our exact knowledge. Richet<sup>2</sup> has made many valuable contributions on this subject. In his first report made with Portier in 1902, he worked with an extract from the tentacles of a muscle and showed that an injection of this made the animal much more susceptible to a second one. Unfortunately, he coined the word anaphylaxis as most suitable to cover this condition of increased susceptibility. He used this word understanding it to mean "without protection," and indicating that the first injection destroyed any natural resistance that the animal might possess against the poison. Now, we know that the condition of sensitization is essential to certain forms of immunity, as was first indicated by Vaughan and Wheeler,<sup>3</sup> and the inappropriateness of the term anaphylaxis is self-evident. However, the word has come into general use, and with this explanation we will continue it. V. Pirquet<sup>4</sup> proposed and has continued the use of the word "allergie," meaning altered energy. This is much more suitable, inasmuch as it simply expresses a fact and binds no one to any theory. However, "allergie" has not been usually employed, and we will use "protein sensitization," "hypersensitiveness," "anaphylaxis," and "allergie" as synonyms.

<sup>1</sup> Zentralbl. f. Bakt., 1904, xxxvii; Münch. med. Woch., 1906; Derm. Zentralbl., 1906; Berl. klin. Woch., 1907.

<sup>2</sup> Compt. rend. de la Soc. biol., 1902; Ann. de l'Institut Pasteur, 1907, xxi, 497; *ibid.*, 1908, xxiii; *ibid.*, 1909.

<sup>3</sup> Jour. Infect. Dis., 1907.

<sup>4</sup> Münch. med. Woch., 1906.

The fact that animals which have once received an injection of protein are liable to sudden death after a second injection of the same kind has been known for many years. Ever since the opening of the Hygienic Laboratory of the University of Michigan (1888), animals once used have been segregated and kept in cages marked "used animals," which indicated that conclusions could not be safely drawn from results obtained when these animals were employed a second time. In the standardization of diphtheria antitoxin it soon became evident that the guinea-pigs that survived one test could not be relied upon in a second one. In the late nineties, Parke, Davis & Co., large manufacturers of antitoxins, ascertained this fact and offered to supply the Hygienic Laboratory of the University of Michigan with "used" guinea-pigs at a small price. The offer was accepted, but the animals were found dear at any price, as they suddenly and unexplainably died when treated with horse serum.

This condition evidently was observed by others, and Theobald Smith mentioned it to Ehrlich, who set Otto to work to find the explanation. Otto<sup>1</sup> published his results under the title "Das Theobald Smithsche Phänomen der Serumüberempfindlichkeit." However, simultaneously with these observations on animals used in the standardization of antitoxin, the profession had occasion to observe the effects of injections of antitoxin in human beings. As early as 1903, v. Pirquet<sup>2</sup> wrote concerning certain clinical effects following antitoxin treatment, and in 1905 he and Shick published a monograph "the serum disease," "Die Serumkrankheit."

**Definition.**—Friedemann<sup>3</sup> offers the following definition: "We speak of anaphylaxis when the organism, in consequence of a previous treatment with an antigen, after a period of incubation becomes hypersensitive to the same or to a closely related substance, and when this condition

<sup>1</sup> V. Leuthold Gedenkschrift, 1906.

<sup>2</sup> Wien. klin. Woch.

<sup>3</sup> Jahresb. u. d. Ergeb. d. Immunitätsforschung, 1910, vi.

can be passively transferred to fresh animals by the serum or organ extracts of the sensitized animal." Biedl and Kraus,<sup>1</sup> omitting passive anaphylaxis, give the following: "By anaphylaxis we mean *that* state of specific hypersensitiveness induced in animals by protein injections, and in which symptoms of poisoning follow subsequent injections of the same protein in doses which would have no effect upon untreated animals." ✓ With some explanation to be given later we accept these definitions as quite satisfactory. In the meantime it is desirable to have a clear understanding of the meaning of the terms employed in discussing this subject. The substance which induces the anaphylactic state is generally known as the "antigen." This implies that it gives rise to the production of an antibody, and the selection of this word has been determined by an attempt to correlate the phenomena of anaphylaxis with the theory evolved by Ehrlich in explanation of the production of antitoxins by treatment with toxins. In truth the "antigen" of anaphylaxis is not a toxin, nor is the new substance generated in the body of the treated animal an antitoxin. The term "anaphylactogen" is unobjectionable, since it is applicable to any substance which induces the anaphylactic state. Sensitizer is a good word, and commits one to no theory. The same is true of the term "sensibilisinogen" used by our French confrères. The sensitizer causes the body cells of the treated animal to elaborate a specific proteolytic ferment which digests or splits up the sensitizer. Again, following the nomenclature of Ehrlich, this ferment elaborated as a consequence of the introduction of the sensitizer is generally designated as the "antibody." It would be equally rational to speak of pepsin as an antibody to beefsteak, because the former digests the latter. The theory evolved by Ehrlich in his studies on toxin immunity is the product of a genius of the highest order. It has stimulated research, which has resulted in discoveries of the greatest importance, but the attempt to explain all

<sup>1</sup> Kraus and Levaditi's Handbuch d. Technik u. Methodik d. Immunitätsforschung. Ergänzungsband.

physiological and pathological processes by this theory, and to describe them in the nomenclature of this theory is unscientific. To say that anaphylaxis is the result of protein—antiprotein reaction—is to talk jargon. When foreign proteins are taken into the alimentary canal they must be digested before they are absorbed. This means that their large molecules must be split into smaller ones, and this must be continued until there are no more protein molecules left. (Every protein molecule contains a poisonous group, and in normal, alimentary digestion this group is rendered non-poisonous by further cleavage before absorption takes place. When foreign proteins find their way into the blood and tissues they must be digested. This is accomplished, as it is in the alimentary canal, by proteolytic ferments, but the danger from the poisonous group in the protein molecule is evidently greater in parenteral than in enteral digestion.) Both enteral and parenteral digestion are physiological processes. Every living cell has its own proteolytic ferments, otherwise it could not live. When stimulated it pours out this ferment, and it does so only when stimulated. The function of a cell ferment depends upon the kind of cell elaborating it, and to a certain extent upon the stimulating substance. (The proteins are the normal stimulants to cell secretion. When a foreign protein is introduced into the blood or tissue it stimulates certain body cells to elaborate that specific ferment which will digest that specific protein. When such a protein first comes in contact with the body cells the latter are unprepared to digest the former, but this function is gradually acquired. The protein contained in the first injection is slowly digested, and no ill effects are observable. When subsequent injections of the same protein are made, the cells, prepared by the first injection, pour out the specific ferment more promptly and the effects are determined by the rapidity with which the digestion takes place. The poisonous group in the protein molecule may be set free so rapidly and in amount sufficient to kill the animal. This in brief is an explanation of the phenomena of anaphylaxis.

**The Sensitizer.**—The sensitizing agent most thoroughly studied is blood serum. When a small dose of blood serum is injected into a guinea-pig intravenously, subcutaneously, intracranially, or intra-abdominally, and, after a period of twelve days or longer has elapsed, a second injection is made, the animal develops the symptoms of anaphylactic shock, which, in the majority of instances, terminate fatally. This reaction is specific. The animal is sensitized to the blood serum of the species of animal from which the blood was taken and not to the sera of other species. The amount of serum necessary to sensitize a guinea-pig is surprisingly small. Rosenau and Anderson found 0.000001 c.c. of horse serum sufficient. Besredka places the minimum amount necessary to secure uniform results at 0.001 c.c. while one-tenth of this proved sufficient in a considerable percentage of the animals. The sensitizing dose of horse serum ordinarily employed in experiments upon guinea-pigs is 0.01 c.c. Large doses sensitize, but a longer time is required. When 5 c.c. is given the time which elapses before complete sensitization results may be as long as three months. The larger the dose the longer the time essential to sensitization. Besredka is inclined to the opinion that when large doses are given there is no sensitization until the greater part of the injected protein is eliminated. If he means that it is eliminated unchanged, he is certainly wrong. The protein of the first injection is slowly digested, and the larger the amount the longer the time required for the digestion, and complete sensitization does not occur until all the protein of the first injection has been disposed of and the cells have had time to accumulate a reserve of the proferment. At least this is our explanation of this point. The second dose, in order to produce a fatal result, must be considerably larger than the minimum sensitizing dose. The proportion between the minimum sensitizing and minimum fatal dose has been placed by Doerr and Russ at 1 to 1000. The second dose, in order to kill the animal promptly, must contain at least a fatal dose of the protein poison, but it may contain many times

this amount and not kill. Whether the second dose kills or not depends not only upon the amount of poison it contains but upon the rapidity with which the poison is set free.

There has been some difference of statement concerning the effect of heat on the sensitizing properties of blood serum. Rosenau and Anderson<sup>1</sup> found that animals could not be sensitized with serum which had been heated at 100°. Doerr and Russ<sup>2</sup> placed the point at which loss of sensitizing properties occurs at 80°. Kraus and Volk<sup>3</sup> raised it to 90°. Besredka has straightened out this matter and has correctly shown that the sensitizing properties of a protein are in part at least dependent upon its physical state, and that diluted serum may be heated even to 120° without losing its capability of sensitizing. It is probable that no protein completely sensitizes the body cells unless it be in at least partial solution. Heating undiluted blood coagulates the protein, and in this way leads to a decrease of its stimulating effects upon the body cells. Besredka has shown that the sensitizing property of blood serum is thermostabile. Wells<sup>4</sup> has very properly pointed out that it is the physical change induced in the protein by coagulation and not chemical alteration, which decreases its efficiency as a sensitizer, and he calls attention to the fact first shown by Besredka that proteins not coagulated by heat, do not decrease in their sensitizing effects when their solutions are boiled. This is true of casein, for instance, but when milk sours and coagulation of the casein results it is not so ready a sensitizer. Wells, furthermore, shows that other methods of coagulation, as by precipitation with alcohol, lessen the sensitizing properties. He suggests that the finely coagulated particles of protein may be seized upon by phagocytes and destroyed. In confirmation of this we have found that proteins insoluble in water, such as edestin, sensitize more efficiently when dissolved in salt solution than when sus-

<sup>1</sup> Hygienic Laboratory, Bulletin No. 45.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, i, 110.

<sup>3</sup> *Ibid.*, 731.

<sup>4</sup> Jour. Infect. Dis., v.

pended in water. Furthermore, we have found that bacterial proteins suspended in normal salt solution and heated to 154° in the autoclave under 2 kilos of pressure are more efficient sensitizers than the unheated suspensions. All these facts support the theory that body cells are best sensitized when the protein comes in intimate contact with them. Possibly cell permeation is necessary for the most complete sensitization.

Besredka finds that when the protein of the second injection is heated it is less likely to kill, and he concludes that proteins contain a thermostabile, sensitizing, and a thermolabile toxic component. We fail to see how such a conclusion follows his findings. If the physical condition of a protein affects its sensitizing properties, why should it not affect its toxic action on reinjection? The poison is set free by the digestive action of the specific ferment elaborated as a result of the first injection. Why should not the physical state of the protein affect the rapidity and thoroughness with which it is digested, and consequently the amount of the protein poison set free or activated at one time? Doerr and Russ have apparently answered this question in a satisfactory manner. By carefully conducted experiments they show that heat affects the sensitizing and toxic properties of proteins in the same ratio.

It should be understood that temperatures high enough to disrupt and destroy proteins are destructive to their sensitizing properties. According to Rosenau and Anderson a temperature of 200° removes every trace of the sensitizing property of proteins.

The influence of the digestive ferments of the alimentary canal on the sensitizing properties of proteins is an interesting and important subject, since it bears upon the possibility of sensitization by administration through the digestive tract. This point has been especially studied by Wells<sup>1</sup> and Pick and Yamanouchi.<sup>2</sup> The former submitted egg

<sup>1</sup> Loc. cit.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, i, 676; Wien. klin. Woch., 1908, 1513.

albumen to tryptic digestion and found that as the digestive action advanced the sensitizing property receded. Some have claimed to sensitize animals with peptone and even with amino-acids, but since the minutest quantity of protein suffices to sensitize, it is more reasonable to suppose that the peptone and amino-acid preparations were not absolutely free from protein. Vaughan and Wheeler have shown that the poisonous portion of the protein molecule does not sensitize in either small or large doses. Franceschelli<sup>1</sup> found that when tissue is autolyzed for months, and until every trace of the biuret reaction is lost, the fluid shows no diminution in its sensitizing properties. This agrees with the finding of Vaughan and Wheeler, that their non-poisonous portion of the protein molecule sensitizes even when it does not respond to the biuret reaction. All this suggests that the sensitizing group in the protein molecule is not itself a protein, or at least not a biuret, body. However, the sensitizing group is destroyed in normal digestion, and it is only under abnormal conditions that protein sensitization results through the alimentary canal. We will return to this subject later.

Whether or not all proteins contain the sensitizing group cannot as yet be answered with certainty. According to Doerr and Russ the globulin of the blood serum is the only protein in that fluid which sensitizes, while Wells concludes that in egg-white the albumen is the only active agent. Wells purified the albumen of egg-white by recrystallization after the method of Hopkins and Pinkus, and he found that the purer his albumen, the smaller the amount necessary to sensitize. Gay and Adler<sup>2</sup> attempted by fractional precipitation of blood serum with ammonium sulphate to separate the anaphylactogenic from the other protein constituents, and they obtained an euglobulin which sensitizes but does not prove toxic on the second injection. Quite naturally it seemed to them that they had succeeded in isolating the sensitizing constituent of blood serum, and

<sup>1</sup> Archiv f. Hygiene, 1909, lxx, 163.

<sup>2</sup> Jour. Med. Research, xviii, 433.

they proposed to call it "anaphylactin." But ammonium sulphate alters the chemical nature of proteins, and Armit<sup>1</sup> has shown that after precipitation with this reagent the poisonous group in it cannot be extracted by the method of Vaughan and Wheeler. It is probable therefore that the "anaphylactin" of Gay and Adler contains the poisonous group, but so combined that it is not set free either *in vitro* or *in vivo*.

Besredka has pointed out that in anaphylactic experiments with milk the fluid should be boiled for about twenty minutes, the injections should be made into the peritoneal cavity, larger guinea-pigs of from 300 to 400 grams' weight should be used, and a period of from sixteen to twenty days allowed to elapse between the sensitizing and test injections. When these conditions are complied with, an exquisite sensitization with uniform results is secured. Besredka was not able to sensitize guinea-pigs with milk given by mouth or rectum. We have found rabbits, especially young ones, easily sensitized by either of these avenues. In our work we have observed that hungry rabbits will eat milk when mixed with other food and seldom are sensitized, but when the milk is introduced into the stomach of a fasting rabbit through a tube, or injected into the rectum, the milk can soon be detected in the heart's blood, and the animal becomes sensitized. Evidently when the milk is taken normally into the stomach, it is digested; when forcibly fed through a tube, it is in part, at least, absorbed undigested. Like the blood, milk as between different species of animals shows a strictly specific action. Animals sensitized to woman's milk do not react when treated with cows' milk, and *vice versa*. By this method we have identified the source of milk stains deposited on wood for months. The evidence concerning the difference between the proteins of the milk and those of the blood of the same animal is somewhat conflicting. Besredka<sup>2</sup> found that

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910, v. 703.

<sup>2</sup> Compt. rend. Soc. biol., lxiv, 888; Ann. de l' Institut Pasteur, 1909, xxiii.

animals sensitized to cows' serum were not affected on the subsequent injection of cows' milk, and *vice versa*. Wells<sup>1</sup> obtained results that were not constant. Uhlenhuth and Händel<sup>2</sup> and Thomsen<sup>3</sup> did sensitize to serum with milk and *vice versa*; the latter used woman's milk and human serum. Bauer<sup>4</sup> by fixation of the complement method seems to have shown that the albumin and globulin of milk are closely related to the same constituents of the blood, while the casein of the milk is a protein unlike any in the blood. This is probably correct and explains the inconstancy in the experiments.

The differences between the proteins of the blood serum and those of the erythrocytes have been demonstrated by the anaphylactic reaction. This has been shown uniformly by the experiments of H. Pfeiffer,<sup>5</sup> Pfeiffer and Mita,<sup>6</sup> Thomsen,<sup>7</sup> Doerr and Moldovan,<sup>8</sup> and Uhlenhuth and Händel.<sup>9</sup> These investigators have found it impossible to sensitize guinea-pigs against blood serum with erythrocytes and *vice versa*. In demonstrating this fact it is necessary to fully separate the corpuscles and serum. The corpuscles should be well washed in order to accomplish this, and provision must be made against solution of the corpuscles in the serum. The corpuscles of each species contain specific proteins and therefore those of one species do not sensitize to those of another.

According to Dunbar,<sup>10</sup> the sexual cells are as specific as blood sera. The proteins of organ extracts are specific as between different species of animals, with some exceptions to be noted later, but as between different organs from the same species, and between the blood serum and organ

<sup>1</sup> Loc. cit.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, iv, 761.

<sup>3</sup> Ibid., iii, 539.

<sup>4</sup> Münch. med. Woch., 1908, No. 16; Zeitschr. f. exper. Path. u. Ther., 1909, vii.

<sup>5</sup> Zeitsch. f. Immunitätsforschung, 1910, viii.

<sup>6</sup> Ibid., vi; *ibid.*, v.

<sup>7</sup> Ibid., i; *ibid.*, iii.

<sup>8</sup> Zeitsch. f. Bakt. Ref., v.

<sup>9</sup> Zeitsch. f. Immunitätsforschung, iv, 761.

<sup>10</sup> Ibid., 740; vii, 454.

extracts they are not strictly specific. It will be readily understood that it is difficult to obtain organ extracts wholly free from the blood of the same animal.

That the crystalline lens contains proteins different from those found in any other part of the body was demonstrated some years ago by Uhlenhuth, by the precipitin reaction, and it was believed that the proteins of the crystalline lens are identical in all animals. This was apparently confirmed by anaphylactic tests, as shown by the work of Andrejew<sup>1</sup> and that of Kraus and Sohma.<sup>2</sup> Guinea-pigs can be sensitized with the proteins of their own lenses, or with those of other animals, and sensitization with the proteins of the lens from any animal responds to the same from any animal. The question whether the proteins of the crystalline lens are identical in all animals is of the greatest biological interest. The precipitin and the first sensitizing tests seemed to establish this belief, but quantitative experiments, such as those of Kapsenberg,<sup>3</sup> indicate that there are slight differences in the proteins of the crystalline lens from different species. Kapsenberg, after reviewing the literature and detailing his own work, concludes: (1) Guinea-pigs are easily and uniformly sensitized with the lens substance of other animals. The dose on reinjection necessary to induce fatal anaphylactic shock is small.<sup>4</sup> (2) Guinea-pigs may be sensitized to the protein of their own lenses, but this is done with difficulty and the fatal dose on reinjection is large (40 mg.). (3) The proteins of the crystalline lens are specific, but not markedly so. Dunbar finds that the specificity of fish proteins is not so marked as those from mammals.

Rosenau and Anderson<sup>5</sup> succeeded in sensitizing guinea-pigs to placental tissue from the same animal, and Lockemann and Thies<sup>6</sup> sensitized rabbits to the serum of the

<sup>1</sup> Arb. aus d. Kaiserl. Gesundheitsamte, xxx, 450.

<sup>2</sup> Wien. klin. Woch., 1908, 1084.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, 1912, xv, 518.

<sup>4</sup> In one instance as low as 2.5 mg. of protein but usually 6 mg.

<sup>5</sup> Hygienic Laboratory Bulletin, No. 45.

<sup>6</sup> Biochem. Zeitsch., xxv.

rabbit fetus. Gozony and Wiesinger<sup>1</sup> passively sensitized rabbits with the blood serum to amniotic fluid in two cases of eclampsia.

Some years ago Obermayer and Pick<sup>2</sup> found that the serum of rabbits treated with proteins radically changed by being iodized, nitrified, or diazoized, did not precipitate the native protein, but did act upon the altered protein with which the animal had been treated, and this occurred without reference to the original sources of the protein. Wells<sup>3</sup> and Pick and Yamanouchi<sup>4</sup> were not able to sensitize animals with iodized protein to an iodized protein obtained from another species.

Sensitization to egg-white has been studied by Vaughan and Wheeler<sup>5</sup> also by Wells.<sup>6</sup> The former used in most of their experiments egg-white diluted with an equal volume of salt solution. Guinea-pigs sensitized to egg-white from chickens responded to test injections of egg-white from tame ducks, though less energetically and less constantly, and still less to egg-white from robins. Vaughan and Wheeler by the method already described (p. 98) split egg-white into a non-poisonous, sensitizing portion and a poisonous, non-sensitizing portion. They believe that a similar cleavage occurs as the result of ferment action on the second injection in sensitized animals. This work forms the basis of their theory of anaphylaxis, which will be discussed later.

All bacterial proteins are anaphylactogens. Indeed, the Koch reaction with tuberculin is an anaphylactic test, but this will be discussed later. Bacterial proteins act as anaphylactogens, whether living or dead, formed or in solution. On account of the physical state of the protein the reaction is generally less pronounced, and strong than with proteins in solution. Bacterial anaphylaxis has been studied by

<sup>1</sup> Orvosi hetilap, liii, 418.

<sup>3</sup> Loc. cit.

<sup>4</sup> Zeitsch. f. Immunitätsforschung, i, 676.

<sup>5</sup> Jour. Infect. Dis., June, 1907.

<sup>6</sup> Loc. cit.

<sup>2</sup> Wien. klin. Woch., 1906.

Rosenau and Anderson,<sup>1</sup> Kraus and his students,<sup>2</sup> and others. The first mentioned sensitized animals with subtilis, colon, typhoid, anthrax, and tubercle bacilli. The second dose given after eleven days or longer induced anaphylactic symptoms. In some instances repeated injections seem to be necessary in order to induce a high degree of sensitization. The evidence concerning the specificity of bacterial anaphylaxis is somewhat conflicting. Kraus and Doerr sensitized guinea-pigs with intraperitoneal injections of one loop or less of cultures of typhus, dysentery, cholera, v. Nasik and v. El-Tor. A second injection of a maceration of homologous cultures given intravenously after from twenty to twenty-five days was followed by marked dyspnea, discharge of urine and feces, and coma. Some recovered, but others died within ten minutes. These investigators found this reaction strictly specific. In another experiment they found that animals sensitized with a maceration of the dysentery bacillus did not respond to the toxin of this bacillus, but did to a second treatment with the maceration. There is no proof that toxins sensitize. Delanoe sensitized guinea-pigs to the typhoid bacillus. He secured the most marked effects when sensitization was induced by repeated injections, and one month or longer elapsed before the test injection. He did not find the reaction markedly specific. Vaughan and Wheeler sensitized guinea-pigs to colon, typhoid, and tubercle proteins, and in this way secured a certain degree of immunity to living cultures. They also sensitized animals with the non-poisonous proteins of the colon and typhoid bacilli, and secured the same degree of immunity to living cultures. This subject will be enlarged when we discuss the relation of anaphylaxis to the infectious diseases.

The purest known proteins act as sensitizers. Even the crystallized proteins as hemoglobin, crystalline egg-white, and such pure vegetable proteins as edestin and excelsin

<sup>1</sup> Loc. cit.

<sup>2</sup> Wien. klin. Woch., 1908, Nos. 18, 28, and 30.

act as exquisite sensitizers. Moreover, it has been found that the more thoroughly a protein is purified the more perfectly it sensitizes and the smaller the dose necessary to sensitize or to kill on reinjection. Wells found that purified casein acts more perfectly and in smaller doses than a corresponding quantity of milk, and the sensitizing dose of crystallized egg-white is less than one one-hundredth that of native egg-white, and the killing dose on reinjection less than one-fifth. These facts have led Wells to suggest that the mixed albumins may contain substances which antagonize the anaphylactic reactions. Since pure proteins sensitize and kill on reinjection, it seems reasonable to conclude that the sensitizing and poisonous groups are constituents of the same molecule. Edestin in its most highly purified form is believed to be a chemical unit, and not a mixture of proteins. This can be split by our method into sensitizing and poisonous portions. It is true that the amount of the non-poisonous portion necessary to sensitize is larger than that of the unbroken molecule necessary to accomplish the same purpose, and it is possible that sensitization with this product is due to the fact that it contains a trace of the unbroken molecule, but the fact that no amount of this portion induces the slightest anaphylactic symptoms on reinjection is not in harmony with this view. It seems more reasonable to assume that in the process of cleavage, which is crude, a large part of the sensitizing group is destroyed. It is certain that the poisonous portion does not sensitize to either itself or the unbroken molecule. By our method the molecule is disrupted and in so doing both portions are largely destroyed. The final word on this matter cannot be spoken until we know that we have absolutely pure proteins to start with, and we have more perfect methods for the cleavage of the protein molecule. However, it seems certain that the sensitizing properties of the protein molecule reside in a group or in groups which are destroyed by digestion long before the poisonous group is markedly impaired. The sensitizing group seems more labile than the poisonous one.

Through the courtesy of White and Avery we have been permitted to read an unpublished research of theirs on "Some Immunity Reactions of Edestin." From this we excerpt the following findings: (1) The smallest sensitizing dose of pure crystallized edestin given intraperitoneally is 0.0001 mg. Guinea-pigs sensitized with this dose react fatally when the reinjection intravenously is not less than 50 mg. When the sensitizing dose is from 0.1 to 5 mg., 0.5 mg. causes a fatal dose on intravenous reinjection. (2) Guinea-pigs sensitized to edestin do not react on intravenous injection of gliadin, or globulins from squash seed, castor bean, and the hazel-nut. Two animals reacted, one fatally, to intravenous injections of flaxseed globulin. The fatal dose of flaxseed globulin was, however, from forty to one hundred and twenty times the minimum fatal intoxicating dose of edestin. (3) Guinea-pigs from a sensitized mother inherit sensitization, though in a lessened degree. (4) The intraperitoneal injection of from 0.05 to 0.1 c.c. of edestin immune serum into a guinea-pig sensitizes the latter to such an extent that it reacts fatally to an intravenous injection of edestin on the following day. (5) "When edestin is hydrolyzed by an alcoholic solution of sodium hydrate by the method of Vaughan, a substance is formed which produces a fatal intoxication in the guinea-pig apparently identical with true anaphylactic shock. The intravenous injection of one part of this poison to forty thousand parts of guinea-pig by weight constitutes the minimum fatal dose." It should be stated that this is the crude poison. (6) "When suitable amounts of edestin and edestin-immune serum are allowed to remain in contact for a given length of time, a precipitate is formed which, when washed with salt solution and mixed with fresh guinea-pig complement and incubated at body temperature, yields a substance or substances which when injected into a guinea-pig intravenously produces a fatal intoxication, apparently identical in every way with the anaphylactic reaction. Fresh complement, when allowed to act under similar conditions with edestin alone, yields no poisonous

substance. From edestin, therefore, by the action of immune serum and complement, under the experimental conditions noted, a toxic product is obtained which seems to correspond with the anaphylatoxin of Friedberger."

It seems most probable that anaphylactogens, agglutinogens, precipitinogens, and lysinogens are identical. In other words, one group in the protein molecule causes the animal cells to develop a substance which under certain conditions may act as an agglutinin, a precipitin, or a lysin. We are inclined to the belief—not yet positively demonstrated—that the same ferment may under varied conditions act as an agglutinin, a precipitin, a lysin, or it may cause a deeper cleavage in the protein molecule, resulting in the liberation of the protein poison. Through the researches of Friedberger, Doerr and Russ, and others, it has been made quite certain that anaphylactogens and precipitinogens are identical, and that these properties reside in the same intramolecular group. As proteins are altered by heat or digestion, their properties as anaphylactogens and precipitinogens are decreased in the same ratio. The protein obtained by one-third to one-half saturation of serum with ammonia sulphate is strongly active both as a precipitinogen and as an anaphylactogen, while that obtained by full saturation is inactive in either direction. Whether this is due to physical or chemical alteration has not been determined.

We may condense our statements concerning anaphylactogens as follows: They are proteins which when introduced parenterally into animals stimulate the body cells to elaborate specific ferments for the purpose of their digestion. When introduced into a sensitized animal they are digested so rapidly that the split products, some of which are poisonous, produce certain more or less violent and characteristic symptoms which may terminate in death. All anaphylactogens are proteins, and all proteins contain a certain poisonous intramolecular group. This group is physiologically the same in all proteins, hence the identity of the symptoms of anaphylactic shock whatever the protein

by which it is induced. All anaphylactogens contain a sensitizing intramolecular group which is not the same in any two kinds of proteins, hence the specificity of sensitization. We have succeeded in splitting some proteins into non-poisonous, sensitizing, and into poisonous, non-sensitizing portions. Whether all proteins contain a sensitizing group or not has not been determined. Our views concerning anaphylactogens differ from those held by others. They think that in mixed proteins, such as blood-serum, corpuscles, organ cells, egg-white, etc., there is some one protein which sensitizes and some other one which is toxic. We hold that the sensitizing and toxic proteins are groups in the same molecule. We think that we have demonstrated this by obtaining both groups from such pure proteins as edestin. Artificially crystallized proteins, such as egg albumen prepared by the method of Hopkins and Pinkus, are not suitable for this work because they are changed chemically by the ammonium sulphate, and are not split up by our method. From our researches we conclude that the sensitizing group of the protein molecule is much more complicated in its chemical structure than the toxic group. Further discussion along this line will be indulged in when we take up the poisonous portion.

We are aware of the claims made by Bogomoletz<sup>1</sup> and by Pick and Samanouchi,<sup>2</sup> that lipoids may act as anaphylactogens, but they have not convinced us that their preparations were wholly free from proteins.<sup>3</sup> Besides, it is possible that a non-protein may act indirectly as an anaphylactogen. This may be due to the substance causing some cleavage in the proteins of the body and these products may sensitize. This question will arise again when we discuss hypersensitiveness to certain medical agents.

**Volatile Sensitizers.**—Rosenau and Amos<sup>4</sup> have demonstrated that the exhaled air contains a substance which

<sup>1</sup> Zeitsch. f. Immunitätsforschung, v and vi.

<sup>2</sup> Ibid., i, 676.

<sup>3</sup> Thiele and Embleton, Zeitschr. f. Immunitätsforschung, 1913, xvi, 160, have investigated the claim of Bogomoletz that lipoids act as anaphylactogens and have been unable to confirm his work.

<sup>4</sup> Jour. Med. Research, 1911, xxv, 35.

sensitizes animals to the blood serum. The exhaled breath of men condensed and injected into guinea-pigs sensitizes these animals to subsequent injections of man's serum. Of 99 guinea-pigs submitted to this test, 26 manifested recognizable symptoms of anaphylactic shock, and 4 of these died on injection of human serum. "The fact that a number of our experiments resulted negatively may mean either that the organic matter is present in the expired air in exceedingly small amounts, or that the guinea-pigs with which we worked did not come from a very sensitive race. There are indications in our work which suggest that the expired breath from certain persons contains more organic matter than from other persons; also that the amount varies with conditions. We obtained a greater percentage of reactions in the guinea-pigs injected with the liquid condensed from the expired breath of females than in those injected with the liquid condensed from the expired breath of males. Whether this is a mere coincidence or not may be determined only by collecting more extensive data.

"The logical conclusion from our results is that protein substances under certain circumstances may be volatile. It seems unlikely that such a complex molecule should possess the power of passing into the air in a gaseous form. The volatility, however, now in question, may resemble that solubility which deals with particles in suspension in a physicochemical state (colloidal suspension). The protein may simply be carried over in 'solution' in the water vapor.

"A comparatively large number of the guinea-pigs inoculated subcutaneously with the condensed liquid from the expired breath developed sloughs at the site of the injection. It is not certain whether this was due to the pressure of the relatively large amount of liquid injected, or to some irritating principle contained in the liquid. Occasionally the local effects may have been due to the fact that the liquid was cold when injected. The injection of the condensed liquid caused no other untoward symptoms upon the animals, which is quite contrary to the observation on rabbits of Brown-Séguard and others."

Later, Rosenau has announced that guinea-pigs kept in stables with horses become sensitized to horse serum.

Wells and Osborne<sup>1</sup> have studied the anaphylactic reactions of some pure vegetable proteins, such as the globulins from castor bean, flax-seed, and squash-seed, edestin from hemp-seed, excelsin from Brazil nuts, legumins from peas and vetch, vignin from cow peas, glycinin from soy beans, gliadin from wheat and rye flour, hordein from barley, and zein from maize. "It has been found that all these proteins cause typical anaphylactic reactions in sensitized animals, with all features essentially the same as when serum and other animal materials containing proteins are used. The minimum doses which produce sensitization and the time of incubation are about the same as with animal proteins but as a rule the symptoms are of somewhat slower onset and less stormy course than are those obtained with foreign sera, and the minimum intoxicating doses are larger. There are also considerable differences in the toxicity of the several vegetable proteins to sensitized animals, but the reasons for these differences have not yet been investigated. The most toxic proteins as measured by the frequency of severe and fatal reactions, were the globulin of squash-seed, vignin, excelsin, and castor-bean globulin, which usually caused death when given in 0.1 gram doses to properly sensitized animals. Edestin caused the least severe reactions of any of the proteins, while hordein and glycinin seldom caused fatal reactions. Nevertheless the minimum sensitizing and intoxicating doses of edestin and squash-seed globulin are essentially the same. The influence of the food of the guinea-pig upon the anaphylactic reaction is of particular importance in experiments with vegetable proteins, since the natural food of the guinea-pig is vegetable. Experiments showed that continuous feeding with a vegetable protein rendered guinea-pigs immune to this protein, so that they could not be sensitized to it. Although brief feeding with animal proteins (cows' milk, foreign sera, egg albumen)

<sup>1</sup> Jour. Infect. Dis., 1911, viii, 66.

renders the animal sensitive to the corresponding animal protein, probably sufficiently protracted feeding with animal proteins will likewise confer immunity. The sensitization through feeding is specific for the protein food, showing that during the processes preceding and including absorption of the food protein no change takes place which robs it entirely of its biological specificity. The close similarity, if not identity, of the legumins of the pea and vetch was shown by the interreaction of these proteins, and the close relation to viginin from the pea was also indicated. The near relation or probable identity of the gliadins from wheat and rye was also shown."

This is in accord with our findings of some years ago, when we demonstrated that vegetable, bacterial, and animal proteins contain the same poisonous group.

**The Sensitizing Group in the Protein Molecule.**—As has been stated (Chapter V) we have split proteins into poisonous and non-poisonous portions. This has been done with proteins of most diverse origin, bacterial, vegetable, and animal, and we have found no true protein which has failed to undergo this cleavage. Certain pseudoproteins, like gelatin, do not respond to this test, but all true proteins, so far as tested, have been split into poisonous and non-poisonous portions. This is the foundation stone of our theory of protein sensitization. All true proteins are sensitizers, and so far it has not been shown that sensitization can be established by any non-protein substance. All sensitizers develop symptoms of poisoning on reinjection. These symptoms induced by reinjection are identical in manifestation and sequence with those induced in the fresh animal by the injection of the poison split off from the protein molecule by chemical agents, or by the ferments in the serum or organ extracts of sensitized animals. Therefore, we have concluded that anaphylactic shock is due to the cleavage of the molecule of the protein sensitizer on reinjection, and the liberation of the protein poison, and this cleavage is due to a specific proteolytic enzyme developed in the cells of the animal body as a result of the first injec-

tion. We have repeatedly shown that the poisonous group obtained from the protein molecule by cleavage with chemicals or with ferments does not sensitize animals. This is contrary to the generally accepted view, and our claim on this point has met with either silence or denial, but we have tested this matter so often and with poisons obtained from so many and such a variety of proteins that we have no hesitancy in affirming that the poisonous group in the protein molecule does not sensitize animals. But it is said that toxins are necessary to elaborate antitoxins, and that the latter can be produced in no other way. This is true, but the protein poisons are not toxins, and they lead to the elaboration of no antibodies. The toxins are specific; the protein poisons are not. The blood serum of an animal treated properly with a toxin neutralizes the toxin both *in vitro* and *in vivo*, while the blood serum of a sensitized animal renders the protein with which the animal has been treated, when brought in contact with it under proper conditions, either *in vitro* or *in vivo*, poisonous. It seems to us that it has been positively demonstrated that the sensitizing and toxic groups in the protein molecule are not the same. It might be argued that in ordinary protein mixtures, such as blood serum and egg-white, one protein may contain the sensitizing group and another the toxic group. This may be true, but when pure proteins, such as edestin, are used the two groups must exist in the same molecule. The specificity of proteins is demonstrated in sensitization. The toxic group shows no specificity. This property characterizes the sensitizing group, and it is in these groups that the fundamental and characteristic property of each protein resides. The exact structure and chemical nature of neither the sensitizing nor the poisonous groups have been determined. The latter seems to be physiologically the same in all proteins, the former is specific in every protein. By our method, detailed in Chapter V, the poisonous group is easily obtained; not in a chemically pure condition, but so that its presence can be demonstrated. The poisonous group, being the same in all proteins,

is obtained from all by the same or by like methods. The sensitizing group, being the same in no two proteins, cannot be isolated from all by the same method. We have been able to obtain specific sensitizing groups from colon, typhoid, and tubercle protein quite uniformly. From the pneumococcus and related organisms we have never succeeded in obtaining a sensitizing group. From egg-white we have rarely succeeded, generally failed. It seems evident to us that the sensitizing groups in many proteins are highly labile bodies, probably of such delicate structure that they easily fall to pieces.

If sensitizers are ever to have a legitimate place in the treatment of disease, it will be of the highest importance to obtain them free from the poisonous group. Every time an unbroken protein is introduced into the body it carries with it, and as a part of it, a poison. From the very careless, rash, and unwarranted way in which "vaccines" of most diverse origin and composition are now used in the treatment of disease, this matter certainly cannot be understood or its danger appreciated by those who subject their patients to such risks. It should be clearly understood that all proteins contain a poisonous group—a substance which in a dose of 0.5 mg. injected intravenously kills a guinea-pig. This poison is present in all the so-called "vaccines" now so largely used, and it is not strange that death occasionally follows the use of "phylacogen" or similar preparations. Not only do these proteins contain a poison, but when introduced parenterally the poison is set free, not in the stomach, from which it may be removed, but in the blood and tissues. It is possible that vaccine therapy may become of great service in the treatment of disease. Even now there are occasional brilliant results which are reported while the failures and disasters are not so widely advertised. But before sensitization can be of great service in a therapeutical way we must secure sensitizers free from poisonous constituents. Until recently the existence of, or the possibility of preparing non-toxic sensitizers has been made evident only by our work.

Recently, confirmation of our studies along this line have come: (1) From White and Avery,<sup>1</sup> who have prepared by our method a sensitizing group from tubercle cell substance. (2) From Zunz,<sup>2</sup> who, as the result of a most exhaustive research, has shown that one of the primary albumoses (the synalbumose of Pick) sensitizes, but does not induce anaphylactic shock on reinjection. Zunz states: Both active and passive anaphylaxis can be induced by the three so-called primary proteoses (hetero-, proto-, and synalbumose), but not by thioalbumose, nor the other so-called secondary proteoses, nor by Siegfried's pepsin-fibrin-peptone- $\beta$ , nor by any of the abiuret products of peptic, tryptic, or ereptic digestion.

Animals sensitized with hetero-, proto-, or synalbumose develop anaphylactic shock on reinjection with the original serum, acid albumin, hetero- or proto-albumose, but *not* after reinjection with synalbumose, thio-albumose, the other secondary proteoses, pepsin-fibrin-peptone- $\beta$ , or any of the abiuret products of peptic, tryptic, or ereptic digestion. The hetero- and proto-albumoses both sensitize and induce anaphylactic shock, while synalbumose sensitizes only. It follows, therefore, that sensitization and the production of anaphylactic shock are due to different groups in the protein molecule.

Wells and Osborne,<sup>3</sup> working with the purest vegetable proteins known, hordein from barley, glutinin from wheat, and gliadin from both wheat and rye, find that: "Guinea-pigs sensitized with gliadin from wheat or rye give strong anaphylactic reactions with hordein from barley, but these are not so strong as the reactions obtained with the homologous protein. Similar results are obtained if the sensitizing protein is hordein, and the second injection is gliadin. We have here a common anaphylaxis reaction developed by two chemically distinct but similar proteins of different biological origin, thus indicating that the specificity of

<sup>1</sup> Jour. Med. Research, 1912, xxvi, 317.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, 1913, xvi, 580.

<sup>3</sup> Jour. Infect. Dis., 1913, xii, 341.

the reaction is determined by the chemical constitution of the protein rather than by its biological origin. This is in harmony with the fact that chemically closely related proteins have, as yet, been found only in tissues biologically nearly related.

“From the results of these experiments it seems probable that the entire protein molecule is not involved in the specific character of the anaphylaxis reaction, but this is developed by certain groups contained therein, and that one and the same protein molecule may contain two or more such groups.”

Evidently the view that the protein molecule contains a sensitizing group, one or more, is finding strong experimental support. In our opinion this view was demonstrated by Vaughan and Wheeler<sup>1</sup> as early as 1907, but recent work, such as that by Zunz, Gay, Wells and Osborne, and others, strengthens the evidence then offered. According to our theory every protein molecule contains a chemical nucleus, key-stone or archon. This is the protein poison, and is physiologically much the same in all proteins. One protein differs from another in its secondary or tertiary groups. In these resides the biological specificity of proteins. Biologically related proteins contain chemically related groups, and in these are found the sensitizing agents. The chemical structure of the protein molecule determines its biological differentiation and development. It is not, therefore, surprising to find that a pure protein from wheat sensitizes to another closely related protein from such a biologically closely related grain as rye. This, however, does not indicate that the proteins from the two grains are wholly identical in chemical structure. It only shows that the two protein molecules contain among their secondary groups identical or closely related atomic combinations. The same can be said of the fact that certain non-pathogenic acid-fast bacteria may, at least partially, sensitize animals to the tubercle bacillus. Biological relationship is deter-

<sup>1</sup> Jour. Infect. Dis., iv, 476.

mined by the chemical structure of the protein molecule. We hold this to be true of all specific biological tests for proteins, whether they be agglutination, precipitin, lytic, complement deviation, or anaphylactic tests. The chemical structure of the protein molecule determines all of these. The form and function of every cell is determined by the chemical structure of its constituent proteins. That the sensitizing agent in the protein molecule resides in its secondary groups is shown by: (a) The fact that sensitization is within limits specific; (b) The fact that the residues left after stripping off these secondary groups by proteolytic digestion or by the action of dilute bases and acids, do not sensitize. Peptones, polypeptids, amino-acids, and the protein poison do not sensitize to either themselves or to the unbroken proteins from which they have been derived.

**The Animal.**—Guinea-pigs give the most striking results. They are easily sensitized and anaphylactic shock develops promptly and violently. It is worthy of note that the work of Besredka in France and of Rosenau and Anderson showed great difference in the reaction in guinea-pigs in the two countries. In this country practically 100 per cent. of the animals sensitized to horse serum die on the second injection, made intraperitoneally; while in France the highest percentage of fatality following the same procedure is 25. It was at first supposed that this difference is due to the race of horse supplying the serum, but Rosenau and Anderson, using Besredka's serum, obtained the same results as with the American serum. The same investigators say that the difference is not due to races of guinea-pigs. In our work with egg-white we noted a much higher percentage of mortality with short-haired, smooth-coated animals than with the long, curly-haired ones.

Doerr and Russ,<sup>1</sup> using ox serum, with the second dose constant at 0.2 c.c., found the following comparative results by varying the sensitizing dose, both doses being

<sup>1</sup> Zeitsch. f. Immunitätsforschung, ii, 109; *ibid.*, iii, 181.

administered intravenously: (1) With a sensitizing dose of from 0.01 to 0.001 c.c., the second dose was followed uniformly by sudden death. (2) With the sensitizing dose reduced to from 0.0001 to 0.00001, the period of incubation was prolonged, but after this the results of the second dose were the same as in the former instance. (3) When the doses were further reduced to 0.000001 c.c., the animals were not sensitized. Pfeiffer and Mita found that 0.1 c.c. of a 10 per cent. suspension of red corpuscles uniformly sensitized guinea-pigs. Vaughan and Wheeler found that 1 c.c. of a solution of egg-white in an equal volume of salt solution sensitized all guinea-pigs, and the result was death within thirty minutes or less when the second dose consisted of from 2 to 5 c.c. of the same solution. Wells found the minimum sensitizing dose of the purest crystalline egg albumen which he obtained to be 0.05 mg. Kraus and Doerr employed from  $\frac{1}{2}$  to 1 loop of agar cultures of bacteria for the sensitization of guinea-pigs, but Holobut and Delanoë found repeated injections more efficient.

In guinea-pigs subcutaneous, intraperitoneal, and intravenous injections of soluble proteins are practically alike in sensitization. Rosenau and Anderson sensitized guinea-pigs by feeding them horse serum. The question of sensitization by way of the alimentary canal will be discussed more fully later.

Rabbits are not so easily and uniformly sensitized as guinea-pigs. Friedemann<sup>1</sup> recommends the following method for the complete sensitization of rabbits: The intravenous injection of 1 c.c. of a heterologous serum per kilo; repetition of the same after one month, and the giving of same dose in the same way eight days later. When this is done the animals are found to be highly anaphylactized. We have found rabbits highly anaphylactized when treated daily with very small intravenous injections for a week, and then after from three to six months later given a like intravenous injection.

<sup>1</sup> Zeitsch. f. Immunitätsforschung, ii.

Doerr and Russ were unable to sensitize mice, but Braun succeeded after repeated injections and Ritz after a single treatment, when the reinjection was made intravenously.

Goats and sheep have been sensitized by Friedemann and Isaac,<sup>1</sup> and horses and birds by Doerr.

Dogs are not easily anaphylactized. Friedemann and Isaac, also Remlinger,<sup>2</sup> failed, and the former suggested that it was due to the fact that this animal is largely carnivorous, but Biedl and Kraus,<sup>3</sup> using the finer method of measuring anaphylactic shock by fall in blood pressure, has made this animal of great value in studying the phenomena of anaphylaxis. The dog has also been employed by Arthus,<sup>4</sup> by Manwaring, Pearce, Edmunds, and others.

Vaughan and Wheeler failed to sensitize cats by a single intra-abdominal injection of egg-white, but repeated injections were not tried. More recently it has been shown that the cat can be easily sensitized. Up to the present time no animal thoroughly tested has failed to respond to protein sensitization.

We are without sufficient data to determine with any certainty the relative susceptibility of man to protein sensitization. As with other animals, man's susceptibility evidently varies within wide limits with the protein supplying the anaphylactogen. Pirquet and Shick have shown that a high degree of sensitization may result from a single relatively small dose of a heterologous serum. The high degree of sensitization shown by many in hay fever and in susceptibility to certain foods raises questions which will be discussed later.

**Period of Incubation.**—By the period of incubation we indicate the interval of time between the introduction of the anaphylactogen and that time when the body is recognizably disturbed by a reinjection of the same or a closely related protein. It is quite properly designated as the

<sup>1</sup> *Zeitsch. f. Exp. Path. u. Ther.*, i, 513.

<sup>2</sup> *Compt. rend. de la Soc. biol.*, lxii, 23.

<sup>3</sup> *Wien. klin. Woch.*, 1909, 363.

<sup>4</sup> *Compt. rend. de l'Acad. Sci.*, cxlviii, 1002.

pre-anaphylactic state. It covers the time necessary for the development of anaphylaxis. The first injection of the foreign protein is without manifest effect upon the animal, but in reality it has a most profound effect. It induces changes which may continue throughout life, and may be transmitted from mother to offspring. The limits of the pre-anaphylactic state have been studied only in the guinea-pig sensitized to horse serum. In these studies it appears that the shortest time required for the development of the anaphylactic state is from six to nine days, and the usual time from ten to twelve days. Otto, Rosenau and Anderson, and Gay and Southard uniformly found that large doses of the anaphylactogen (5 c.c. or more of horse serum) prolonged the pre-anaphylactic state or delayed the full development of sensitization. Friedberger and Burkhard<sup>1</sup> have apparently contradicted this finding, but since the maximum dose employed by the latter was only 1 c.c., we fail to see that there is any conflict. Evidently there is a maximum amount of anaphylactogen which the body cells can dispose of within six or eight days, and that this for horse serum in the guinea-pig is something more than 1 c.c., and something less than 5 c.c. As has been stated, Doerr and Russ found that when the sensitizing dose was less than 0.001 c.c. of ox serum the pre-anaphylactic stage is also prolonged. It seems rational to conclude from all the evidence at hand that with sensitizing doses of 0.001 to 1 c.c. of serum the average duration of the pre-anaphylactic state is from ten to twelve days, with a minimum period of six days. With sensitizing doses above or below these limits the period of incubation may be prolonged.

**The Anaphylactic State.**—Rosenau and Anderson, also Gay and Southard, found that guinea-pigs sensitized to horse serum remain in this condition for at least two years. It may possibly continue throughout life. Vaughan and Wheeler found that guinea-pigs lose their anaphylactic

<sup>1</sup> Zeitsch. f. Immunitätsforschung, iv, 690.

state to egg-white after about one year, and after this time they can be resensitized. The same investigators found that guinea-pigs sensitized to colon or typhoid proteins begin to lose their sensitization after thirty days. Pirquet and Shick report the continuance of the sensitized state in man after treatment with diphtheria antitoxin up to more than seven years, and Currie<sup>1</sup> up to five years.

**The Reinjection.**—This term has come to have in this connection a restricted and definite meaning. Repeated injections may be employed in inducing anaphylaxis, but by "reinjection" we mean the one made after the anaphylactic state has been established. While subcutaneous, intra-abdominal, and intravenous methods of administration are alike suitable and effective in inducing anaphylaxis, the intravenous reinjection is much the most effective. Besredka has been partial to the intracerebral introduction of the "reinjection," and he claims that it has advantages over the intravenous, although the latter is the most effective. With a set of highly sensitized guinea-pigs and with the same serum he obtained the following comparative results with the different methods of administration: Fatal dose, intravenously,  $\frac{1}{40}$  to  $\frac{1}{20}$  c.c.; intracranially,  $\frac{1}{10}$  to  $\frac{1}{8}$ ; intraperitoneally, only about one-half the animals responded to 5 c.c., and subcutaneously this amount had scarcely any effect. Besredka prefers intracranial injections because they are not so delicate as the intravenous, and more easily measured. In conjunction with Steinhardt<sup>2</sup> he has developed a method of standardizing sera. He has found that sera differ widely in toxicity, as tested on sensitized guinea-pigs by intracerebral injections, the fatal dose varying from  $\frac{1}{4}$  c.c. in a sample thirteen years old, to  $\frac{1}{128}$  c.c. in some fresh samples. This variation is in part due to age and in part to the horses from which they are taken. His studies on the effects of age on the toxicity of sera as tested upon

<sup>1</sup> Jour. of Hygiene, viii, 35.

<sup>2</sup> Ann. d. l'Institut Pasteur, 1907, 157.

anaphylactized animals are interesting and important. He finds that sera from the different horses of the Pasteur Institute, all of the same race, and on the same food, show some, but not marked, variations. On the day that it is drawn, horse serum is highly toxic tested by this method. During the first ten days the toxicity rapidly decreases, after that time more slowly. In making these tests he uses guinea-pigs already used in the standardization of diphtheria antitoxin, and thus saves expense. The standards for therapeutic sera, established by the Frankfurt Institute are as follows: (1) It must be clear and contain no marked deposit. (2) It must not contain bacilli. (3) The highest phenol content must be 0.5 per cent. (4) It must contain no free toxin, especially tetanus toxin. Besredka thinks a fifth requirement should be made, namely, that the D. L. should be less than  $\frac{1}{20}$  c.c., as tested intracerebrally on sensitized guinea-pigs. He makes the following statement concerning the average serum of the Pasteur Institute: The first day it is hypertoxic (D. L. is  $\frac{1}{32}$  c.c.); on the eleventh day it has fallen to one-half (D. L. is  $\frac{1}{16}$  c.c.); by the forty-fifth day the last-mentioned dose induces severe symptoms, but does not kill; after two months it has fallen to  $\frac{1}{8}$  c.c., and after this the decrease is very slow. He thinks it wise not to use a serum less than two months old. In France all therapeutic sera are heated to  $50^{\circ}$  before distribution, and Besredka states that cases of serum disease are less frequent, and when they do occur, less serious than in countries in which unheated sera are employed. The temperature cannot be raised above  $60^{\circ}$  without weakening the antitoxin. Rosenau and Anderson have tried many chemicals and ferments with the hope of destroying the anaphylactic toxicity of therapeutic sera without injuring the antibody, but with wholly negative results. Other methods of averting the dangers of serum disease will be discussed elsewhere.

Besredka finds that milk may be heated to  $100^{\circ}$  for twenty minutes, or to  $120^{\circ}$  for fifteen minutes without appreciable loss in its anaphylactic toxicity. It begins to lose, however,

when the temperature reaches  $130^{\circ}$ ; at  $135^{\circ}$  to  $140^{\circ}$  it becomes gelatinous and is no longer toxic when tested on sensitized guinea-pigs.

**Symptoms.**—The symptoms induced by the reinjection of a homologous or closely related protein into an anaphylactized animal vary within certain limits in different species of animal, but in the same species are constant, whatever the protein used. This in itself is a strong argument in favor of the claim made by us that the anaphylactic poison is the same, in its physiological action at least, whatever the protein be. In other words, it is strongly in favor of the view that all proteins, at least all which possess the capability of sensitizing animals, contain the same poisonous group and the symptoms are due to the liberation or activation of this poison.

When a sensitized guinea-pig receives a reinjection of the same protein to which it has been sensitized after a proper interval of time, certain characteristic and practically invariable symptoms develop; generally within five or ten minutes, sometimes as late as thirty or forty minutes. These symptoms develop in three stages, which are best studied when they do not proceed too rapidly. For this reason the reinjection should be made intraperitoneally. When given intravenously the symptoms develop so rapidly that a study of the different stages may be difficult or quite impossible. The first stage is that of peripheral irritation. The animal is excited and evidently itches intensely, as is shown by its attempts to scratch every part of its body that it can reach with its feet. The second stage is one of partial paralysis. The animal lies upon its side or belly, with rapid, shallow, and difficult breathing. It is disinclined to move, and when urged to do so shows more or less incoördination of movement, and muscular weakness, with partial paralysis, especially observable in the posterior extremities, which it drags. Rarely the animal dies in this stage. The third, or convulsive stage, begins with throwing the head back at short intervals. The convulsions become general, more frequent and violent,

and the animal having reached this stage, usually dies in a convulsion or immediately following one. Expulsion of urine and feces is frequent in the convulsive stage. Recovery after reaching the convulsive stage is exceedingly rare. When this stage is not reached, recovery usually occurs, and is so prompt and complete that after a few hours, or at most by the next day, the animal cannot be distinguished from its perfectly healthy fellows.

This is an exact reproduction of the picture of poisoning an untreated guinea-pig with the protein poison of Vaughan and Wheeler, and another indication that this and the anaphylactic poison are one and the same.

In dogs the first two stages, as seen in the guinea-pig, occur with some variations. The first stage is one of excitement. The animal moves about uneasily and cries. He retches and sometimes vomits. Expulsion of urine and feces frequently occurs. In the second stage, one of great muscular weakness, he lies flat on his side or belly, with his head on the table. When placed on his feet he stumbles, falls, and lies with extended legs, as if paralyzed. There may be marked expiratory spasms with retching, and repeated expulsion of feces. There is finally suppression of urine. The animal remains in this state of depression for many hours, and then dies or slowly and completely recovers, so that the next day it seems as well as ever. Again, this is a duplication of the poisoning produced in an untreated dog with the protein poison.

Acute anaphylactic shock is seen in men being treated with sera or other albuminous fluids. We saw it repeatedly some years ago when we treated tuberculosis with yeast nuclein, and the tuberculin reaction is one of sensitization. It is not our purpose to go into detail concerning the anaphylactic phases seen in man under various conditions. That part of our subject will be dealt with later. At present we are to speak only of acute anaphylactic shock in man. When the homologous protein is injected into a man sensitized by disease or by previous treatments, symptoms develop promptly, often within a few minutes, usually

within a few hours. The stage of peripheral irritation is characterized by the sudden appearance of a rash. The rashes that occur most promptly are urticarial or erythematous. We have seen such a rash rapidly spread over the surface like a blush in every direction from the point of injection, and soon involve the entire surface. The lips and tongue seem swollen, and often the backs of the hands are swollen. The individual becomes apprehensive, says that he cannot breathe, and falls into a state of more or less marked collapse. In extreme instances there is retching, and occasionally vomiting. The second stage, that of great muscular weakness, continues for a variable time and usually rapidly passes away. In rare instances speedy death results.

**The Mechanism of Anaphylaxis.**—Gay and Southard<sup>1</sup> were the first to study the pathological changes induced by anaphylactic shock in guinea-pigs. They reported minute hemorrhages in the pleura and in the mucous membrane of the stomach, and showed that the lungs are inflated after death. Auer and Lewis<sup>2</sup> made plain that death in guinea-pigs from anaphylaxis is not due to effects on the central nervous system, but is due to tetanic contraction of the smooth muscles of the bronchioles. They also demonstrated that these spasms could be averted and life saved by preventive injections of atropine. These findings have been fully confirmed by subsequent researches, especially those of Biedl and Kraus. It is to the last-named investigators that we owe the most complete and satisfactory demonstration of the mechanism of anaphylactic shock. Biedl and Kraus<sup>3</sup> have summed up their own and the researches of others on this point up to the time of their writing (1910). We will first follow the summary and then review the work done since that time.

In dogs, fall in blood pressure is a characteristic and

<sup>1</sup> Jour. Med. Research, 1908.

<sup>2</sup> Jour. Amer. Med. Assoc., 1909.

<sup>3</sup> Kraus and Levaditi, Handbuch d. Technik u. Methodik d. Immunitätsforschung, Ergänzungsband, i.

constant result of the reinjection. When sensitization is not complete, fall in pressure may be the only symptom. In all cases there is complete parallelism between the clinical symptoms and the fall in blood pressure. As the latter proceeds the former increase in intensity, and in recovery rise in pressure accompanies the return to the normal. With a normal pressure of from 120 to 150 mm. of mercury, soon after the reinjection, and as the pulse grows smaller and faster and the general depression deepens, the blood-pressure in the femoral artery falls to 80 or 60, sometimes to 40 or even less. The character of the curve changes, the effects of respiratory movements become less marked, and cease altogether as the pressure approaches the lowest point. Now, only the movements of retching and expiratory spasms cause transitory rises in the curve. When the lowest point is reached the depression is greatest and recovery is indicated by and accompanies rise in pressure. The corneal and cutaneous reflexes remain intact throughout, and exclude both a central narcosis and peripheral muscular paralysis. The absence of marked respiratory disturbances is an additional indication in the same direction, and, furthermore, shows that the respiratory function of the red corpuscles is not at fault.

The genesis of the fall in blood pressure becomes an interesting question. The type of the fall and the accompanying condition of the pulse show that it is not due to weakness of the heart's action. More than fifty years ago it was shown by Marey that a fall in blood-pressure accompanied by increased frequency of the pulse is not due to a decrease in the strength of the heart, but in all probability to decreased peripheral resistance. Both in the course of the fall and after it has reached the lowest point there is no irregularity in the action of the heart. On the contrary, while in non-narcotized dogs immediately after the reinjection the heart beat becomes slower and sometimes irregular, as the pressure falls the heart becomes and remains regular. That the heart is not injured is furthermore shown by the fact that with spasmodic expirations in which the abdominal

viscera are compressed, the pressure invariably rises. It follows that the low blood pressure in anaphylactic shock is due to decreased peripheral resistance from marked peripheral vasodilatation.

The next question is to determine whether the dilatation is due to paralysis of the vasomotor centre or that of the periphery. At first it seemed that the trouble might be central, because stimulation of the vasomotor centre failed to increase the blood pressure. But this is negated by the fact that stimulation of the peripheral vasomotor apparatus otherwise than through the centre also failed to increase the pressure. Not only did irritation of the terminal end of the splanchnic fail to raise the pressure, but the intravenous injection of from 0.1 to 0.2 mg. of adrenalin, which in normal animals is followed by marked increase in pressure, in anaphylactic shock is either wholly without effect or has but slight influence.

It is generally held that the capability of increasing blood pressure possessed by adrenalin is due to its action on the nervous apparatus in the vessel walls, and possibly in part on the vessel muscle. It follows that the vasodilatation of anaphylactic shock is due to paralysis of the peripheral vasomotor apparatus. Stimulation of the vasomotor centre naturally fails to raise the pressure because the end apparatus does not work. It should be stated that this failure of adrenalin to raise the pressure occurs only in the stage of deep depression when the pressure is low. If the pressure begins to rise, as recovery begins, then the administration of adrenalin carries it up rapidly. That the fall in blood pressure in anaphylactic shock is due to a transitory paralysis of the peripheral vasomotor apparatus seems to be quite conclusively demonstrated.

As has been shown by Boehm, barium chloride causes a marked and fairly persistent increase in blood pressure, which is due to its stimulating effect upon the smooth muscles of the vessel walls. In anaphylactic shock even when the pressure has fallen to the lowest point, the administration of barium chloride causes a marked rise. Moreover,

when barium chloride is given before the reinjection, the latter does not cause a fall in blood pressure. Still more striking is the fact that when barium chloride is given in anaphylactic shock, as the pressure rises the symptoms disappear; also, when this substance is given in doses which cause in normal animals a marked and persistent increase in pressure, before the reinjection, the latter induces no anaphylactic symptoms. That the animals upon which these observations were made were in a sensitized state was proved by inducing passive anaphylaxis in normal animals with their sera. It will be seen from the above that in experimental anaphylaxis in dogs, barium chloride is efficient both as a preventive and a curative agent.

The antagonistic action of barium chloride demonstrates the peripheral genesis of anaphylactic vasodilatation, but it does not wholly settle the question as to whether the dilatation is due to the effect of the poison on the nerves or on the smooth muscle. The failure of adrenalin and the success of barium chloride in raising the pressure in anaphylactic shock render it highly probable that the anaphylactic poison lowers the blood pressure by paralysis of the smooth muscle of the vessel walls. It seems quite certain that barium chloride and the anaphylactic poison act upon the same peripheral apparatus, that the action of the former is stimulating, and that of the latter is paralyzing, and the former is the stronger and able to prevent or replace the latter.

Having established the fact that fall in blood pressure is a marked and constant result of the anaphylactic poison, the symptoms become easily explainable. The resulting anemia of the brain explains the disturbances of respiration, the retching, the expulsion of urine and feces, the great depression and muscular weakness, and the speedy recovery, when death does not result.

Biedl and Kraus give as additional phenomena of anaphylactic shock the following: (1) On reinjection the coagulability of the blood falls markedly or wholly disappears. Before the reinjection is made the blood of a

sensitized dog coagulates like that of a normal animal, while that in anaphylactic shock remains fluid for hours and even days. (2) During anaphylactic shock the polynuclear leukocytes wholly disappear from the blood, while the lymphocytes and platelets remain. (3) A second reinjection made in the depression phase or some hours later, or on the next day after recovery, is wholly without effect. This is true whether the amount of serum employed in the second reinjection is small or large. Furthermore, the animal is anti-anaphylactic after the shock has been either prevented or relieved, by the administration of barium chloride. However, Biedl and Kraus did find one dog which rapidly recovered under barium chloride responsive to a second reinjection made the next day.

Biedl and Kraus compare anaphylactic shock with the poisoning of normal dogs with Witte's peptone, and find that even in the minutest details they are not only similar, but identical. The intravenous injection of Witte's peptone in normal dogs in doses from 0.3 to 0.03 grams per kilo causes fall in blood pressure, loss of coagulability of the blood, the disappearance of polynuclear leukocytes, and peptone immunity. Moreover, peptone poisoning can be prevented or relieved by injections of barium chloride. They conclude that anaphylactic intoxication is caused by a poison which is physiologically identical with the active constituent of Witte's peptone. This is of the highest importance to us because we hold that the protein poison of Vaughan and Wheeler is the active principle of Witte's peptone, and in fact of all proteins which contain anaphylactogens. We have prepared this poison from Witte's peptone as well as from other proteins, bacterial, vegetable, and animal. We will return to this point.

It should be understood that the above extracts from the researches of Biedl and Kraus refer only to serum anaphylactic intoxication, as observed in dogs. As has been stated, the cause of death from anaphylactic shock in guinea-pigs was discovered by Gay and Southard and more fully studied by Auer and Lewis, and has been confirmed by subsequent

investigators. There is spasmodic contraction of the muscles of the bronchioles. This is independent of central injury, or, in other words, is due to peripheral action, and is prevented or relieved by the intravenous administration of atropine in doses of from 1 to 10 mg., provided the drug is given before the heart stops. In anaphylactic shock in guinea-pigs there is a primary rise in blood pressure, which after an intravenous reinjection lasts from thirty seconds to two minutes. This is followed by a sudden fall which may go as low as 20 or even 10 mm. of mercury. But the fall in blood pressure is not the cause of death. It is on account of the difference in action of the anaphylactic poison in guinea-pigs and dogs that the symptoms in the two species vary. The sudden onset, the stormy progress, and the fatal ending of the symptoms in guinea-pigs are seldom or never seen in dogs. In the former, spasmodic contraction of the bronchioles prevents the expiration of the air, and when the lungs are laid bare they are seen to fill the thoracic cavity; they do not collapse, and are pale and bloodless. Biedl and Kraus have shown that these conditions, characteristic of anaphylactic intoxication in guinea-pigs, result also when guinea-pigs are poisoned with peptone. Besides, fatal poisoning with peptone may be prevented by the intravenous injection of atropine. Thus, it is shown that in these animals also the anaphylactic poison is identical, physiologically at least, with the active constituent of Witte's peptone. In dogs this poison paralyzes the vessel muscles of the splanchnic region, while in guinea-pigs it stimulates the constrictor muscles of the bronchioles.

Biedl and Kraus, having come to the conclusion that anaphylactic intoxication and peptone poisoning are identical, discuss the poisonous property of peptone. Pick and Spiro state that there are peptones which do not lower the blood pressure or lessen the coagulability of the blood, and that there are digestive products containing no albumose or peptone, or only traces of either, which do induce these poisonous effects. They conclude that they are peptones

devoid of peptone action, and there may be peptone action without peptone. They think that in the peptic digestion of proteins there is formed in small amount a highly poisonous body for which they propose the name peptozym. Popielski,<sup>1</sup> who has made a chemical and physiological study of Witte's peptone, states that the albumose contained in it is without effect, and that peptone prepared from it by the method of Pick has the same, but less marked, action as the original Witte's peptone. From this he concludes that the active agent is not peptone. He also concludes that, in peptic digestion, a highly poisonous substance is formed along with the peptone, and on account of its action he proposes the name "vasodilatin." This he obtained in an impure state by fractional precipitation of aqueous solutions of Witte's peptone with hot, absolute alcohol. This substance is highly active and contains relatively small amounts of albumose and peptone, and no cholin. This agrees well with our own work. As has been stated, we have prepared our protein poison from Witte's peptone, but Nicolle and Abt<sup>2</sup> could not obtain it by our method from Defresne's peptone, and we subsequently confirmed this. Gastric digestion is a progressive process, and it progresses through its successive stages at widely differing rates. When it is arrested as in the manufacture of peptones, the product may contain the poisonous group, either in combination or free, or the digestion may have continued to the destruction of the protein poison. This seems a simple and rational explanation of the above-mentioned findings, and reconciles their apparent contradictions. If this be the correct explanation, one batch of peptone may contain the poison, while another from the same manufacturer may contain no trace of it. The protein poison is a group in the protein molecule; at each successive step in the digestive process it exists in a smaller and more labile molecule, and finally it itself is broken up and rendered inert.

<sup>1</sup> Arch. f. Exp. Path., lvi; Pflüger's Archiv, cxxxvi.

<sup>2</sup> Ann. d. l'Institut Pasteur, 1907.

We have tried to extract the protein poison from Witte's peptone by long-continued shaking with absolute alcohol, but with only negative results. It is probable that the poison as it exists in peptone is in the form of a larger molecule than is split off by our method, and consequently is not soluble in absolute alcohol.

**Passive Anaphylaxis.**—The serum of a sensitized animal introduced into a fresh animal renders the latter susceptible. The second animal may be of the same or another species. In the former case the condition induced by the transference of the serum is known as homologous, and in the second as heterologous passive anaphylaxis. On account of the ease and completeness with which it is sensitized the guinea-pig is most frequently the recipient, whatever be the species of the donor. The transfer of the condition of sensitization from the mother to her offspring is an illustration of homologous passive anaphylaxis. This has been studied especially by Rosenau and Anderson, Gay and Southard, and Otto. The latter has found the young sensitive at forty-four days after birth. Gay and Southard<sup>1</sup> were the first to demonstrate experimental passive anaphylaxis. These investigators found their recipients first sensitive on the fourteenth day. This indicated a somewhat long period of incubation for the development of the anaphylactic state in the recipient, and this was not easily explainable. Otto and Friedemann<sup>2</sup> injected the anaphylactic serum subcutaneously and the antigen intraperitoneally twenty-four hours later. With shorter intervals they failed to obtain any response. Braun<sup>3</sup> was the first to inject the anaphylactic serum intravenously, but even with this method a short period of incubation seemed to be necessary. By injecting both sera intravenously, Doerr and Russ<sup>4</sup> cut down the supposed period of incubation to four hours, but their most striking and constant results

<sup>1</sup> Jour. Med. Research, 1907, xvi, 143.

<sup>2</sup> Münch. med. Woch., 1907.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, 1910, iv.

<sup>4</sup> Ibid., iii, 181.

at that time were obtained by injecting the anaphylactic serum intraperitoneally and the antigen intravenously, twenty-four hours later. Later it was shown by Doerr and Russ, also by Biedl and Kraus, that acute symptoms, with death, may result from the simultaneous intravenous injection of anaphylactic serum and antigen. There is therefore no period of incubation in passive anaphylaxis, and this condition loses much of the theoretical importance which was attached to it so long as it seemed to require a period of incubation for its development. As we shall see later, the serum and organ extracts of sensitized animals mixed *in vitro* in proper proportions with the antigen produce a poison which kills fresh animals in anaphylactic shock.

It has been found by Gay and Southard that 0.1 c.c. of serum suffices to render the recipient passively anaphylactic.

In reference to passive homologous anaphylaxis in rabbits, Friedemann<sup>1</sup> makes a statement of which the following is a summary: Among the old authorities, Weichard with a mixture of placental cells and the antiserum, Batelli with laked blood and the corresponding hemolysin, and Nicolle with horse serum and anti-horse serum, succeeded. More recent authorities<sup>2</sup> have failed to secure passive homologous anaphylaxis in rabbits. The divergencies are probably explained by Friedemann, who recommends the following: (1) Antigen and antiserum should be injected intravenously and simultaneously. When the antigen is introduced twenty-four hours after the serum there is no marked reaction. (2) There is an optimum proportion between anti-serum and antigen. By employing 2.5 c.c. of anti-serum, Friedemann obtained no results when the antigen varied from 2.5 to 0.25 c.c., but did obtain positive effects when the amount of antigen was reduced to from 0.025 to 0.0025 c.c.

This corresponds exactly with our researches on anaphylaxis *in vitro* (see p. 274).

<sup>1</sup> Jahresbericht u. d. Ergeb. d. Immunitätsforschung, 1910, vi, 67.

<sup>2</sup> Braun, Kraus, and others.

Doerr and Russ<sup>1</sup> attempted to measure the antibody in sera by the following methods: (1) A series of guinea-pigs received intraperitoneally 1 c.c. of antiserum and twenty-four hours later decreasing amounts of the antigen intravenously. By this method they determined the smallest amount of antigen necessary to induce sudden death. (2) A series of guinea-pigs received intraperitoneally decreasing doses of the antiserum, and twenty-four hours later a constant dose (0.01 to 1 c.c.) of the antigen intravenously. In this way they determined the smallest amount of serum necessary to induce sudden death. In our opinion these experiments, while of great value, do not give results which justify standardization of the so-called antibodies. Anaphylactic shock is not determined, at least wholly, by the amount of antigen given, nor yet by the amount of antibody in the animal, but by the proportion between the two.

We have stated that Gay and Southard were the first to discover passive anaphylaxis, and that their work was done with guinea-pigs as both donors and recipients. Our French confrères generally credit Maurice Nicolle<sup>2</sup> with this discovery, and in his work rabbits served both as donors and recipients. It seems that the work of Nicolle was done before that of Gay and Southard, but not published until after the work of the Americans appeared in print. This is the statement made by Levaditi.<sup>3</sup> However, the work done in one country was quite independent of that done in the other, and Nicolle showed that rabbits which were being treated daily by intraperitoneal or intravenous injections of 1 c.c. of horse serum furnished a serum which, when injected into the peritoneal cavity of a fresh rabbit, sensitized the latter, as was demonstrated by the subcutaneous injection twenty-four hours later of 1 c.c. of horse serum, this injection giving rise to an inflammatory edema—the Arthus phenomenon (see p. 262). When the reinjection

<sup>1</sup> Zeitsch. f. Immunitätsforschung, iii, 181.

<sup>2</sup> Ann. de l'Institut Pasteur, 1907, xxi, 128.

<sup>3</sup> Jahresb. u. d. Ergeb. d. Immunitätsforschung, 1907, iii, 40.

was made into the brain, sudden death resulted. It will be seen from the above that passive anaphylaxis may be demonstrated in the recipient not only by general reaction, as anaphylactic shock, but by local reaction also. Even before the work of either Gay and Southard, or that of Nicolle, v. Pirquet and Shick<sup>1</sup> had, by a reversed method, demonstrated passive anaphylaxis. To each of three rabbits they administered 10 c.c. of horse serum; twenty-four hours later, two received each 2 c.c. of rabbit-antihorse serum, and 1 c.c. of rabbit serum, all subcutaneously in the ear. In the first two, edema resulted, while in the third there was no effect. By this reversed method anaphylactic shock may be induced. Pick and Yamanouchi<sup>2</sup> injected 2 c.c. of ox-serum subcutaneously in young rabbits of about 700 grams weight, and 14 days later 5 c.c. of rabbit anti-ox serum intravenously, causing anaphylactic shock and death.

Weil-Hallé and Lemain<sup>3</sup> have observed both local and general symptoms of anaphylaxis in both guinea-pigs and rabbits when simultaneously on one side rabbit-antihorse serum and on the other normal horse serum, were injected. A few hours after such injections, the tissue around the point of injection of the antiserum becomes edematous, infiltrates, and becomes necrotic. Either speedy death follows, or the animal becomes cachectic and dies after two or three weeks.

Kraus, Doerr, and Sohma<sup>4</sup> found that the blood-serum of rabbits sensitized to the proteins of the crystalline lens, renders the recipients anaphylactic to the same proteins, and that this sensitization is strictly specific, and Doerr and Kraus<sup>5</sup> have made a similar showing in bacterial anaphylaxis. Furthermore, Richet<sup>6</sup> has made like demonstrations with the serum of animals sensitized to mytilocongestion and crepitin.

<sup>1</sup> Die Serumkrankheit, 1906.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, i, 676.

<sup>3</sup> Compt. rend. de la Soc. biol., 1907.

<sup>4</sup> Wien. klin. Woch., 1908, No. 30.

<sup>5</sup> Ibid., No. 28.

<sup>6</sup> Ann. de l'Institut Pasteur, 1907-08.

The following additional facts concerning passive anaphylaxis are of importance and will be referred to again when we come to discuss the theories of anaphylaxis:

1. Otto<sup>1</sup> has shown that blood-serum taken from an animal in the pre-anaphylactic stage (eight days after the first injection of horse serum) sensitizes recipients.

2. Gay and Southard and others have shown that the blood-serum taken from animals in the so-called anti-anaphylactic state sensitizes the recipients.

3. Otto has shown that a third species of animal may be used to demonstrate anaphylaxis; thus, a guinea-pig which has been treated with rabbit-antihorse serum manifests anaphylactic symptoms when subsequently treated with horse serum. We<sup>2</sup> have shown that under proper conditions the anaphylactic poison may be generated *in vitro*.

Besredka<sup>3</sup> in discussing passive anaphylaxis states that results are inconstant. In our opinion this is due to the difficulty in securing the proper proportion between the antigen and the anaphylactic serum introduced into the recipient.

**Anti-anaphylaxis.**—We wish to join Friedemann<sup>4</sup> in a protest against the use of this term. However, as Friedemann states, it is so deeply embedded in the literature of anaphylaxis that it cannot be omitted. German and American authors are not the only ones who object to the term anti-anaphylaxis, and the explanation of it given by Besredka and Steinhardt. Levaditi<sup>5</sup> has produced potent arguments against the views of his confrères. The work done by Besredka and Steinhardt is of the highest value, but we differ from them in the explanation of their results. As Levaditi states, we owe the discovery of this condition to Otto and to Rosenau and Anderson, but the name and

<sup>1</sup> Münch. med. Woch., 1907, No. 39.

<sup>2</sup> Zeitschr. f. Immunitätsforschung, xi, 673.

<sup>3</sup> Kraus and Levaditi, Handbuch d. Technik u. Methodik d. Immunitätsforschung, Ergänzungsband i, 240.

<sup>4</sup> Jahresbericht u. d. Ergeb. d. Immunitätsforschung, 1910, vi, 54.

<sup>5</sup> Ibid., iii, 37.

the most thorough study of it have come from the researches of Besredka and Steinhardt.

Theobald Smith<sup>1</sup> stated that the guinea-pigs which had received the largest doses of diphtheria toxin-antitoxin mixture more frequently survived the second dose than those that received smaller doses. Rosenau and Anderson<sup>2</sup> found that animals to which they gave reinjections before the end of the period of incubation (before twelve days) were not responsive when another reinjection was made at the end of twelve days, and that a longer period than another twelve days had to pass before they became responsive. All who have tested this point in serum anaphylaxis have found that the larger the sensitizing doses employed, the longer must be the period before typical anaphylactic shock appears on reinjection. Moreover, all who have experimented with anaphylactic shock, whatever the antigen employed, and whatever the avenue of administration, have found that animals which survive the first reinjection are for a time thereafter, which is variable, refractory to a second reinjection. Besredka and Steinhardt observed that it was easy to develop the refractory state in guinea-pigs by either of the following methods: (1) The intracerebral injection of 0.25 c.c. of horse serum before the expiration of the period of incubation (twelve days). (2) The intracerebral injection of less than the fatal dose ( $\frac{1}{40}$  to  $\frac{1}{450}$  c.c.) after the period of incubation. (3) The intraperitoneal injection of 5 c.c., after the period of incubation. The last method seems to apply only to French guinea-pigs, which, as we have already stated, are not so easily sensitized as those of other countries. (4) Rectal injections. The rectum is cleansed with a glycerin-enema and then 10 c.c. of a dilution of serum with an equal volume of normal salt solution is injected. This never affects sensitized guinea-pigs, and after twelve hours they generally prove refractory to poisonous doses given intracerebrally. Besredka<sup>3</sup> secures

<sup>1</sup> Jour. med. Research, 1904, xxii, 3.

<sup>2</sup> Hygienic Laboratory Bulletin, 1906. No. 29.

<sup>3</sup> Kraus and Levaditi, Handbuch d. Ergeb. d. Immunitätsforschung, Ergänzungsband, i.

the refractory state in sensitized guinea-pigs by these methods. He accomplishes a similar result by giving the reinjection while the animal is deeply narcotized with either ether or alcohol. He finds that in this state many animals survive the reinjection made at any time and by any method, and that after recovery they are completely, but only temporarily, refractory. Of these methods he prefers the rectal injection, or better still, the subcutaneous injection of a less than fatal dose. For the reinjection he prefers the cerebral method. Since these investigations, made by Besredka, are of the highest importance both theoretically and practically, we must study them more in detail. They are of theoretical importance because he holds that mixed proteins contain not only sensitizing and toxic substances, but also a vaccinating body and the last mentioned of these he claims vaccinates sensitized animals, thus rendering them immune to reinjections. This part of his study he has carried out most thoroughly with milk, and along this line we will follow him. Milk heated for fifteen minutes at  $120^{\circ}$  still sensitizes and kills on reinjection, but when heated for fifteen minutes at  $130^{\circ}$  it neither sensitizes nor kills sensitized animals on reinjection, but does render sensitized animals refractory to reinjections of milk heated to only  $120^{\circ}$ . From these results he concludes: (1) That the sensitizing and toxic components of milk behave alike under the influence of the temperatures mentioned. (2) That the vaccinating component can be separated from the other two. In other words, the milk heated to  $130^{\circ}$  vaccinates or immunizes against anaphylaxis, while it can neither sensitize fresh animals nor kill sensitized ones. In order to determine the nature of the vaccinating substance he coagulates the milk with Bulgarian lactoferment and separates the coagulum from the whey by the centrifuge or by filtration through paper. The whey immunizes sensitized animals, but is without toxic action. The whey is neutralized with soda and the flocculent precipitate which forms is separated from the supernatant fluid by decantation of the latter, and then made into a gelatinous mixture with

salt solution. This mixture renders sensitized animals refractory, and Besredka finally concludes that the vaccinating constituent of milk is lactoprotein, which is not destroyed by heating to  $130^{\circ}$  nor removed from solution by coagulation with the Bulgarian ferment. Against this conclusion we must call attention to the following facts demonstrated by Besredka himself: (1) Milk or serum introduced into the stomach, rectum, or peritoneal cavity of sensitized animals, renders them refractory to a cerebral reinjection. (2) Small, non-fatal, doses of milk or serum given subcutaneously or in any other way has a like effect. Why, therefore, is it not reasonable to say that the vaccinating property of the whey or of the precipitate obtained from it is not due to the small amount of casein which it undeniably contains? Especially is this query pertinent, since as Besredka states, certain food authorities hold that casein is the only protein in milk. We must conclude that while Besredka's work along this line is most interesting and valuable, he has failed to prove the existence of a vaccinating component in milk.

Besredka's work on the refractory state, which he calls anti-anaphylaxis, is of practical value in pointing out a possible way by which sensitized individuals may be saved from anaphylactic shock in the therapeutic administration of sera. We will return to this point later (see Chapter XV).

We wish now to inquire whether there is any condition that may properly be designated as anti-anaphylaxis. In discussing passive anaphylaxis we have seen that serum taken from animals while in the refractory state, whether it be before the complete development of sensitization or after recovery from a non-fatal reinjection, and transferred to normal animals renders the recipient susceptible. It hardly seems proper to say that an animal has been desensitized when its blood-serum has this effect. If the blood-serum of refractory animals rendered sensitized animals refractory, the term anti-anaphylaxis might be proper, but this is in no case true. The refractory animal is still sensitized, but the degree of sensitization has been

so lowered that it no longer manifests itself in anaphylactic shock when a reinjection is made. Moreover, in all instances in which it has been tested, the refractory state is only temporary, and sooner or later the sensitized condition is sufficiently restored to be recognizable by anaphylactic shock. We will take up this point again when we discuss the theories of anaphylaxis.

**The Arthus Phenomenon.**—This phenomenon, already referred to, deserves more detailed study. Arthus<sup>1</sup> observed that a single injection of horse serum into rabbits, whether the amount was small or large, whether the injection was made subcutaneously, intraperitoneally, or intravenously, whether the serum was unheated or heated to 57°, had no recognizable effect at the time or later, but that repeated injections were followed by certain constant results. When daily subcutaneous injections of 5 c.c. were made, the following effects were observed: After the first three injections the serum was readily absorbed in a few hours; after the fourth there appeared a soft infiltration about the point and this disappeared after two or three days; after the fifth the infiltration, which appeared, was hard, edematous, and required five or six days for its absorption; after the sixth there appeared a hard, compact, aseptic mass which remained unchanged for weeks; after the seventh, there was the same condition much accentuated. The skin over the swelling became red, then whitish and dry; the tissue became gangrenous and finally dropped out, leaving a deep wound which slowly contracted into a scar. This local reaction became more marked, and more extended, with further repetitions of the injections. This reaction is strictly specific, inasmuch as animals first treated with horse serum do not react to subsequent treatment with other sera or with milk. Subsequent studies show that this reaction can be obtained in the same way in guinea-pigs, rats, and pigeons. It is not necessary that the sensitizing and the reinjections be made in the same way. If

<sup>1</sup> Compt. rend. de la Soc. biol., 1903, 817.

the former be subcutaneous, the latter may be intraperitoneal or intravenous, or *vice versa*. However, when the animal has been sensitized by several (six to eight) subcutaneous or intraperitoneal injections, and the reinjection consisting of 2 c.c. is made into the vein, the animal, in many instances, dies from anaphylactic shock. Immediately after the reinjection it shakes its head, and evidently becomes anxious. Breathing is frequent, polypnea reaching sometimes as high as 200 to 250 respirations per minute. There is expulsion of stool. Immobility, apnea, and exophthalmia appear and the animal may die within three minutes after receiving the reinjection. Obduction shows the heart in systole and the blood fluid.

The rabbits which recover after manifesting disturbances of respiration pass into a long-continued cachectic state. Rabbits which have received daily intravenous injections and then, after a period of time receive the reinjections by the same route, generally, but not invariably, die in anaphylactic shock. Sensitization by repeated intravenous injections and subcutaneous reinjections, invariably result in the production of the Arthus phenomenon.

Nicollé<sup>1</sup> found that rabbits in which Arthus' phenomenon has been developed become highly receptive to, and readily succumb to, infection. The same investigator produced the Arthus reaction in guinea-pigs, though he found these animals less suitable than rabbits for the demonstration of this form of anaphylaxis. Lewis<sup>2</sup> also demonstrated in guinea-pigs the phenomenon of Arthus. Remlinger<sup>3</sup> has done similar work. He injected massive doses (10 c.c.) of horse serum, at intervals of one week, from six to eight times, and one month after the last, gave a reinjection. On doing this his animals either developed general symptoms or passed into a cachectic condition and died. Vaughan and Wheeler<sup>4</sup> killed guinea-pigs by daily intraperitoneal injections of egg-white.

<sup>1</sup> Ann. d. l'Institut Pasteur, 1907, xxi, 128.

<sup>2</sup> Jour. Exper. Med., 1908.

<sup>3</sup> Compt. rend. de la Soc. biol., 1907, lxii, 23.

<sup>4</sup> Jour. Infect. Dis., June, 1907.

Arthus and Brun<sup>1</sup> studied the microscopic changes in the tissues in this reaction. A fluid containing a few polynuclear leukocytes first infiltrates the subcutaneous tissue. Later, the infiltration approaches the surface, and forms a line of cleavage between the stratum corneum and the stratum lucidum. The subcutaneous connective tissue is converted into a homogeneous mass, and there are extravasations of blood. Finally, the process leads to necrosis with a sharp line of demarcation. "It is an aseptic necrosis which first involves the connective tissue and vessels, and finally the epidermis."

From a study of the Arthus phenomenon we draw two conclusions: (1) A prolonged period of incubation is not necessary in order to induce the anaphylactic state. Such a period is necessary in order to secure the explosive manifestation of anaphylaxis, but the development of the specific "antibody" begins soon after the first injection of the anaphylactic protein. (2) There is no such thing as a condition of antianaphylaxis. If there were, certainly animals which are receiving daily injections should manifest it, but the only effect is to suppress the explosive character of anaphylaxis. We will return to these questions when we take up the theories.

**Anaphylaxis and Toxic Sera.**—It is well known that a single injection, even in very small amount, of certain sera into animals of another species proves fatal. One of the most highly poisonous sera is that of the eel, a very minute quantity of which injected into a guinea-pig causes death. Doerr and Raubitschek<sup>2</sup> have studied the toxic and anaphylactic effects of eel serum on guinea-pigs. They find that heating this serum to 58° destroys its toxic action. A single dose of this heated serum has no apparent effect upon guinea-pigs, but does sensitize them so that a second dose of the same is followed by anaphylactic shock. This demonstrates that the toxin and the anaphylactogen of eel

<sup>1</sup> Compt. rend. de la Soc. biol., 1903, 1478.

<sup>2</sup> Berl. klin. Woch., 1908, No. 33.

serum are distinct substances, the former being thermolabile and the latter thermostabile. The same investigators demonstrated the same thing in another way. The toxin of eel serum is destroyed by acidifying the serum with hydrochloric acid, and is not restored on neutralization (differing in this last respect from certain other toxins, such as those of cobra poison and of diphtheria). Eel serum when acidified with from 0.4 to 1 per cent. of hydrochloric acid and then neutralized has no poisonous action in a single dose on guinea-pigs, but does sensitize them to a second dose of the serum treated in the same way. Precipitation of eel serum by saturation with ammonium sulphate carries down both the toxin and the anaphylactogen. Ox serum behaves in a similar way with eel serum on guinea-pigs, and it also is robbed of its toxic property when heated to 60°. The blood serum of guinea-pigs treated with unheated eel serum contains both antitoxin and the substance produced by the anaphylactogen, and with this serum fresh animals may be protected against unheated eel serum and anaphylactized to heated serum.

It follows from these researches that the substance elaborated in the organism by an anaphylactogen is not an antitoxin. This does not mean that the animal which dies from the first dose of a toxic serum and the one that dies from the second dose of a heated serum do not die from the effects of the same poison. The toxic serum owes its toxicity to a ferment which splits up the proteins of the animal's body, setting a poison free. The unheated serum leads to the elaboration in the animal's body of a ferment which splits up the protein of the heated serum on the second injection, setting a poison free. With the toxic serum the ferment is introduced into the guinea-pig, and splits up the proteins of the body. When the heated serum is injected the cells of the guinea-pig elaborate a ferment which splits up the proteins of the heated serum on its second injection. In both instances the poison is generated by the parenteral digestion of proteins, and in all probability is the same.

*ferments*  
**The Toxogens.**—There has been marked diversity of opinion concerning the nature of the substance developed in the body under the influence of the anaphylactogen. Most German authorities, following the nomenclature introduced by Ehrlich in his masterly studies of toxins and antitoxins, have designated the sensitizing proteins as antigen and the substance elaborated in the organism as antibody. It is evident that if antigen be appropriate for the sensitizing substance, the substance produced under its influence must be the “antibody.” We have already expressed our opinion concerning the unfitness of the word “antigen.” The inappropriateness of the term “antibody” is equally evident. The anaphylactogen, instead of rendering the organism resistant to subsequent injections, renders it more sensitive. It is true, as we shall see later, that this increased sensitiveness may be a most delicate and efficient means of subsequent protection. It sharpens the agents of defence, but it does not blunt the implements of attack. It prepares the body cells for subsequent contests, but it does not disarm the invader. It places in the hands of the defender more efficient means of warfare, but it does not impair the equipment of the attacking force. It is not a shield for protection, but a sharpened sword for battle.

The blood serum of an animal which has been treated with a toxin, mixed *in vitro* with the toxin in proper proportion, may be injected into a fresh animal without effect. The blood serum of an animal treated with an anaphylactogen, mixed *in vitro* in proper proportion with the anaphylactogen and injected into a fresh animal kills it. Surely there is no justification for the use of the terms “antigen” and “antibody” in explaining the phenomena of sensitization. Moreover, their employment confuses and misleads, and in our opinion they should be discarded. However, like many other terms improperly used in scientific research, they have become so deeply engrafted into the literature that they cannot be eliminated, but their inappropriateness should be clearly understood.

Not all the German authorities have used the term "antibody" in indicating the substance elaborated in the organism in the development of the anaphylactic state. Otto calls it the "reaction-body," and to this there can be no objection. V. Pirquet, as we have seen, used "allergy," meaning altered reaction instead of anaphylaxis, and "allergin" for the substance which reacts with the foreign protein on reinjection. Besredka calls the sensitizing agent sensibilisinogen, and the substance developed under its influence, sensibilisin. Nicolle uses the term, albuminolyisin and Richet the word toxogen. All of these are free from the objections which we have urged to the term "antibody." We have adopted Richet's term, but this does not imply condemnation of the others. Indeed, the word "toxogen" needs some explanation in order to prevent error following its use. As we shall see later, the anaphylactic poison is not a toxin. The word "toxogen" is used by us as meaning a generator of poisons, and these poisons are not toxins, inasmuch as they do not lead to the elaboration of antitoxins when introduced into the animal body. The toxogen is a ferment.

Pfeiffer<sup>1</sup> long before the word anaphylaxis had been coined really discovered the fundamental fact which later research has confirmed. This is known as Pfeiffer's phenomenon. He found that when cholera vibrios are injected into the abdominal cavity of a guinea-pig, which has previously been immunized by repeated injections of non-fatal doses of the living culture, they are dissolved like sugar or salt in water. This destruction of the vibrios can be demonstrated by microscopic study, but notwithstanding the destruction of the bacteria, the animal is poisoned, and dies. In fact the more powerful the lytic serum and the more rapid and complete the destruction of the bacteria, the more certain and prompt is death. Later, it was shown by Bordet that with fresh lytic serum the vibrios may be dissolved *in vitro*. Furthermore, it was shown by Pfeiffer

<sup>1</sup> Zeitsch. f. Hygiene, 1903.

that living cultures of the cholera, typhoid, colon, and many other bacilli secrete no toxin, but that the cellular proteins of these organisms are themselves poisonous. By these experiments Pfeiffer laid the foundation of our knowledge of lytic immunity, which, as we shall see, is the chief protective function in the anaphylactic state. The anaphylactogen which he used was the cellular protein of the cholera bacillus. This caused the elaboration of the toxogen, which is contained in his lytic serum, and this digesting the anaphylactogen on the second injection, split it up with the liberation of the poison. From these researches Pfeiffer developed his theory of endotoxins, which we will discuss later.

The next important work done along this line was that of Weichardt.<sup>1</sup> He extracted the proteins from placental cells, and found that the blood serum of rabbits which had received repeated injections of such extracts when mixed with the anaphylactogen either *in vitro* or *in vivo*, produced a poison which killed rabbits with the typical symptoms of anaphylactic shock.

The toxogen exists in the blood serum and in the tissues of sensitized animals, and with the former it may be transferred to normal animals, thus establishing passive anaphylaxis. As we have seen, passive anaphylaxis may be induced in either homologous or heterologous animals. In the study of anaphylactic sera one observation has, in our opinion, led several authorities astray. It has been found that passive anaphylaxis, in some instances at least, may be induced with anaphylactic serum, either unheated or heated (56°). From this it has been inferred that the toxogen is thermostabile. In fact, the toxogen consists of amboceptor and complement, and the latter is destroyed by a temperature of 56°; but when heated, anaphylactic serum is injected into a fresh animal the recipient does or may furnish the complement. Whether it does or does not, determines the degree of success in inducing passive anaphylaxis, which, as we have seen, is not constantly accomplished.

<sup>1</sup> Berl. therap. Woch., 1903, No. 1.

Reference has already been made to the endotoxin theory of Pfeiffer. This assumed the existence of a preformed poisonous body in the cell, and cytolysis resulted in setting it free. It was believed to be an intracellular toxin, an independent and separate molecule, and not a group in a more complex molecule. This theory was applicable only to cellular proteins. An endotoxin, as understood by Pfeiffer, could not exist in a soluble protein, and since soluble proteins are most efficient as anaphylactogens, the theoretical endotoxin cannot be the anaphylactic poison. Indeed, there is now no reason for believing in the existence of the endotoxin. The brilliant work of Friedemann<sup>1</sup> has shown that red blood corpuscles may be dissolved without setting free any active poison, and, on the other hand, the poisonous group from the hemoglobin molecule may be extracted without dissolving the corpuscles. Hemoglobin is not an active poison.<sup>2</sup> Animals are not affected by a large amount of it given in a single dose, but it is an anaphylactogen which means, according to our understanding at least, that its molecule contains a poisonous group which is liberated on reinjection through the cleavage action of the toxogen. Friedemann showed that the poisonous group can be extracted from the proteins of the red corpuscles without dissolving them. He used 3 c.c. of a heavy suspension of washed ox-corpuscles. To this he added an equal volume of a highly active anaphylactic serum, and after a time separated the corpuscles in the centrifuge. The corpuscles were again washed and incubated for a short time with fresh rabbit serum, then placed in an ice-box, then centrifuged, and the colorless fluid injected into fresh animals induced anaphylactic shock. By this method Friedemann was the first to prepare the anaphylactic poison *in vitro*. This work has been confirmed, and it has been shown fully that the formation of the anaphylactic poison is quite independent of hemolysis. Thomsen<sup>3</sup>

<sup>1</sup> Zeitsch. f. Immunitätsforschung, ii.

<sup>2</sup> This is Friedemann's statement, not ours. We have found hemoglobin quite poisonous, even to the species supplying it.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, i, 741.

has demonstrated that in guinea-pigs sensitized with erythrocytes, there is no recognizable hemolysis on reinjection, although anaphylactic shock occurs. It is generally believed, as first taught by Bordet, that in hemolysis the stroma only is involved. When unbroken corpuscles are used the anaphylactic poison may come from either the hemoglobin or the stroma, or from both. We have anaphylactized animals with hemoglobin and with stroma. The former is more easily done on account, we presume, of its more ready solubility. We have found the stroma difficult to dissolve without using so much alkali that the preparation is not suitable for animal injection, and suspensions, as a rule, do not so readily sensitize as solutions. Friedberger and Vallardi<sup>1</sup> have found that only by having stroma, amboceptor, and complement in proper portions can they prepare the anaphylactic poison, an excess of any one giving negative results. Moreover, while the poison is quickly generated under proper conditions from the unbroken corpuscles a much longer time is required when the stroma only is used. It has been shown by Neufeld and Dold<sup>2</sup> that the anaphylactic poison can be extracted from bacteria without cytolysis. Furthermore, they have found that the anaphylactic poison is more easily extracted from those bacteria which are least susceptible to lytic influences. For instance, the pneumococcus which is highly resistant to lytic influences easily yields its anaphylactic poison, even at 0°, while no poison is obtained at 37°, and the cholera bacillus, which is highly labile, yields the poison with more difficulty. Friedberger and Schütze<sup>3</sup> found that the tubercle bacillus, which is highly resistant to lysis, readily supplies the anaphylactic poison. We have shown that the tubercle bacillus from which the protein poison has been extracted, leaves a residue which is not only not poisonous, but sensitizes fresh animals, and this has been confirmed by the later researches of White and Avery.<sup>4</sup>

<sup>1</sup> Zeitsch. f. Immunitätsforschung, vii, 94.

<sup>2</sup> Ueber Bakterienempfindlichkeit u. ihre Bedeutung f. Infektion.

<sup>3</sup> Berl. klin. Woch., 1911, No. 9.

<sup>4</sup> Jour. Med. Research, 1912, xxvi, 317.

Friedberger's excellent work on the extraction of the anaphylactic poison from bacteria shows the necessity of attending to the quantitative proportions between the bacteria, amboceptor, and complement, also that the poison may be destroyed by prolonged digestion. The amount of cellular substance necessary to supply a fatal dose of the poison is smaller than the lethal dose of the living, unbroken cells. This confirms our work, for we demonstrated some years ago that the protein poison is only a part of the larger molecule which is a part of the cell. The greater part of the bacterial cell is, after the removal of the poisonous portion, wholly without toxic action. Neufeld and Dold have shown that there is no relation between the amount of poison in a given bacillus and its pathogenic action, and Friedberger and Goldschmidt<sup>1</sup> have obtained the anaphylactic poison from the prodigious and other non-pathogenic bacteria. All of this is confirmatory of work we did many years ago. In 1902 we published the following findings:

THE EFFECTS OF INTRAPERITONEAL INJECTIONS OF THE AIR-DRIED CELLS OF THE BACILLUS PRODIGIOSUS IN GUINEA-PIGS

| No.         | Weight in gm. | Dose in mg. | Result. |
|-------------|---------------|-------------|---------|
| 1 . . . . . | 260           | 50          | +       |
| 2 . . . . . | 305           | 50          | +       |
| 3 . . . . . | 287           | 20          | +       |
| 4 . . . . . | 272           | 10          | +       |
| 5 . . . . . | 260           | 5           | +       |
| 6 . . . . . | 270           | 3           | +       |
| 7 . . . . . | 252           | 2           | —       |
| 8 . . . . . | 252           | 1           | —       |

THE EFFECTS OF INTRAPERITONEAL INJECTIONS OF THE AIR-DRIED CELLS OF THE BACILLUS VIOLACEUS IN GUINEA-PIGS

| No.         | Weight in gm. | Dose in mg. | Result. |
|-------------|---------------|-------------|---------|
| 1 . . . . . | 220           | 30          | +       |
| 2 . . . . . | 230           | 20          | +       |
| 3 . . . . . | 255           | 15          | +       |
| 4 . . . . . | 265           | 10          | +       |
| 5 . . . . . | 210           | 5           | —       |

<sup>1</sup> Zeitsch. f. Immunitätsforschung, vi, 299.

THE EFFECTS OF INTRAPERITONEAL INJECTIONS OF THE AIR-DRIED CELLS  
OF *SARCINA AURANTIACA* IN GUINEA-PIGS

| No.         | Weight in gm. | Dose in mg. | Result. |
|-------------|---------------|-------------|---------|
| 1 . . . . . | 240           | 25          | +       |
| 2 . . . . . | 300           | 15          | +       |
| 3 . . . . . | 305           | 10          | —       |

THE EFFECTS OF INTRAPERITONEAL INJECTIONS OF THE FINELY-GROUND  
CELLS OF THE COLON BACILLUS IN GUINEA-PIGS

| No.          | Weight in gm. | Dose in mg. | Result. |
|--------------|---------------|-------------|---------|
| 1 . . . . .  | 172           | 4.09        | +       |
| 2 . . . . .  | 170           | 4.05        | +       |
| 3 . . . . .  | 165           | 3.66        | +       |
| 4 . . . . .  | 195           | 2.60        | +       |
| 5 . . . . .  | 135           | 1.80        | +       |
| 6 . . . . .  | 145           | 1.45        | +       |
| 7 . . . . .  | 165           | 0.825       | +       |
| 8 . . . . .  | 200           | 0.10        | —       |
| 9 . . . . .  | 175           | 0.085       | —       |
| 10 . . . . . | 162           | 0.081       | —       |

As is well known, rabbits repeatedly treated with some foreign protein, such as horse serum, furnish a serum which precipitates the foreign protein *in vitro*. The rabbit at the same time, and by the same treatment, is sensitized. Quite naturally one suspects that toxogens and precipitins are identical. Friedemann was the first to test this question experimentally. He mixed the blood serum of sensitized rabbits *in vitro*, in varying proportions with the homologous anaphylactogen. The precipitate which formed was collected and washed in the centrifuge; then it was digested with fresh rabbit serum in order to supply the complement. Such preparations after varying periods of digestion, were injected intravenously into rabbits, but with negative results. Friedberger,<sup>1</sup> using guinea-pigs instead of rabbits, succeeded fully in producing the anaphylactic poison by this method. For two reasons the guinea-pig is better suited for this work than the rabbit. The blood of the former is richer in complement, and this animal is the more susceptible to the action of the anaphylactic poison. However, in

<sup>1</sup> Zeitsch. f. Immunitätsforschung, iv, 636.

carrying out this work, even with the guinea-pig, the results are not constant, and variations in the quantitative relations of the solutions concerned in the reaction may lead to failure. An excess of the anaphylactogen prevents, apparently at least, the action of the ferment, and no poison is formed. Here lies an important question which we will briefly discuss. Some years ago when we were testing the lethal doses of certain bacterial cellular proteins,<sup>1</sup> we frequently observed that a small dose killed, while two or three or more times this amount did not; or the smaller dose killed within a shorter time than the larger. The proteins were administered intra-abdominally, and in suspension. Finally, we demonstrated that the more finely powdered cell substance was ground the more poisonous it became. By this we mean that smaller doses killed. Later we found, much to our surprise, that high temperatures increased the toxicity of the suspensions of cellular proteins. We came to the following conclusions: (1) The toxicity of the bacterial suspensions is determined by the rapidity and completeness with which the cells are split up by the ferments of the body. (2) Other things being equal, the rapidity and completeness with which the cells are digested depend upon the proportion of surface exposed to the action of the ferment. (3) Grinding the powder more finely increases the surface exposure of a given weight, and therefore leads to the liberation of a larger amount of the poison in a given unit of time. (4) When the bacterial suspensions are heated the proteins contained in the cells are partly dissolved, or at least the molecular surface is extended, digestion is more rapid and complete, and the substance becomes more efficient as a poison, not because more poison is generated, but because that contained in the cell is made more available. Later in our work on protein fever<sup>2</sup> we came upon the same thing in a new guise. We found that intra-abdominal and intravenous injections, single or repeated, of egg-white in large doses in rabbits

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, ix, 458.

had but little or no effect on the temperature of the animal, while small doses frequently repeated caused rapid elevation of temperature, and death within ten to twelve hours. Here, again, small doses kill while larger ones are without visible effect. The explanation is in our opinion the same as that given for the bacterial suspensions. When 1 c.c. of the egg-white dilution is injected into the ear vein of the rabbit and diluted with all the blood in the animal body, the molecular surface of the foreign protein is immensely greater than when 10 c.c. of the egg-dilution is injected. The egg-white has no poisonous action until it is split up by ferments, and the rapidity and completeness with which this is done is determined in part at least by the extent of the molecular surface of the substrate. The same thing is seen in the action of the precipitins. An excess of the antigen prevents precipitation. We believe the matter of molecular surface exposure to be of great importance in the various phenomena of anaphylaxis. The greater it is in the anaphylactogen the more potent is its action both in sensitizing and on reinjection.

Friedberger suggests that in the preparation of the anaphylactic poison *in vitro*, excess of the anaphylactic serum or prolonged time exposure may carry digestion beyond the formation of the poison; itself being split up. This is in accord with our findings as reported below.

The formation of the anaphylactic poison from soluble proteins *in vitro* was first done in our laboratory.<sup>1</sup> The importance of this matter leads us to reproduce the experimental part of our report:

Our method of procedure is as follows: Experimentation has so far been confined to guinea-pigs. The chest of the etherized animal is opened and the blood is drawn from the heart into sterilized tubes and thus the serum is obtained. The animal dies from bleeding. The organs are rubbed up in a conical glass with sand, stirred with 30 c.c. of physiological salt solution, and allowed to stand for subsidence

<sup>1</sup> Zeitsch. f. Immunitätsforschung, xi, 673.

one hour. The supernatant fluid is then removed and is known as the organ extract. Egg-white and horse serum are diluted with physiological salt solution until 0.1 c.c. contains the amount of protein desired in the individual experiment, but in case more than 10 mg. of protein is used, a multiple of 0.1 c.c. constitutes the solvent. These solutions are freshly prepared for each experiment, and everything is done aseptically. The volume of serum or organ extract used is 5 c.c., and the amount injected into the heart of the animal is, unless otherwise noted, 4 c.c. Further details will appear in the record of the experiments.

1. One milligram of egg-white incubated for thirty minutes in 5 c.c. of the serum or organ extracts of a normal, unsensitized guinea-pig is without marked effect when injected into the heart of another unsensitized guinea-pig.

TABLE XXIV

| No.         | Fluid. | Effect.           |
|-------------|--------|-------------------|
| 1 . . . . . | Serum  | None              |
| 2 . . . . . | Liver  | None              |
| 3 . . . . . | Kidney | Slight scratching |
| 4 . . . . . | Spleen | None              |

2. One milligram of egg-white incubated for thirty minutes in 5 c.c. of the serum or organ extracts of a guinea-pig killed three days after sensitization to egg-white is without marked effect when injected into the heart of a fresh guinea-pig.

TABLE XXV

| No.         | Fluid. | Effect.           |
|-------------|--------|-------------------|
| 1 . . . . . | Serum  | Slight scratching |
| 2 . . . . . | Liver  | None              |
| 3 . . . . . | Kidney | None              |
| 4 . . . . . | Spleen | None              |
| 5 . . . . . | Brain  | None              |

3. With the conditions the same as in Table XXV, except that the animal supplying the serum and organ extracts had been sensitized to egg-white fourteen days before being killed, the effects were marked as showed in Table XXVI.

TABLE XXVI

| No.         | Fluid.         | Effect.                |
|-------------|----------------|------------------------|
| 1 . . . . . | Serum (3 c.c.) | Dead in four minutes   |
| 2 . . . . . | Liver          | Dead in four minutes   |
| 3 . . . . . | Kidney         | Dead in four minutes   |
| 4 . . . . . | Spleen         | Convulsions, recovered |

It should be remarked that in all instances in which symptoms followed they were characteristically those of the protein poison.

4. With the serum or extract of the same animal employed in Table XXVI, but with the incubation prolonged to ninety minutes, the effects were less marked, as recorded in Table XXVII.

TABLE XXVII

| No.         | Fluid.         | Effect.                 |
|-------------|----------------|-------------------------|
| 1 . . . . . | Serum (3 c.c.) | First and second stages |
| 2 . . . . . | Liver          | Convulsions, recovery   |
| 3 . . . . . | Spleen         | Slight                  |
| 4 . . . . . | Kidney         | First and second stages |

We infer from this that the digestion continued until the poison was in part destroyed.

5. With the serum and extracts from the same animal employed in Tables XXVI and XXVII, but the fluids after the addition of the egg-white kept in the cold room for twenty-four hours, the effects were not marked, as shown in Table XXVIII.

TABLE XXVIII

| No.         | Fluid. | Effect.           |
|-------------|--------|-------------------|
| 1 . . . . . | Serum  | None              |
| 2 . . . . . | Liver  | None              |
| 3 . . . . . | Kidney | Slight scratching |
| 4 . . . . . | Spleen | None              |

6. With the serum and extracts obtained from an animal killed seventeen days after being sensitized to egg-white, the effects varied with the time of incubation, as recorded in Table XXIX.

TABLE XXIX

| No.       | Fluid. | Time of incubation. | Effect.                 |
|-----------|--------|---------------------|-------------------------|
| 1 . . . . | Serum  | 15 min.             | Dead in 30 minutes      |
| 2 . . . . | Serum  | 30 min.             | Dead in 6 minutes       |
| 3 . . . . | Liver  | 15 min.             | First and second stages |
| 4 . . . . | Liver  | 30 min.             | Dead in 6 minutes       |
| 5 . . . . | Kidney | 15 min.             | First and second stages |
| 6 . . . . | Kidney | 30 min.             | Dead in 5 minutes       |
| 7 . . . . | Spleen | 15 min.             | First stage             |
| 8 . . . . | Spleen | 30 min.             | First and second stages |

It seems from this that fifteen minutes is too short a time for the full development of the poison.

7. The ferment passes through hardened filter paper. The serum and organ extracts from an animal killed twenty days after sensitization to egg-white were filtered through hardened paper. To each 5 c.c. of the filtrates 1 mg. of egg-white protein was added, incubated for thirty minutes, and then injections were made intracardiacly in fresh guinea-pigs, with the results shown in Table XXX.

TABLE XXX

| No.       | Fluid.         | Effect.                 |
|-----------|----------------|-------------------------|
| 1 . . . . | Serum (3 c.c.) | Death delayed 12 hours  |
| 2 . . . . | Liver          | First and second stages |
| 3 . . . . | Kidney         | Dead in 8 minutes       |
| 4 . . . . | Spleen         | Dead in 6 minutes       |

8. The ferment passes through a Berkefeld filter. The serum and organ extracts of an animal killed twenty-two days after sensitization to egg-white were filtered through a Berkefeld V. To each 5 c.c. of these filtrates 1 mg. of egg-white protein was added, incubated for thirty minutes, and then intracardiac injections were made in fresh guinea-pigs with results shown in Table XXXI.

TABLE XXXI

| No.       | Fluid. | Effect.           |
|-----------|--------|-------------------|
| 1 . . . . | Serum  | Dead in 6 minutes |
| 2 . . . . | Liver  | Dead in 9 minutes |
| 3 . . . . | Kidney | Dead in 4 minutes |
| 4 . . . . | Spleen | Dead in 6 minutes |

9. The poison formed by the action of the ferment on the protein passes through hardened filter paper. One milligram of egg-white protein was added to each 5 c.c. of serum and organ extracts obtained from a guinea-pig killed twenty days after sensitization to egg-white; then these portions were incubated for thirty minutes, filtered through hardened paper, and injected intracardiacy in fresh guinea-pigs, with the results shown in Table XXXII.

TABLE XXXII

| No.         | Fluid. | Effect.                 |
|-------------|--------|-------------------------|
| 1 . . . . . | Serum  | Convulsions, recovery   |
| 2 . . . . . | Liver  | Dead in 12 minutes      |
| 3 . . . . . | Kidney | Dead in 2 minutes       |
| 4 . . . . . | Spleen | First and second stages |

10. The poison passes through a Berkefeld V. The serum and organ extracts of a guinea-pig, killed twenty-three days after sensitization to egg-white, were treated with 1 mg. of egg-white protein to each 5 c.c., incubated for thirty minutes, filtered through a Berkefeld V, and the filtrates injected intracardiacy in fresh guinea-pigs, with the results shown in Table XXXIII.

TABLE XXXIII

| No.         | Fluid.         | Effect.                 |
|-------------|----------------|-------------------------|
| 1 . . . . . | Serum (3 c.c.) | First and second stages |
| 2 . . . . . | Liver          | Dead in 6 minutes       |
| 3 . . . . . | Spleen         | Dead in 10 minutes      |
| 4 . . . . . | Kidney         | Dead in 9 minutes       |

11. Serum and organ extracts from sensitized animals are inactivated when heated to 56° for thirty minutes.

In our first experiment on this point the fluids were placed in small Erlenmeyer flasks, and these were set in water at 56° and allowed to stand for thirty minutes; then 5 c.c. portions, to each of which 1 mg. of egg-white protein was added, were kept in the incubator for thirty minutes. The result was that the inactivation was incomplete, as shown by Table XXXIV.

TABLE XXXIV

| No.         | Fluid. | Effect.                 |
|-------------|--------|-------------------------|
| 1 . . . . . | Serum  | First and second stages |
| 2 . . . . . | Liver  | Dead in 4 minutes       |
| 3 . . . . . | Kidney | First stage             |
| 4 . . . . . | Spleen | None                    |

As a control to the experiments of Table XXXIV, animals were treated with the serum and organ extracts from the same animal to which egg-white had been added and incubated for thirty minutes without previous subjection to heat. The results are shown in Table XXXIV A.

TABLE XXXIV A

| No.         | Fluid.         | Effect.                 |
|-------------|----------------|-------------------------|
| 1 . . . . . | Serum (3 c.c.) | Dead in 6 minutes       |
| 2 . . . . . | Liver          | Dead in 6 minutes       |
| 3 . . . . . | Kidney         | Dead in 5 minutes       |
| 4 . . . . . | Spleen         | First and second stages |

In a repetition of this test, the serum and extracts were placed in thin sealed tubes and kept submerged for thirty minutes in water at the temperature of 56°. After this the fluids were treated with 1 mg. of egg-white protein, incubated for thirty minutes, and injected into the hearts of normal guinea-pigs, with the results shown in Table XXXIV B.

TABLE XXXIV B

| No.         | Fluid. | Effect |
|-------------|--------|--------|
| 1 . . . . . | Serum  | None   |
| 2 . . . . . | Kidney | None   |
| 3 . . . . . | Liver  | None   |
| 4 . . . . . | Spleen | None   |

As a control to the experiments of Table XXXIV B, the unheated extracts from the same animal were employed, with results as shown in Table XXXIV c. There was not enough serum to use in the control.

TABLE XXXIV C

| No.         | Fluid. | Effect.           |
|-------------|--------|-------------------|
| 1 . . . . . | Kidney | Dead in 8 minutes |
| 2 . . . . . | Liver  | Dead in 5 minutes |
| 3 . . . . . | Spleen | Dead in 4 minutes |

12. Serum and organ extracts inactivated by heating to  $56^{\circ}$  may be reactivated by the addition of corresponding fluids obtained from an unsensitized animal. The serum and organ extracts obtained from an animal killed twenty-seven days after being sensitized to egg-white were inactivated by being heated to  $56^{\circ}$ , then treated with equal volumes of serum and corresponding organ extracts, egg-white added, 1 mg. to each 5 c.c. of fluid, and incubated for thirty minutes. These fluids when injected into the hearts of unsensitized animals produced the results shown in Table XXXV.

TABLE XXXV

| No.         | Fluid.         | Effect.                 |
|-------------|----------------|-------------------------|
| 1 . . . . . | Serum (3 c.c.) | First and second stages |
| 2 . . . . . | Liver          | Dead in 34 minutes      |
| 3 . . . . . | Kidney         | Dead in 56 minutes      |
| 4 . . . . . | Spleen         | Dead in 45 minutes      |

It will be noticed that when reactivated fluids were used, death was not so speedy.

As a control to the experiments of Table XXXV, the inactivated fluids from the same animal were used without the addition of complement, with results shown in Table XXXV A.

TABLE XXXV A

| No.         | Fluid.         | Effect. |
|-------------|----------------|---------|
| 1 . . . . . | Serum (3 c.c.) | None    |
| 2 . . . . . | Liver          | None    |
| 3 . . . . . | Kidney         | None    |
| 4 . . . . . | Spleen         | None    |

13. The serum or organ extracts of animals sensitized to egg-white do not produce a poison when incubated with

horse serum for thirty minutes. The serum and organ extracts of an animal killed twenty-six days after sensitization to egg-white were incubated with horse serum protein, 1 mg. to each 5 c.c. of fluid, for thirty minutes, and then injected into the hearts of unsensitized animals, with the results shown in Table XXXVI.

TABLE XXXVI

| No.         | Fluid.         | Effect. |
|-------------|----------------|---------|
| 1 . . . . . | Serum (2 c.c.) | None    |
| 2 . . . . . | Liver          | None    |
| 3 . . . . . | Kidney         | None    |
| 4 . . . . . | Spleen         | None    |

14. The serum and organ extracts of an animal sensitized to horse serum do produce a poison when incubated with horse serum. The serum and organ extracts of an animal killed eleven days after sensitization with horse serum were incubated for thirty minutes with 1 mg. of horse serum protein to each 5 c.c. of fluid and then injected into the hearts of fresh animals, with the results shown in Table XXXVII.

TABLE XXXVII

| No.         | Fluid. | Effect.            |
|-------------|--------|--------------------|
| 1 . . . . . | Serum  | Dead in 24 minutes |
| 2 . . . . . | Spleen | Dead in 18 minutes |
| 3 . . . . . | Liver  | Dead in 27 minutes |
| 4 . . . . . | Kidney | Dead in 60 minutes |
| 5 . . . . . | Brain  | Dead in 4 minutes  |

15. The serum and organ extracts of an animal sensitized to typhoid bacilli by the intensive method (described in *Zeitschrift f. Immunitätsforschung*, vol. ix, p. 458) when incubated with living typhoid bacilli for thirty minutes do produce a poison. This animal had been thus treated twenty-seven days before. One-tenth of a loop from an agar slant four days old was added to each 5 c.c. of fluid. The results are shown in Table XXXVIII.

TABLE XXXVIII

| No.       | Fluid. | Effect.            |
|-----------|--------|--------------------|
| 1 . . . . | Serum  | Dead in 4 minutes  |
| 2 . . . . | Spleen | Dead in 16 minutes |
| 3 . . . . | Brain  | Dead in 4 minutes  |
| 4 . . . . | Liver  | Dead in 4 minutes  |
| 5 . . . . | Kidney | Dead in 6 minutes  |

16. A like experiment with the serum and organ extracts of an animal treated twenty-eight days before with the bacillus of cholera gave the results shown in Table XXXIX.

TABLE XXXIX

| No.       | Fluid. | Effect.                 |
|-----------|--------|-------------------------|
| 1 . . . . | Serum  | Dead in 14 minutes      |
| 2 . . . . | Liver  | Dead in 6 minutes       |
| 3 . . . . | Brain  | Dead in 8 minutes       |
| 4 . . . . | Kidney | Convulsions, recovery   |
| 5 . . . . | Spleen | First and second stages |

17. Sensitization with egg-white in the guinea-pig does not continue indefinitely. Vaughan and Wheeler found that these animals, when the second injection was made four hundred days or longer after the first, proved not to be in a condition of sensitization, and that injections made after this time resensitized. Apparently in cases of sensitization with egg-white the serum first loses the specific ferment. A guinea-pig killed thirty-four days after sensitization to egg-white furnished a serum and organ extracts which when incubated with egg-white and injected into the hearts of fresh animals gave the results shown in Table XL.

TABLE XL

| No.       | Fluid.         | Effect.               |
|-----------|----------------|-----------------------|
| 1 . . . . | Serum (3 c.c.) | None                  |
| 2 . . . . | Liver          | Dead in 7 minutes     |
| 3 . . . . | Kidney         | Dead in 18 minutes    |
| 4 . . . . | Brain          | Convulsions, recovery |
| 5 . . . . | Spleen         | Convulsions, recovery |

18. When the amount of egg-white protein added to 5 c.c. of the fluid before incubation was varied, the results as shown in the promptness and intensity of the effect of the poison were found to vary. The serum and organ extracts of two guinea-pigs, one killed twenty-eight days and the other thirty days after sensitization to egg-white, were divided into portions of 5 c.c., to which varying amounts of egg-white were added, incubated for thirty minutes, and injected into the hearts of fresh animals, with the results recorded in Table XLI.

TABLE XLI

| No. | Fluid. | Amount of egg-white. | Effect.                 |
|-----|--------|----------------------|-------------------------|
| 1   | Serum  | 0.5 mg.              | Slight                  |
| 2   | Spleen | 0.5 mg.              | Dead in 6 minutes       |
| 3   | Liver  | 0.5 mg.              | Dead in 4 minutes       |
| 4   | Kidney | 0.5 mg.              | Dead in 8 minutes       |
| 5   | Serum  | 5.0 mg.              | Convulsions, recovery   |
| 6   | Liver  | 5.0 mg.              | Dead in 2 hours         |
| 7   | Kidney | 5.0 mg.              | Dead in 5 minutes       |
| 8   | Spleen | 5.0 mg.              | Dead in 70 minutes      |
| 9   | Brain  | 5.0 mg.              | Dead in 2 hours         |
| 10  | Serum  | 10.0 mg.             | Convulsions, recovery   |
| 11  | Brain  | 10.0 mg.             | First and second stages |
| 12  | Spleen | 10.0 mg.             | First and second stages |
| 13  | Kidney | 10.0 mg.             | Dead in 80 minutes      |
| 14  | Liver  | 10.0 mg.             | Dead in 60 minutes      |
| 15  | Serum  | 20.0 mg.             | None                    |
| 16  | Brain  | 20.0 mg.             | Slight convulsions      |
| 17  | Spleen | 20.0 mg.             | Dead in 1 hour          |
| 18  | Kidney | 20.0 mg.             | Dead in 95 minutes      |
| 19  | Liver  | 20.0 mg.             | Dead in 65 minutes      |

19. The protein poison prepared from egg-white by cleavage with a 2 per cent. solution of sodium hydroxide, and still by no means pure, kills guinea-pigs when injected into the heart in doses of 0.5 mg. The minimum fatal dose of the pure poison must be much less than this. The symptoms are the same as those induced by injections of the serum and organ extracts from guinea-pigs sensitized to egg-white after incubation with egg-white for thirty minutes.

It will be seen that the serum and organ extracts of sensitized guinea-pigs contain an agent which when mixed with homologous anaphylactogens *in vitro* in proper proportions and incubated for the proper time produces a poison which when injected intracardiacly into fresh animals causes typical anaphylactic shock and sudden death. This poison-producing agent is a ferment and is inactivated by a temperature of  $56^{\circ}$  and reactivated on the addition of serum or organ extracts from normal animals. Like the toxins and many other ferments this one consists of amboceptor and complement. The latter is destroyed by a temperature of  $56^{\circ}$ , but being a constituent of normal serum and organ extracts, its loss is made good on the addition of these substances. The ferments formed in anaphylaxis are strictly specific, their specificity being determined by the anaphylactogen and residing in the amboceptor. The ferment elaborated in anaphylaxis, like the toxins, consists of amboceptor and complement. The anaphylactogen is not a toxin and the substance produced in the body under its influence is not an antitoxin. The anaphylactogen does not even contain a toxin group; it contains a poison, and it is this that is set free on reinjection. As we have stated, there is no more justification in calling the anaphylactic ferment an antibody than there would be in designating the proteolytic ferments of the alimentary canal antibodies.

**The Poison.**—While anaphylactogens and anaphylactic ferments are specific the poison is not specific. It is one and the same thing whatever the anaphylactogen, and in our opinion it is the poisonous group in the protein molecule. Our studies on the protein poison, done before the phenomena of anaphylaxis were known, demonstrated the presence of a poisonous group in widely diversified proteins, and it probably exists in all true proteins. We found it in bacteria, both saprophytic and pathogenic, and, as has been stated, we then were convinced that the pathogenicity of bacteria bears no relation to the poison content of the molecule of its cellular protein. The pathogenicity of a

bacterium is determined by its capability of growing in and ultimately sensitizing the animal body. Furthermore, we demonstrated, to our own satisfaction at least, that the symptoms and lesions of the infections are not directly due to the multiplication of the bacteria in the body, but to their destruction by the sensitized cells of the animal body, because at the time when the growth and multiplication of the bacteria proceed most rapidly—in the period of incubation—there are no symptoms and no lesions. The onset of the disease marks the time when sensitization becomes manifest.

We found the protein poison in diverse animal and vegetable proteins, and with the few substances in which we did not find the protein poison, such as gelatin and some peptones, we were not able to sensitize guinea-pigs.

The reasons we have for holding that our protein poison is identical with the anaphylactic poison may be stated as follows:

1. The protein poison exists in all true proteins, so far as they have been tested; consequently it exists in all anaphylactogens.

2. Whatever the protein from which the poison is obtained, its physiological action is the same. While there may be and probably are chemical differences in the protein poison as obtained from diverse proteins, physiologically there is no difference. Likewise the symptoms in anaphylaxis are the same whatever the anaphylactogen.

3. The symptoms induced in fresh animals by the protein poison are identical in every detail with those observed in sensitized animals after reinjection. They come on in the same time, proceed in the same order, and terminate alike.

4. Friedberger has shown that guinea-pigs killed with the protein poison show the Auer-Lewis phenomenon in the lungs.

5. Edmunds has shown that dogs killed with the protein poison manifest the same symptoms as those studied in anaphylactic shock. The lowered blood pressure found in anaphylactic shock and in peptone poisoning in dogs is

just as marked in those under the influence of the protein poison.

6. Our poison is the active principle in peptone, and when it has been extracted from peptone the residue is no longer poisonous.

7. When the poison has been removed from an anaphylactogen the residue may or may not sensitize, but in no case does it induce the symptoms of anaphylaxis on reinjection.

8. The activity of the protein poison is progressively increased to a certain point in proteolytic digestion. Peptone is more poisonous than the protein from which it is formed, and the same is true of some of the products of tryptic digestion. The protein poison in ordinary proteins is not active because it is combined with other groups, and as these groups are detached it becomes more and more poisonous. The protein molecule is a highly complex organic compound made up of many groups, some of which are basic, and some acid in character, and at least one which, when detached from the others, is highly poisonous, and it is poisonous because of the avidity with which it disrupts the proteins of the body. To make it simpler we may say that the protein molecule is a neutral or basic salt, and as the basic elements are split off it becomes an acid salt, and finally a free acid, and with each step its poisonous action increases because its capability of depriving other salts of their basic elements increases. Finally the acid, itself a complex body, becomes disrupted and loses its poisonous properties.

9. Since proteolysis is a progression in which complex molecules are broken into simpler and still simpler ones, in all proteolytic digestion there is an increase in the activity of the protein poison up to a given point, when it ceases to be a poison. It follows, therefore, that whatever the specificity of the proteolytic ferment, at some stage in the process the poison is more or less freed from the groups which tend to prevent its action. The protein molecule has definite lines of cleavage, and is disrupted only along these lines,

and in all cases its poisonous group is at some stage of the process activated as it were. If it were not for the fact that the poisonous group is not readily diffusible through animal membranes, and especially through the walls of the alimentary canal, all proteins would be poisonous to us even when taken by the mouth, because the protein poison is set free in alimentary digestion, but not being readily diffusible, it is split up and rendered inert as digestion proceeds. When, however, digestion is parenteral, escape from the effect of the protein poison is impossible, and the ultimate effect upon the organism is determined wholly by the amount rendered active at one time. When it is set free with explosive rapidity and in relatively large amount it induces anaphylactic shock, and possibly death. When set free slowly and in small amount, we have fever or fall in temperature, according to the amount of the poison liberated. When set free either in the circulating fluid or when it passes into this fluid immediately we have systemic effects. When set free locally we have inflammation in the adjacent tissue. Narrowly used the term anaphylaxis refers to the symptoms of anaphylactic shock. In a wider sense it covers all the phenomena of parenteral protein digestion. Some think that parenteral digestion is always abnormal, either artificially induced or due to pathological conditions. We doubt the truth of this assumption. By inhalation, through abrasions and possibly through the alimentary canal, man must be frequently, almost constantly, taking into his blood and tissues very minute traces of undigested proteins, but ordinarily the amounts thus taken in are so infinitesimally small that the body cells are not sensitized, and no harm comes. While, as we have seen, some anaphylactogens sensitize in very small doses, these are not infinitesimal, and there are measurable doses which do not sensitize. The limits vary with the protein and the animal.

Friedemann and Isaac,<sup>1</sup> also Pfeiffer and Mita,<sup>2</sup> think

<sup>1</sup> *Zeitsch. f. exp. Path.*, 1905, i, 513; 1906, ii; 1908, iv, 830,

<sup>2</sup> *Zeitsch. f. Immunitätsforschung*, 1909, iv.

that the poison does not come, solely at least, from the protein used in the reinjection. Friedemann says that it is generally held that the poison comes from the antigen, but that this is pure hypothesis. He holds that the poison may come from any one of the factors in the reaction—the anaphylactogen, the amboceptor, and the complement. He holds that the minimum killing dose of the protein on reinjection is so small that it cannot be supposed to furnish a fatal quantity of the poison, and he thinks that the ferment once set in action by the reinjection may go on and digest the proteins of the animal body. Friedemann has done most valuable work on metabolism in anaphylaxis, and he holds that the increase in the nitrogen output is greater than all of this element contained in the reinjection, and therefore he thinks that the evidence that the whole of the poison at least does not come from the foreign protein is incontrovertible. He is undoubtedly right in his finding that nitrogen metabolism in anaphylaxis is far beyond that which can be accounted for by the nitrogen in the foreign protein, and in this he has been confirmed by others. Our own work<sup>1</sup> proves the same thing, but in our opinion this does not show that the poison itself has any other source than the protein of the reinjection. In the first place, as we have seen, the minimum of the protein necessary to produce anaphylactic shock is much greater than that necessary to sensitize. Rosenau and Anderson sensitized one guinea-pig with 0.000001 c.c. of horse serum, and Besredka found sensitizing doses under 0.001 c.c. uncertain, and he found the smallest killing dose to be  $\frac{1}{40}$  c.c. even when given intravenously. It will be seen from these figures that there is a big difference between the sensitizing and the killing dose. One-fortieth of a cubic centimeter of horse serum contains about 2 mg. of protein. We found in our work that serum albumin yields about one-third its weight of poison, then 2 mg. would yield 0.66 mg., and the protein poison obtained by us, in a crude

<sup>1</sup> Jour. Amer. Med. Assoc., 1909.

way and far from pure, kills guinea-pigs when injected intracardiacy in doses of 0.5 mg. The minimum fatal dose of the pure poison as split off by the ferment in the body must be much less than this. It will be seen from this that the proteins of the reinjection, even when the smallest fatal dose is used, probably contain enough of the poison to kill. In the second place the ferment developed in anaphylaxis is specific. It splits up its own anaphylactogen and no other protein. There is no reason for supposing that it can digest the body proteins. It seems to us that this supposition is wholly untenable. It is contrary to all we know about the specificity of the anaphylactic ferment. How then may we account for the greatly increased nitrogen metabolism? When the foreign protein is split up the split products chemically react with the protein molecules of the animal's body. The liberated poison tears off the basic groups from the body molecules, and this goes on to the extent and during the time that the cleavage continues. We see this in its most marked form in the Arthus phenomenon, for in this the process is more localized. We have shown that foreign proteins injected intravenously in rabbits soon disappear from the circulating blood, and after this they may be detected in the skin and in other tissues. We have already seen (p. 262) to what extent destruction of tissue may occur in the Arthus phenomenon. There is an additional explanation of the augmented nitrogen metabolism. The protein matter resulting from the disruption of the molecules of the body by the split products from the anaphylactogen is digested by the normal non-specific parenteral ferments, and in this way nitrogen elimination is increased. As has been stated, Friedberger has obtained the poison by digesting precipitates and bacterial cells with homologous anaphylactic sera. As thus obtained and injected intravenously it induces anaphylactic shock and death. He has obtained like results by digesting bacterial cells with the normal serum of guinea-pigs. It might be assumed from this that Friedberger's poison is not the true anaphylactic poison, but in our

opinion, it is the poisonous group in the protein molecule, and this is the anaphylactic poison, it matters not what the agent be which has detached it from the other groups. This agent may be wholly chemical, such as we have used in the retort, and it may be any proteolytic ferment, the ferment of the gastric juice, that of a specific or a non-specific serum. As we have stated, since proteolysis consists in the successive and progressive disruption of the protein molecule, in at least one stage of this process, whatever causes it, the protein poison must be released from combination with those groups which in the original molecule neutralize it. The sera of many animals, possibly of all, contain proteolytic ferments; some are more active than others; some act upon certain while some act upon other proteins. The products of proteolysis resulting from different ferments certainly differ, and even the poisonous group as detached from the non-poisonous groups by different ferments probably differs in its molecular structure, but the poisonous principle is the same in all cases. Even its physiological action may be slightly modified, though there is no evidence of this, by variation in the lines of cleavage along which the protein molecule is disrupted. The pieces into which the large protein molecule is split depend upon the shape, weight, and force of the hammer that strikes it, and the point where the blow falls. Some of the pieces are large and some small, and when the blow is especially effective the pieces may be so small that the poisonous group is broken and rendered inert; but even when protein is fused with caustic alkali the cyanogen group is still in evidence.

It seems to us that Friedberger's work has only confirmed our contention, first published in 1907, that the anaphylactic poison is the poisonous group in the protein molecule. Friedberger calls his poison "anaphylatoxin." We join Friedemann in protesting against this name. The substance is not a toxin, as we now understand that word. Friedberger has demonstrated this fact himself, inasmuch as he has shown that his poison does not induce immunity, nor

does it cause animals treated with it to elaborate an anti-toxin.

That the anaphylactic poison is a protein derivative is certain; whether it is still a biuret body has not been determined. The chemistry of the protein poison has been discussed (p. 101).

*$\beta$ -iminazolylethylamin.*—This amin is produced by splitting off carbon dioxide from histidin, and this may be done by either chemical or bacterial agencies. It was first prepared synthetically by Windam and Vogt,<sup>1</sup> and then by Ackermann<sup>2</sup> by the action of putrefactive bacteria on histidin. About the same time it was detected in ergot, and its physiological action investigated by Barger and Dale.<sup>3</sup> In the same year Kutscher<sup>4</sup> isolated from ergot a substance which chemically could not be distinguished from this amin, but which was believed to have a somewhat different physiological action.  *$\beta$ -iminazolylethylamin*, hereafter designated by the abbreviation  *$\beta$ -i*, was suggested as a possible agent in inducing anaphylactic shock by Dale and Laidlaw,<sup>5</sup> who made a thorough study of its physiological action. It is highly poisonous, 0.5 mg. being sufficient to kill a guinea-pig, with all the symptoms of anaphylactic shock, when administered intravenously. Dale and Laidlaw describe its action on guinea-pigs as follows: "In large guinea-pigs, weighing 800 to 1000 grams, injection of 0.5 mg. into the external saphenous vein caused death in a few minutes. The immediate effect was a marked respiratory impediment, resulting in violent but largely ineffective inspiratory efforts, during which the lower ribs were drawn in. After a time the respiratory convulsions ceased, and the animal lay comatose, though the heart continued to beat for some time longer. *Post mortem*: The lungs were found permanently distended. If the fatal amount were

<sup>1</sup> Berichte, 1907, xl, 3691.

<sup>2</sup> Zeitsch. f. physiol. Chem., 1910, xlv, 504.

<sup>3</sup> Proc. Chem. Soc., 1910, xxvi, 128.

<sup>4</sup> Zentralbl. f. Physiol., 1910, xxiv, 163.

<sup>5</sup> Journal of Physiology, 1911, lxi, 318.

given more slowly, as in two doses of 0.25 mg., after the second of which death ensued rapidly, the final condition of pulmonary distention was extreme. Death was clearly due to asphyxia, evidently resulting from progressive obstruction to the respiration, sufficient in its early stages to prevent the exit of air sucked into the lungs by the violent inspiratory spasms, and later becoming complete. The larger the initial dose, and, therefore, the earlier the obstruction became complete, the less pronounced the distention of the lungs. Such an effect could only be due to constriction of the bronchioles by spasm of their muscular coats, though the effect would be aided by increased bronchial secretion. Preliminary injection of atropine, though it did not abolish the action, had decided protective value. After 5 mg. of atropine a dose of 1 mg. of  $\beta$ -i intravenously had the normal effect, but another guinea-pig, which received a preliminary injection of 5 mg. of atropine, recovered from subsequent intravenous injections of 0.5 mg., 0.25 mg., and again 0.5 mg. of  $\beta$ -i given in fairly rapid succession; whereas one dose of 0.5 mg. was, in our experience, invariably fatal when given intravenously to a guinea-pig untreated with atropine. Whether atropine actually weakens the bronchial spasm, or merely modifies the effect by preventing secretion, must remain uncertain. We were unable to remove the obstruction when once developed by a subsequent injection of atropine."

In dogs and cats  $\beta$ -i causes a marked fall in blood-pressure, and in this respect also agrees with the anaphylactic poison. On the smooth muscle, notably on that of the virgin uterus, it has a markedly stimulating effect. It does not, according to the findings of the English investigators, affect the coagulability of the blood.

In a later paper Barger and Dale<sup>1</sup> make a further comparison between the physiological action of  $\beta$ -i and peptone poisoning, especially with the action of the "vasodilatin" of Popielski, and they state their conclusions as follows:

<sup>1</sup> Journal of Physiology, 1911, lxi, 499.

“The hypothetical vasodilator must, therefore, be regarded as consisting of at least two substances:

“1.  $\beta$ -iminazolythalamine, causing fall of blood-pressure, and the other characteristic effects on plain muscle and gland cells, but not affecting coagulation of the blood.

“2. Another substance, or other substances, which renders the blood incoagulable, and which may or may not play some part in the other effects.”

Friedberger and Moreschi<sup>1</sup> conclude from its behavior toward alkalies that it is not the true anaphylactic poison which they believe to be the anaphylatoxin of Friedberger.

Biedl and Kraus<sup>2</sup> hold, contrary to Barger and Dale, that  $\beta$ -i does delay the coagulation of blood in dogs. They say: “In dogs 3 mg. of this substance causes immediate fall in blood-pressure, retards the coagulation of the blood, and induces the phenomena of anaphylaxis.”

With our poison,  $\beta$ -i seems to agree closely. Both induce bronchial spasm and distention of the lungs in guinea-pigs, and cause prompt and marked fall in blood pressure in dogs. Neither destroys the coagulability of the blood. In the purest form in which we have obtained it our poison kills guinea-pigs intravenously in doses of 0.5 mg., and this is the fatal dose of  $\beta$ -i. When the active agents in our crude poison are isolated we shall not be surprised if  $\beta$ -i or some closely allied body is among them.

$\beta$ -i has been prepared by Barger and Dale from the mucosa of the small intestine of the ox by boiling with 0.1 per cent. of hydrochloric acid, and further treatment with silver nitrate, and excess of baryta, according to the method of Kutscher. In regard to this work they make the following statement: “We have no evidence with regard to the origin of the  $\beta$ -i in the extract of intestinal mucosa. All possible precautions were taken to avoid putrefaction before the material was worked up. Moreover, a piece of intestine removed immediately after death, or even during life, from

<sup>1</sup> Berl. klin. Woch., 1912, No. 16.

<sup>2</sup> Zeitsch. f. Immunitätsforschung, 1912, xv, 447.

an anesthetized animal, washed, scraped, and worked up immediately gives an extract with the characteristic physiological action of  $\beta$ -i. Bayliss and Starling showed that the depressor substance could be extracted from fresh mucous membrane of the dog's intestine by alcohol. It must probably, then, be regarded as a normal product of intestinal mucosa, though whether it is present in living cells, or only formed when these are killed and disintegrated, remains uncertain."

$\beta$ -i has recently become a commercial product under the name "ergamin;" it is also known as "histamin."

Mellanby and Twort<sup>1</sup> have confirmed Ackerman's findings<sup>2</sup> that histidin is converted into ergamin by bacterial agencies, and have demonstrated that it is formed in this way in the alimentary canal. They have isolated a bacillus which causes this conversion: "It is a small bacillus with rounded ends, non-motile, and Gram-negative. It will grow aërobically or anaërobically on the ordinary laboratory media. The optimum temperature is about 37°. The growth on gelatin, agar, and broth is similar to that of bacillus coli. Milk is clotted and no liquefaction of gelatin takes place. Acid and gas are produced in media containing glucose, lactose, or dulcete." "In the alimentary canal of a guinea-pig, at least, and probably in that of most mammals, the bacillus capable of producing  $\beta$ -i from histidin is present from the duodenum downward. It is legitimate, therefore, to assume that the presence of the histidin base, described by Barger and Dale, is due to bacterial decomposition going on in the intestine." It grows and produces ergamin in alkaline Ringer's solution containing 0.1 per cent. of histidin. When the concentration of the histidin is greater, the growth is not so prompt nor the conversion so complete. "It is evident that the toxic symptoms produced by the substance together with its presence in the alimentary tract must bring it under consideration as a possible cause

<sup>1</sup> Jour. Physiol., 1912, xlv, 53.

<sup>2</sup> Zeitsch. f. physiol. Chemie, 1910, lxxv, 504.

of pathological conditions. It is probable that under normal conditions the liver can deal adequately with  $\beta$ -i, as it can with the amines of tyrosin and tryptophan, and render it innocuous; but if this defensive mechanism of the liver breaks down for any reason, then many toxic symptoms will no doubt follow. For instance, one of us has elsewhere suggested that the condition of cyclic vomiting in children may be due to the excessive accumulation of such substances as  $\beta$ -i in the intestine, causing, from time to time, an exacerbation of symptoms. In any case a fact which would appear to point to means of lessening the formation of this substance in the alimentary canal is worth consideration. This base is not produced in an acid medium, and this fact is additional support to the medical treatment, as advocated by Metchnikoff, involving the injection of lactic acid producing bacilli. It is necessary, however, to point out that the colon bacillus responsible for the production of the toxic product is not killed by the acidity of a medium, but its energies are only directed along other lines, so that as soon as an alkaline reaction returns the production of the histidin base continues."

**The Kyrins.**—These bodies have been studied and described by Siegfried,<sup>1</sup> who regards them as intermediate products between the proteins and the amino acids. His method of preparation consists in digesting the protein (fibrin, casein, etc.) for three weeks at 38° to 39°, with from 12 to 16 per cent. hydrochloric acid. This mixture is filtered and the filtrate precipitated with phosphomolybdic acid. The precipitate is extracted with dilute sulphuric acid and precipitated with alcohol. Solution and precipitation with these reagents are repeated about fifteen times, but after the ninth a substance of constant composition is secured, and this is a kyrin. With the cleavage process carried one step farther these bodies are converted into amino acids. It will be seen from the method of preparation that they are closely related to the diamino acids (arginin

<sup>1</sup> Zeitsch. f. physiol. Chem., 1906, xlviii, 54.

and lysin). The precipitate with phosphomolybdic acid may be crystallized, and the picrate differs from the corresponding salts of arginin and lysin by its solubility in alcohol. The kyrins give the biuret reaction, the color differing from that given by peptone in being more distinctly a Bordeaux red. Siegfried states that the kyrin formed from fibrin splits on further cleavage into lysin, arginin, and glutamic acid.

The kyrins are said to be highly poisonous, and Kammann<sup>1</sup> has suggested that they, or similar bodies, may be the active agents in the production of anaphylactic shock. However, we have been unable to find any record of thorough studies of the poisonous action of these cleavage products. Schittenhelm and Weichardt<sup>2</sup> have studied two kyrins, one prepared from hemoglobin and the other from gelatin. The latter is not poisonous, and this agrees with our work in which we failed to obtain the poisonous group from gelatin. The globinokyrin is moderately active, but not so poisonous as the protamins.

**Anaphylatoxin.**—Friedberger<sup>3</sup> treated rabbits with lambs' serum until he obtained abundant precipitates with the sera of these animals. These precipitates were deposited in a centrifuge, washed with salt solution, and then digested with normal guinea-pig serum for some hours in the incubator. When this was done and the serum decanted and injected into normal guinea-pigs, the animals promptly died with all the symptoms of anaphylactic shock. A poison had already been obtained in a similar manner by Weichardt from placental tissue, and by Friedemann from blood corpuscles (see p. 269). Friedberger named the poisonous substance which he obtained by the digestion of specific precipitates with normal serum, anaphylatoxin. Designating this substance as a toxin might be criticized, but at that time Friedberger believed in his theory of sessile receptors, and it is plain that he regarded the poison which

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1911, xi, 659.

<sup>2</sup> Ibid., 1912, xiv, 609.

<sup>3</sup> Ibid., 1910, iv, 636.

he had prepared as a toxin. One of the conclusions stated in the paper in which he reported this work is as follows: "Die Bildung eines Antitoxin gegen das Anaphylatoxin ist mir bisher noch nicht einwandfrei gelungen, jedoch ist sie wahrscheinlich." He also concluded that the poisonous action of this substance is destroyed by a temperature of 65°. So far he has not announced the successful preparation of an antitoxin, and further research has convinced him that anaphylatoxin is thermostable. In these respects, therefore, anaphylatoxin does not differ from the poison obtained by the cleavage of proteins with chemical agents or ferments. Later, Friedberger<sup>1</sup> became convinced that anaphylatoxin is not a specific body; it is a common product of the cleavage of diverse proteins. This, also, distinguishes it from toxins, one invariable characteristic of which is their specificity.

One of the most important contributions made to the literature of anaphylaxis is the paper by Friedberger and Vallardi.<sup>2</sup> In this contribution Friedemann is properly credited with having been the first to produce the anaphylactic poison by ferment action *in vitro*. The following statement is made: "Concerning the nature of anaphylatoxin we know nothing, but we are justified in assuming its close relationship to the split product obtained by Vaughan and Wheeler through the action of alkaline alcohol on proteins, also the similarity of its action with that developed by poisoning with peptone, as shown by Biedl and Kraus, and Pfeiffer and Mita, is evident."

With specific precipitates, the stroma of blood corpuscles, and with whole corpuscles, under the action of amboceptor and complement, anaphylatoxin was developed, and its effect on fresh animals was demonstrated. The specific precipitates were obtained by treating rabbits with lambs' blood and then mixing the sera from these animals. Such precipitates were collected in a centrifuge, washed

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910. vi. 179.

<sup>2</sup> Ibid., 1910 vii. 94.

twice with salt solution, and then incubated with the serum of a normal guinea-pig. The ferment in the serum split up the precipitate with the liberation of the poison, and when the serum containing the poison was injected into a fresh guinea-pig the animal promptly died, with all the symptoms of anaphylactic shock. Washed stroma treated in a similar way yielded the same poison. With whole red corpuscles the results are complicated by the poisonous action of the liberated hemoglobin, which is an active poison without further cleavage. Friedberger designates the precipitates, stroma, and corpuscles subjected to the action of the normal serum as antigens. We have a marked antipathy to the use of this term in discussing the phenomena of anaphylaxis, and would designate the substances submitted to the action of the normal serum as substrates, and regard the serum as containing the ferment. It might be said that the terms we use are of but little importance, and the meaning is the one important thing. This is true, but we use words to express ideas, and we hold that the term "antigen" in this connection confuses and tends to lead to gross misconception. As has been shown by Friedberger and others, anaphylatoxin may be obtained by incubating various proteins with normal serum, and why should we call the substances thus split up by the ferment in the serum an antigen? Would it not be just as proper to denominate starch which is converted into sugar by amylase an "antigen?" But this is a digression, and we will return to the work of Friedberger and Vallardi. They found that when specific precipitates and stroma were used the amount of the poison obtained was not in proportion to the amount of substrate used. With a larger amount of substrate, that of the serum (ferment) being constant, they obtained no poison. With the amount of substrate very small, they obtained either no poison or at least not enough to demonstrate its presence by its effect on the animal. They obtained positive results, as shown by anaphylactic death only when the amount of substrate but slightly exceeded that necessary to kill a

sensitized animal on reinjection. When the amount of substrate employed was very small they obtained either too little of the poison to affect the animal, or, what is more probable, the digestion was so active that the poison itself was destroyed. When whole blood corpuscles were used the amount of poison obtained did increase with an increase in the substrate, but the poison thus formed in greater abundance was hemoglobin. At least this is our explanation of their results. On the theory of Friedberger, his own results are difficult, or, as we think, impossible of explanation, while on our theory they explain themselves, and, in fact, are exactly what might have been expected.

Friedberger and Vallardi find that both the subjective and objective symptoms of poisoning with anaphylatoxin are identical with those of both active and passive anaphylaxis, and in this we quite agree with them. Biedl and Kraus have held, and still hold, that anaphylatoxin cannot be the true anaphylactic poison because, as they claim, the condition of the lungs after death in guinea-pigs from this poison is not the same. We agree with Friedberger, who holds that the distention of the lungs after anaphylactic death, first mentioned by Gay and Southard, and more fully emphasized by Auer and Lewis, is found after death from naturally poisonous sera, from poisonous antisera, from peptone as shown by Biedl and Kraus, from the poison of Vaughan and Wheeler, from the  $\beta$ -i compound of Barger and Dale, and possibly after poisoning from other substances as well. Lung distention due to constriction of the bronchioles is, in guinea-pigs at least, a constant result of the protein poison, but should not be considered as pathognomonic of this poison. Friedberger and Jerusalem<sup>1</sup> attempted to isolate and study the physical and chemical properties of anaphylatoxin. It should be understood that the poison as they had it is in guinea-pig serum. They state their conclusions as follows: (1) The solution can be evaporated to dryness (*in vacuo*) without loss of

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910, vii, 748.

toxicity. (2) By evaporation and resolution in smaller volume it can be concentrated. (3) It cannot be extracted from the serum by ether or chloroform. (4) It can be precipitated without loss of toxicity by alcohol.<sup>1</sup> (5) Anaphylatoxin is not a globulin. (6) It can be obtained by the action of complement upon heated (as well as unheated) precipitates.

Biedl and Kraus claim that anaphylatoxin cannot be the true anaphylactic poison because it does not induce anaphylactic shock when injected into the brain, and Besredka has shown that with serum the reinjection of a very small dose into the brain causes the shock. To this Friedberger very properly replies that the only form in which he has obtained the poison is in solution in guinea-pig serum, and he cannot introduce a large enough quantity of this into the brain without doing mechanical injury, and, moreover, the minimum reinjection dose in the brain is not smaller than that required intravenously. It should always be borne in mind that Friedberger's anaphylatoxin is a solution of a poison whose physical and chemical properties are not known in blood-serum.

Friedberger<sup>2</sup> states that after death from anaphylatoxin the blood does not coagulate. This seems to complete the identity of the action of this poison with that formed in anaphylaxis. The symptoms and all the postmortem findings seem to be identical.

Friedberger<sup>3</sup> and his students showed that various bacteria, such as the vibrio of Metchnikoff, the prodigiosus, the typhoid and tubercle bacillus, when incubated with normal guinea-pig serum, furnish a soluble poison which, when injected into normal animals intravenously, causes anaphylactic shock and death. The poison obtained from these diverse bacterial proteins as well as that obtained from

<sup>1</sup> It will be understood that the proteins of the serum were precipitated by the alcohol and the poison carried down with the precipitate. It does not mean that the poison would necessarily be precipitated from aqueous solution by alcohol.

<sup>2</sup> *Zeitsch. f. Immunitätsforschung*, 1910, viii, 239.

<sup>3</sup> *Ibid.*, 1911, ix, 369.

specific precipitates, blood corpuscles, stroma, and other proteins, is in all instances the same in its physiological action. It matters not whether the bacteria submitted to the action of the serum be heated or unheated, the result is the same. In other words, Friedberger and his students accomplished with the proteolytic ferments in blood-serum by the cleavage of proteins just what we did nearly ten years earlier by chemical agents. We demonstrated that all proteins, living or dead, formed or without form, contain a poisonous group, and that the physiological action of this group is the same whatever the protein from which it is obtained. We split up the pathogenic and non-pathogenic bacteria, vegetable and animal proteins of the most diverse kind, and obtained from each and every one the same poison, and now the same has been accomplished by ferments. This we regard as a confirmation of our statement made many years ago that the protein molecule contains at least one poisonous group. Besides, the poison obtained by us, when we split up proteins with chemical agents, is the same or very closely related to that now obtained by the cleavage of the same proteins by the more delicate agency of ferment action. As we stated at the time, our method was crude, and the poison was obtained only at great loss, but the principle is the same. From our work we developed the theory of the relation of the split protein products to immunity and disease, which was formulated in 1907, and which, in our opinion, is confirmed in every particular by the work of Friedberger and others. We fail to see why he and our German confrères in general still use the Ehrlich nomenclature in discussing the protein split products. Bacterial cellular substance is submitted to a ferment *in vitro*, and broken up, and why should this substance be called "an antigen" and the ferment an "antibody?" The theory of sessile receptors, the only theory, so far as we know that Friedberger ever originated, was long ago demonstrated to be false by his own work. He has adopted another theory, one which he certainly did not originate, but for the establishment of which he

has done much, and still he employs the language of his own theory long since discarded by himself.

Friedberger and Nathan<sup>1</sup> showed that by the action of normal guinea-pigs' serum on normal horse serum, or *vice versa*, in proper proportions, a poison is set free. The serum that is to serve as substrate is inactivated by being heated to 56°, and this is then acted upon by the ferment in the unheated serum. It will be understood that the amount of the substrate must be small. In fact, it was found that the poison is produced when the substrate contained not more than 1 mg. of protein. When guinea-pig serum was used as the ferment, it was found to act best in quantities of about 6 c.c. For instance, when inactivated horse serum in quantities of from 0.01 to 0.0005 c.c. was incubated with 6 c.c. of normal guinea-pig serum for eighteen hours, and then 4 c.c. of this injected intravenously into guinea-pigs of about 200 grams, the animal promptly died from anaphylactic shock. On the other hand, when guinea-pig serum was used as the substrate and horse serum as the ferment, somewhat larger quantities of each were needed. For instance, with 0.1 c.c. of inactivated guinea-pig serum incubated with 8 c.c. of horse serum for twenty-four hours a fatal amount of the poison was obtained, while with the substrate reduced to 0.01 c.c. no symptoms were induced. Friedberger, in reporting this work, expressed astonishment that from a very small amount of protein, enormous quantities of which, in its unbroken state, could be injected into animals without recognizable effect, there could be obtained a potent poison, and still years before we had split up these proteins by chemical means and obtained the same poison. What we had done with chemical agents, Friedberger did with ferments. We claimed ten years ago that we had split the protein molecule along definite lines of cleavage, and that our product was not a mere degradation body. This demonstration that ferments split the protein molecule along the same lines is a justification of our claim.

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1911, ix, 567.

This work of Friedberger, in our opinion, confirms another claim which we put forth some years ago. There are two kinds of parenteral proteolytic enzymes in the body, or capable of being developed in the body. One of these is non-specific and the other specific. The former is found in the normal blood serum, and the latter is developed by protein sensitization. The normal serum of this guinea-pig under proper conditions splits up most diverse proteins with the liberation of the poisonous group. The blood-serum and organ extracts of the sensitized animal contain specific ferments which cleave the special protein to which the animal has been sensitized. The non-specific protein takes care of the small amounts of foreign protein which are constantly finding their way into the blood without having undergone digestion. Ordinarily, these enter the blood in such small amounts that they are rapidly and fully digested beyond the poisonous stage by these non-specific proteolytic ferments. Among its other functions the blood is a digestive fluid, and it exercises this function not only on the unbroken proteins which find their way into it from the outer world, but also upon certain substances which are constantly coming into it as a result of tissue metabolism.

Another important research from Friedberger's laboratory is reported by him and Girgolaff.<sup>1</sup> Rabbits and guinea-pigs were treated with homologous proteins, bacteria, and sera, and after the animal had developed the specific ferment (antibody) it was bled to death by opening the aorta and transfused with salt solution until all the blood was washed out. Then a portion of some organ from this animal was implanted in the abdominal cavity of another, and after recovery from the operation this animal was found to be sensitized. A few illustrations will best explain this work. A guinea-pig of 200 grams received 1 c.c. of lambs' serum intravenously. Fourteen days later this animal was exsanguinated and washed out with salt solution. Then two pieces of its spleen—about half of this organ—were implanted in the

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1911, ix, 575

abdominal cavity of a fresh guinea-pig of about the same weight. Fourteen days later this animal received intravenously 1.5 c.c. of lamb serum, and promptly died of anaphylactic shock. Autopsy showed the lungs distended, the heart still beating, and the blood of the right heart had not coagulated at the expiration of ten minutes.

The organ may be implanted into another species, as is shown by the following: A rabbit was treated with lamb serum until the precipitation titer of the serum was 1 to 1000; then the rabbit was exsanguinated and perfused with salt solution, and two pieces of its spleen implanted in the abdominal cavity of a guinea-pig. Fourteen days later this guinea-pig received intravenously 1.5 c.c. of lamb serum, and died of anaphylactic shock. Guinea-pigs thus treated were found to be sensitized not only to lambs, but also to rabbit serum, thus proving that the implanted organs continued to secrete both their normal and their specifically developed proteolytic ferments (antibodies). Like results were obtained when pieces of kidney were transplanted. Moreover, the animals thus sensitized by the receipt of transplanted organs retained their sensitized condition for a time at least after the removal of the implanted tissue. The following is an illustration. A guinea-pig received intravenously 1 c.c. of lamb serum. Five days later it had two subcutaneous injections of 1 c.c. of lamb serum. Eight days later it was killed and transfused, and portions of its spleen and kidney implanted in a fresh guinea-pig. Six days later the implanted tissues were wholly removed, and after the guinea-pig had fully recovered from this operation it was found to be still sensitized to lamb serum. Evidently the implanted tissue had not only continued to develop its specific ferment, but had discharged it in part at least into the blood. These experiments are of the highest value for two reasons: (1) They caused Friedberger to wholly abandon his theory of sessile receptors, and (2) they show that the specific ferment developed in protein sensitization is a cellular product, and that the cells of the spleen and kidney, possibly of other organs as well, elaborate it.

Later this work was continued by Girgolaff,<sup>1</sup> who discusses the possible explanation of these findings. (1) It might be possible that the recipient is passively anaphylactized by the transfer of some serum from the donor. This supposition is held untenable for two reasons. First, the washing out is so thoroughly done, and second, the volume of the piece of organ transferred is too small. Moreover, if it were passive anaphylaxis, the recipient should be most responsive to reinjection within a day or two, while in fact it is not responsive until after seven or eight days. (2) It might be suggested that some of the protein used in sensitizing the first animal is carried over to the second and actively sensitizes it. This is highly improbable on account of the small amount of protein used in sensitizing the first animal; the length of time (in some cases fourteen days) elapsing before the transfer of the tissue, and the thorough washing out given the first animal. Besides, this was shown to be impossible because in one instance a rabbit was killed and its organs transferred to another rabbit three hours after the former had received a bacterial suspension, just at the time when the bacilli should have been most abundant in the tissue, and these animals were not sensitized. (3) The only conclusion which seems to have any justification is that the cells of the tissue removed, having acquired a new function while in their normal location, continue to exercise this function in their new location. We regard this as a most complete verification of our theory of anaphylaxis, in which we hold that a new function is developed in certain cells of the animal body by the sensitizer. Moreover, it does seem that this work should lead to the discarding of all theories involving an "antigenrest," about which much has been said.

Vaughan, Vaughan, Jr., and Wright<sup>2</sup> demonstrated that the serum and organ extracts of normal guinea-pigs do not form a poison when incubated with egg-white, but

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1912, xii, 401.

<sup>2</sup> *Ibid.*, 1911, xi, 673.

that corresponding preparations from guinea-pigs sensitized to egg-white do (p. 274).

Friedberger and Mita<sup>1</sup> show that summer frogs can be anaphylactized. From 0.1 to 0.5 c.c. of lamb serum was given by the abdominal vein, or into the dorsal lymph sac as a sensitizing dose. From one to four weeks later a reinjection causes characteristic symptoms. The animal soon becomes stupid and lies with extended limbs. When placed on its back it does not regain its normal position. Sudden death does not follow, and the animal usually survives from twelve to twenty-four hours. When the heart is watched through a fenestrated chest, the pulse is seen to grow slow and irregular, and finally the heart stops in diastole. Anaphylatoxin was found to have a similar action on the isolated heart.

Friedberger and Scymanowski<sup>2</sup> show that the presence of leukocytes lessens the formation of anaphylatoxin, and apparently destroys it when abundantly formed. They question whether this is due to an activity of the leukocyte or to its absorption of the poison. We suggest that the leukocytes destroy the poison by digesting it and converting it into a harmless body.

More than ten years ago (see p. 46) we showed that the poison contained in the cellular substance of the diphtheria bacillus is a wholly different thing from the toxin elaborated by the same organism. This convinced us that the protein poisons—substances obtained by the cleavage of the protein molecule—are not toxins. When the diphtheria bacillus grows it elaborates and excretes a soluble ferment known as diphtheria toxin. When injected into animals in sufficient doses this toxin kills after from two to five days. When repeatedly injected in smaller doses the body elaborates an antibody—an antitoxin. When the cellular substance of the diphtheria bacillus is split up by our method a poison is obtained. This is not a toxin, but a poison. When injected into

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1911, x, 362.

<sup>2</sup> *Ibid.*, 1911, xi, 485.

animals in sufficient doses it kills within a few minutes—not after days. When repeatedly injected in non-lethal doses the animal body does not elaborate an antibody—an antitoxin. Diphtheria toxin is specific; it is an exclusive product of the diphtheria bacillus, and animals treated with it in the proper way produce a specific antibody. The cellular poison of the diphtheria bacillus is not specific; the same poison is contained in other proteins, and it gives rise to no specific antibody. We convinced ourselves many years ago that the protein poisons and the toxins are not related bodies, and we demonstrated that diphtheria toxin gives no protection against poisoning with the active substance contained in the protein molecules making up the cell substance of the diphtheria bacillus. For this reason we have never discussed the phenomena of sensitization due to protein poisons in terms applicable only to toxin. Years after we did this work Friedberger and Reiter<sup>1</sup> confirmed it by showing that the protein poison obtainable from the cellular substance of the dysentery bacillus is a wholly different substance from the toxin of the same bacillus, but they make no mention of our work in this connection, and they still call the protein poison a toxin and speak of the antibody.

It has been fully demonstrated that the protein poison can be detached from its combination in the molecules of specific precipitates, blood corpuscles, stroma, many bacteria, etc., by incubation with normal guinea-pig serum. Other proteins require for their disruption and for the liberation of the poisonous group, sera in which specific ferments have been developed by sensitization. The proteolytic ferment in normal guinea-pig serum is not specific. It is capable of digesting many, but not all, proteins. When a guinea-pig has been sensitized to a given protein, its serum contains not only the general, non-specific proteolytic ferment normal to it, but in addition, the specific ferment. Whether the latter is a wholly new

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1911, xi, 493.

product or is due to a modification in the former we cannot say. For the present we will confine our attention to the general, non-specific proteolytic ferment in normal guinea-pig serum. Like all ferments, it is supposed to consist of two parts. (1) A thermostabile part, known as the amboceptor, and (2) a thermolabile part, known as complement or alexin. The latter is destroyed by a temperature of  $56^{\circ}$ , and serum heated to this point is inactivated. It has been found by most observers that inactivated serum from the guinea-pig, or, in fact, any inactivated ferment solution, does not function. Seitz<sup>1</sup> found in a few instances that by incubating certain bacteria with inactivated serum he obtained a free poison, but this is contradicted by the experience of so many investigators that we must conclude that his technique was defective.<sup>2</sup> There are, however, some points of real interest in connection with this reaction. Since bacteria and certain other proteins, when incubated with normal serum, yield a soluble and active poison, why does this reaction not occur when these proteins in the unbroken condition are injected directly into the blood? The only answer to this question seems to be that the ferment is in a more readily available form in the serum than it is in blood. The ferment in the serum probably comes largely from the breaking down of leukocytes. When an unbroken protein is injected into an animal usually the first effect upon the blood is a leukopenia. Certainly there is for a time a diminution of the leukocytes in the peripheral blood. After a time there is a hyperleukocytosis, and this is generally believed to be for the purpose of breaking up the foreign protein. There is, however, another possible explanation. It may be that in the circulatory blood the disruption is carried beyond the point of setting free the poison. It may itself be disrupted and converted into relatively harmless substances. There is also the possibility that the inclusion of the foreign protein by the phagocyte may delay the disruption of the former.

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1911, xi, 588.

<sup>2</sup> See article by Lura, *ibid.*, 1912, xii, 467.

It seems that all bacteria, both pathogenic and non-pathogenic, at least so far as tested, yield a poison when incubated with normal serum of the guinea-pig. Some are acted upon or disrupted more promptly and quickly than others, but with prolonged incubation all yield enough of the poison to affect animals to a recognizable extent. This confirms our work in which it was shown that all proteins, so far as tested, yield a poisonous fraction when properly disrupted by chemical agencies. It was this work that led us to conclude that every protein molecule contains a poisonous group. Dold and Aoki<sup>1</sup> have obtained the poison by incubation with serum from streptococci, meningococci, gonococci, *b. mallei*, pestis, pneumoniae, paratyphus, chicken cholera, swine erysipelas, yeast cells, actinomyces, the spirochetes of chicken spirillosis, and Russian relapsing fever. They did fail to obtain it from the spores of certain molds, but this does not prove that these spores do not contain a poison. It simply shows that the proteins of these spores are resistant to the cleavage action of the ferment contained in the normal serum of the guinea-pig.

Boehneke and Bierbaum<sup>2</sup> find that repeated, alternate freezing and thawing have no effect upon either the substrate or the ferment in the production of the poison, and no effect on the poison itself.

Bessau<sup>3</sup> sensitized animals simultaneously with ox and horse sera. The injections were made on each side of the thorax subcutaneously. After full sensitization had been developed, as was demonstrated by reinjection of controls which had been sensitized to only one serum, those doubly sensitized were given sublethal reinjections of one of the sera, and after recovery from the effect induced they were found to be less susceptible to reinjections of the second serum. He also determined the minimum fatal dose of anaphylatoxin prepared from typhoid bacilli on fresh

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1912, xii, 200.

<sup>2</sup> Ibid., 1912, xiv, 130.

<sup>3</sup> Centralbl. f. Bakteriol., 1911, lx, 637.

guinea-pigs, and found that animals sensitized to a serum, and given a non-fatal reinjection of the homologous serum, after recovery survived the minimum fatal dose of anaphylatoxin. From these experiments Bessau reached the following conclusions: (1) The condition or state of anti-anaphylaxis is not specific. (2) It is not due to absorption of the ferment (antibody). (3) It is due to increased tolerance of, or lessened susceptibility to, the poison. Friedberger and his students<sup>1</sup> have taken up these points, and by exact quantitative experiments have demonstrated that the state of anti-anaphylaxis, like that of anaphylaxis, is strictly specific, but that it is true that increased tolerance does play a part in the experiments as made by Bessau. When an animal is simultaneously sensitized to two sera, and after the condition of sensitization has been fully developed, a non-fatal reinjection of one of these sera renders the animal after recovery absolutely insusceptible to any dose of the serum which has been employed in the reinjection, but leaves it still susceptible to the second serum in doses only slightly larger than those required to kill control animals sensitized to that serum only. We can make this plainer by the following statement. When an animal is sensitized to two sera, two specific proteolytic ferments are developed. When an animal in full sensitization to both sera is treated with a non-lethal reinjection of one of them, the specific ferment for this serum is exhausted, and a certain amount of the poison is set free, not enough to kill the animal, but enough to give the animal increased tolerance to the poison. Consequently the fatal dose of the other serum necessary to kill on reinjection, say, twenty-four hours later, is larger than the minimum fatal dose when the animal has been sensitized to only one serum. We demonstrated (see page 139) many years ago not only that tolerance to the protein poison can be increased, but that resistance to living cultures of pathogenic bacteria may be increased by repeated doses of the poison. Furthermore, we showed

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1912, xiv, 371.

that in neither of these instances is the action specific, nor does the poison have the action of a toxin, nor is the increased tolerance of it due to the production of antitoxin. Repeated treatment of animals with the poison, beginning with a sublethal dose and gradually increasing the dose, may enable the animal to bear three or four times the minimum lethal dose, as tested on fresh animals, but the effect induced is never quantitatively comparable to that obtained by similar treatments with increasing doses of toxin. Besides, we were never able to find any evidence of the presence of an antitoxin in the blood serum of the treated animal. For these reasons we decided years ago that the protein poison is not a toxin. Moreover, we found that the increased resistance to typhoid infection came just as promptly and was as marked when the animal was treated with poison obtained from egg-white as that obtained by repeated treatments with the poison split off from the cellular substance of the typhoid bacillus. This demonstrated that the tolerance obtained to the protein poison is not specific. This is another clear proof that the poisonous group contained in the protein molecule is not a toxin. Years after our work had been done and reported, Friedberger<sup>1</sup> found that after a guinea-pig had recovered from severe poisoning with his anaphylatoxin, it would bear a certainly fatal dose of the same, and at that time he thought that he had secured a toxin-antitoxin immunity. Later still H. Pfeiffer<sup>2</sup> found that the urine of an anaphylactized guinea-pig is highly poisonous; also, that treating a sensitized guinea-pig with such urine made it more resistant on reinjection; also, that after recovery from anaphylactic shock, guinea-pigs are more resistant to the poison in the urine of anaphylactized animals. He also thought that he had established a toxin-antitoxin immunity, but if we read their later works with correct interpretation, neither Friedberger nor H. Pfeiffer now believe that the protein

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910, iv, 636.

<sup>2</sup> Ibid., 1911, x, 550.

poison is a toxin, in the sense of diphtheria or tetanus toxin, though both continue to call it a toxin. The work of Bessau and Friedberger confirms ours and establishes beyond any doubt that the increased tolerance brought about by repeated administrations of the protein poison by direct injection or by recovery from anaphylactic shock is not of the nature of a toxin-antitoxin immunity.

It seems to us that there is one point about anti-anaphylaxis which both Bessau and Friedberger fail to see. When a sensitized animal is reinjected with the homologous protein and recovers, it immediately loses, for a time at least, its responsiveness to the same protein. As Friedberger has shown, injections of two hundred times the amount necessary to kill the sensitized animal is without effect. Indeed, the animal seems to be returned suddenly to the condition of a fresh animal, one which has never received a protein injection. The usual explanation is that all the specific ferment in the sensitized animal has been exhausted by the non-fatal reinjection. Bessau thinks it due, as we have seen, to a decreased susceptibility to the poison, or, as we say, to an increased tolerance of the poison. Both of these are undoubtedly factors, and they may be the most important factors in the sudden development of the anti-anaphylactic state, but they are not the only factors, and we are inclined to the opinion that they are not the most important. That the specific ferment (the antibody of other writers) is not wholly exhausted is shown by the fact that the blood serum of an animal in the anti-anaphylactic state, when transferred to a fresh animal, sensitizes the recipient. This could not be if the ferment had been wholly used up. There must still be active ferment in the portion of blood serum transferred. The factor which we suspect of being of greatest importance in the production of the anti-anaphylactic state is the changed relation between the amount of ferment and the substrate. This is one of the most important and interesting problems connected with protein sensitization. In our earliest work with the cellular bacterial poisons,

when suspensions were injected into the abdominal cavity we found that large doses often failed to kill, or killed slowly, while smaller doses killed more certainly and more promptly; then we found that grinding our cellular substances more finely increased their toxicity; later we found that the introduction of large quantities of egg-white into fresh animals was without visible effect, while the repeated injection of very small doses produced prompt effects and speedily killed. Later still we ascertained that when a small amount of the blood serum of a guinea-pig sensitized to egg-white was incubated with from 1 to 5 mg. of egg-white *in vitro* we obtained an active poison, but when the amount of egg-white present was greatly increased there was no evidence of the production of a poison. Friedberger has repeatedly met with the same thing in preparing his anaphylatoxin. With a small amount of ferment and an excessive amount of substrate the reaction is impeded. Then the presence and accumulation of the products of fermentation retard the fermentative process. The concentration of the ferment, the substrate, and the products of fermentation all influence the rapidity with which the fermentative process proceeds, and all of these are altered when a few cubic centimeters of the blood serum of an animal in the anti-anaphylactic state is transferred to a fresh animal and the latter receives an injection of the proper protein. Besides, it is possible that in the preparation of the serum the amount of available ferment is increased by disruption of the leukocytes.

Friedberger at first stated that his anaphylatoxin is thermolabile. If this be true it cannot be identical with or very closely related to our protein poison, which is thermostabile. Later, Friedberger found that in acid solution anaphylatoxin is thermostabile. It is well to see how these differences can be reconciled. It must be understood that anaphylatoxin has never been isolated, not even partially, from the serum in which it is formed, and of course the serum is alkaline. Years ago we showed that our poison in alkaline solution decreases in toxicity, and that this

decrease takes place rapidly at high temperature. We showed this by making aqueous solutions of the poison alkaline with sodium bicarbonate and keeping them in the incubator for varying periods. Friedberger finds that when he heats the alkaline serum containing anaphylatoxin to 65°, its toxicity decreases, but when the serum is made acid it may be heated to 100° without appreciable loss in toxicity. It will be seen, therefore, that the two substances behave in the same manner when heated in alkaline solution. We never supposed that the heat destroyed our poison, but that on combination with alkali, which combination is hastened by heat, it becomes less poisonous, and Friedberger has failed to show that this is not true of anaphylatoxin.

There is another striking point of similarity between our poison and Friedberger's anaphylatoxin, and in this particular both substances show a close relationship to peptone. It has long been known that when an animal is quite fully under the influence of peptone the further administration of peptone has but little effect. This is true of both our poison and anaphylatoxin. We have designated this as tolerance, but it must be admitted that it is an unusual form of tolerance and it needs further investigation.

Besredka, Ströbel, and Jupilli<sup>1</sup> refuse to accept anaphylatoxin as the true anaphylactic poison because its administration to sensitized animals does not induce the so-called anti-anaphylactic state. Of course it does not and should not be expected to do so. The anti-anaphylactic state is due to the partial exhaustion of the specific proteolytic ferment, and the retarding effects of the products of digestion on the remaining ferment. Administration of the poison itself, already formed, uses up none of the ferment, and the other products of the cleavage action, besides itself, are not present. Another reason the French investigators give for concluding that anaphylatoxin is not the true anaphylactic poison is that the former is not specific in origin and may be obtained equally from diverse proteins;

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1913, xvi, 250.

certainly it can be obtained from all true proteins, as we demonstrated many years ago. The specificity of an infectious disease does not lie in the poison which is formed, but in the ferment by which it is formed. The same poison is contained in all bacteria, pathogenic and non-pathogenic, indeed, in all proteins, but there are specific ferments which break up one protein more readily and more completely than other ferments. The specificity lies in neither the substrate, except that it must be a protein, nor in the cleavage product, but in the agent that effects the cleavage.

**Physiological Action of the Protein Poison.**—Edmunds<sup>1</sup> has made the most thorough study of the protein poison, as prepared by Vaughan and Wheeler, reported up to the present time. His experiments were made on dogs and with the "crude soluble poison" made from casein. This preparation contains something less than 10 per cent. of the poison in the purest form in which, so far, we have been able to obtain it, and this is not chemically pure. On account of its importance we make the following, somewhat lengthy, abstract from the paper by Edmunds.

Intravenous injections in intact dogs are reported as follows: "The most prominent symptoms were a marked depression, disturbance of the alimentary canal, and some respiratory disturbances, the latter consisting of slight acceleration with a slightly labored expiration. In some animals the respiratory symptoms, with the exception of the slight acceleration, were scarcely noticeable. A study of these symptoms shows that they resemble closely those exhibited by dogs which are suffering from anaphylactic shock, although they are milder than those described by Pearce and Eisenbrey and others."

The effect on the circulatory system was studied upon dogs anesthetized with morphine and paraldehyde. Blood pressure was measured from the carotid, and the respiration recorded by a tambour resting against the chest wall and connected with a second one by which the movements were

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1913, xvii, 105.

traced upon blackened paper. Injection into the external jugular vein of the soluble portion of 100 mg. of the crude poison was followed immediately by a slow decline in blood pressure, amounting to from 6 to 8 mm. Hg.; after about twenty seconds the fall became rapid, passing from a normal of about 72 mm. to about 20 mm. Synchronously with the fall in pressure the heart-beat was at first slightly accelerated, passing from 138 or 140 to 144, but when the pressure reached the low point the heart-rate dropped to 92. The respiration was but little changed in rate, becoming slightly slower with the fall in pressure, but the strength was considerably decreased, neither inspiration nor expiration being as complete as normal.

The blood pressure was slow to recover. In some instances there was an increase of only a few millimeters after thirty minutes. When under the full influence of the poison, stimulation of either the sciatic or the great splanchnic nerve with the induced current elicited no response, showing peripheral paralysis of the vasomotors. In pithed animals (with the brain and cord destroyed) the effect upon blood-pressure was the same as on whole animals, only that the initial pressure being small, the fall did not measure so many millimeters. "That this action was peripheral to the ganglia along the course of the constrictor fibers was proved by the use of large doses of nicotine, sufficient being given to paralyze them. When this stage was reached an injection of the poison still produced the characteristic fall.

"The localization of the point of action of the poison upon nerve ending, receptive substance, or muscle wall was studied with the aid of nicotine, epinephrin, and digitalis. The action of nicotine was greatly weakened by the previous injection of the poison. Where before the poisoning, nicotine had given a marked increase in pressure in the characteristic manner, following 300 mg. of the poison 5 mg. of nicotine raised the pressure from 16 mm. to only 62 mm. The heart-rate was increased by the nicotine in the usual manner, from 120 per minute to 216. The pressure curve

from the nicotine was not only lower than that usually seen with such doses, but was much altered in shape, there being a very slow rise in place of the precipitous increase commonly obtained. The injection of nicotine was followed after a short time by a dose of epinephrin which raised the pressure from 22 to 220 mm. Further injections of large doses of the poison were followed by repeated injections of epinephrin which raised the pressure from 18 to 225 mm. These experiments seemed to point conclusively to the nerve endings as being the structure primarily acted upon by the poison, as evidently the receptive substance which is stimulated by the epinephrin had not been paralyzed by the poison. In addition to epinephrin, both digitalis and barium chloride raised the lowered blood pressure very satisfactorily, demonstrating that the muscle cell in these cases was not affected by the doses of poison given. In some animals, however, the use of large doses of the poison was followed by a lessened response to epinephrin and digitalis, thus showing that while the nerve ends are first affected, the effect of large doses is not necessarily confined to these structures, but may spread to the receptive substance and contractile substance proper."

Small doses (25 mg.) of the poison have but little effect upon the heart, beyond a temporary increase in systole and diastole in both auricle and ventricle. This increase is followed in about a minute by some weakening in both chambers, most marked in the auricle, both systole and diastole being decreased. With larger doses (100 to 150 mg.) the same changes are produced, the only difference being one of degree. The increase in systole in both auricle and ventricle is quite marked in some cases, while the change in the extent of the dilatation is not so great, but is present in most cases. These changes lead to an increased amplitude of beat which lasts usually from one to two minutes, until the blood-pressure has reached its lowest limit. The fall in pressure coming on while the strength of the beat is increased finds no explanation in the behavior of this organ.

“The changes which the heart undergoes, following the increase in systole described, consist in a weakening in both chambers, while the extent of dilatation may be still further increased, or it may show little change. Two factors come in to complicate the changes produced by the poison. First, the great fall in blood pressure produced by it decreases the resistance against which the heart has to contract; and second, the changes in respiration, which at times produce a mild degree of asphyxia. It happened in some cases, before the poison was given, the artificial respiration had seemed entirely adequate, after its administration the lungs did not inflate so well and the blood showed signs of deficient aeration. The heart changes were therefore studied further in animals in which all other organs, save the lungs, were excluded from the circulation. This experiment was carried out on a large bulldog anesthetized in the usual way. Under artificial respiration the sternum was cut lengthwise, the two halves pulled apart, and the heart exposed. The large vessels at the base of the heart were dissected free and a clamp placed in position on the aorta just below the origin of the left subclavian artery, this and the right subclavian being tied. A loose clamp was also placed on the inferior vena cava just above the diaphragm. Cannulas were placed in both common carotids and external jugular veins. The cannulas in the left carotid and the right jugular were paraffined and connected by a short paraffined rubber tube, thus providing a channel for the blood from the left to the right heart. The cannula in the right carotid was connected with the mercury manometer to record the blood pressure, and that in the left jugular was used to inject the poison. The clamps on the aorta and the vena cava were now closed, thus shutting off the circulation in all parts of the body, except the heart and lungs. The pericardium was opened and sewed to the sides of the chest to form a sort of cradle for the heart. The myocardiograph was attached to the right auricle and ventricle in the usual manner, and arranged to record their movements on the kymograph.” After recovery from the opera-

tion the pressure stood at 105 mm. Injection of the poison caused a fall of only 2 or 3 mm. Hg. In this way it was shown that the heart and lungs were not responsible for the fall in pressure observed in the intact animals.

Isolated organs were perfused with solutions of the poison and a dilatation of the vessels, probably due to paralysis of the vasomotor mechanism, was observed. This paralysis did not disappear after subsequent washing with Ringer's solution, but did so promptly on the use of epinephrin. The perfusion experiments, therefore, indicate a local paralyzing effect upon the vessel walls.

Edmunds demonstrated by careful experimentation<sup>1</sup> that the liver dilates with the fall in blood-pressure. This seems to settle the question of the distribution of the blood as the pressure falls. "The fall is due primarily to a peripheral paralysis of the vasomotors running in the splanchnic nerves." The spleen, kidneys, and intestine do not show increase in volume, as the blood is drained from these organs into the capacious blood channels of the liver. Other vascular areas besides those innervated by the splanchnics are affected. This was shown by the fact that when the poison was injected into white dogs the skin over the thorax and abdomen and down on the legs became bright pink. When the liver was excluded from the circulation the fall in blood pressure occurred, but less promptly.

Respiratory changes in the dog, due to the poison, are not marked. The usual effects are slight acceleration and weakening. With the chest walls open and under artificial respiration, there would be, at times, signs of asphyxiation which were easily relieved by a slightly stronger pressure on the bellows. The most marked change in the blood picture observed was a diminution in the eosinophiles, both relatively and absolutely.

It has been observed by all who have studied the action of peptone and the protein poison, that after the blood-

<sup>1</sup> The details can be found in *Zeitsch. f. Immunitätsforschung*, 1913, xvii, 105.

pressure has fallen to the lowest limit the further administration of the peptone or poison is without effect.

Edmunds closes his studies with the following conclusions: "The toxic portion of the split protein molecule as described by Vaughan and Wheeler produces in dogs when injected intravenously the same symptoms as are seen in these animals when suffering from acute anaphylactic shock. An analysis of the changes shows the same rapid fall in blood pressure due mainly to paralysis of the vasomotor endings of the splanchnic nerves. The blood does not accumulate at the time of the fall in pressure in the intestines or kidneys, but is drained from them into the liver, and probably into the large abdominal veins. There is no evidence of a constriction of the pulmonary vessels, nor of lack of blood to the left side of the heart. In these points the action of the protein poison agrees with the changes described in anaphylactic shock, but whereas with the latter the ability of the blood to coagulate may be lost, this is not affected by the split product."

**General Physiological Action of Proteins.**—Schittenhelm and Weichardt<sup>1</sup> conclude a study of this subject as follows: The compound proteins, as such, are relatively inactive. In the doses employed they give rise to no symptoms and do not affect blood pressure. Their components (the globulins, histons, and protamins) are highly poisonous compared with the native simple proteins. They cause a marked fall in blood-pressure, delay blood coagulation, influence respiration and temperature, and in small doses may cause death. This is true even when they are of homologous origin. From the composition of the protamins and histons it has been inferred that their poisonous action is connected in some way with their large diamino acid content, but the globins do not contain a large amount of these acids. On the other hand, as has been stated, the kyrins contain a large amount of diamino acids and are not so poisonous as the protamins and histons. It should

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1912, xiv, 609.

be remarked that while the globins do not contain a large amount of the diamino acids, they are rich in the closely related body, histidin, and the relation of this to the highly poisonous  $\beta$ -i body of Barger and Dale has been mentioned. Certainly there is reason for suspecting that the poisonous group or groups in the protein molecule has some close chemical relationship to the diamino acids.

**Sensitization is Cellular.**—Dale<sup>1</sup> has shown that the plain muscle of a sensitized guinea-pig contracts when touched with a dilute solution of the homologous protein. This demonstrates that sensitization is cellular. Dale states his conclusions as follows: “(1) Plain muscle from an anaphylactic guinea-pig, freed from all traces of blood and serum, has a very high degree of sensitiveness to the specific sensitizing protein. The plain muscle of the virgin uterus is essentially suited to the demonstration of the condition, and exhibits a definite rise of tonus in response to extreme dilutions of the antigen. (2) The effect is practically immediate, *i. e.*, the delay is not obviously more than can be attributed to the method of application of the antigen. (3) The response is not a mere exaggeration of the reaction which normal, plain muscle gives to fresh sera in general. Preparations of purified protein can be obtained (*e. g.*, serum globulin precipitated by Gibson’s method, or egg albumen crystallized by Hopkins’ method) which have no effect on the normal plain muscle, but are as toxic for the anaphylactic plain muscle as the native proteins. (4) One dose of the specific antigen, in sufficient concentration to produce “maximal” response of the anaphylactic plain muscle, completely desensitizes the latter to further doses of any dimensions, provided that the experiment is not complicated by the use of an antigen preparation of normal toxicity. Either normal or anaphylactic plain muscle gives repeated responses to successive large doses of a normally toxic serum or other native protein, but this phenomenon is not anaphylactic response. (5) When sensitizing doses

<sup>1</sup> Jour. of Pharm., 1913, iv, 167.

of several antigens are given, a multisensitization of the plain muscle can be demonstrated. Desensitization of the muscle to one antigen is not without effect on its sensitiveness to the others. (6) The washed plain muscle from guinea-pigs immunized to an antigen by a series of injections, is sensitive to the antigen, like that from anaphylactic pigs. But the sensitiveness is in this case less rigidly specific, *e. g.*, plain muscle from a guinea-pig immunized to horse serum showed a subsidiary sensitiveness to sheep serum. (7) The sensitiveness of the washed, plain muscle is seen in passive as in active anaphylaxis, whether the serum producing passive sensitization is obtained from sensitive or immune guinea-pigs. (8) The actively or passively sensitized plain muscle after being desensitized *in vitro* can be resensitized *in vitro* by mere contact for some hours with a not too great amount of sensitive serum. It has not been found possible to sensitize normal plain muscle in exactly the same way; but perfusion of a normal uterus for five hours, with diluted serum from sensitive guinea-pigs, produced a decided passive sensitization. (9) The response to the specific antigen of the bronchioles of the anaphylactic guinea-pig is not impaired by excluding the abdominal viscera and the brain from the circulation, and is produced with apparently undiminished vigor in the isolated lungs perfused with Ringer's solution."

Dale suggests that the response of plain muscle to its specific sensitizer might be used for medico-legal purposes. The suspected material might be used to sensitize a guinea-pig. After allowing time for full sensitization the uterus could be excised and a suspended horn tested with various sera until the one giving the typical response was detected. With the other horn the limits of the response might be determined. A second method might be as follows: "Guinea-pigs could be sensitized with a small injection of known serum from the suspected species, *e. g.*, human serum. After the usual incubation period one pig would be killed, the uterus excised, and the degree of sensitiveness of the first horn to human serum tested. If the sensitiveness

were not of a high order, another pig could be tried at once, or after a few days longer incubation period. When a uterus was found which gave a large and clear response to, say, 1 in 100,000 human serum, the other horn, kept meanwhile in warm oxygenated Ringer, could be suspended in a small volume such as 10 c.c. of Ringer's solution. A dose of the suspended material could then be added, and if no reaction were produced it would be clear that the dose contained less than 0.0001 c.c. of human serum, which should be sufficient evidence, in any ordinary case, that the blood under examination was not human. If, on the other hand, a decided response were produced, it would only be necessary to test further the action of the specimen on a normal uterus, so as to exclude primary non-specific toxicity."

It has been demonstrated by Manwaring,<sup>1</sup> and confirmed by Voegtlin and Bertheim<sup>2</sup> that dogs sensitized to horse serum do not respond on reinjection when the liver is excluded from the circulation.

**Theories.**—Hamburger and Moro at one time suggested that the first injection leads to the formation of precipitins, and that on reinjection precipitates are formed, and induce the anaphylactic symptom-complex by the formation of capillary emboli. The formation of specific precipitins is a reaction which occurs *in vitro*, but not *in vivo*. Besides, the symptoms of anaphylaxis are not those due to emboli, and finally, no emboli are formed.

Gay and Southard thought that as a result of the first injection there remains in the circulation a protein rest which they named "anaphylactin," and that this continues to stimulate the cells, creating an abnormal affinity for the homologous protein which on reinjection leads to anaphylactic shock. The transfer of this "anaphylactin" to a fresh animal was supposed to explain passive anaphylaxis, a phenomenon first studied by these investigators.

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910, viii, 1.

<sup>2</sup> Jour. Pharm., 1911, ii, 507.

Richet held that sensitizers contain a substance which he called "congestin," and that this develops in the animal another substance known as "toxogenin." The reaction between the latter and the homologous protein on reinjection sets free a poison "apotoxin," which on account of its effect on the nervous system, develops the symptoms of anaphylaxis. Considering Richet's toxogenin a ferment, we can accept this theory as essentially correct.

Besredka taught that the sensitizer contains two substances—"sensibilisinogen" and "antisensibilisin." On the first injection the former develops in the animal body a substance, "sensibilisin," and on reinjection the sensibilisin and the antisensibilisin combine to form a poison which acts on the nervous system. Besredka has not been able to produce satisfactory proof of the existence of antisensibilisin. His work along this line has already been referred to (p. 260).

For reasons which will become evident as we proceed, it is desirable to go somewhat into detail in considering the theory of Friedberger. This was first published in 1909,<sup>1</sup> and in this publication Friedberger clearly and unequivocally set forth his theory. It may be known as the theory of sessile or fixed receptors. The following is an abstract of the statement: On the first injection the protein finds but few groups with which it can combine, and for this reason it is not poisonous, even in large doses, just as happens when tetanus toxin is injected into a naturally immune animal. During the period of incubation the animal cells develop specific receptors for the homologous protein. With frequent injections at short intervals, as when the object is to obtain a highly active precipitating serum, the newly formed receptors are in large part cast off into the blood. When a single small dose is given, as in sensitization, less receptors are cast off into the blood, and more remain attached to the cells. In this way an organism relatively insusceptible to a given foreign protein is made

<sup>1</sup> Zeitsch. f. Immunitätsforschung, ii, 208.

highly susceptible, and on the second injection the protein is firmly anchored to the cell, just as the cells of an animal susceptible to tetanus anchor the tetanus toxin. The only difference is that in the latter instance the receptors are preformed, while in the case of sensitization they are developed as a result of the first injection. It is, as Ehrlich's theory explains, the same substance, the receptor, so long as it remains attached to the cell, that is the cause of the poisoning, and which becomes the cause of cure when detached from the cell, and cast off into the blood. The only difference, as has been stated, is that the substance attached to the cell (the receptor) is not, at least in sufficient quantity, preformed, and must be developed by the first injection. Protein (toxin) immunity and anaphylaxis, therefore, are alike save in the proportion and location of the antibodies. When the precipitin is already in the body fluids the injection of the homologous protein is without effect; when the precipitin is still attached to the cell in sufficient quantity the reinjection of the homologous protein is followed by the phenomena of anaphylaxis. The antibodies exist in two places: (1) As free antibodies in the serum (known as precipitins in test-tube experiments). (2) As sessile antibodies attached to the cells. In cases of local sensitization, as in Arthus phenomenon, the local cells only are affected because they are the only ones which bear the sessile receptors. The animal escapes anaphylactic shock because the cells of the body as a whole, and especially those of the nervous system, do not carry the specific sessile receptors. Friedberger, in his theory, explains antianaphylaxis as follows: An animal is rendered anti-anaphylactic when it receives a large reinjection before the period of incubation is complete. In this case the reinjection uses up the sessile receptors already developed, but these are not enough to lead to anaphylactic shock, and at the end of the period of incubation the new crop of sessile receptors is not sufficiently developed to give rise to the symptoms of anaphylaxis. Again, an anaphylactized animal may be rendered antianaphylactic by a small dose

of the antigen. This is explained by Friedberger by supposing that the small dose uses up a part of the sessile receptors, and that there are not enough left to induce anaphylactic shock when another injection is made. In short, he concludes: "In every case antianaphylaxis is nothing more than anaphylaxis refracta dosi." Passive anaphylaxis is explained by Friedberger by supposing that the free receptors in the blood of an anaphylactized animal become, on injection into a fresh animal, anchored to the cells, thus forming fixed or sessile receptors. This is Friedberger's theory. It is clean cut and clearly stated by its distinguished author, but at present it has no support, and is clearly out of harmony with known facts, some of the most important of which have been discovered by the researches of its own author. It was an attempt to make the facts of anaphylaxis fit Ehrlich's theory of the action of toxins and the production of toxin immunity, while the trend of later research is to show that the two sets of phenomena have but little in common. Friedberger's theory would make the action of sensitizers, such as serum albumin, egg-white, edestin, bacterial proteins, etc., identical with that of diphtheria and tetanus toxin, abrin, ricin, the venoms, etc. There is nothing in the theory about the development of the proteolytic ferments, and the liberation of a protein poison by the parenteral digestion of the sensitizer on reinjection. If we read his works with correct interpretation, Friedberger has abandoned his own theory largely, if not wholly. Indeed, in Contribution VI,<sup>1</sup> Friedberger plainly discards his own theory.

According to Friedberger's theory all sensitizers act like the toxins; although at first only mildly toxic, they become more so by developing the receptors, and thus rendering the animal more susceptible. It is in a way proper for Friedberger to speak of anaphylaxis as a "protein-anti-protein" reaction. Friedberger calls the sensitizer an antigen and the substance developed in the animal an

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1910, vi, 179.

antibody. According to our theory these terms are not only inappropriate, but are confusing and misleading.

The theory of Vaughan and Wheeler was first published in 1907,<sup>1</sup> two years before that of Friedberger, and while it has been developed and, in our opinion, confirmed by later investigations, there has been no material alteration in it. To one who has read this chapter thus far the essentials of this theory must be already fairly understood, but a concise statement of its fundamental points must be made here even if some repetition be necessary. The proteins taken into the alimentary canal are broken up by the digestive ferments into non-protein split products, mostly amino-acids. During or after absorption these pieces are resynthesized, in part at least, to form the body proteins peculiar to the species. The precipitin test shows that with the exception of the proteins of the crystalline lens, those of all the fluids and tissues of the body are peculiar to the species. Those of one species differ from those of all other species. Just where this synthesis occurs in the animal body we are not sure, but that the species proteins are formed from the split products of the proteins of the food has been positively demonstrated. Every protein molecule contains a poisonous group. In the whole molecule this group is saturated with other non-poisonous groups. As the whole molecule undergoes cleavage as a result of enzyme action, the poisonous group is more or less completely liberated, and in this process it becomes activated. In alimentary digestion the poisonous group becomes most active at or about the stage of the formation of peptone. As the digestive process proceeds, the poisonous group is itself disrupted, and ceases to be a poison. The protein poison is not readily diffusible, and for this reason it is retained in the alimentary canal until it is broken up and rendered inert. When an unbroken or undigested protein finds its way into the blood or tissues it must be digested. There are two kinds of proteolytic digestion:

<sup>1</sup> Jour. Infect. Dis., June, 1907.

(1) Enteral, (2) parenteral. Proteins that escape enteral digestion and find their way into the body must be digested by the body cells. When some foreign protein, like the blood serum of another animal, milk, egg-white, bacterial proteins, etc., are injected into the blood or tissues they must be digested. There is only one way in which this can be accomplished, and that is by the cells of the body, and there is only one way in which these cells can do this, and that is by elaborating a specific proteolytic ferment which will digest and destroy the foreign protein.

In experimental anaphylaxis the first injection introduces into the body a foreign protein. This must be digested and the body cells slowly elaborate a specific proteolytic ferment which slowly digests it. In doing this certain body cells acquire a new function. The protein of the first injection is slowly digested usually without the development of recognizable effects, and consequently we conclude that the animal has not been affected or had its functions altered in any way. But this is a mistake. The animal has been profoundly affected. It has developed a new function which it may retain quite indefinitely, and which may be transmitted from mother to offspring. The foreign protein is digested and its poisonous group set free, but this has been done so slowly and gradually that the effects have not come within the range of our powers of recognition. After the protein of the first injection has been disposed of, the new ferment in the form of a zymogen continues to be formed in the cells, and on the second injection after the proper interval, this zymogen is activated and splits up the protein so promptly and so abundantly that the liberated poison induces the symptoms of anaphylactic shock.

The following statements formulated in 1907, in our opinion, still hold good:

1. Sensitization consists in developing in the animal a specific proteolytic ferment which acts upon the protein that brings it into existence, and on no other.

2. This specific proteolytic ferment stored up in the cells of the animal as a result of the first treatment with the protein remains as a zymogen until activated by the reinjection of the same protein.

3. Our conception of the development of a specific zymogen supposes a rearrangement of the atomic groups of the protein molecules of certain cells, or an alteration of their molecular structure. In other words, we regard the production of the specific zymogen not as the formation of a new body, but as resulting from an alteration in the atomic arrangement within the protein molecule, and a consequent change in its chemism.

4. Some proteins in developing the specific zymogen produce profound and lasting changes in molecular structure, while the alterations induced by others are slighter and of temporary duration, the molecular structure soon returning to its original condition.

5. Bacteria and protozoa are living, labile proteins, while egg-white, casein, serum albumin, etc., are stabile proteins. The proteins of one group are in an active, while those of the other are in a resting state, but both are essentially proteins made up of an acid or poisonous chemical nucleus, and basic, non-poisonous groups. Bacterial immunity and protein sensitization, apparently antipodal, are in reality the same, and each consists in developing in the animal body the capability of splitting up specific proteins. If the living protein be split up before it has time to multiply sufficiently to furnish a fatal quantity of the poison, the animal lives and we say it has been immunized. If the stabile protein be introduced into the animal body it leads to the development of a specific proteolytic ferment, and if enough of it to supply a fatal dose be reinjected after this function has been developed, the animal dies.

6. We are compelled to change our ideas concerning the causation of the lesions of the infectious diseases. Formerly, we believed the structural changes to be due wholly to the living, growing, feeding microorganisms. For instance, we were sure that the intestinal ulcerations

of typhoid fever are caused by the living bacilli. Now we know that these lesions follow the intravenous injection of dead proteins. As has been stated, each foreign protein has its predilection tissue in which it is largely deposited, whose cells it especially sensitizes, and where it is disrupted. This explains the characteristic lesions and symptoms of the different infectious diseases. Bacterial inflammation is essentially a chemical process, or is due to the disruption of cell molecules through the chemical affinity between certain groups in the bacterial cell and certain groups in the cell of the animal. So long as the bacterial cells are alive the chemism that holds the living molecule together tends to resist this process of disintegration. The pathogenic bacterium assimilates the nutritious constituents of the fluids of the animal body, builds them into its own tissue, converts them into substances foreign to the host, and finally, when the bacterial cell goes to pieces either from spontaneous dissolution, or through the aggressive action of some animal cell, these reconstructed chemical groups are set free and poison the animal, inducing lesions in various tissues, and, in many instances, so interrupting the vital functions as to cause death. It is in harmony with these statements that Friedberger has been able to induce aseptic pneumonia by spraying horse serum into the lungs of guinea-pigs sensitized with the same, and Schittenhelm and Weichardt have established "enteritis anaphylactica" by the reinjection of egg-white into sensitized dogs. It is more than probable that cholera infantum and the kindred summer diarrheas result from the absorption of undigested milk and consequent sensitization. The designation "protein diseases" might be used to cover the majority of bacterial and protozoal diseases, and many of these hitherto regarded as autogenous.

7. It seems to be a physiological law that the specific ferments elaborated by living cells are determined by the proteins brought into contact with them, but as yet we know but little concerning these bodies which we call ferments. That they are labile chemical bodies resulting

from intramolecular rearrangement in the protein molecules of the cell seems a plausible theory, but at present it is only a theory. We know but little of the action of these so-called ferments upon their homologous proteins. Our knowledge of the chemistry of protein sensitizers is exceedingly limited, and as we have pointed out, it is highly desirable that work in this direction should be prosecuted with vigor, because we need sensitizers free from the poisonous group. Furthermore, there is the question why small doses of protein induce fever while large doses have no such effect. At present we have no satisfactory answer to this question. If it could be conclusively demonstrated that the toxins are ferments, the subject of the etiology of disease would be greatly simplified. We have elsewhere (see Chapter XV) given our reasons for holding that the toxins are ferments, and at this point we wish to formulate what we believe to be two biological laws:

(a) When the body cells find themselves in contact with, or permeated by, foreign proteins they tend to elaborate specific ferments which digest and destroy the foreign proteins.

(b) When body cells are attacked by destructive ferments they tend to elaborate antiferments the function of which is to neutralize the ferments and thus protect the cells.

Zunz<sup>1</sup> finds the proteoclastic (protein-splitting) properties of blood-serum, as tested on the sensitizing protein, increased in the anaphylactic state. This increase becomes measurable in the pre-anaphylactic stage, usually about the fifth day after the injection, and continues to be measurable to from the twentieth to the sixtieth day. It is not recognizable in blood-serum taken during or soon after anaphylactic shock. Zunz concludes that the increased proteoclastic property of the blood serum is not sufficient to fully account for the phenomena of anaphylaxis. In this conclusion we fully agree with the distinguished Belgian investigator. In our opinion the failure of the blood serum taken during

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1913, xvii, 241.

or soon after anaphylactic shock to show measurable proteoclastic effect is due to the accumulation of the cleavage products in the blood. That all the phenomena of anaphylaxis are not due to cleavage ferments in the blood-serum must be evident to all who have followed us thus far. The work of Pfeiffer and Mita, that of Zunz, our own, and that of others agree in showing that the property of splitting up the sensitizing protein, in measurable quantity, at least, disappears from the blood-serum of the sensitized animal long before the anaphylactic state disappears. When a guinea-pig is sensitized to horse serum, the blood-serum of this animal loses the power to split up horse serum *in vitro* in appreciable amount after from twenty to sixty days, but the animal remains sensitive to horse serum for at least two years, as shown by Rosenau and Anderson, and probably so long as the animal lives. We must therefore agree with Zunz that the increased proteoclastic property of the blood-serum of the sensitized animal is not sufficient to account for all the phenomena of sensitization. Our theory of sensitization takes this into account. We hold that sensitization develops in certain body cells a new function—that of elaborating a new specific, proteoclastic ferment. The duration of this new function varies with the sensitizing protein and with the cells in which it is developed. In a given cell this function must be limited by the life of the cell. We do not know just what cells develop this new function, but we do know that the animal may remain in a sensitized state long after the blood-serum fails to show any cleavage action on the sensitizing protein *in vitro*. It may be that the specific ferment present in the blood-serum of recently sensitized animals comes from the white corpuscles, or it may come in part, or altogether; from fixed cells. It seems justifiable to conclude that the ferment which manifests its action in animals long after it is absent from the blood must come from fixed cells, and that these are stimulated to elaborate this ferment only when the specific protein is brought into contact with them, probably only when they are permeated by the specific protein. All the facts which

have been ascertained in regard to this matter indicate that sensitization is secured only by alteration in the cell and that in some cells the newly developed function is more persistent than in others.

The facts of cross-sensitization seem in harmony with our view that the protein molecule contains one or more special sensitizing groups. The white of hen's eggs sensitizes to itself, less perfectly to the white of duck's eggs, and very imperfectly or not at all to the white of robin's eggs. The serum of man's blood sensitizes to itself and less fully to that from the ape. Horse serum sensitizes to itself, and less fully to that of the donkey. Certain non-pathogenic acid-fast bacteria sensitize in some degree to the tubercle bacillus. In short, the phenomenon of sensitization, like that of precipitation, may be employed to show biological relationship. All this seems in harmony with the view that the specificity of sensitization depends upon the similarity or dissimilarity in the chemical structure of protein molecules from different sources.

Doerr<sup>1</sup> makes the following statement concerning Friedberger's anaphylatoxin: So far as the matrix of the poison is concerned it is highly improbable that it comes from the bacteria or other antigens. That the most diverse proteins should yield the same poison seems improbable. The theory that the poison comes from the amboceptor, as held by Wassermann and Keysser, is still less probable. That the anaphylatoxin comes from the blood-serum, the one constant factor in all the experiments in its production, is most probable. During its formation or in the process of blood coagulation, a poison is formed, but the serum obtained, after coagulation is complete, is not poisonous on account of the presence of antibodies. When the serum is digested with bacteria, precipitates, etc., the latter absorb the antibodies and thus the serum again becomes poisonous. That anaphylatoxin can be obtained when there are no formed elements present, as for instance when inactivated horse serum is

<sup>1</sup> Handbuch d. path. Mikroorganismen by Kolle and Wassermann, second edition, ii, 947.

digested with fresh guinea-pig serum, does not contradict this theory because the colloidal bodies may serve as absorption agents.

This explanation of the easy production of anaphylatoxin, given by Doerr, is worthy of consideration. His failure to understand how the same poison can be obtained from the most diverse proteins has no weight with us since we have prepared a poison which has, grossly at least, the same physiological action, from the most diverse proteins, bacterial, vegetable, and animal. The protein molecule, wherever found, must have some common nucleus, and this we believe to be the poison. But that a poison may be liberated in the process of blood coagulation does not seem to us to be beyond the range of possibility. Blood coagulation is a fermentative process and that there is no cleavage in the protein molecule in this process has not been shown.

As Doerr points out, so long ago as 1877 Köhler showed that fresh defibrinated blood, whether homologous or heterologous, is an active poison. This has been confirmed by others, and recently it has been reinvestigated by Moldovan, who has shown that blood freshly defibrinated by shaking with glass beads causes acute death when injected intravenously into guinea-pigs and rabbits. In the former animals the typical anaphylactic lungpicture after death is seen. When the dose is slightly sublethal there is marked fall in temperature with subsequent fever. When the doses are smaller there is marked fever. On standing for fifteen to forty-five minutes defibrinated blood loses in toxicity. Serum obtained by rapid centrifugation of defibrinated blood is poisonous. The same is true of the deposited and once-washed corpuscles. When coagulation is delayed by the presence of sodium citrate, neither the supernatant fluid nor the corpuscles are poisonous, but both become so when coagulation has been induced by shaking with porcelain beads. Later, Doerr has shown that blood received in paraffined vessels becomes poisonous; but when coagulation is complete the toxicity disappears. When coagulation is made to proceed slowly by the addition of hirudin solution or a 0.7 per cent.

solution of colloidal silicic acid, it retains its toxicity for several hours. The source of the poison in coagulation blood has been discussed and variously explained. Köhler thought the fibrin ferment is the poison, but this was shown not to be true by Boggs. Studzinski suggested that the poison might come from the mechanical disruption of the red corpuscles, but Moldovan and Doerr have shown that the plasma or serum may be absolutely free from both hemoglobin or cells and still be poisonous. Freund thought that the poison might come from the disrupted blood platelets, but in rapid centrifuges even the platelets may be removed and even then the plasma or serum may be poisonous. However, the role that the platelets play in coagulation still suggests that they may furnish the matrix for the poison.

The toxicity of extracts of normal tissue, especially of the lungs and lymph nodes, is most interesting in this connection. If the normal lungs of a rabbit or guinea-pig be digested for two hours in physiological salt solution, the solution kills promptly on intravenous injection. Homologous organ extracts are more active than heterologous. Rabbits are the most susceptible animals used so far. The poison seems to be destroyed when heated to 70°; it does not pass through a Berkefeld filter, and is absorbed by kaolin as is anaphylatoxin. The addition of serum either homologous or heterologous seems to destroy or neutralize the poison after contact of one hour or more. Doerr states that the fresh vaccine growth just scraped from a calf with a curette furnishes an extract which kills rabbits instantly on intravenous injection. These extracts seem to owe their effects to a coagulating ferment.

Section of a dying animal shows the left heart, and the pulmonary arteries and veins filled with coagula. Doerr admits that poisoning with organ extracts from normal animals is quite unlike death from anaphylactic shock. In the former the blood is coagulated; in the latter it is fluid. But Doerr holds that the formation of thrombi during life is not the sole cause of death after the administration of extracts from normal tissues or after the intravenous

injection of fresh defibrinated blood. He states that Bianchi failed to find intravascular thrombi after sublethal doses of the extracts, and that Moldovan met with a like observation after poisoning with defibrinated blood. To us the difference between poisoning with normal tissue extracts and the effects of anaphylactic shock seem quite clear. The organ extracts do not contain a chemical poison, but a ferment. This ferment coagulates the blood and leads to the formation of thrombi. This is a process of protein digestion, and whether a protein poison is set free in it remains for future research to determine. In anaphylactic poisoning the ferment is in the body cell and splits up the protein introduced with the liberation of a protein poison.

In this connection the work of Blaizot<sup>1</sup> is of interest. When dog's serum is treated with an extract from the intestinal mucosa of the dog, or rabbit's serum with an extract of the intestinal mucosa of the rabbit, after a few minutes of contact, if either preparation be injected intravenously into a guinea-pig, acute death results. Extensive thrombi are found in the heart and large vessels. The serum of the guinea-pig is not rendered poisonous by homologous extracts, but is made poisonous by heterologous extracts.

We must, however, admit with Doerr, that the matrix of Friedberger's anaphylatoxin remains undetermined, with much probability in favor of the possibility of its being in the so-called complement, or the serum of the guinea-pig, the one constant factor in its production. This does not mean that it is not a protein poison. It must be borne in mind that anaphylatoxin is recognized only by its effect, and it has never been even partially isolated from the serum. Our protein poison comes certainly from the protein molecule. It cannot be a ferment as we understand ferments at present. It is thermostabile, and it elaborates no antibody and yet it may be identical with anaphylatoxin, for whether the latter comes from bacterial cells or from the serum it is of protein origin.

<sup>1</sup> Compt. rend. Soc. biol., 1910-11-12.

Friedberger, Mita, and Kumagi<sup>1</sup> have prepared anaphylatoxin by the action of the normal serum of the guinea-pig on the crude toxins of tetanus and diphtheria, and the venom of the cobra. They assume that the poison comes from the cleavage of the toxins, an assumption which seems to us wholly without warrant. Some years ago we precipitated the crude toxins of tetanus and diphtheria with alcohol and split up the precipitate with chemical agents by our method, and obtained the protein poison, but we never felt justified in even supposing that the poison came from the toxins. Crude toxins and venoms are complex protein substances, and because the protein poison can be obtained by the cleavage of these is far from proof that the poison comes from the toxin constituent of such a mixture. Indeed, one does not know that the active principle in these mixtures is a protein. Until the toxins have been obtained in something like a pure state it is useless to speculate concerning their split products.

Bordet<sup>2</sup> has shown that anaphylatoxin can be obtained by the action of the normal serum of the guinea-pig on agar, and this has been confirmed by Nathan.<sup>3</sup> One-half gram of agar is added to 100 c.c. of 0.85 per cent. salt solution and sterilized by boiling. From 0.5 to 1 c.c. of this agar solution is incubated with 5 c.c. of normal serum from the guinea-pig at 37° for from one to twenty-four hours and then centrifuged. Many tubes may be employed and the supernatant fluid from these mixed. From 3 to 5 c.c. of this fluid injected intravenously into a guinea-pig of about 250 grams kills with typical anaphylactic symptoms in from three to five minutes. Even 0.1 c.c. of the agar solution furnishes enough poison to kill. When the amount of agar solution employed is greater than 1 c.c. or less than 0.1 the amount of poison formed is, as a rule, not sufficient to kill, but may induce anaphylactic symptoms of varying intensity. When the

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1913, xvii, 506.

<sup>2</sup> Compt. rend. Soc. biol., 1913, lxxiv, No. 5.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, 1913, xvii, 478.

serum is inactivated by being heated for one-half hour at 55° no poison is formed. Experiments of this kind, as well as those with kaolin already referred to, have led to various suggestions. They have caused many to suspect that the poison comes from the serum. It has been suggested: (a) That the agar or kaolin or bacteria absorbs the complement from the serum and that this renders the serum poisonous. (b) That the poison is preformed in the serum but that its action is neutralized by some other constituent of the serum which is absorbed by the agar or kaolin. (c) That the absorption of some constituent of the serum by the agar, kaolin, or bacteria leads to a disturbance of the equilibrium of the protein constituents of the serum which as a consequence break up with the liberation of the poison. These suggestions assume that the poison comes from the serum, and this may be true. Further experimentation will determine this, but it must be borne in mind that agar contains small amounts of protein, and this has a large surface exposure and is in a physical state most favorable to the action of a proteolytic ferment in the serum.

Zinsser<sup>1</sup> has shown that the action of complement "upon typhoid bacilli strongly sensitized or not at all sensitized may be carried on, at body temperature, for considerably longer than twelve hours, without leading to a destruction of the poisons, and that this is true when the quantities of the bacteria used vary within the wide range of from one to twelve agar slants. It has been found, in fact, that in the case of this microorganism prolonged exposure at the higher temperature of considerable quantities of bacteria constitute an unfailing method of regularly obtaining powerful poisons. The results obtained by the use of smaller quantities and the less vigorous action at low temperatures are far less regular or satisfactory." He thinks that his results throw more weight on the assumption that anaphylatoxins are responsible to a large extent for the toxic manifestations of typhoid fever. He also states: "If we

<sup>1</sup> Jour. Exp. Med., 1913, xvii, 117

leave out of consideration bacteria which, like the diphtheria bacillus, produce true secretory poisons, it would be the ability to gain a foothold in the body, the degree of invasive power, the predilection in the choice of a path of entrance, and the specific local accumulation, upon which the speed and quantity of toxin production and absorption would depend, and which consequently would give character to variations in the clinical pictures of different diseases. Besides, simplifying considerably our comprehension of bacterial toxemia, the point of view suggested by this work again brings out the great importance of the work of Vaughan and Vaughan and Wheeler on the non-specific poisonous fraction obtained by hydrolysis of bacterial and other proteins, and makes it desirable that the particular conditions of anaphylatoxin and endotoxin production in the case of individual pathogenic bacteria should be carefully studied."

We regard the work of Jobling and Bull<sup>1</sup> as confirmatory of our studies in every particular. These investigators have studied the action of the cellular substance of the typhoid bacillus and its split products, produced by the action of a proteolytic ferment obtained from leukocytes, and state their findings as follows: "Freshly washed, unheated typhoid bacilli intravenously injected into dogs cause the development of definite symptoms as early as twenty minutes after the injection. Boiling for ten minutes does not destroy the toxic effects of a freshly washed bacterial emulsion. Complete solution of the bacteria (in dilute alkali) of a fresh emulsion does not prevent the removal of the toxic substance with the coagulable proteins. The action of leukoprotease splits the toxic substance to a non-coagulable state, the digested mixtures being toxic after removing the coagulable portion. The mere presence of the leukocytic ferment is not responsible for the toxicity of the filtrate from the digested mixture, and continued digestion destroys the toxicity of a previous toxic mixture. From these observations it is concluded that the toxic properties of freshly

<sup>1</sup>Jour. Exp. Med., 1913, xvii, 453.

washed typhoid bacteria are not entirely due to preformed secretory toxic bodies that are stored in the bacterial bodies, but that these properties are due largely to products formed by hydration of the bacterial proteins through the agency of ferments present in the circulation of the animal previous to the injection, or which become mobile subsequent to the entrance of the foreign bodies into the blood-stream. Since leukocytic ferments can attack the bacterial proteins *in vitro*, it is possible that the leukocytes are a source of the ferments which are active in experimental and natural cases of intoxication with the whole bacteria."

Nolf<sup>1</sup> has stated a theory of anaphylaxis which has come to be known as "the physical theory." It supposes that the active constituent of proteins is a thromboplastic substance which disturbs the colloidal equilibrium of the blood and leads to the deposition on the surfaces of the leukocytes and the endothelial cells of capillaries, of a delicate film of fibrin. Thus stimulated, these cells pour out an unusual amount of antithrombin. On account of the consumption of a part of the fibrinogen and the increased formation of antithrombin the blood fails to coagulate after anaphylactic shock or peptone poisoning. On account of the coagulation deposits on the endothelial cells the viscosity is increased and the leukocytes adhere to the vessel walls, thus accounting for the leukopenia observed after protein injections. The endothelial cells are injured and the walls of the capillaries become more permeable, thus accounting for the local edema often seen in anaphylaxis. The fine capillaries of a given area may be occluded by thrombi, and this explains the necrosis characteristic of the Arthus phenomenon. The irritation of the endothelial cells extends to the smooth muscle, and this leads to vasoparalysis, and the characteristic fall in blood-pressure. The affinity of the endothelial cells for the protein is stimulated by the first injection, and acts in a fulminating way on reinjection, and thus the suddenness of anaphylactic shock is explained.

<sup>1</sup> Archiv. intern. de physiol., 1910.

This is a plausible and attractive statement, and we are inclined to believe that there is truth in it, but we fail to see any reason for designating it as a "physical theory." It starts out with the assumption that proteins contain a poison and the theory is an explanation of the *modus operandi* of the chemical poison. The endothelial cells are sensitized and pour out a ferment, antithrombin, in increased quantity. That the endothelial cells are involved in sensitization we held as long ago as 1907. That the permeability of the walls of the capillaries is increased under the action of protein poison has been frequently demonstrated by the general diapedesis. We found this true even when rabbits died as the result of a single large dose of egg-white.

There is, however, one very important point in which the theory of Nolf differs from ours. In his theory the cause of death on reinjection is not due to the cleavage of the protein introduced, but is due to the action of the antithrombin on the blood. He holds that the fact that intravenous reinjections are so much more effective both in dose and in time than intraperitoneal and subcutaneous administrations is in favor of his theory, and we are inclined to agree with him on this point. However, anaphylactic shock cannot be due wholly to rendering the blood non-coagulable, because this may be done by injections of hirudin without shock. Doerr objects to our theory on the ground that anaphylactic shock follows reinjection so quickly that there is not time for a ferment to split up the injected protein and liberate a poison; but Nolf's theory also depends upon ferment action. The sensitized endothelial cells must be awakened by the reinjection, must pour out their abnormally accumulated ferment, and this must act, not on the injected protein, as we suppose, but on the blood. According to either theory, certain cells are sensitized and store up zymogen, which is activated on reinjection. This ferment acts either upon the injected protein or on the proteins of the blood. It seems to us that the time objection made by Doer to our theory is quite as applicable to that of Nolf. The latter is, after all, only a modification of the former.

## CHAPTER XII

### THE PARENTERAL INTRODUCTION OF PROTEINS<sup>1</sup>

FOR a long time it was thought that the proteins of our food undergo but slight modification before absorption through the walls of the alimentary canal. The studies of Beaumont laid the foundation of the scientific investigation of proteolytic digestion, and soon it was shown that the digestive juices convert proteins into peptones.

After experiments had demonstrated that peptone is formed in alimentary digestion and had shown the comparatively ready diffusibility of the digestive products, several questions arose. Among these may be mentioned the following: (1) Is all the protein converted into peptone in the alimentary canal, or is part of it absorbed in unaltered form? (2) What is the fate of peptone after absorption?

Brücke,<sup>2</sup> whose studies on pepsin and its action made him one of authority in this matter, held that only a part of the protein is converted into peptone in alimentary digestion, and that much of the soluble protein of the food is absorbed unchanged. Furthermore, he taught that the fate of the two after absorption is different. The peptone, he taught, is rapidly oxidized and serves as a source of energy, but is not utilizable in the building of tissue, the latter function devolving solely on the protein absorbed in unaltered form. Brücke's arguments in support of this theory may be briefly stated as follows:

<sup>1</sup> This is largely taken from an article by Vaughan, Cumming, and McGlumphy (*Zeitsch. f. Immunitätsforschung*, 1911, ix, 16).

<sup>2</sup> *Sitzungsber. d. k. Akad. d. Wissensch. zu Wien*, 1859, Band xxxvii, *ibid.*, Band lix.

(a) At best the gastric juice forms peptone slowly, and the time during which the food is detained in the stomach does not permit of its complete peptonization. It will be understood that the action of the pancreatic juice was not known, nor had erepsin been discovered when Brücke wrote. (b) In an animal killed while in digestion, Brücke found forty-eight hours after death coagulable protein not only in the chyle vessels in the intestinal walls, but in the intestinal villi, and he concluded that this could come only from the absorption of unaltered protein. (c) Brücke argued that the absorption of unbroken protein is quite as possible as that of fat, since the molecule of the former could not be larger than that of the latter. This argument assumed that the absorption of both proteins and fats is simply a process of filtration.

Diakonow<sup>1</sup> supported the theory of Brücke because peptone cannot be found in large amount in the blood. Voit and Bauer<sup>2</sup> and Eichhorst<sup>3</sup> concluded that unaltered protein is absorbed because they found that the introduction of protein in the large intestine is followed by increased elimination of urea. This certainly is proof that the protein is absorbed, but not proof that it is absorbed unaltered. Eichhorst showed that a glycerin extract of the mucous membrane of the large intestine had no digestive action, but he did not show that the large intestine did not contain any pancreatic juice and this might have digested the proteins. Fick<sup>4</sup> took an aqueous solution of peptone and precipitated it with alcohol, then dissolved the precipitate in water and injected it into nephrotomized dogs. He found that the blood after this treatment yielded a larger amount of nitrogenous material soluble in alcohol and precipitable with mercuric nitrate, and he concluded that peptone introduced into the blood is speedily converted into urea without being employed in tissue building, while unaltered

<sup>1</sup> Hoppe-Seyler's med. chem. Untersuchungen, 1867.

<sup>2</sup> Zeitsch. f. Biol., 1869, v

<sup>3</sup> Pflüger's Arch., 1871, iv, 570.

<sup>4</sup> Ibid., 1872, v, 40.

protein is used in tissue metabolism. Fick's conclusions support Brücke's hypothesis. Maly<sup>1</sup> pointed out a possible error in Fick's work, showing that while peptone may be precipitated from aqueous solution by alcohol, it is not wholly insoluble in this menstruum, and the increase of alcohol soluble nitrogen in the blood might be due to peptone and not to the conversion of this into urea.

Evidently if Brücke's theory were true, the animal body could not maintain its health and vigor if fed exclusively on peptone, which according to the theory is not utilizable by the animal in the repair of tissue. Plösz<sup>2</sup> fed animals exclusively, so far as their nitrogenous food is concerned, on peptones and found that they did not loose weight or suffer in any detectable way. Maly<sup>3</sup> confirmed this finding which has been repeated many times and under divers conditions, so that now Brücke's contention that the absorption of unaltered protein is essential to health has no support.

The second question, What is the fate of the absorbed peptone, became for a time one of much importance. Plösz and Gyergyai<sup>4</sup> injected from 200 to 300 c.c. of a 10 per cent. solution of peptone into the stomachs of fasting dogs and after periods of from one to four hours searched for the peptone in the blood and tissues of various organs. The method of recognizing peptone consisted in the application of the biuret and Millon tests to the filtrate after the removal of all the coagulable protein by acid and heat. They found the largest amount of peptone in the mesenteric veins and in extracts of the mesentery, much less in the liver, and only traces in hepatic and carotid blood. Next, they injected dogs and cats intravenously with a 10 per cent. solution of peptone, employing from 100 to 200 c.c., and introducing it at the rate of from 2 to 3 c.c. per minute. A dog received 200 c.c. during one and one-half hours, and after three hours the carotid blood showed only a small

<sup>1</sup> Pflüger's Arch., ix, 605.

<sup>3</sup> Loc. cit.

<sup>2</sup> Ibid., 325.

<sup>4</sup> Pflüger's Arch., 1875, x, 536.

amount of peptone which had wholly disappeared after four hours. When larger amounts were used a small quantity appeared in the urine, but the proportion eliminated in this way was only a small part of that injected. They also transfused certain organs and tissues with blood to which peptone has been added, and found that the peptone soon disappeared from the blood. These investigators concluded that peptone is soon so changed in the organism that it can no longer be detected by the method which they employed. Whether it is changed directly into albumin or is so altered by cell activity by combining with other substances, they could not decide. They were of the opinion that the capability of effecting this change is not confined to any one organ or tissue, but that it may occur in the liver, muscle, or other tissue. They were quite convinced, however, that the conversion is not essentially one of oxidation, since the amount of oxygen in the blood did not affect it.

Schmidt-Muhlheim<sup>1</sup> injected from 5 to 10 grams of peptone into the jugular vein of dogs and found that the peptone disappeared from the blood within sixteen minutes after completing the injection. He also concluded that the injected peptone undergoes a rapid conversion into albumin and globulin.

According to Hofmeister,<sup>2</sup> peptone, when injected into the blood, does quickly disappear from that fluid, but is not converted into albumin or globulin. It quickly diffuses through all the tissues undergoing a dilution which is determined by the total fluid in the body, and which is so great that its detection by chemical tests is impossible. Diffusion into the brain results in certain characteristic symptoms, the most marked of which are muscular weakness and somnolence. These symptoms may be observed in a 10 kg. dog after the subcutaneous injection of from 0.2 to 0.4 gram of peptone, but the fatal dose is large; as

<sup>1</sup> Du Bois Reymond's Arch. f. Phys., 1880.

<sup>2</sup> Zeitsch. f. phys. Chemie, 1881, v, 127.

high as 1 gram per kilo. Injections of peptone lead to lowered blood pressure and much of the peptone, according to Hofmeister's finding, may be deposited in the tissue where it may be detected at a time when there is none in the blood. He found one-seventh of the peptone injected into the blood in the kidneys, which organs were equivalent to only  $\frac{1}{192}$  of the body weight, and concludes that the peptone injected into the circulation has a special predilection for renal tissue. When the amount of peptone injected is large, Hofmeister recovered as much as 84 per cent. of it from the urine.

Neumeister<sup>1</sup> reviewed the literature of this subject up to that time, and made some additional contributions. He stated that some proteins are absorbed unchanged, that others need only to be dissolved, and that still others must be digested. He stated that the compound proteins, as casein and hemoglobin, when injected into the blood, act like foreign bodies, and are eliminated in the urine, while the simple and denatured proteins, when injected into the blood, do not cause albuminuria. Stockvis did not observe albuminuria after injecting dog, rabbit, or frog serum into dogs or rabbits, but did when he used egg albumen. Lehmann invariably induced albuminuria by injecting egg albumen intravenously in dogs, but failed to do so when he employed sodium albuminate, or syntonin, prepared from frogs' muscle, myosin, or fibrin. Ponfick found that dogs bear astounding amounts of lamb serum, free from corpuscles, when the injections are made slowly, and under gentle pressure. "The amount of urine was not appreciably increased, although the color became darker, owing to the greater concentration, while not a trace of albumin could be detected." There is no statement concerning the effect upon the elimination of nitrogen; Forster injected large amounts of horse serum into dogs, while the urine remained free from albumin. Neumeister injected into the jugular veins of dogs without inducing albuminuria, the following

<sup>1</sup> Zeitsch. f. Biol., 1891, xxvii, 309

proteins in large amounts: Syntonin and albuminate from egg albumen, syntonin from ox flesh, crystalline phyto-vitellin from pumpkin seed, and pure serum albumin from the ox.

According to Neumeister, Salviolo was the first to show that peptone is transformed by the living intestinal wall, but this investigator only demonstrated that peptone disappears when placed in an intestinal loop and cannot be found in either the blood from the part, or within the loop. The nature of the transformation was not determined. The fact that peptone is synthesized into albumin seems to have been first suggested by two women, students of Kronecker, Nadine Popoff and Julia Brinck. It was thought by these investigators that this synthesis is accomplished partly by the epithelial cells of the intestinal wall, and partly by a microorganism, to which Julia Brinck gave the name *micrococcus restituens*.<sup>1</sup> Hofmeister<sup>2</sup> suggested that the leukocytes in the intestinal wall might combine with peptone much as hemoglobin does with oxygen in the lungs, and Heidenhain<sup>3</sup> thought that the leukocytes might play a part in the absorption of peptones, but that it could not be as suggested by Hofmeister,<sup>4</sup> otherwise the leukocytes in the circulating blood would combine with peptone injected intravenously.

As early as 1874 Tschiriew,<sup>5</sup> working under Ludwig's direction, found that dog serum transfused into another dog increased the elimination of urea much more slowly than when given to the dog by mouth, but Forster<sup>6</sup> found that horse serum affects the urea output in dogs equally, both in amount and time, whether given intravenously or by mouth.

Zunz and von Meering<sup>7</sup> injected solutions of peptone

<sup>1</sup> Zeitsch. f. Biol., 1889, vii, 427, 453.

<sup>2</sup> Zeitsch. f. phys. Chemie, 1881, v, 151.

<sup>3</sup> Pflüger's Arch., 1888, liii.

<sup>5</sup> Arb. a. d. phys. Inst. zu Leipzig.

<sup>6</sup> Zeitsch. f. Biol., 1895, ii, 496.

<sup>7</sup> Pflüger's Archiv, 1883, xxxii, 173.

<sup>4</sup> Loc. cit.

and egg-white intravenously in rabbits. The injections were single and too large to be followed by marked rise, and too small to result in marked depression of temperature. However, that they noticed the ill effects of these injections is shown by the following quotation: "The poisonous action of peptone was unknown to us at the time when the experiments were made, but some of our rabbits died soon after rather large injections of peptone. Moreover, unchanged egg-white is not an indifferent substance, and it has long been known that its direct introduction into the blood may cause albuminuria and deep-seated changes in the kidneys."

Gürber and Hallauer<sup>1</sup> employed casein for the reason that it may be distinguished from other proteins by the action of rennin. Solutions of this protein were injected intravenously into rabbits and the casein was detected in the bile. Evidently the foreign protein was on its way to the intestines where it might be properly digested. These authors quite properly point out that because a protein injected into the blood does not appear, or appears only in part, in the urine, is no proof that it has been assimilated in unchanged form by the tissues, because it may have been carried to the intestine and there properly digested. They also brought out another point, confirmed later by our own work, that when the foreign protein diffuses from the blood it carries with it some of the blood proteins. This is true whether it is poured out into the intestine or eliminated through the kidneys.

The results obtained by Gürber and Hallauer have been confirmed by Burckardt,<sup>2</sup> who injected hemielastin intravenously and found it in the wall of the small intestine. He concluded that it had been brought to this locality preparatory to its being properly digested and fitted for assimilation.

Friedemann and Isaac,<sup>3</sup> after extensive experimentation,

<sup>1</sup> *Zeitsch. f. Biol.*, 1904, xlv, 372.

<sup>2</sup> *Zeitsch. f. physiol. Chem.*, 1907, li, 506.

<sup>3</sup> *Zeitsch. f. exp. Path. u. Ther.*, 1907, iv, 830.

concluded that both homologous and heterologous proteins when parenterally administered are digested and probably assimilated. They say: In fasting animals the parenteral administration of protein leads to increased protein metabolism. The increased nitrogen elimination is the same whether the injected protein be the serum of the same or of a different species, or egg albumen. In dogs in nitrogen equilibrium protein parenterally administered behaves the same as that given by mouth. Carbohydrates hinder increased nitrogen elimination while on a carbohydrate free diet there is increased nitrogen elimination. In herbivorous animals (goats and sheep) there seems to be a tendency to retain some of the nitrogen given parenterally, but the results obtained were not constant. We cannot speak of a toxic protein metabolism unless symptoms immediately follow the administration. If heterologous proteins be poisonous, when administered parenterally, we have not been able to demonstrate it in fasting animals. If this does not exclude a toxic metabolism it renders its assumption wholly hypothetical. In our experiments increased nitrogen elimination corresponds to increased administration, and a toxic protein metabolism is characterized by the fact that nitrogen ingestion and elimination bear no relation to each other. The parenteral administration of protein has an advantage over the enteral, because in the former we know just how much protein enters the blood. When blood serum is introduced there can be no increased concentration of proteins in the blood, but in fasting animals the introduction of serum, either homologous or heterologous, does lead to increased nitrogen elimination. The transfusion of blood even in large amount from one dog to another is not followed by any marked nitrogen elimination by the recipient. It seems, therefore, that even homologous serum behaves like a foreign protein, possibly on account of the changes that have taken place in it during coagulation. In sensitized animals the parenteral introduction of the homologous protein leads to explosive-like increase in nitrogen elimination.

In the light of later research, some criticism of the above given conclusions reached by Friedemann and Isaac may be made. Foreign-proteins when introduced parenterally are poisonous. In some, the poisonous action is due to ferments, but all are poisonous, even when the ferment action has been destroyed by heat. It is only a question of dosage. Even egg-white or horse serum injected intravenously in sufficient doses will kill dogs and rabbits. Saturation of the body cells with any foreign protein interrupts their function. The protein foods of the body cells are carefully prepared physiologically. The foreign proteins eaten by the animal are broken into non-protein bodies, and these pieces are put together again after a model which is peculiar to that species of animal to which the feeder belongs. In this way the specific albumins and globulins of the blood of each species are constructed, and these supply the normal protein foods for the body cells. Foreign proteins parenterally introduced are digested, if the amount be not too large. This digestion is carried out in part in the intestines, and other body cells acquire the function of digesting a limited amount of the protein introduced. Certainly the digestive products formed in the intestine are fit for assimilation, and it may be that those formed in other parts are also utilizable, but the capability of the body of taking care of proteins parenterally introduced is limited, and in large doses all foreign proteins thus administered are poisonous. Whether this is also true of homologous sera we do not have sufficient data to determine.

Bankowski and Szymanowski<sup>1</sup> find that normal human blood, when injected intravenously into guinea-pigs, kills with the symptoms of anaphylactic shock in doses of 0.5 per cent. of the body weight of the animal. In typhoid fever the minimum fatal dose falls to 0.25 and in scarlet fever and measles to 0.13 per cent. of the body weight of the animal. Fetal human blood is well-nigh atoxic; when

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1913, xvi, 330.

the dose is as much as 2.5 per cent. of the body weight it kills slowly. The mother's blood kills in doses of 0.5 per cent., and fetal blood in doses of 2.5 per cent. This is not due to biological differences between the blood of the mother and that of the fetus, because one sensitizes to the other as well and in as small doses as to itself, but is due to the relative freedom of the fetal blood from ferments. When human blood is injected into the blood of a guinea-pig the former carries the ferment and the latter supplies the substrate. In the infectious diseases the proteolytic ferment in the blood is increased and consequently the minimum fatal dose is decreased.

Dehne and Hamburger<sup>1</sup> state that white mice do not produce a precipitin when treated with horse serum, and Celler and Hamburger<sup>2</sup> find that white rats fail to respond to ox serum by elaborating a precipitin.

Uhlenhuth<sup>3</sup> and Michaelis and Oppenheimer<sup>4</sup> found that when rabbits are repeatedly fed through a tube with egg-white or serum, they develop precipitins. Celler and Hamburger say that this may be due: (1) To injury of the esophagus or stomach by the tube, and the introduction of the protein in this way. (2) To the failure of the secretion on account of the unnatural method of feeding. (3) To the direct introduction of the protein into the intestine where, according to Oppenheimer and Rosenberg,<sup>5</sup> serum proteins resist tryptic digestion.

Celler and Hamburger found that in forced or tube feeding the protein may be absorbed unchanged, on account of the lack of digestive juice.

Chiray<sup>6</sup> has studied the effects of the administration of heterologous proteins. The intravenous injection of a very small amount of egg-white in rabbits causes, after from ten to thirty minutes, a transitory albuminuria with increase in the

<sup>1</sup> Wien. klin. Woch., 1904, No. 29.

<sup>2</sup> Ibid., 1905, xviii, 271.

<sup>3</sup> Deutsch. med. Woch., 1900, p. 734.

<sup>4</sup> Arch. f. Phys., phys. Abt., Suppl.-Band, p. 336.

<sup>5</sup> Hofmeister's Beiträge, 1903, v, 412.

<sup>6</sup> Thèse de Paris, 1906; Jahresber. d. Tierchemie, 1907, xxxvi, 805.

volume of urine, but without glycosuria, hemoglobinuria, or hematuria. The intravenous, subcutaneous, or intraperitoneal injection in increasing doses, even with long intervals, causes in rabbits a gradual decrease in weight. When the egg-white is injected into the portal vein the albuminuria appears much later than when the injection is made into the general circulation. Subcutaneous injections induce an albuminuria which appears later, is less marked, and persists longer than when the injection is made intravenously. The intramuscular injection of 2 c.c. of egg-white in man was without effect, but when tried upon one who had renal deficiency, albumin appeared in the urine from fourteen to twenty-four hours later. In cases of marked albuminuria injections of egg-white did not materially affect the excretion of albumin. In rabbits, dogs, and men the introduction of large amounts of egg-white into the stomach was followed by albuminuria, though this did not invariably occur in the men. The injection of egg-white into the rectum of rabbits was followed by an albuminuria which appeared later and was more persistent than when it was given by the stomach. Egg-white administered by rectum to man, especially to convalescents from infectious diseases, was followed by an albuminuria, but this did not occur when the albumin was mixed with an active trypsin before injection. In some, not in all, the administration of peptone by rectum was followed by albuminuria as well as peptonuria. Injections of egg-white in rabbits decrease the proteins of the blood, as shown by the refractometer. It appears that not only a part of the foreign protein, but also a part of the blood proteins passes into the muscles. All of the injected protein, probably not the greater part, does not pass through the kidney, at least not within the time of an observation, but much is withdrawn from the blood and held in the tissues. Repeated injections of egg-white lead to marked structural changes in the kidneys. Alimentary albuminuria is not due to poisons resulting from the splitting of the protein, but the absorption and elimination of the unbroken protein which is a foreign and poisonous

body. When a milk diet is presented, casein may in some instances be found in the urine. The prohibition of egg diet in albuminuria is justified.

The statement that the injection of a foreign protein leads to the exudation of the normal proteins of the blood, as made by Chiray, is interesting, and if confirmed it may be found to be of marked importance. It has been practically confirmed by Wolf,<sup>1</sup> who found that the proteins of the plasma were diminished by the injection of Witte's peptone in 11 out of 14 tests.

Oppenheimer<sup>2</sup> estimated that as much as 49 per cent. of egg-white injected intravenously or intra-abdominally in rabbits is eliminated in the urine. However, he does not claim any great exactness for this work, because he is aware of the fact that all the protein in the urine does not come from that injected, and that a part of it is serum albumin. It is probable that the latter makes up the larger part.

Castaigne and Chiray<sup>3</sup> hold that heterologous proteins injected subcutaneously are absorbed and eliminated in the urine unchanged. They act as poisons, causing destruction of the proteins of the blood and increased elimination of nitrogen, urea, and sulphur. The decrease in the normal proteins of the blood may be as high as from 1 to 3 per cent. This is not due to hydremia as shown by determination of total solids. Repeated injections of heterologous proteins, either subcutaneously or intravenously, lead to cachexia.

Nobecourt<sup>4</sup> introduced egg-white into the alimentary canal of rabbits. He used 46 animals, 31 adults, weighing from 1650 to 2270 grams and 15 young, weighing from 320 to 1030 grams. These received by the stomach or rectum from 5 to 13 c.c. of egg-white, at each injection at intervals of one, three, seven, ten, and fifteen days. The mortality was as follows:

<sup>1</sup> Arch. int. Phys., iii, 343.

<sup>2</sup> Hofmeister's Beiträge, iv, 263.

<sup>3</sup> Compt. rend. Soc. biol., 1906, lx, 218.

<sup>4</sup> Ibid., 1909, xlvi, 850.

| Intervals.                   | Stomach. | Per cent. of mortality: |          |           |
|------------------------------|----------|-------------------------|----------|-----------|
|                              |          | In adults:              |          | In young: |
|                              |          | Rectum.                 | Stomach. | Rectum.   |
| Every day . . . . .          | 0        | 0                       | 100      | 100       |
| Every three days . . . . .   | 100      | 0                       | 100      | 100       |
| Every seven days . . . . .   | 50       | 50                      | 33       | 33        |
| Every ten days . . . . .     | 50       | 0                       | 33       | 33        |
| Every fifteen days . . . . . | 0        | 50                      | 33       | 33        |

From the above test, 24 animals, 20 adults and 4 young survived. After a rest of from twenty to forty-four days each of these received rectal injections, every seven days of from 3.3 to 9.6 c.c. of egg-white.

| Interval in first series.    | Per cent. of mortality.                   |                        |
|------------------------------|---|------------------------|
|                              | Method of administration in first series. |                        |
|                              | Stomach.                                  | Rectum.                |
| Every day . . . . .          | 66  | 0                      |
| Every three days . . . . .   | 66 adults                                 | 0                      |
| Every seven days . . . . .   | { 50 adults<br>50 young                   | 75 adults<br>100 young |
| Every ten days . . . . .     | 0   | 50                     |
| Every fifteen days . . . . . | 0   | 0                      |

From the second ordeal, 13 animals, 12 adults and 1 young, survived. After a rest of from seventeen to forty days these received every seven days rectal injections of from 5.2 to 8.9 c.c. of egg-white, with the following results:

| Intervals in first series.   | Per cent. of mortality.                   |         |
|------------------------------|---|---------|
|                              | Method of administration in first series. |         |
|                              | Stomach                                   | Rectum. |
| Every day . . . . .          | 0   | 50      |
| Every three days . . . . .   | 0   | 100     |
| Every seven days . . . . .   | { 50 adults<br>100 young                  | 100     |
| Every ten days . . . . .     | 100                                       | 100     |
| Every fifteen days . . . . . | 100                                       | 0       |

From this test, 4 animals, all adults, survived. After a rest of from twenty-four to forty-five days these received every seven days from 4.6 to 7.5 c.c. of egg-white. Three died.

It appears from the experiments of Uhlenhuth and Nobecourt that egg-white is absorbed, at least in some instances, unchanged from the stomach and intestines of rabbits.

When tubes are used for the introduction of the egg-white it is possible that a small amount of the material may be introduced through some slight wound or abrasion in the mucous membrane. Sensitization might be induced in this way, but it is hardly conceivable that subsequently enough would be introduced in this way to kill the animal. It seems, therefore, that we must conclude that in forced feeding, at least, unbroken egg-white may be absorbed from the alimentary canal of the rabbit. It must be understood, however, that apart from any injury to the mucous membrane, the conditions of forced feeding are not exactly the same as those in natural feeding. Celler and Hamburger have called attention to this point. By continued tube-feeding of rabbits with the serum and blood of the ox, in only one instance did they obtain a precipitin for the serum, and an hemolysin for the corpuscles, while they found that rabbits after fasting took the serum and blood willingly when mixed with milk, and in none of these was there any evidence of absorption without digestion. They admit the possibility of wounding the mucous membrane with the tube, or of carrying the material through the tube into the intestine, but they are inclined to the opinion that in the unnatural tube feeding the digestive secretions are not poured out so freely or are less effective than in natural feeding. This is in accord with the findings of Pawlow, who holds that desire for food is an important factor in securing thorough digestion.

With this brief and imperfect review of the literature of the subject, we turn to our own experimental work. Our method is to inject egg-white into the animals and test for its presence in the blood and extracts of tissue by sensitizing guinea-pigs, having first demonstrated that the blood of the rabbit and extracts from its tissue do not sensitize guinea-pigs to egg-white. The details of the method will be developed in the report of the experiments. Our findings are as follows:

1. Egg-white injected into the stomach of a rabbit may be in part absorbed unchanged.

December 7, 1909, at 9.30 A.M., 50 c.c. of egg-white was introduced through a tube into the stomach of a rabbit that had been kept without food for two days. Neither at the time nor subsequently did this have any recognizable effect upon the rabbit. 3 c.c. of blood was drawn from the heart of this rabbit at 10.30 and 11.30 A.M., and at 12.30 P.M., and 4.30 P.M., and each of these portions of blood was injected intraperitoneally in a fresh guinea-pig. January 3, 1910, each of these pigs received intra-abdominally 5 c.c. of a dilution of egg-white with an equal volume of physiological salt solution.

Only one of these pigs developed symptoms of sensitization and this one received blood drawn from the rabbit's heart three hours after the introduction of the egg-white into the stomach. Neither the blood drawn earlier nor that drawn later sensitized guinea-pigs.

January 8, 1910, at 8 A.M., 50 c.c. of a dilution of egg-white with physiological salt solution (1 to 1) was introduced through a tube into the stomach of a rabbit which had not been kept without food.

Hourly 2.5 c.c. of blood was drawn from the heart of this animal, and injected intra-abdominally into guinea-pigs.

January 22, 1910, these pigs were treated each with 5 c.c. of the egg-white dilution intra-abdominally. The first, second, and third hour pigs showed no sensitization; the fourth and fifth hour ones were sensitized, while the sixth, seventh, and eighth were not.

That absorption from the stomach of the fed animal should have been more tardy than from the fasting one is easily understood.

2. Egg-white injected into the rectum of a rabbit may be, in part at least, absorbed unchanged.

January 8, 1910, at 8 A.M., 50 c.c. of egg-white diluted with physiological salt solution (1 to 1) was introduced through a tube into the rectum of a rabbit. Hourly, 2.5 c.c. of blood was drawn from the heart and injected intra-abdominally into guinea-pigs.

January 22, 1910, these pigs were tested and all from the

first to the seventh hour were found to be sensitized to egg-white.

It appears from this that egg-white may be absorbed from the rectum of a rabbit without being so far altered as to destroy its specific sensitizing properties and that absorption into the blood begins within the first hour and continues for at least seven hours.

3. Egg-white injected into the peritoneal cavity of a rabbit may be absorbed unchanged.

December 7, 1907, at 9.30 A.M., a rabbit received intraperitoneally 50 c.c. of a dilution of egg-white with an equal volume of physiological salt solution. Hourly, 2.5 c.c. of blood was drawn from the heart of this animal and injected intra-abdominally into guinea-pigs.

January 3, 1910, these pigs were treated with the egg-white dilution given intraperitoneally.

All, from the first to the fourth hour, died, the first two in fifteen and the latter in twenty minutes. The fifth hour one was not sensitized. It should be stated that in all these experiments guinea-pigs found not to be sensitized to egg-white were subsequently tested and found to be sensitized to the blood serum of the rabbit.

4. Egg-white injected intravenously in rabbits quickly disappears from the circulating blood.

January 3, 1910, a rabbit received intravenously 50 c.c. of a dilution of egg-white with physiological salt solution (1 to 1). Every half hour blood was drawn from the heart of this animal and injected intra-abdominally into guinea-pigs.

January 12, 1910, these pigs were tested with the egg-white dilution.

The first two were found to be sensitized to egg-white while the others were not. The pig that received blood drawn at the end of the first hour died in a typical way within thirty minutes, while the blood drawn at the expiration of one and one-half hour failed to sensitize.

December 6, 1909, a rabbit received intravenously 50 c.c. of the egg-white dilution (1 to 1). Hourly blood was

drawn from the heart and injected into guinea-pigs. The first two were found to be sensitized while the others were not.

5. Egg-white injected intravenously in rabbits may be detected in the peritoneal cavity after it has disappeared from the circulating blood.

January 3, 1910, a rabbit received intravenously 50 c.c. of the egg-white dilution. Two and one-half hours later and after the egg-white had disappeared from the heart's blood, as was shown by a subsequent test, some physiological salt solution was injected into the peritoneal cavity, withdrawn and injected into a guinea-pig, which later was found to be sensitized to egg-white.

6. Egg-white injected intravenously into rabbits may be detected in the bile.

November 15, 1909, a rabbit received intravenously 50 c.c. of the egg-white dilution, one and one-half hours later the abdominal cavity was opened, the animal being under ether, and small amounts of bile and washings from the small intestine were injected into guinea-pigs, all of which later were found to be sensitized to egg-white. Of four pigs thus treated all but one died, and this one developed marked symptoms when treated with the egg-white dilution.

7. Egg-white when injected intravenously into a rabbit may be detected by the sensitization test in certain organs after it has disappeared from the circulating blood.

December 6, 1909, at 8.45 A.M., a rabbit received intravenously 50 c.c. of a dilution of egg-white with an equal volume of physiological salt solution. 5 c.c. of blood was drawn from the heart of this animal at 9.45, 10.45, and 11.45 A.M., and at 1.45 P.M. Each of these portions was injected into the abdomen of a guinea-pig and one hour after the last blood was taken the rabbit was killed with ether, and extracts of the brain, liver, kidney, and spleen, with physiological salt solution were made and injected into other fresh pigs. December 17, 1909, each of the pigs that had been treated with the blood of the rabbit had 5 c.c. of egg-white dilution (1 to 1) intra-abdominally.

The only pig that gave any evidence of sensitization was the one that had received the first blood, drawn one hour after the injection of the egg-white. The symptoms in this animal were slight and transitory. The other pigs showed no indications of having been sensitized. It seems from this that after one hour there was no egg-white in the circulating blood of the rabbit. All of these pigs had been sensitized to the proteins of the rabbit's blood as was shown by treating them with rabbit serum.

January 4, 1910, the guinea-pigs that had received the extracts of the organs were treated with the egg-white dilution. All were affected within a few minutes.

The one that had received the kidney extract was most seriously disturbed and passed to the convulsive stage, but ultimately recovered. The one that had the spleen extract came next in the severity of the symptoms developed. The first and second stages were well-marked in this animal, somewhat less so in the pig that had received the extract from the brain. Much to our surprise, the pig that had received the extract from the liver was least affected. However, failure to sensitize with the extract from an organ does not necessarily mean that the tissue of the organ contained none of the foreign protein. It may combine with certain tissues so firmly that it is not removed by a simple solvent, like physiological salt solution. The fact that from certain organs the extracts did sensitize the pigs shows that these tissues had absorbed the egg-white, but failure to sensitize or to sensitize so fully with other extracts does not conclusively show that such tissues have not absorbed the protein.

8. Egg-white carried into the tissue after intravenous injection may be washed back into the blood current by transfusion with salt solution.

A rabbit received intravenously 50 c.c. of the egg-white dilution. After one hour egg-white had disappeared from the circulating blood. Two and one-half hours after the injection of the egg-white, the animal was transfused with physiological salt solution. During the transfusion,

2 c.c. portions of the fluid were drawn from the heart and injected into guinea-pigs. The last of these portions was drawn after one liter of the salt solution had passed through. All of these portions sensitized the guinea-pigs. After the transfusion, the brain, liver, spleen, kidney, and the deltoid muscle were removed, and rubbed up with physiological salt solution. 2 c.c. of each of these extracts, after filtration, was injected into guinea-pigs. The one having the brain extract was not affected by the subsequent injection of egg-white. The one having the liver extract was in convulsions within five minutes after receiving the egg-white. The ones having the extracts from the spleen and muscle developed first and second stages of sensitization, but recovered, while the one that received the kidney extract was not affected.

We conclude from this that the brain and kidney were washed free of the egg-white by the transfusion, while the muscle, liver, and spleen held the egg-white more tenaciously.

9. The injection of egg-white intravenously in rabbits decreases after a few hours the total protein in the blood.

In reviewing the literature we have referred to the finding of Chiray that the intravenous injection of foreign proteins decreases the total proteins of the blood. His experiments were made with a refractometer. We deemed this of sufficient importance to justify further study. On one day blood was drawn from a rabbit and the serum obtained. On the next day this animal received 50 c.c. of the filtered egg-white dilution intravenously. Each cubic centimeter of this dilution of egg-white contained 26 mg. of protein, as calculated from a nitrogen determination. In other words, with the dilution there was introduced into the blood of the rabbit 1.3 grams of foreign protein. On the day after the injection more blood was drawn and the serum from this secured. The total nitrogen in these sera was determined and the protein content calculated with the following results:

|   |       |
|---|-------|
| Per cent. of protein in the blood serum before the injection of egg-white . . . . . | 10.50 |
| Per cent. of protein in the blood serum after the injection of egg-white . . . . .  | 8.18  |
| Loss . . . . .  | 2.32  |

This experiment was repeated on a second animal with the following results:

|   |      |
|---|------|
| Per cent. of protein in the blood serum before the injection of egg-white . . . . . | 9.33 |
| Per cent. of protein in the blood serum after the injection of egg-white . . . . .  | 7.36 |
| Loss . . . . .  | 1.97 |

In a third animal the following results were obtained:

|   |      |
|---|------|
| Per cent. of protein in the blood serum before the injection of egg-white . . . . . | 7.90 |
| Per cent. of protein in the blood serum after the injection of egg-white . . . . .  | 6.30 |
| Loss . . . . .  | 1.40 |

It appears from these figures that the injection of egg-white intravenously in rabbits is followed by the disappearance of an appreciable amount of the normal proteins from the circulating blood. This confirms the finding of Chiray.

10. The injection of a large amount of egg-white intravenously in rabbits proves fatal.

No. 1. 35 c.c. of undiluted egg-white filtered through cotton was slowly injected into the ear vein. The respiration was immediately embarrassed, and with a slight convulsive movement the animal died before it could be removed from the table. On opening the thorax the heart was found to be still beating and irregularly distended. The right side was dilated and filled with dark fluid blood. Markedly anemic areas were plainly seen in the lungs.

No. 2. 32 c.c. of the same was injected more slowly and through a finer needle. The result was practically the same.

No. 3. 40 c.c. was injected. The respiration became difficult and the animal quite limp. The right side was found to be paralyzed, but the animal lived for two hours, when it died with failure of respiration, and without a movement. The heart was dilated and contained dark, fluid blood. Anemic areas were seen in the lungs and the muscles also were anemic.

Van Alstyne and Grant<sup>1</sup> injected dilute egg-white intravenously into a dog and sensitized guinea-pigs with blood drawn from one-quarter to seventy-two hours. Pearce<sup>2</sup> injected foreign proteins intravenously into rabbits, and sensitized guinea-pigs with organ extracts. His conclusions are stated as follows: "Extracts of the kidneys of normal rabbits prepared one, two, three, and four days after the intravenous injection of egg albumen and horse serum have the power to sensitize guinea-pigs to a second injection of these proteins. The sensitization by first- and second-day extracts was constant and intense, that by the third-day extracts was less marked and sometimes was not evident, and that by the fourth-day extracts was only occasional, and when present was always weak. Comparative studies of the power of the blood, liver, and kidney to sensitize indicate that this sensitization depends upon the content of the foreign protein in the circulatory blood and not upon its accumulation or fixation in the tissues of an organ. This opinion is supported by other experiments in which the sensitizing power of the blood and of the extracts of unwashed kidneys was compared with the sensitizing power of washed kidneys. The weak sensitizing power of washed kidney extract is taken as evidence that foreign proteins of the kinds used are not held in the tissues of the kidney, and if these results may be applied to nephrotoxic proteins, it follows that nephritis is not due to selection and persisting fixation of a protein by the renal cells, but is due to the action of such proteins merely during the process of elimina-

<sup>1</sup> Jour. Med. Research, 1911, xxv, 399.

<sup>2</sup> Jour. Exp. Med., 1912, xvi, 349.

tion. In experimental acute nephritis of the type due to uranium nitrate, the power of sensitization to egg albumen is prolonged for twenty-four hours, and in the chromate type for forty-eight hours, thus indicating that in nephritis of the acute type at least, the elimination of a foreign protein is delayed."

In our opinion the possibility of harm coming to the kidney or any other organ from the deposition of a foreign protein in it is not due to any directly poisonous effect of the foreign protein but to the liberation of the poisonous group when the body cells become sensitized and split up the foreign protein.

Abderhalden<sup>1</sup> has shown by both dialysis and by the polariscope that foreign proteins injected into animals are digested by ferments. However, he does not find evidence that specific proteolytic ferments are formed. Indeed, it still remains a question whether the sensitizer leads to the development of an entirely new ferment or causes the common non-specific proteolytic ferment of the blood to develop specific properties. We regard this question as only of academic value. In either case the proteolytic ferment becomes specific, whether formed by an altered rearrangement in the molecules of the cells or by alteration in the molecular structure of a non-specific proteolytic ferment. Abderhalden believes that the ferment is always present in the blood, and that it is a secretion of the leukocytes. We agree with him insofar as the non-specific proteolytic ferment of the blood is concerned. The blood is a digestive fluid, but we believe that specific ferments are developed in various fixed cells under the influence of foreign proteins or sensitizers. Abderhalden holds that the ferment is always present in the blood, and that the ferment and the sensitizer may both be present as they are on first injection, but that for the production of anaphylactic shock a third and unknown factor is necessary. He seems to be influenced in this belief largely by the

<sup>1</sup> Schutzfermente, 1912.

phenomena of so-called antianaphylaxis, but he admits that rapid digestion in this state may be prevented by the accumulated products of digestion. He says: "From recent studies we know that the ferment forms a compound with the substrate before the equilibrium of the latter is destroyed. After the cleavage the ferment is again free, so far as it is not bound by the cleavage products." It seems to us that this is all that is necessary to explain the known facts in so-called antianaphylaxis.

In his work on the digestive action of blood serum Abderhalden has largely employed polypeptids and purified peptones. Of course, he does not expect these denatured proteins to act as sensitizers and lead to the development of specific ferments, but they are especially suited for digestive experiments, because the split products as soon as formed are easily recognized by their effect on the rotation of light. In this way he has shown that peptones and polypeptids are quickly split into their constituent amino-acids by the proteolytic ferment normally present in serum. In other instances he has employed native proteins as sensitizers. In one case he divided a lot of guinea-pigs sensitized to egg-white into three groups. The members of the first group while in the sensitized state were bled, and the serum thus obtained was digested with egg-white, and it was demonstrated both by dialysis and the optical method that the egg-white was digested by the serum. Now, had this been done with the serum of normal guinea-pigs there would have been no recognizable digestion. It must follow, therefore, so far as we can see, that the blood-serum of the sensitized guinea-pig contains a ferment which is not present in the blood-serum of the fresh guinea-pig. Furthermore, had it been tried, it would have been found that the blood-serum of the guinea-pig sensitized to egg-white would either have no digestive action, or but slight effect, on other proteins. It follows, therefore, that the sensitized animal differs from the unsensitized in the fact that its body cells elaborate a specific ferment which digests the protein by which it was called into existence, and no other. It will,

of course, be understood that the non-specific proteolytic ferments are capable of digesting a more or less extended group of proteins, but with this ferment the digestive process proceeds slowly, and it is not supposable that all proteins would be digested by it.

From the second set of sensitized guinea-pigs Abderhalden took the serum and dialysed it by itself, the purpose being to see if, while in the sensitized state, the blood-serum contains any diffusible biuret body. The serum from six sensitized guinea-pigs was tested in this way, and only in one instance did the dialysate respond to the biuret test. On the eighteenth day after sensitization the remaining guinea-pigs (6) were reinjected and blood was taken five, fifteen, thirty, forty-five, sixty, and ninety minutes after the reinjection. The serum obtained from the samples of blood was dialyzed and the dialysate subjected to the biuret test. The samples taken five and fifteen minutes after reinjection failed to yield dialysates which responded to the biuret test, while the remaining four did. This experiment demonstrates that at the very moment when anaphylactic shock is being developed, peptone-like bodies are being formed in the blood. It is probable that the specific digestion of the protein begins at the very moment that the reinjection is made, but that further time is necessary for the digestive product to accumulate in a few cubic centimeters of blood serum in recognizable amount. The biuret test is not a highly delicate means of recognizing proteins.

H. Pfeiffer and Jarisch<sup>1</sup> have repeated diffusion experiments. The method of procedure may be briefly described as follows: Guinea-pigs are sensitized with horse serum, and after varying intervals they are bled and serum obtained. The serum of the sensitized animals is mixed with varying amounts of horse serum, and the mixture incubated in small dialyzers. If digestion takes place, peptone-like products are formed, diffuse through the membrane, and

<sup>1</sup> *Zeitsch. f. Immunitätsforschung*, 1912, xvi, 38.

are detected in the dialysate by the biuret test. First, it was shown that the serum of normal guinea-pigs with or without mixture with horse serum does not supply biuret bodies. After guinea-pigs had been sensitized to horse serum for six days, then for the first time serum obtained from them and mixed with horse serum and the mixture dialysed, did the biuret test, when applied to the dialysate, prove positive. This does not mean, as we take it, that the formation of the specific ferment begins on the sixth day after the injection of the sensitizer. It means that with the amount of the sensitizer employed, the specific ferment had accumulated sufficiently and was efficient enough when brought into contact with horse serum *in vitro* to digest it enough to show its action by the biuret test when applied to the dialysate. The serum of sensitized animals continues to digest the homologous protein (that to which the sensitization is due) up to about the thirtieth day. This does not mean that the formation of the ferment ceases after this time. We know that this is not the case, because Rosenau and Anderson have shown that guinea-pigs sensitized to horse serum remain in this condition for two years at least, and probably throughout life. This is an important point and one which H. Pfeiffer nowhere discusses, so far as we can find, although it has been brought out by his work more prominently than by anyone else. In order that it may be understood, we will try to state it plainly. Guinea-pigs sensitized to horse serum furnish, from about the sixth to about the thirtieth day, serum which *in vitro* digests horse serum, as is shown by the formation of diffusible biuret bodies. After the thirtieth day or thereabouts, the serum of the sensitized animal no longer has this digestive action on horse serum *in vitro*; at least such action is not demonstrable. And yet the guinea-pig remains sensitive to horse serum. This, in our opinion, is due to the fact that certain fixed cells in the animal body remain sensitive and responsive to reinjection long after the leukocytes lose their sensitization. Pfeiffer and Jarisch found that in the so-called antianaphylactic state the blood-

serum of the guinea-pig does not digest the horse serum, at least not to the extent of supplying the dialysate enough of the digestive product to be detectable by the biuret test, and that no response to this reaction can be obtained until the third or fourth day after the reinjection. This is not due to the absence of the specific ferment in the blood-serum, but is due to the accumulation of the digestive products, leading to an increase in the antitryptic titer of the serum. It has been known for some time that the addition of normal blood-serum to a mixture of casein and trypsin prevents or arrests the digestive action of the latter on the former. This phenomenon has been investigated by Rosenthal,<sup>1</sup> who concluded that the antitryptic action of blood-serum is not due to the presence of antiferment. His reasons for this conclusion may be stated as follows: (1) It takes at least twenty-four hours to produce an antiferment, and this effect of blood-serum on tryptic digestion is immediate. (2) The antitryptic action of blood-serum is not increased by ligature of the pancreatic duct, and it should be if it were due to increased formation of anti-trypsin. (3) The antitryptic constituent of blood-serum is thermostabile and non-specific, and it would be thermolabile and specific were it an antiferment. (4) The antitryptic action of blood-serum is increased in full digestion, and in those diseases in which there is excessive protein metabolism, and is decreased in hunger. Rosenthal concluded that the antitryptic action of blood serum is due to the presence of digestive products. When these are abundant the digestive effect of blood serum is decreased or its antitryptic property is increased. When the blood is relatively poor in the products of protein metabolism the digestive property of this fluid is increased or its antitryptic property is decreased. This is a striking illustration of at least one of the ways in which the parenteral digestion of proteins is regulated, and it seems to us quite sufficient to explain the phenomena of so-called antianaphylaxis.

<sup>1</sup> *Folia Serologica*, 1910, vi, 285.

Rusznjak<sup>1</sup> has shown that when a sensitized animal recovers from a reinjection or is in the so-called antianaphylactic state, its blood is laden with digestive products. He has demonstrated this by showing that as early as thirty minutes after the reinjection the antitryptic titer of the blood-serum is greatly increased. In this way the animal body strives to protect itself from the effects of unusual parenteral digestion. Parenteral digestion is a normal process. It is continuous and the protein poison in small amount is being formed constantly in the body, and in part converted into a harmless substance by further digestion, and in part eliminated as such in the urine. After anaphylactic shock it is found in the urine in unusual quantity. The regulation of the formation and disposition of this poison is dependent upon the fine adjustment between cell metabolism and the digestive action of the blood. Parenteral digestion, as a physiological process, is carried on by the non-specific proteolytic ferments of the blood, and tissues. When a substance easily acted upon by this non-specific proteolytic ferment is suddenly thrown into the blood, life may be endangered. This is apparently the case when a hemolytic ferment is injected into the body in the form of a foreign active serum, or a venom. The hemolysis, thus caused, results in the liberation of a large amount of protein substance which is readily split up by the normal, non-specific proteolytic ferment, and the poison thus formed may destroy life. However, if the dose be not overwhelmingly great the digestive products retard the action of the ferment and tend to conserve life. H. Pfeiffer and Jarish attempt to distinguish between primary and secondary protein toxicoses. When the protein poison, preformed, as in peptone, urinary residue or  $\beta$ -i (ergamin) is injected into an animal, the antitryptic titer of the serum is decreased. On the other hand, when the protein is broken up in the animal body, as in anaphylactic shock or hemolytic poisoning, the products of the cleavage increase the antitryptic

<sup>1</sup> Deutsch. med. Woch., 1912, No. 4.

titer of the serum, or, in other words, lessen the digestive action of the blood, and they propose to distinguish between primary and secondary protein toxicoses by determining the antitryptic titer of the blood serum.<sup>1</sup> They state that the curve of the antitryptic serum titer in retention uremia after double nephrectomy in rabbits is similar to that of anaphylactic shock and hemolytic poisoning. Before the development of symptoms of poisoning the antitryptic titer rises above the normal (owing to the accumulation of digestion products).

It must be evident that the presence of a specific proteolytic ferment is not necessary in all cases to split up proteins with the liberation of the protein poison. As has been stated, Friedberger and many others have found that the protein poison is liberated from bacterial proteins by digestion with the normal serum of guinea-pigs. In this there can be no question of a specific ferment. The animals supplying this serum have not been sensitized with bacterial or any other proteins. They are normal, untreated animals; besides, there is nothing specific in this reaction, since the same poison is obtained from diverse bacterial proteins. As we have held for years, every protein molecule contains a poisonous group, and whenever and by whatever agent the protein molecule is disrupted, the poisonous group may be set free. The disrupting agent may be a chemical substance, a specific or a non-specific ferment. Failure to grasp this point has, in our opinion, led more than one investigator into error. At one time Friedberger stated that our poison cannot be the true anaphylactic poison because its formation is not specific. If this be true of our poison it is also true of his so-called anaphylatoxin. When a protein is digested or split up there is one stage in the process when the poisonous group is liberated. This may not always be evident because when the cleavage is carried one step farther the poison itself is destroyed. This is true

<sup>1</sup> The details of this procedure are given in their paper, *Zeitsch. f. Immunitätsforschung*, 1912, xvi, 38.

of our poison and of Friedberger's anaphylatoxin. As we have stated more than once, parenteral digestion is a normal, physiological process, and in this process the protein poison is liberated. There are many proteins, not all, which are digested by the normal, non-specific proteolytic ferment of the blood and tissues. In this, in our opinion, lies the explanation of the results obtained by Szymanowski,<sup>1</sup> who has found that the intravenous injection of varied protein-precipitating agents, such as copper nitrate, copper sulphate, mercuric chloride, lead acetate, phosphomolybdic acid, tannin, and picric acid in small doses, may cause all the symptoms of acute anaphylactic shock and death. In our opinion, the most probable explanation of this is that the precipitates formed in the blood by these substances act like foreign proteins and are digested by the non-specific proteolytic ferment of the blood with the liberation of the protein poison.

There has been some difference of opinion as to the source of the protein poison in anaphylaxis. At one time H. Pfeiffer thought that it must come from the proteins of the body. He was led to this conclusion by the smallness of the dose of the anaphylactogen necessary to induce anaphylactic shock on reinjection, but in his latest paper he states that the poison comes from the anaphylactogen (antigen). For like reason Friedemann<sup>2</sup> was inclined to the opinion that the poison is furnished by the serum of the sensitized animal, but he seems now to think that it comes from the anaphylactogen (antigen). Wassermann and Keysser<sup>3</sup> thought that the source of the poison is in the ferment (amboceptor). They shook horse serum with kaolin and then separated the kaolin from the horse serum in a centrifuge and digested the kaolin with guinea-pig serum and obtained a poison. They explained this by supposing that the kaolin absorbed the amboceptor from the horse serum, and when this was acted upon by the

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1912, xvi, 1.

<sup>2</sup> Ibid., ii, 591.

<sup>3</sup> Folia Serologica, 1911, vii.

complement of the guinea-pig serum the poison was formed. But Friedberger showed that kaolin does not absorb amboceptor, but does absorb the protein of horse serum, and this, when acted upon by the ferment in the guinea-pig serum, furnishes the poison. Bauer<sup>1</sup> thinks that the digesting serum becomes poisonous from the loss of its complement; thus, when bacteria are digested with the normal serum of the guinea-pig the bacteria absorb the complement from the serum and on account of this loss the serum becomes poisonous. Bauer thinks that de-complemented serum acts as an anaphylactic poison. This claim is deserving of further study. The ease with which Friedberger and his students prepare anaphylatoxin from all kinds of bacteria by digesting them with normal guinea-pig serum has caused some to suspect some flaw in the experiment. Besredka and Ströbel<sup>2</sup> claimed that the poisonous effect obtained is due to traces of peptone transferred from the medium on which the bacteria have been grown, and that when bacteria grown on peptone-free media were employed the results were negative. They also found that when peptone-agar was digested with normal guinea-pig serum the latter became poisonous. In answer to this communication, Lura<sup>3</sup> stated that bacteria grown on peptone-free agar and those grown on potato furnish the poison, when digested with serum, just as abundantly as those grown on peptone-agar. The work of Lura has been confirmed by others, and if there be a flaw in the preparation of anaphylatoxin by digesting bacteria with normal guinea-pig serum it has not been detected up to the present time.

Pearce and Eisenbrey<sup>4</sup> showed by the following experiment that the specific ferment formed in sensitization is, under certain conditions at least, a product of the fixed cells: "Our procedure has been to exsanguinate, under

<sup>1</sup> Berl. klin. Woch., 1912, 344

<sup>2</sup> Compt. rend. de la Soc. biol., 1911, lxxi.

<sup>3</sup> Zeitsch. f. Immunitätsforschung, 1912, xii, 701.

<sup>4</sup> Journal of Infectious Diseases, 1910, vii, 565.

ether anesthesia, a small normal dog (*A*), and to transfuse this animal by Crile's method, with the blood of a larger sensitized dog (*B*), until the blood pressure reached approximately its original level. After sufficient blood has been obtained from *B* to raise the pressure of *A*, the sensitized dog is then bled to exsanguination and transfused from a third normal dog (*C*) until its pressure reaches its previous normal level. At the proper moment, the normal dog containing the blood of the sensitized dog and the latter containing the blood of the normal dog, each receives intravenously the toxic dose of horse serum. In the former, a fall in pressure does not occur, and in the latter it does, thus proving that the phenomenon of anaphylaxis is due to a reaction in the fixed cells, and not either primarily or secondarily in the blood." That the blood does under certain conditions at least contain the specific ferment is shown by the production of passive anaphylaxis.

## CHAPTER XIII

### PROTEIN FEVER<sup>1</sup>

It is interesting and instructive to read the older literature on fever in the light of the knowledge which has been gained in the study of sensitization. It has long been known that the parenteral introduction of proteins in small amounts, and especially repeated introduction, leads to fever. The older literature on this subject as well as an account of his own work was given in 1883 by Roques.<sup>2</sup> In 1888, Gamaleia<sup>3</sup> showed quite clearly that fever accompanies and results from the parenteral digestion of bacterial proteins, and a year later Charrin and Ruffer<sup>4</sup> confirmed this work and extended it to non-bacterial proteins. In 1890 Buchner<sup>5</sup> produced the characteristic phenomena of inflammation—calor, rubor, tumor, and dolor—by the subcutaneous injection of diverse bacterial proteins. In 1895, Krehl and Matthes<sup>6</sup> induced fever by the parenteral introduction of albumoses and peptones, but they did not obtain constant results, which we now know are secured only by regulation of the size and frequency of the dosage. In 1909, Vaughan, Wheeler, and Gidley<sup>7</sup> demonstrated that any desired form of fever (acute fatal, continued, intermittent, or remittent) can be induced in animals by regulating the size and frequency of the doses of foreign protein administered parenterally, and in 1911, Vaughan, Cumming, and Wright extended the details of this work.

<sup>1</sup> This chapter is taken in part from an article by Vaughan, Cumming, and Wright, *Zeitsch. f. Immunitätsforschung*, 1911, ix, 458.

<sup>2</sup> *Substances Thermogenes*, Paris, 1883.

<sup>3</sup> *Ann. de l'Institut Pasteur*, xii, 229.

<sup>4</sup> *Compt. rend. Soc. de biol.*, 1889, 63.

<sup>5</sup> *Berl. klin. Woch.*, 1890, 216.

<sup>6</sup> *Arch. f. exp. Path. u. Pharm.*, 1895, xxxv, 232.

<sup>7</sup> *Jour. Amer. Med. Assoc.*, August 21, 1909.

When a man drinks water containing typhoid bacilli and proves susceptible, he does not immediately manifest symptoms of this disease. There is a period of incubation which in typhoid fever is about ten days. During this time the bacilli are multiplying in the man's body in great numbers, and are converting his proteins into bacterial proteins. The period of incubation stops and that of the active disease begins when the cells of the man's body become sensitized, elaborate a specific proteolytic ferment, and with this begin to split up the foreign protein.

**The Production of Continued Fever in Rabbits by Repeated Subcutaneous Injections of Dilutions of Egg-white.**—If the above be true it should be possible to cause a continued fever in animals by repeated injections of a foreign protein in small doses. This was first tried on rabbits with egg-white, and the results are shown in Fig. 12.

This animal was kept under observation and its temperature taken for six days before the injections were begun. The temperature of this fore period varied from  $101.8^{\circ}$  to  $102.5^{\circ}$  F. The injections consisted of egg-white with an equal volume of 0.5 per cent. phenol solution, and were freshly prepared each day. The injections were made under the skin over the back and repeated at intervals of two hours from 7 A.M. to 9 P.M. The urine was collected, measured, its specific gravity taken with a picnometer, and its nitrogen content determined by the Kjeldahl method. The animal was weighed once a day. The first injection of 2 c.c. of the egg-white dilution was made at 1 P.M., May 27, 1909. This dose was continued at the intervals stated until 3 P.M., June 1, when it was doubled, and again doubled at 7 A.M., June 4. The animal received 40 doses of 2 c.c. each, 20 doses of 4 c.c. each, and 82 doses of 8 c.c. each of the egg-white dilution; in all 816 c.c. Albumin appeared in the urine when the dose was increased to 4 c.c. The last dose was given at 9 A.M., June 15, after which the albumin in the urine gradually diminished and wholly disappeared June 26. The day before the first dose was given the animal weighed 2525 grams, and on the day of the last dose it

weighed 2180 grams. After discontinuing the treatment, June 15, the weight continued to decrease until June 21, when it reached its lowest, 1850 grams. After this the weight gradually increased until June 26, after which it was not taken.

The following figures give the weight of the animal, the amount of urine, the specific gravity, percentage of ash and percentage of N in the urine:

| Date,<br>1909. | Weight,<br>gm. | Urine,<br>c.c. | Specific<br>gravity. | Ash,<br>per cent. | N.<br>per cent. |
|----------------|----------------|----------------|----------------------|-------------------|-----------------|
| May 26         | 2525           | ..             | ..                   | ..                | ..              |
| May 27         | 2450           | 260            | 1.0106               | 1.61              | 0.16            |
| May 28         | 2440           | 125            | 1.0124               | 2.12              | 0.15            |
| May 29         | 2380           | 54             | ..                   | ..                | 0.16            |
| May 30         | 2330           | 275            | 1.0110               | 1.73              | 0.19            |
| May 31         | 2270           | 266            | 1.0130               | 2.41              | 0.23            |
| June 1         | 2355           | 104            | 1.0294               | 3.75              | 0.53            |
| June 2         | 2335           | 370            | 1.0114               | 1.89              | 0.16            |
| June 3         | 2320           | 290            | 1.0093               | 1.26              | 0.30            |
| June 4         | 2290           | 155            | 1.0101               | 1.55              | 0.28            |
| June 5         | 2290           | 225            | 1.0129               | 1.20              | 0.42            |
| June 6         | 2250           | 95             | 1.0148               | 1.71              | 0.56            |
| June 7         | 2320           | 100            | 1.0165               | 2.00              | 0.66            |
| June 8         | 2350           | 52             | 1.0267               | 2.02              | 2.53            |
| June 9         | 2355           | 115            | 1.0134               | 1.56              | 1.00            |
| June 10        | 2345           | 155            | 1.0106               | 1.66              | 0.56            |
| June 11        | 2230           | 165            | 1.0100               | 1.64              | 0.60            |
| June 12        | 2330           | 95             | 1.0170               | 1.56              | 0.92            |
| June 13        | 2280           | 190            | 1.0140               | 1.81              | 0.54            |
| June 14        | 2250           | 115            | 1.0148               | 1.15              | 0.98            |
| June 15        | 2180           | 140            | 1.0179               | 2.60              | 0.65            |
| June 16        | 2130           | 155            | 1.0194               | 2.99              | 0.66            |
| June 17        | 2080           | 170            | 1.0139               | 2.76              | 0.50            |
| June 18        | 2040           | 205            | 1.0184               | 2.10              | 0.43            |
| June 19        | 1960           | 150            | 1.0198               | 2.80              | 0.64            |
| June 20        | 1880           | 22             | ..                   | ..                | 2.90            |
| June 21        | 1850           | 10             | ..                   | ..                | ..              |
| June 22        | 1885           | 55             | ..                   | ..                | ..              |
| June 23        | 1900           | ..             | ..                   | ..                | ..              |
| June 24        | 1980           | ..             | ..                   | ..                | ..              |
| June 25        | 2070           | ..             | ..                   | ..                | ..              |
| June 26        | 2120           | ..             | ..                   | ..                | ..              |

It must be admitted that this chart bears a striking resemblance to one of typhoid fever. Indeed, we have here a condition practically identical with an infectious

fever, and yet without infection. In the infectious diseases the invading, multiplying cell supplies the foreign protein; in this experiment the supply of foreign protein has been kept up by the frequency of the injections. The amount of urine is variable, but averages less than normal as it does in the continued fever of man. The elimination of nitrogen is increased. During the twenty-four hours immediately following the first dose the temperature kept below the normal average, and each time the dose was doubled a marked rise followed in twenty-four hours. There is the

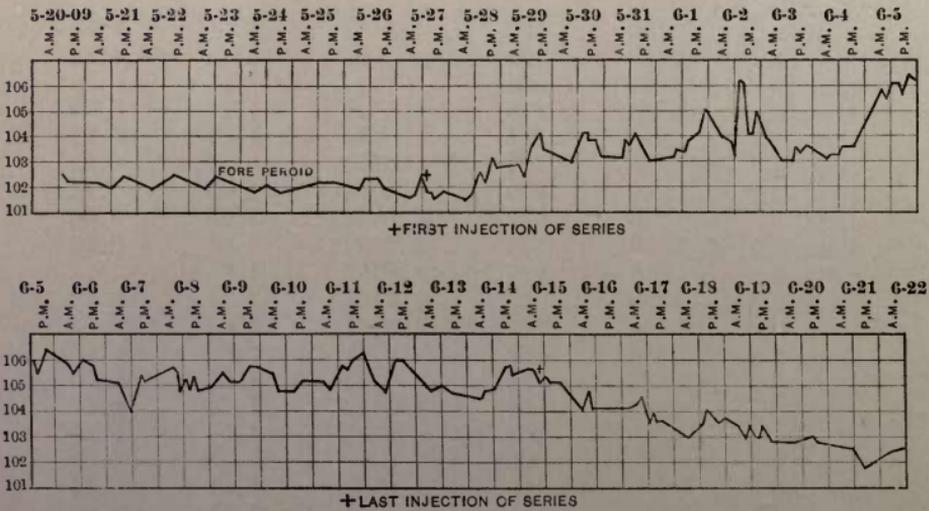


FIG. 12.—The production of continued fever in a rabbit by repeated subcutaneous injections of egg-white.

gradual rise in the morning temperature, so frequently seen in typhoid fever and the fact that for the most part the highest temperature for the day falls in the afternoon is also interesting. When the injections were discontinued the temperature gradually fell and remained somewhat below the normal, as is often observed in convalescence from typhoid fever.

We wished to ascertain the source of the albumin in the urine; did it consist wholly of egg albumen or serum albumin, or did it contain both? In order to determine this we injected 2 c.c. of the filtered urine on June 18, intra-abdomi-

nally, into each of 5 guinea-pigs for the purpose of sensitizing them to whatever proteins the urine might contain. Twelve days later 2 of these animals received intra-abdominally, each 5 c.c. of egg-white dilution (with an equal volume of water); 2 others had, each 5 c.c. of fresh serum from a rabbit, and the fifth had a mixture of 2.5 c.c. of each of these fluids. All were found to be sensitized, thus showing the presence of both egg-white and serum protein in the urine of the febrile rabbit.

It is worthy of note that while these animals developed the three stages characteristic of protein sensitization, the second and third stages were unusually prolonged and less acute than those generally observed in sensitized guinea-pigs. Of the two treated with egg-white, one died at the end of two hours and the other fifteen minutes later. Of the two treated with rabbit serum, one died at the end of one hour, while the other lived for three hours. The one that had the mixture of proteins developed the symptoms more promptly than any of the others, but did not die.

A continued fever was maintained in another rabbit by injections of the same strength of egg-white solution from April 30 to May 18, 1909. In this instance the size of the dose was not altered. The animal received four doses daily from April 30 to May 11, after which five were given until May 15, and then for three days we returned to four doses daily. The fever continued, after the injections were discontinued, until the evening of May 20, when it fell by crisis below the normal, slowly returning to the normal. The urine was collected and nitrogen determined as in the other instance, but the charts are so similar that we do not consider it necessary to present the second one.

**The Production of Continued Fever in Rabbits by Repeated Subcutaneous Injections of the Poisonous Group of the Typhoid Protein.**—Fig. 13 shows the effects of repeated subcutaneous injections of sublethal doses of the poisonous group split off from the cellular substance of the typhoid bacillus with a 2 per cent. solution of sodium hydroxide in absolute alcohol.

The material used was the crude soluble poison containing

about 10 per cent. of the poison in the purest form in which we have been able to obtain it. This crude soluble poison was administered every two hours from 7 A.M. to 9 P.M. from May 3 to May 18, 1909. Each dose consisted of 200 mg. of the crude poison, 300 mg. being a fatal quantity for rabbits of the size used.

When a fatal dose of the protein poison is administered the temperature rapidly falls, but with smaller repeated doses a continued fever results. The more nearly the dose approaches the fatal amount the more speedily will the

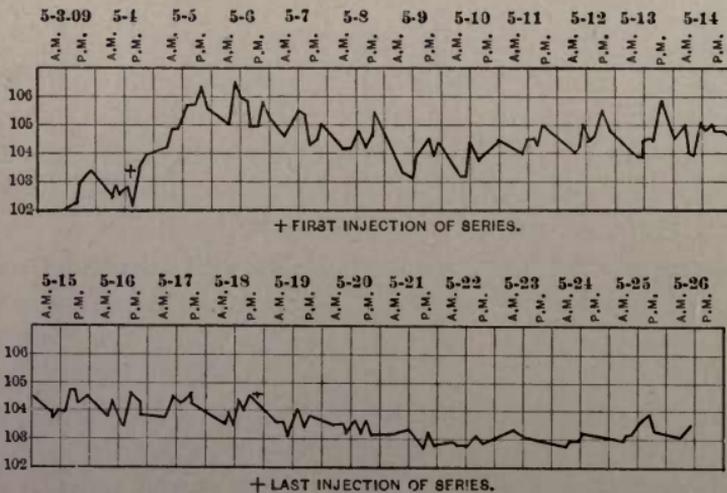


FIG. 13.—The production of continued fever in a rabbit by repeated subcutaneous injections of the poisonous group from the typhoid bacillus.

animal succumb. Death may be sudden and under a high temperature, or it may be slow and preceded by a fall of several degrees below the normal. From sublethal doses of the poison, animals recover quickly and apparently, completely. This seems to indicate that the poisonous effects are quickly neutralized in the animal body, but we are of the opinion that this neutralization is secured by more or less chemical disintegration in the protein molecule of certain cells in the body. The protein poison is acid to litmus, and we have secured continued fever in rabbits by repeated injections of either the acid solution or the same

after neutralization with sodium bicarbonate. Fig. 13 needs no further explanation.

**The Effects of Intra-abdominal Injections of Egg-white.**—Large single or repeated doses of egg-white injected intra-abdominally in non-sensitized rabbits have but little effect on the temperature. Generally the temperature runs slightly subnormal after such injections.

August 22, 1909, we injected the whites of three eggs into the abdominal cavity of a rabbit. The highest temperature of the fore period was 100.9°. After the injection the temperature was taken every two hours from 8 A.M. to 6 P.M. up to September 6. The animal was weighed each day and its urine measured and tested for albumin. There was no fever; indeed, the morning temperature fell some days to 97° and one day to 96.6°. The animal lost in weight, slightly more than one-fifth of its original weight. The volume of urine averaged normal, and at no time did it contain albumin.

On the other hand, 0.05 c.c. of egg-white, filtered through cotton, injected intra-abdominally every half hour from 8 A.M. until 4 P.M. produced the following results:

| Time.      | Dose in c.c. | Temperature. |
|------------|--------------|--------------|
| 8.00 A.M.  | 0.05         | 102.8°       |
| 8.30       | 0.05         | 101.0°       |
| 9.00       | 0.05         | 102.6°       |
| 9.30       | 0.05         | 102.9°       |
| 10.00      | 0.05         | 102.6°       |
| 10.30      | 0.05         | 103.2°       |
| 11.00      | 0.05         | 103.4°       |
| 11.30      | 0.05         | 103.5°       |
| 12.00      | 0.05         | 103.4°       |
| 12.30 P.M. | 0.05         | 104.4°       |
| 1.00       | 0.05         | 104.8°       |
| 1.30       | 0.05         | 105.1°       |
| 2.00       | 0.05         | 105.3°       |
| 2.30       | 0.05         | 105.3°       |
| 3.00       | 0.05         | 105.8°       |
| 3.30       | 0.05         | 105.8°       |
| 4.00       | 0.05         | 105.8°       |
| 4.30       | 0            | 106.6°       |
| 5.00       | 0            | 106.6°       |
| 5.30       | 0            | 106.1°       |
| 8.00       | 0            | 104.1°       |
| 8.00 A.M.  | 0            | 103.0°       |

**The Production of Fever in Rabbits by Repeated Intravenous Injections of Egg-white.**—The injection of a large amount of egg-white intravenously in a single or in repeated doses in non-sensitized rabbits does not cause any marked elevation of temperature.

After keeping a rabbit under observation for three days and finding that its temperature at no time reached  $102^{\circ}$ , we injected into its ear vein every two hours from 8 A.M. to 6 P.M. 4 c.c. of a dilution of egg-white with an equal volume of physiological salt solution, which dilution had been passed through a Berkefeld filter, and each cubic centimeter of which contained 26 mg. of protein as ascertained by a Kjeldahl determination. This dosage was continued for six days. During the greater part of this time the temperature, which was taken before each injection, remained normal, sometimes subnormal, and only once did it reach  $102^{\circ}$ . Then the dose was increased to 10 c.c. and continued for four days. Twenty-four hours after the increase in dose there was an irregular, but not marked elevation of temperature, the highest point reached being  $104.4^{\circ}$ . During the whole of the time the animal seemed quite well. Its greatest weight was observed during the time when the largest injections were being given, and at the same time the daily elimination of urine greatly increased, from 114 c.c., the average of the fore period, to as much as 650 c.c. at the time of the largest injections. The urine was tested daily for albumin with negative results. This experiment was continued from August 6 to 19, 1909. In March, 1910, a single injection of 5 c.c. of the dilution of egg-white was followed by a gradual rise in temperature to  $105.8^{\circ}$  within a few hours.

In order to induce fever in rabbits by the intravenous injection of dilutions of egg-white the doses must be small and the most striking results are obtained when the size of the dose is gradually increased. We have made many experiments along this line and some of them will be detailed.



shows the temperature curve of this animal, including the long fore period.

In No. 5 the highest temperature of the fore period was 101.1°.

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0            | 100.1°       |
| 9.00      | 1            | 100.4°       |
| 10.00     | 2            | 101.3°       |
| 11.00     | 3            | 102.2°       |
| 12.00     | 4            | 102.7°       |
| 1.00 P.M. | 5            | 102.8°       |
| 2.00      | 6            | 103.5°       |
| 3.00      | 7            | 104.7°       |
| 4.00      | 8            | 105.1°       |
| 5.00      | 9            | 105.6°       |
| 6.00      | 10           | 104.0°       |
| 7.00      | 11           | 103.8°       |
| 8.00      | 12           | 104.0°       |
| 8.30      | 0            | Death        |

This animal showed no symptoms until the final convulsive movement.

The following is the record of No. 34.

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 102.8°       |
| 10.00     | 2            | 104.0°       |
| 11.00     | 3            | 105.4°       |
| 12.00     | 4            | 105.2°       |
| 1.00 P.M. | 5            | 105.4°       |
| 2.00      | 6            | 105.4°       |
| 3.00      | 7            | 106.0°       |
| 4.00      | 8            | 105.4°       |
| 5.00      | 9            | 105.3°       |
| 6.00      | 10           | 105.8°       |

By 10 P.M. the temperature had fallen to 102.2°. The record of No. 35 is shown by the following:

| Time      | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 102.8°       |
| 10.00     | 2            | 103.6°       |
| 11.00     | 3            | 105.4°       |
| 12.00     | 4            | 105.6°       |
| 1.00 P.M. | 5            | 106.2°       |
| 2.00      | 6            | 106.7°       |
| 3.00      | 7            | 106.6°       |
| 4.00      | 8            | 106.8°       |
| 5.00      | 9            | 106.6°       |

By 10 P.M. the temperature had fallen to 103.4° and the next morning the animal was apparently normal. Twenty-six days later this animal was treated in the same way, with fatal results. Fig. 15 shows the curve for both treatments.

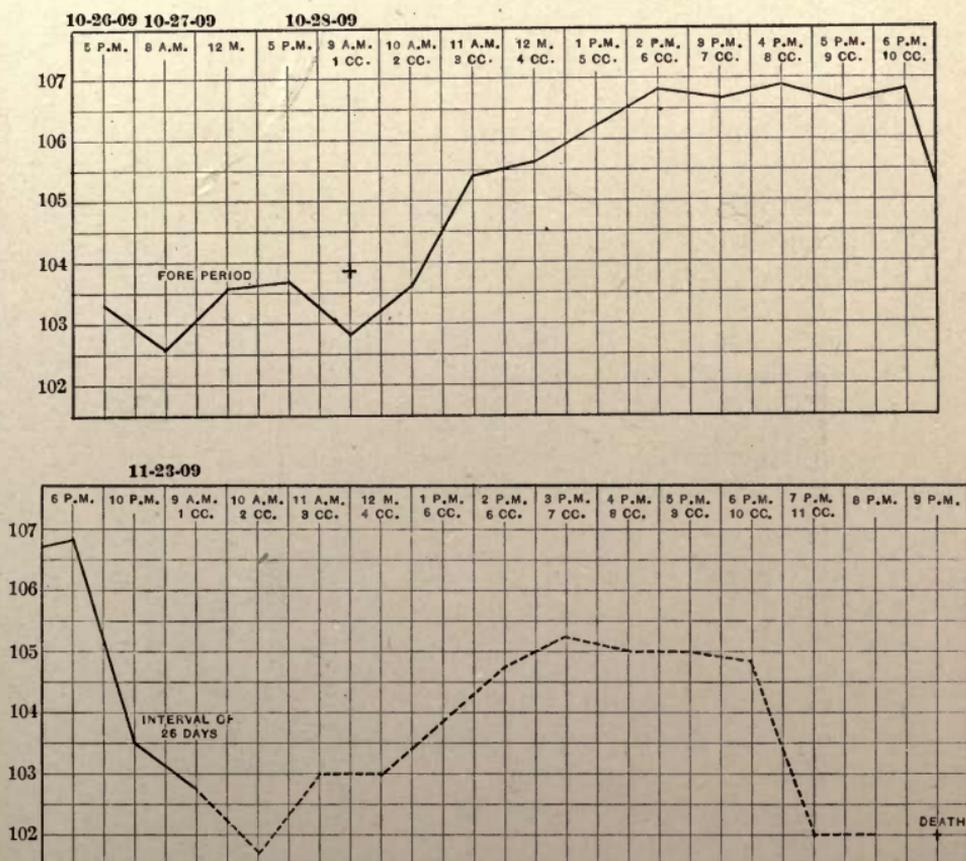


FIG. 15.—Acute fever produced in a rabbit by intravenous injections of egg-white. The continuous line represents the temperature in the non-sensitized animal. The broken line represents the temperature in the same animal, sensitized.

*Group II.*—The dilution used contained 13 mg. of protein in each cubic centimeter.

The following is the record of No. 15:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 102.6°       |
| 10.00     | 2            | 104.0°       |
| 11.00     | 3            | 104.9°       |
| 12.00     | 4            | 105.8°       |
| 1.00 P.M. | 5            | 105.1°       |
| 2.00      | 6            | 105.6°       |
| 3.00      | 7            | 105.6°       |
| 4.00      | 8            | 106.2°       |
| 5.00      | 9            | 105.6°       |
| 6.00      | 10           | 104.2°       |

The temperature gradually fell, and the next morning at 10 it was 101.8°.

The following is the record of No. 16:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 103.5°       |
| 10.00     | 2            | 104.1°       |
| 11.00     | 3            | 103.4°       |
| 12.00     | 4            | 104.2°       |
| 1.00 P.M. | 5            | 104.5°       |
| 2.00      | 6            | 104.5°       |
| 3.00      | 7            | 103.8°       |
| 4.00      | 8            | 103.4°       |
| 5.00      | 9            | 103.2°       |
| 6.00      | 10           | 104.2°       |

The next morning the temperature was 101.8°.

*Group III.*—Each cubic centimeter of the dilution contained 6.5 mg. of protein.

The following is the record of No. 18:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 1            | 103.2°       |
| 9.00      | 2            | 102.0°       |
| 10.00     | 3            | 102.5°       |
| 11.00     | 4            | 103.8°       |
| 12.00     | 5            | 104.0°       |
| 1.00 P.M. | 6            | 104.6°       |
| 2.00      | 7            | 104.0°       |
| 3.00      | 8            | 103.6°       |
| 4.00      | 9            | 104.0°       |
| 5.00      | 10           | 103.8°       |
| 6.00      | 11           | 103.8°       |

The temperature was normal the next morning.

After an interval of 174 days this animal was treated in the same way, with a fatal ending. Fig. 16 shows the curve for both treatments.

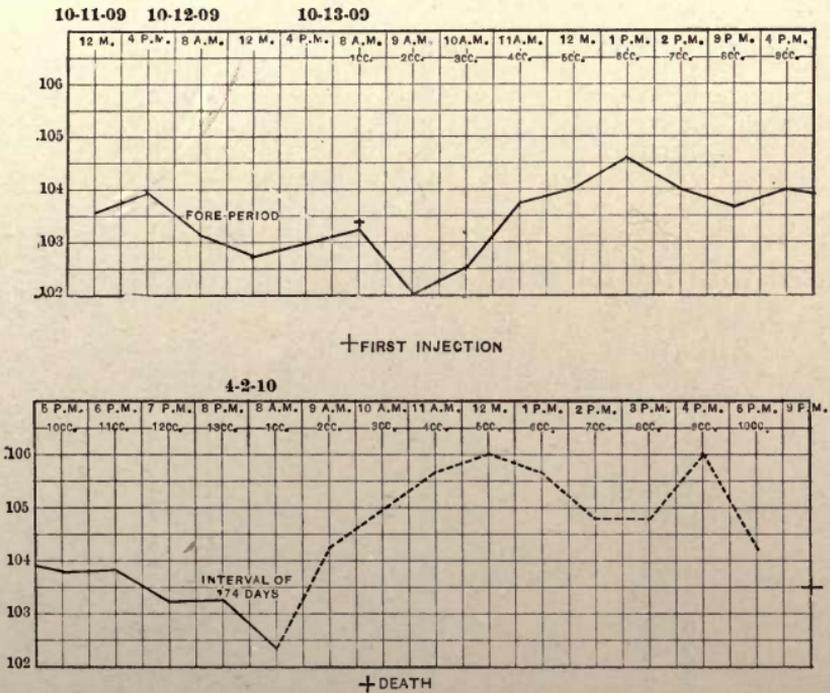


FIG. 16.—Acute fever produced in a rabbit by the intravenous injection of egg-white. The continuous line represents the temperature in the non-sensitized animal. The broken line represents the temperature in the same animal, sensitized.

Group IV.—Each cubic centimeter of the dilution contained 3.25 mg. of protein.

The following is the record of No. 20:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 103.0°       |
| 10.00     | 2            | 103.0°       |
| 11.00     | 3            | 103.4°       |
| 12.00     | 4            | 103.8°       |
| 1.00 P.M. | 5            | 104.0°       |
| 2.00      | 6            | 103.8°       |
| 3.00      | 7            | 104.2°       |
| 4.00      | 8            | 104.4°       |
| 5.00      | 9            | 104.4°       |

The following is the record of No. 21:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 103.0°       |
| 10.00     | 2            | 101.8°       |
| 11.00     | 3            | 102.4°       |
| 12.00     | 4            | 102.8°       |
| 1.00 P.M. | 5            | 103.2°       |
| 2.00      | 6            | 104.0°       |
| 3.00      | 7            | 103.6°       |
| 4.00      | 8            | 103.8°       |
| 5.00      | 9            | 104.0°       |

After an interval of 140 days this animal was again treated with the following record:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 102.0°       |
| 10.00     | 2            | 103.4°       |
| 11.00     | 3            | 105.2°       |
| 12.00     | 4            | 105.8°       |
| 1.00 P.M. | 5            | 106.6°       |
| 2.00      | 6            | 106.4°       |
| 3.00      | 7            | 105.6°       |
| 4.00      | 8            | 106.2°       |
| 5.00      | 9            | 105.6°       |
| 6.00      | 10           | 106.4°       |
| 7.00      | 0            | 106.2°       |
| 8.00      | 0            | 105.6°       |
| 9.00      | 0            | 106.4°       |
| 10.00     | 0            | 103.4°       |

After the second injection Cheyne-Stokes respiration appeared and continued through the day, but the injections were continued until 6 P.M., and the animal recovered.

*Group V.*—In this group but one injection was made each day. The dilution contained 26 mg. of protein in each cubic centimeter. The beginning dose was 3 c.c. and each day it was increased by 2 c.c. In No. 6 the dose was given each day at 10 A.M., and was repeated for seven consecutive days. The beginning dose was 3 c.c., two of the larger doses being repeated. In this way a well-marked intermittent fever of mild type was established. After

each injection the temperature arose within from two to four hours, and returned to normal during the evening, and continued so until the next injection. With increase in the size of the dose the tendency was to take the remittent type, so that at no time of the day did the fall quite reach the normal limit.

*Group VI.*—We have found some rabbits which do not respond to the pyrogenic effect of intravenous injections of egg-white until the size of the dose is reduced. On receiving a new consignment of rabbits, we were surprised to find that these animals did not develop fever on receiving repeated doses of a dilution of egg-white (1 to 1) with salt solution. The following illustrates our experience:

No. 101. This animal, weighing about 2600 grams, was treated with increasing doses of the dilution (1 to 1):

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 9.00 A.M. | 1            | 102.0°       |
| 10.00     | 2            | 100.4°       |
| 11.00     | 3            | 100.5°       |
| 12.00     | 4            | 100.2°       |
| 1.00 P.M. | 5            | 100.0°       |
| 2.00      | 6            | 96.4°        |
| 3.00      | 0            | Death        |

The abdominal cavity was filled with bloody fluid. The urine found in the bladder contained a small amount of albumin.

No. 102. Thinking that No. 101 was an individual exception, No. 102 was treated with the same dilution:

| Time.      | Dose in c.c. | Temperature. |
|------------|--------------|--------------|
| 10.00 A.M. | 1            | 101.2°       |
| 11.00      | 2            | 101.4°       |
| 12.00      | 3            | 101.5°       |
| 1.00 P.M.  | 4            | 101.5°       |
| 2.00       | 5            | 101.8°       |
| 3.00       | 6            | 101.3°       |
| 4.00       | 7            | 101.5°       |
| 4.45       | 0            | Death        |

No. 103. In this experiment the egg-white dilution was reduced (1 to 2):

| Time.      | Dose in c.c. | Temperature. |
|------------|--------------|--------------|
| 10.00 A.M. | 1            | 101.4°       |
| 11.00      | 2            | 101.7°       |
| 12.00      | 3            | 102.0°       |
| 1.00 P.M.  | 4            | 102.5°       |
| 2.00       | 5            | 102.8°       |
| 3.00       | 6            | 102.4°       |
| 4.00       | 7            | 101.8°       |
| 5.00       | 0            | 97.6°        |
| 6.00       | 0            | 97.4°        |
| 6.30       | 0            | Death        |

No. 104. In this experiment the dilution was further reduced (1 to 3).

| Time.      | Dose in c.c. | Temperature. |
|------------|--------------|--------------|
| 7.30 A.M.  | 1            | 101.6°       |
| 8.30       | 2            | 101.2°       |
| 9.30       | 3            | 101.7°       |
| 10.30      | 4            | 101.8°       |
| 11.30      | 5            | 102.6°       |
| 12.30 P.M. | 6            | 103.4°       |
| 1.30       | 7            | 103.8°       |
| 2.30       | 8            | 104.1°       |
| 3.30       | 9            | 104.4°       |
| 4.30       | 0            | 104.5°       |
| 5.30       | 0            | 105.2°       |

These differences in response to the injections of egg-white seem to be characteristic of certain groups or batches of animals and not of individuals within the group. Our laboratory buys its rabbits in lots of from twenty-five to one hundred. If one out of a given lot responds to a certain dose of egg-white we have found that others of the same lot respond in much the same way. Whether this is due to differences in food or in breed we have not determined, but are inclined to believe that breed has much to do with it. Possibly, age is an individual factor.

**A Brief Statement of the Autopsy Findings in Acute Poisoning of Rabbits with Egg-white.**—This work has been turned over to our colleagues of the department of pathology, and

Morse has been kind enough to make a few autopsies for us. We make a short abstract of his report:

Rabbit A received intravenously four doses of 10 c.c. each of a dilution of egg-white with an equal volume of physiological salt solution. The doses were administered at intervals of one hour. During the administration of the fourth dose the animal died in convulsions. A gray rabbit of average size and well-nourished; external orifices are normal; mucous membranes cyanotic; body, cold; rigor mortis, moderate. The peritoneal cavity contains a small amount of blood-tinged, serous fluid. Superficial inspection reveals nothing else of note. There is no displacement of organs, no peritonitis, no area of hemorrhage in the serosa. The pericardial sac is normal. There is no increase of pericardial fluid and the pleural cavities are dry. In the anterior mediastinum there is a moderate amount of pale fat with a few petechial hemorrhages. The thymus is large, swollen, and edematous. It spreads over the anterior mediastinum covering the great vessels and it contains hundreds of miliary hemorrhages. There are no large areas of blood in the tissue. The heart is moderately dilated and filled with red clot. There is no imbibition of hemoglobin in the intima of the great vessels. The heart valves are normal, the myocardium is darker than normal, and drips blood too freely. The lungs are reddish pink, though slightly darker than normal and rather moist on section. There is no pneumonia and no solid areas are seen in the lung tissue. The spleen is slightly congested and darker than normal. The kidneys are dark, congested, and drip blood on section. The adrenals are apparently normal. The stomach and intestine show no abnormality. The bladder is empty and normal in appearance. The liver is large, dark, and bleeds freely on section. In the retroperitoneum there is a moderately large suffusion of blood through the cellular tissue and partially involving the head of the pancreas. The brain appears normal, but section shows the tissue somewhat congested and moist.

The chief microscopic findings may be stated as follows:

The myocardium shows slight increase in hemofuscin, and there seems to be an excessive fragmentation of the muscle bundles (myocardite segmentaire of Renault). However, this may be due to the fixing fluid. The most striking thing is the occurrence of numerous miliary hemorrhages into the muscle substance, forcing the fibers apart in places. There is a diffuse distribution of blood throughout the heart muscle, blood cells being found here and there outside the capillaries in the muscles, forcing the fibers apart as though there had been a general diapidesis. The ventricular cavity shows a homogeneous red clot. The lungs show marked acute, passive congestion and localized areas of moderate congestion. There are a few small hemorrhages near the veins along the bronchi and also beneath the pleura. The liver shows extreme passive congestion, all the capillaries being gorged with blood. The whole liver substance appears as though soaked in blood, which lies everywhere, between the liver cells and in the bile capillaries. Some of the smaller ducts contain blood. The liver cells have a cloudy appearance and the nuclei are farther apart than normal, due to the engorgement with blood. The kidneys show some cloudy swelling and marked acute passive congestion. A condition similar to that seen in the heart and liver is found throughout the kidney. The renal tissue is full of miliary hemorrhages, and appears to be soaked in blood. Everywhere between the tubules and scattered throughout the parenchyma are red blood cells. Many of the glomeruli have red blood cells lying free within Bowman's capsules, and the capillaries of the tufts are extremely dilated. The epithelium of the proximal convoluted portion of the tubules is markedly desquamated. Many of the collecting tubules contain pale blood cells which have lost their hemoglobin. The pelvic fat is in part displaced by large suggillations of blood, and there are a few areas of coagulated blood around the kidney capsule. The pancreas is passively congested and a portion of this organ has been included in a large clot in the retroperitoneal region and is wholly necrotic. There is also marked fat necrosis and infiltration of the tissue with blood.

In sensitized rabbits killed by injections repeated after six months, Morse has found the microscopic lesions of the same character, but much less marked than those described above as resulting from acute poisoning. In fresh rabbits hemolysis and hemorrhage seem sufficient to account for death, but this does not appear to be the case in sensitized animals dying suddenly from relatively small doses.

**The Effects of Intravenous Injections of Laked Human Red Corpuscles on the Temperature of Rabbits.**—The blood was drawn into a solution of sodium citrate, and the corpuscles thrown down in a centrifuge. The corpuscles were repeatedly washed with physiological salt solution, and then dissolved in distilled water and diluted to the volume of the original blood.

One dose of 5 c.c. of this solution was injected into the ear vein of rabbit No. 7. The highest temperature of the fore period was 101.8°. The effects of this injection are shown by the following figures:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0            | 101.8°       |
| 9.00      | 5            | 101.5°       |
| 11.00     | 0            | 104.2°       |
| 12.00     | 0            | 105.2°       |
| 1.00 P.M. | 0            | 106.0°       |
| 2.00      | 0            | 105.6°       |
| 3.00      | 0            | 105.6°       |
| 4.00      | 0            | 104.7°       |
| 5.00      | 0            | 104.2°       |
| 6.00      | 0            | 104.0°       |
| 8.00 A.M. | 0            | 101.8°       |

In No. 10, 2 c.c. of the same solution had the effect shown in the following:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0            | 103.0°       |
| 10.00     | 2            | 103.1°       |
| 11.00     | 0            | 104.4°       |
| 12.00     | 0            | 105.6°       |
| 1.00 P.M. | 0            | 107.0°       |
| 2.00      | 0            | 106.4°       |
| 3.00      | 0            | 106.2°       |
| 4.00      | 0            | 105.6°       |
| 6.00      | 0            | 104.6°       |
| 8.00 A.M. | 0            | 102.8°       |
| 12.00     | 0            | 102.6°       |

It will be observed that this animal had a temperature of 103.1° before the injection was made. Fig. 17 gives this record.

In No. 11, 1 c.c. of the same solution had the following effect:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0            | 102.3°       |
| 9.00      | 1            | 102.0°       |
| 10.00     | 0            | 104.2°       |
| 11.00     | 0            | 104.4°       |
| 12.00     | 0            | 105.4°       |
| 1.00 P.M. | 0            | 104.0°       |
| 2.00      | 0            | 103.0°       |
| 3.00      | 0            | 102.2°       |
| 4.00      | 0            | 102.6°       |

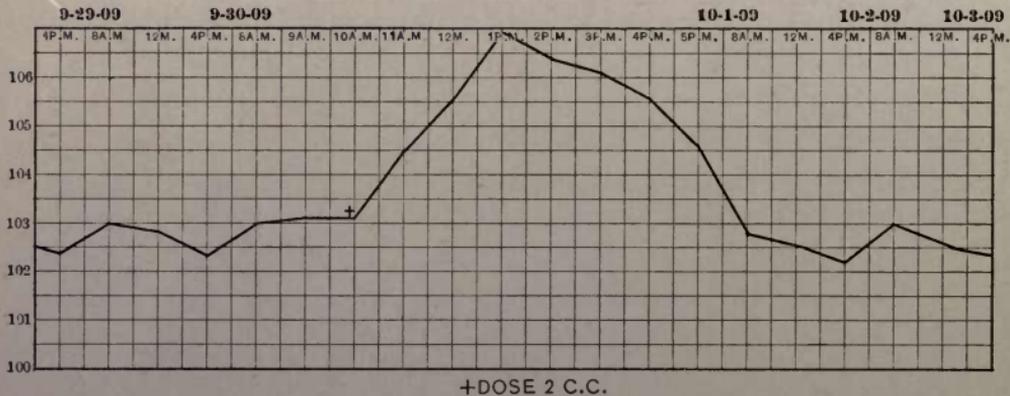


FIG. 17.—Acute fever produced in a rabbit by an intravenous injection of washed human blood cells hemolyzed.

Fig. 18 gives the curve in this case.

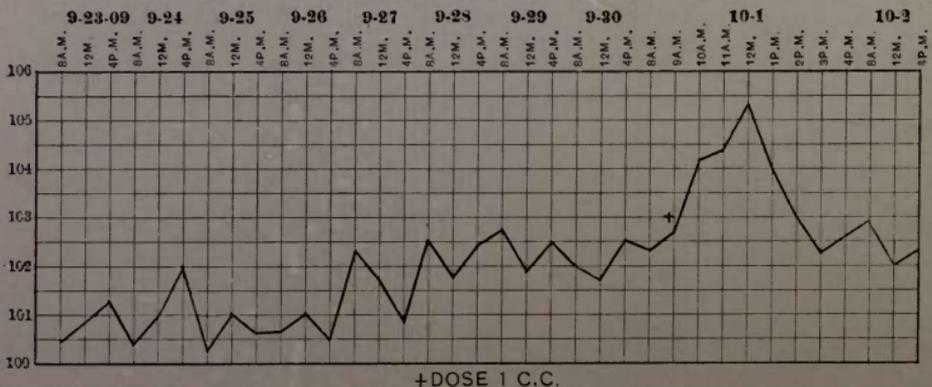


FIG. 18.—Acute fever produced in a rabbit by an intravenous injection of washed human blood cells, hemolyzed.

In No. 12, 0.5 c.c. of the same solution produced the following effects:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0            | 102.4°       |
| 10.00     | 0.5          | 102.0°       |
| 11.00     | 0            | 103.0°       |
| 12.00     | 0            | 104.0°       |
| 1.00 P.M. | 0            | 105.2°       |
| 2.00      | 0            | 104.4°       |
| 3.00      | 0            | 103.4°       |
| 4.00      | 0            | 102.4°       |

The highest temperature in the fore period covering seven days was 102.6°.

In No. 17, 10 c.c. of the same solution caused a precipitate fall in temperature, and death in seven hours.

The laked blood corpuscles of either man or rabbit after filtration cause an elevation of temperature when injected into rabbits either intra-abdominally or intravenously.

In rabbit No. 55, rabbits' corpuscles prepared as already stated and filtered were injected intra-abdominally as shown by the following figures:

| Time.     | Dose in c.c. | Temperature. |
|-----------|--------------|--------------|
| 8.00 A.M. | 0.1          | 102.8°       |
| 9.00      | 0.2          | 102.2°       |
| 10.00     | 0.3          | 102.8°       |
| 11.00     | 0.4          | 102.8°       |
| 12.00     | 0.5          | 104.3°       |
| 1.00 P.M. | 0.6          | 105.2°       |
| 2.00      | 0.7          | 105.1°       |
| 3.00      | 0.8          | 104.8°       |
| 4.00      | 0.9          | 104.4°       |
| 5.00      | 0            | 104.6°       |

In No. 56 the unfiltered laked corpuscles were injected intra-abdominally:

| Time.      | Dose in c.c. | Temperature. |
|------------|--------------|--------------|
| 8.00 A.M.  | 0.05         | 102.0°       |
| 8.30       | 0.10         | 101.8°       |
| 9.00       | 0.15         | 101.6°       |
| 9.30       | 0.20         | 101.8°       |
| 10.00      | 0.25         | 101.8°       |
| 10.30      | 0.30         | 101.8°       |
| 11.00      | 0.35         | 101.8°       |
| 11.30      | 0.40         | 102.4°       |
| 12.00      | 0.45         | 102.4°       |
| 12.30 P.M. | 0.50         | 103.0°       |
| 1.00       | 0.55         | 103.2°       |
| 1.30       | 0.60         | 103.2°       |
| 2.00       | 0.65         | 103.8°       |
| 2.30       | 0.70         | 104.6°       |
| 3.00       | 0.75         | 105.0°       |
| 3.30       | 0.80         | 105.2°       |
| 4.00       | 0.85         | 105.4°       |
| 4.30       | 0.90         | 105.4°       |
| 5.00       | 0.95         | 105.4°       |
| 5.30       | 1.00         | 105.6°       |
| 6.00       | 1.05         | 105.4°       |
| 6.30       | 1.10         | 105.7°       |
| 7.00       | 1.15         | 104.0°       |
| 7.30       | 1.20         | 105.0°       |
| 8.00       | 1.25         | 105.2°       |
| 8.30       | 1.30         | 105.2°       |
| 9.00       | 1.35         | 105.0°       |
| 10.00      | 0            | 105.6°       |
| 10.00 A.M. | 0            | 105.2°       |
| 2.00 P.M.  | 0            | 105.0°       |
| 4.00       | 0            | 103.6°       |
| 6.00       | 0            | 104.6°       |
| 8.00 A.M.  | 0            | 103.4°       |
| 9.00       | 0            | 103.6°       |

**The Destination of Egg-white Introduced into the Circulating Blood of the Rabbit.**—We have shown (p. 357) that egg-white injected into the ear vein of a rabbit soon disappears from the circulating blood and diffuses through the various tissues, from which it may be extracted with physiological salt solution and its presence demonstrated by sensitizing guinea-pigs. Since reporting on this we have found that the distribution of egg-white, injected into the blood, through the tissues extends not only to the organs mentioned

in the article referred to, but also to the skin and walls of the alimentary canal.

We have also attempted to determine how long after injection egg-white can be detected in the tissues.

Three rabbits received intravenously 25 c.c. of egg-white dilution (1 to 1). One was killed twenty-four hours later, and sections of its skin, kidney, brain, liver, spleen, and intestinal and stomach walls rubbed up with salt solution. After standing in the cold room overnight these emulsions were filtered and the filtrates injected intra-abdominally into guinea-pigs. The second rabbit was killed after forty-eight hours, and the third after seventy-two hours, and their tissues treated in the same way. All the guinea-pigs that received extracts from the first and second rabbits were found to be sensitized, though none died. Choking symptoms were very marked, most pronounced in those that received extracts from the spleen and kidney. The symptoms were quite as marked in those that received the extracts from the second rabbit as in those treated with the extracts from the first. The pigs that received the extracts from the third rabbit showed absolutely no symptoms.

From these experiments we conclude that egg-white diffused through the tissues after injection into the blood becomes, sometime between two and three days, either so far changed as to lose its identity or so fixed in the tissue that it cannot be washed out with salt solution. This time interval probably varies with the kind and amount of foreign protein introduced, and in different species of animals.

**The Digestive Action of the Blood Serum of Rabbits in Which Fever Has Been Induced with Egg-white.**—The following illustrates some of our experiments on the digestive action of the blood serum: The temperature of two rabbits was raised to 106° by hourly intravenous doses of a dilution of egg-white (1 to 1). One hour after the last injection both of these animals were bled to death from the jugular vein and the serum obtained.

Two cubic centimeters of this fever serum without any

addition, after standing for twenty-four hours in the incubator, was diluted to 10 c.c. with normal salt solution and deprived of normal proteins by acetic acid and heat. The filtrate gave a slight biuret test but no Millon. At 11.15, 2.5 c.c. of the filtrate was injected intracardiacally into a guinea-pig. Temperature before the injection was 98.8°. At 11.30, 97.9° and at 11.40, 98.4°. The animal was not visibly disturbed, with the exception of slight tremor.

A second sample of 2 c.c. of this serum which had been mixed with 2 c.c. of milk and kept in the incubator for twenty-four hours was treated in the same way. The biuret was slight and the Millon negative. The guinea-pig was not disturbed nor the temperature lowered.

A third portion of the serum mixed with an equal volume of a 2 per cent. solution of Witte's peptone was tested in the same way. The filtrate gave a beautiful biuret, but no Millon. The temperature of the pig fell 2.6° in ten minutes, but otherwise the animal was not affected.

A fourth portion of the serum mixed with an equal volume of a dilution of egg-white was tested in the same way. The filtrate gave a splendid biuret and also a good Millon. The pig received only 1.25 c.c. of the filtrate, half the quantity given to the others, but it immediately developed the symptoms characteristic of the protein poison and died within five minutes. Postmortem examination showed no injury and the heart-apex still beating.

We took a mixture of 2 c.c. of the fever serum and 10 c.c. of the egg-white dilution (1 to 1), which had stood in the incubator for five days. This was diluted to 20 c.c. and heated, after being made distinctly acid with acetic acid. After the removal of the normal blood proteins the filtrate gave both the biuret and the Millon tests very distinctly. Five cubic centimeters of the filtrate was evaporated on the water-bath and the yellowish residue extracted with 20 c.c. of absolute alcohol. The portion insoluble in alcohol was extracted with 5 c.c. of salt solution. The part soluble in salt solution responded feebly to both the biuret and the Millon tests. The part insoluble in both alcohol and salt

solution, when suspended in water, did not give the biuret, but did give an intense Millon reaction, while the part soluble in alcohol gave neither. A duplication of this experiment gave identical results.

We are not ready to conclude from our work, which has been more extensive than detailed here, that the ferment of the fever serum is strictly specific. An exhaustive measurement of its specificity will take much time and close work. The digestive products probably vary much, in amount at least, with conditions, and a close study of parenteral digestion offers a promising field for research. We are inclined to think that the rabbit is a good animal in which to study parenteral digestion, and we suspect that this form of digestion is not altogether abnormal in this animal. We have already shown (p. 355) that egg-white introduced into the stomach or rectum of a rabbit is, in part at least, absorbed unchanged into the blood. Besides, we have observed that our laboratory rabbits when abundantly supplied with food often show a rectal temperature of  $103^{\circ}$  or over, and when the food supply is limited the temperature is lower and more constant.

**The Production of Acute Fever, Followed by Immunity, by Repeated Intra-abdominal Injections of Bacterial Suspensions.** In this group of experiments guinea-pigs have been used. The usual method has been to take a standard loop from an agar slant four days old, suspend this in 10 c.c. of normal salt solution, and with a beginning dose of 0.1 c.c. of this suspension, the dose is increased by 0.1 c.c. each time and is repeated every half hour. As a basis for these experiments two guinea-pigs were treated, in the manner described, with the salt solution alone. The result in one of these is shown in Fig. 19. The total range in this case covers  $2.7^{\circ}$ , and it is possible that these injections stimulate parenteral digestion slightly. The general agreement of the curve in kind with those to be presented later is quite as interesting, though not so striking as its difference from them in quantity. It is worthy of note that during the continuance of these injections the temperature did not fall below the initial.

Figs. 20, 21, 22, and 23 are illustrations of the results obtained by this mode of treating guinea-pigs with sus-

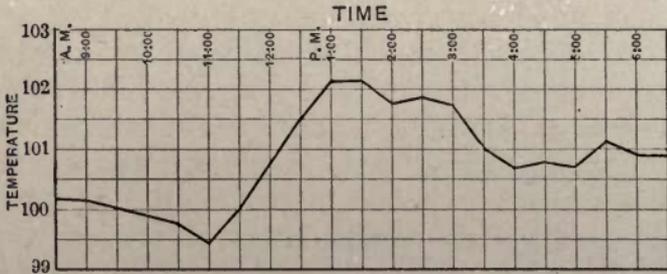


FIG. 19.—Showing effect of repeated injections of physiological salt solution.

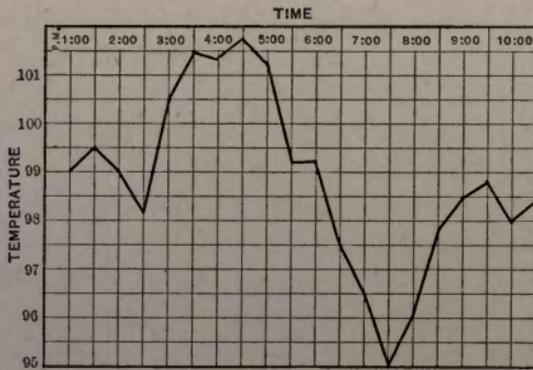


FIG. 20.—Showing effect of repeated injections of bacillus subtilis



FIG. 21.—Showing effect of repeated injections of bacillus prodigiosus.

pensions of living bacteria. All the animals whose temperatures are shown in these curves recovered. Additional information concerning these experiments is given in tables XLII to XLV. The first one or two animals of each group were treated with a beef-tea culture of the bacillus

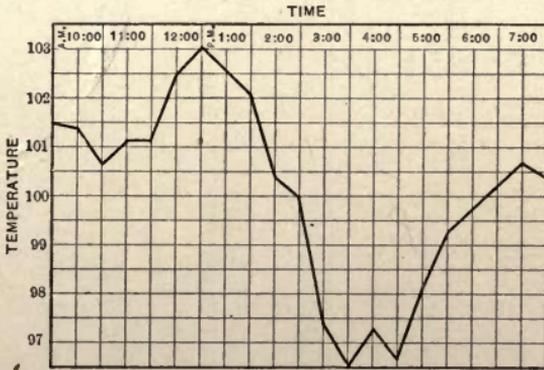


FIG. 22.—Showing effect of repeated injections of bacillus cholerae.

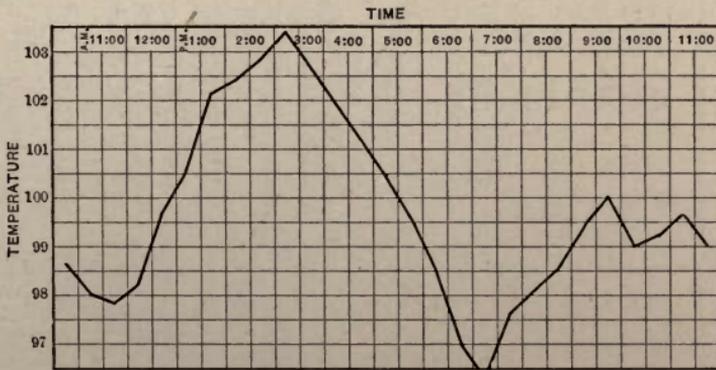


FIG. 23.—Showing effect of repeated injections of bacillus typhosus.

twenty-four hours old. After this, we changed to the suspension in salt solution and the exact dilution used in each animal is shown in the tables. The average bacterial content of each loop is also given in the tables, and the first dose contained one one-hundredth of this number, in case the dilution was 10 c.c.

TABLE XLII.—*BACILLUS PRODIGIOSUS*

| No. | Strength of suspension.        | Initial temp. | First fall. | Rise. | Second fall. | No. of injec'tns | Result of series.         | Days interval | Second injection. | Result. |
|-----|--------------------------------|---------------|-------------|-------|--------------|------------------|---------------------------|---------------|-------------------|---------|
| 5   | 24 hrs. beef tea               | 99.8          | 0.8         | 1.3   | 6.0          | 24               | Died after last injection |               |                   |         |
| 10  | 1 loop in 10 c.c. <sup>1</sup> | 99.6          | 0.7         | 1.6   | 6.0          | 17               | " " "                     |               |                   |         |
| 16  | " " 10 "                       | 99.0          | 0.4         | 2.9   | 3.3          | 24               | Recovered                 |               |                   |         |
| 20  | " " 20 "                       | 101.0         | 1.4         | 4.0   | 4.9          | 26               | "                         |               |                   |         |
| 131 | " " 10 "                       | 100.4         | 0.5         | 1.0   | 11.2         | 11               | "                         | 12            | 2 M.L.D. Cholera  | Lived   |
| 132 | " " 10 "                       | 100.8         | 0.2         | 2.0   | 9.8          | 11               | Died 12 hrs. after series |               |                   |         |
| 134 | " " 10 "                       | 100.4         | 0.0         | 2.5   | 14.2         | 21               | Recovered                 | 8             | 3 M.L.D. Cholera  | Lived   |
| 136 | " " 10 "                       | 100.2         | 0.0         | 0.0   | 9.7          | 20               | "                         | 8             | 3 M.L.D. Typhosus | Lived   |

<sup>1</sup> The average loop contained 38,645,000 bacteria.

TABLE XLIII.—*BACILLUS SUBTILIS*

| No. | Strength of suspension.        | Initial temp. | First fall. | Rise. | Second fall. | No. of injec'tns | Result of series.         | Days interval | Second injection. | Result. |
|-----|--------------------------------|---------------|-------------|-------|--------------|------------------|---------------------------|---------------|-------------------|---------|
| 4   | 24 hrs. beef tea               | 98.9          | 0.8         | 2.4   | 3.6          | 25               | Recovered                 |               |                   |         |
| 6   | 24 " "                         | 99.4          | 2.0         | 1.7   | 6.0          | 17               | Died after last injection |               |                   |         |
| 9   | 1 loop in 10 c.c. <sup>1</sup> | 100.8         | 0.7         | 1.8   | 2.3          | 26               | Recovered                 |               |                   |         |
| 15  | " " 10 "                       | 100.4         | 0.4         | 2.0   | 2.6          | 24               | "                         |               |                   |         |
| 19  | " " 20 "                       | 98.6          | 0.6         | 0.7   | 1.7          | 23               | "                         |               |                   |         |
| 135 | " " 10 "                       | 102.2         | 0.5         | 1.8   | 4.1          | 21               | "                         | 8             | 2 M.L.D. Typhosus | Lived   |
| 133 | " " 10 "                       | 101.4         | 0.8         | 1.6   | 4.9          | 21               | "                         | 8             | 2 M.L.D. Cholera  | Lived   |

<sup>1</sup> Average loop contained 16,320,000 bacteria.

TABLE XLIV.—BACILLUS TYPHOSUS

| No. | Strength of suspension.        | Initial temp. | First drop. | Rise. | Second drop. | No. of injec'tns | Result of series.                      | Days interval. | Second injection. | Result. |
|-----|--------------------------------|---------------|-------------|-------|--------------|------------------|--|----------------|-------------------|---------|
| 3   | 24 hrs. beef tea               | 98.7          | 0.8         | 2.5   | 6.0          | 23               | Died 30 min. after series              | 6              | 1.0 M.L.D.        | Lived   |
| 8   | 1 loop in 10 c.c. <sup>1</sup> | 98.6          | 0.0         | 5.8   | 1.4          | 26               | Recovered                              | 12             | 1.5 M.L.D.        | Lived   |
| 14  | " 10 "                         | 98.7          | 0.2         | 2.5   | 5.1          | 26               | "                                      | 7              | 2.5 M.L.D.        | Lived   |
| 25  | " 10 "                         | 97.4          | 3.6         | 3.4   | 3.5          | 24               | "                                      |                |                   |         |
| 26  | " 10 "                         | 98.7          | 2.7         | 1.5   | 6.0          | 24               | Died 16 hrs. after series <sup>3</sup> | 7              | 2.0 M.L.D.        | Lived   |
| 27  | " 10 "                         | 97.8          | 2.5         | 3.4   | 2.2          | 24               | Recovered                              |                |                   |         |
| 54  | " 10 "                         | 99.7          | 0.0         | 0.7   | 3.4          | 23               | Died 30 min. after series <sup>3</sup> | 11             | 4.0 M.L.D.        | Lived   |
| 55  | " 10 "                         | 99.8          | 0.2         | 4.7   | 3.7          | 23               | Recovered                              | 9              | 2.0 M.L.D.        | Lived   |
| 18  | " 20 "                         | 100.6         | 0.8         | 1.4   | 0.6          | 23               | "                                      | 8 <sup>2</sup> | 2.0 M.L.D.        | Lived   |
| 139 | " 10 "                         | 100.4         | 0.0         | 2.7   | 6.4          | 21               | "                                      | 8 <sup>2</sup> | 3.0 M.L.D.        | Lived   |
| 140 | " 10 "                         | 100.1         | 0.0         | 2.0   | 10.2         | 21               | "                                      |                |                   |         |

<sup>1</sup> Average loop contained 49,720,000 bacteria. <sup>2</sup> Second injection was B. cholerae. <sup>3</sup> Heart's blood contained typhoid bacilli.

TABLE XLV.—BACILLUS CHOLERÆ

| No. | Strength of suspension.             | Initial temp. | First drop. | Rise. | Second drop. | No. of injections | Result of series.                      | Days interval.  | Second injection. | Result. |
|-----|-------------------------------------|---------------|-------------|-------|--------------|-------------------|--|-----------------|-------------------|---------|
| 1   | 24 hrs. beef tea                    | 99.4          | 2.0         | 2.5   | 6.0          | 19                | Died 10 hrs. after series              |                 |                   |         |
| 2   | 24 " "                              | 98.7          | 0.8         | 2.5   | 6.0          | 23                | " 20 " "                               | 6               | 1 M. L. D.        | Lived   |
| 7   | 1 loop in 10 c.c. <sup>1</sup>      | 100.6         | 1.5         | 3.8   | 4.4          | 26                | Recovered                              |                 |                   |         |
| 13  | 1 " 10 "                            | 99.8          | 0.2         | 1.0   | 6.0          | 26                | Died 8 hrs. after series               |                 |                   |         |
| 17  | 1 " 20 "                            | 101.6         | 1.5         | 1.8   | 3.8          | 23                | " 4 days " "                           | 7               | 3 M. L. D.        | Lived   |
| 22  | 1 " 15 "                            | 99.8          | 1.0         | 1.4   | 6.0          | 24                | Recovered                              |                 |                   |         |
| 23  | 1 " 15 "                            | 98.3          | 1.0         | 2.1   | 6.0          | 24                | Died 36 hrs. after series              | 6               | 2 M. L. D.        | Lived   |
| 24  | 1 " 15 "                            | 98.9          | 0.4         | 3.4   | 6.0          | 24                | Recovered                              | 6               | 2 M. L. D.        | Lived   |
| 43  | 1 " 15 "                            | 98.5          | 0.3         | 4.4   | 0.6          | 21                | " "                                    |                 |                   |         |
| 45  | 1 " 15 "                            | 100.6         | 0.8         | 2.6   | 2.4          | 21                | Died 12 hrs. after series <sup>3</sup> | 11              | 3 M. L. D.        | Lived   |
| 44  | 1 " 15 "                            | 98.8          | 0.0         | 5.8   | 1.2          | 21                | Recovered                              |                 |                   |         |
| 46  | 1 " 15 "                            | 101.2         | 0.8         | 3.6   | 10.6         | 11                | Died 2 days after series               | 11              | 4 M. L. D.        | Lived   |
| 47  | 1 " 15 "                            | 99.8          | 0.7         | 4.5   | 0.5          | 11                | Recovered                              | 7               | 5 M. L. D.        | Lived   |
| 52  | 1 " 13 " each dose 1 c.c.           | 100.8         | 0.6         | 1.7   | 3.7          | 21                | " "                                    |                 |                   |         |
| 53  | 1 loop in 10 c.c., each dose 1 c.c. | 99.8          | 0.2         | 1.8   | 6.2          | 23                | Died 36 hrs. after series              |                 |                   |         |
| 141 | 1 loop in 10 c.c.                   | 100.8         | 0.0         | 2.4   | 11.8         | 21                | Died 12 hrs. after series              |                 |                   |         |
| 142 | 1 " 10 "                            | 100.7         | 0.0         | 3.5   | 14.6         | 21                | " 12 " "                               | 10 <sup>2</sup> | 2 M. L. D.        | Lived   |
| 143 | 1 " 20 "                            | 99.2          | 1.4         | 3.2   | 1.8          | 20                | Recovered                              | 10 <sup>2</sup> | 3 M. L. D.        |         |
| 144 | 1 " 20 "                            | 101.6         | 1.3         | 2.1   | 5.5          | 20                | " "                                    |                 |                   |         |
| 145 | 1 " 20 "                            | 100.8         | 2.3         | 3.0   | 3.0          | 20                | Died after series                      |                 |                   |         |

<sup>1</sup> Average loop contained 53,310,000 bacteria. <sup>2</sup> Second injection typhoid. <sup>3</sup> Heart's blood contains cholera bacilli.

It will be seen that in the large majority of the animals the first effect is a slight fall in temperature. We designate this as the primary or short fall. It should be understood that we estimate the falls and rises from the initial temperature. The short fall is followed by the primary rise. In a few instances the primary fall does not occur, or what is more probable, has not been detected. In one animal (No. 136, Table XLII) neither primary fall nor primary rise was detected. The rise is followed by the secondary or long drop. We call attention to some of the great drops; for instance, Nos. 131 and 134, Table XLII; No. 140, Table XLIV; and Nos. 46, 141, and 142, Table XLV. We did not suspect that this treatment would give immunity, and for this reason in our earlier work the recovered animals were not tested, nor have we as yet determined the limits of the immunity secured by these treatments. As is shown by the tables, the immunity is not, qualitatively at least, specific. Animals treated with subtilis or prodigious bear, at least from two to three M. L. D.'s of cholera or typhoid bacilli, and the immunity secured by the latter is interchangeable.

When treated animals are inoculated with living cultures they become sick some hours before the controls, and we are reminded of the immunity induced some years ago in this laboratory with the haptophor, or non-poisonous groups of the colon and typhoid bacilli, and reported by V. C. Vaughan, Jr. (p. 144), by Vaughan and Wheeler (p. 157), and by Vaughan.<sup>1</sup>

**The Production of Fever by Repeated Injections of Vegetable Proteins.**—As was shown in this laboratory some years ago, the vegetable proteins contain the same poisonous group found in bacterial and animal proteins; consequently there seemed no reason why these should not induce fever, and such we have found to be true. We will give here only one illustration. We extracted 10 grams of oat-meal with 100 c.c. of normal salt solution, and with a beginning dose of 0.1 c.c. of the slightly opalescent fluid

<sup>1</sup> Zeitsch.-f. Immunitätsforschung, 1909, i, 263.

thus obtained we have induced fevers similar to those already described in this chapter.

We have made many experiments on the production of fever with non-protein bodies, giving special attention to the amino-acids, the xanthin group, inorganic salts of ammonia, and certain carbohydrates, but a report upon these findings must be postponed.

**General Conclusions.**—Protein fever, and this includes the great majority of clinical fevers, results from the parenteral digestion of proteins. Bouillaud<sup>1</sup> was practically right when he said: “La fièvre est une maladie, dont la nature est toujours la même.” Proteins, living and dead, occasionally find their way into the body. They may come from without or from within. Crushed bone, muscle, or other tissue, on being deprived of its vitality or detached from its normal surroundings, becomes foreign material and must be broken up preparatory to its elimination. Under certain conditions proteins taken into the alimentary canal escape enteral digestion and are in part absorbed unbroken. When this happens, they are disposed of by parenteral digestion. In a finely divided form, as in the pollen of plants, proteins are absorbed from the respiratory tract and give rise to the condition designated as hay or rose fever. But in the great majority of instances proteins gain entrance to the body in unbroken form, as living proteins, bacteria, or protozoa. The parenteral proteolytic ferments are of two kinds, non-specific and specific. The former are normally present in the blood and tissues, especially in the former, of all animals. They differ in kind in different species and in amount and efficiency in individuals. Their purpose is to break up foreign proteins that find their way into the blood and tissues. They are, within limits, general proteolytic ferments, as are those of the alimentary canal; though the variety of proteins upon which they can act is more limited. They constitute the most important factor in racial and individual immunity.

<sup>1</sup> *Traité clinique et expérimentale des Fièvres*, 1826.

Man is immune to most bacteria, not because they do not elaborate poisons, for every protein molecule contains its poisonous group, but because they are destroyed by the general proteolytic enzymes as soon as they enter the tissue and consequently are not permitted to multiply in man's body. These non-specific, parenteral proteolytic enzymes are probably secretions of certain specialized cells. Under natural conditions these enzymes are capable of digesting those proteins upon which they do act only in small amounts, but the cells which elaborate them may be stimulated to increased activity by proper treatment, and the method detailed in this paper seems to accomplish this purpose. Whether or not these enzymes become qualitatively specific under such treatment as we have detailed can be determined only by further study. The immunity secured by these enzymes is limited in extent and transitory in duration.

The specific, parenteral proteolytic ferments are not normal products of the body cells, but are brought into existence under the stimulation of those proteins, introduced into the blood and tissues, which on account of their nature or amount escape the action of the non-specific ferments. It is to the development of these ferments that the phenomena of sensitization (wrongly called anaphylaxis) are due. A protein introduced into the blood and not promptly and fully digested by the non-specific enzymes is discharged from the blood current and deposited in some tissue, the cells of which after a time develop a specific ferment which splits up this protein and is not capable of acting upon any other. For certain proteins there are certain predilection organs and tissues in which they are stored, either exclusively or most abundantly: the pneumococcus in the lungs; the typhoid bacillus in the mesenteric and other glands; the viruses of the exanthematous diseases in the skin, etc. For the development of the specific proteolytic ferments time is required, and this varies with the protein and probably with the tissue in which it is deposited. The development of these ferments necessitates

changes in the chemical constitution of the protein molecules of the cell, and by this means the cell acquires a new function, which subsequently is brought into operation only by contact with that protein to which its existence is due. As a result of this rearrangement in molecular structure, the cell stores up a specific zymogen which is activated by contact with its specific protein. This explanation of the phenomena of sensitization originated in this laboratory,<sup>1</sup> and was not simply a fortunate guess, as has been assumed by some. The same is true of the statement made at the same time, that protein sensitization and bactericidal immunity are identical, and not antipodal, as they may appear to the superficial observer. A close study of the split products of bacterial, vegetable, and animal proteins, and especially of the poisonous group found in all proteins, had already been made in this laboratory. A study of the symptoms induced by the protein poison and of those following a second administration to the sensitized animal was certainly good and sufficient ground for concluding, as then stated, that the man that dies from the administration of morphine and the one that dies from opium both owe their death to the same poison. The most valuable experiments of Friedberger and his assistants, and of Pfeiffer and Mita, have, in our opinion, fairly established the validity of this explanation.<sup>2</sup>

Whether the products of digestion with the non-specific ferments and those elaborated by the specific enzymes are identical or not remains to be ascertained. The presence of a poisonous group in the protein molecule is disclosed in both enteral and parenteral digestion, as well as by our process of splitting up the protein with dilute alkali in absolute alcohol. In the first case it appears in the peptone

<sup>1</sup> Jour. of Infect. Dis., June, 1907.

<sup>2</sup> In 1910 Friedberger (Berl. klin. Woch., Nos. 32 and 42) made very plain the relation between sensitization and the infectious diseases, and in his address at the meeting of the German naturalists at Königsberg in September, 1910 (Münch. med. Woch., 1910, Nos. 50 and 51), he dwelt most instructively upon the wide application of the facts learned in his studies of sensitization.

molecule, which is large and complex. By the chemical process it is obtained as a less complex, more diffusible, and consequently more active body. Between the two there are probably several intermediate substances. The promptness in action manifested by all proteolytic ferments is determined, in part at least, by the proportion between the surface of the substrate and the mass. We observed some years ago<sup>1</sup> that the more finely divided the cellular substance of bacteria is, the smaller the dose which proves fatal. This is due to the greater surface exposure, and the same apparently holds good for colloids in solution. When soluble proteins are expelled from the blood and diffused throughout the animal body, the conditions for their rapid cleavage are most favorable, and consequently the fulminating phenomena observed after the second injection into a sensitized animal.

When a protein deposited in mass is rapidly acted upon by the parenteral enzymes, more or less marked inflammation results. This may be demonstrated by injecting suspended, dead, bacterial cellular substance into the peritoneal cavity of a guinea-pig when a diffuse peritonitis results, and we wish to suggest that the exantheams are due to the rapid digestion of proteins deposited in the skin. We admit that this is largely theoretical, but we have found, as already stated, that egg-white is in part deposited in the skin of rabbits after intravenous injection. This may be an explanation of the Arthus phenomenon.

The fact that every protein molecule contains a poisonous group does not mean that the products of protein digestion must contain a poison, for the poison itself may be split up and rendered inert, as happens when the proteins in the alimentary canal are broken up into amino-acids. It may therefore happen that in certain forms or stages of parenteral digestion no poison is formed.

The low temperature seen in some of our charts undoubtedly indicates the liberation of the poisonous

<sup>1</sup> Trans. Assoc. Amer. Phys., 1902.

group, and consequently the subnormal as well as the high temperature is a result of parenteral digestion, and it is in this stage that the greater danger to the life of the animal lies, as is plainly shown in our results. However, there is danger to life in the high temperature in and of itself. A rabbit is not likely to survive a temperature above  $107^{\circ}$ , and this was reached in at least one of our experiments, and closely approached in many others.

Fever must be regarded as a conservative process, although like many of nature's processes it often leads to disaster. But its purpose is the disposal of foreign and dangerous material, and therefore must be regarded as beneficent.

In parenteral digestion the following sources of heat production must be evident: (1) The unaccustomed stimulation and consequent increased activity of the cells which supply the enzymes must be the source of no inconsiderable increase in heat production. (2) The cleavage of the foreign protein means the liberation of heat. (3) The reaction between the products of the digestion and the tissues, especially when an active and irritant poison is liberated, must lead to increased heat production. We regard the first and last of these as the more important sources of the overproduction of heat in the febrile state.

**Special Conclusions.**—1. Large doses of unbroken protein administered intra-abdominally, subcutaneously, or intravenously have no effect upon the temperature; at least, do not cause fever.

2. Small doses, especially when repeated, cause fever, the forms of which may be varied at will by changing the size and the interval of dosage.

3. The effect of protein injections on the temperature is more prompt and marked in sensitized than in fresh animals.

4. The intravenous injection of laked blood corpuscles from either man or the rabbit causes in the latter even in very small quantity, either in single or repeated doses, prompt and marked elevation of temperature.

5. Laked corpuscles after removal of the stroma by filtration have a like effect.

6. Protein fever can be continued for weeks by repeated injections, giving a curve which cannot be distinguished from that of typhoid fever.

7. Protein fever is accompanied by increased nitrogen elimination and gradual wasting.

8. Protein fever covers practically all cases of clinical fever.

9. Animals killed by experimentally induced fever may die at the height of the fever, but, as a rule, the temperature rapidly falls before death.

10. Fever induced by repeated injections of bacterial proteins and ending in recovery is followed by immunity.

11. The serum of animals in which protein fever has been induced digests the homologous protein *in vitro*.

12. Fever results from the parenteral digestion of proteins.

13. There are two kinds of parenteral proteolytic enzymes, one specific and the other non-specific.

14. The production of the non-specific ferment is easily and quickly stimulated.

15. The development of the specific ferment requires a longer time.

16. Sensitization and lytic immunity are different manifestations of the same process.

17. Foreign proteins, living or dead, formed or in solution, when introduced into the blood soon diffuse through the tissues and sensitize the cells. Different proteins have predilection places in which they are deposited and where they are, in large part at least, digested, thus giving rise to the characteristic symptoms and lesions of the different diseases.

18. The subnormal temperature which may occur in the course of a fever or at its termination is due to the rapid liberation of the protein poison, which in small doses causes an elevation, and in larger doses a depression of temperature.

19. Fever *per se* must be regarded as a beneficent phenomenon, inasmuch as it results from a process inaugurated by the body cells for the purpose of ridding the body of foreign substances.

20. The evident sources of excessive heat production in fever are the following: (a) That arising from the unusual activity of the cells supplying the enzyme; (b) that arising from the cleavage of the foreign protein; (c) that arising from the destructive reaction between the split products, from the foreign protein and the proteins of the body.

In 1910 Friedberger<sup>1</sup> studied the effects of graduated doses of foreign proteins on the temperature of both normal and sensitized animals. With lambs' serum intravenously administered to normal guinea-pigs he obtained the following results:

|       |  |
|-------|--|
| 5.0   | c.c. equal fatal dose.                     |
| 0.5   | c.c. equals limit for fall in temperature. |
| 0.01  | c.c. equals upper constant.                |
| 0.005 | c.c. equals fever plane.                   |
| 0.001 | c.c. equals lower constant.                |

In sensitized guinea-pigs the above figures were changed to the following:

|          |                              |
|----------|------------------------------|
| 0.005    | c.c. equals fatal dose.      |
| 0.0005   | c.c. equals limit for fall.  |
| 0.00001  | c.c. equals upper constant.  |
| 0.000005 | c.c. equals limit for fever. |
| 0.000001 | c.c. equals lower constant.  |

In 1911 Schittenhelm, Weichardt, and Hartmann<sup>2</sup> experimented upon the effect of the parenteral administration of diverse proteins on animal temperature and came to the following conclusion, which in our opinion is well stated: "In severe experimental anaphylaxis there is a fall in temperature; in the lighter manifestations there is fever." We regard this as a confirmation of our conclusion reached some years earlier. "Small, especially repeated, doses of the protein poison cause fever, while large doses depress the temperature."

Some years ago Friedmann and Isaak<sup>3</sup> showed that after the parenteral introduction of foreign proteins the increase in nitrogen elimination is greater than can be accounted

<sup>1</sup> Berl. klin. Woch., 1910, No. 42.

<sup>2</sup> Zeitsch. f. exp. Path. u. Ther., 1911.

<sup>3</sup> Ibid., 1905, 1906, and 1908.

for by the protein injected. This has been confirmed by the work of Schittenhelm and Weichardt,<sup>1</sup> and as has been stated, we found the same in protein fever. Our explanation for the marked increase in nitrogen elimination has been given.

In intermittent and remittent fevers and in relapses in all infectious diseases the phenomena of protein sensitization are fully demonstrated. In the different forms of malaria, chill and fever correspond to the discharge of foreign protein into the blood, just as promptly as anaphylactic symptoms follow the injection of the homologous protein in a sensitized animal. The moment the blood cells rupture and the protozoal protein is disseminated the sensitized cells discharge the lytic ferment by which the foreign protein is disrupted and destroyed, but in this process the poison is liberated.

Local sensitization is frequently established in the mucous membrane of the air passages and of the alimentary canal, also in the skin for two reasons. In the first place, foreign proteins are frequently brought into direct contact with these tissues, and in the second place, foreign proteins introduced into the blood are frequently deposited in the skin and in the walls of the alimentary canal. These local sensitizations characterize many of the infectious diseases. The work of Dunbar and Weichardt on hay fever is a good illustration. These investigators injected each other subcutaneously with minute quantities of pollen suspension. Immediately Dunbar, being a hay-fever subject, became dizzy, and within a few minutes began to sneeze, then a whooping-like cough began. The eyes were congested, and an abundant secretion flowed from the nose. The face became swollen and cyanotic, and soon the body was covered with an urticarial rash. After twenty-four hours these symptoms subsided. Weichardt, not being a hay-fever subject, was not affected. That this and kindred affections are not benefited by antisera was abundantly and positively demonstrated by the failure of the so-called hay-fever

<sup>1</sup> Zentralbl. f. d. ges. Physiol. u. Pathol. d. Stoffwechsel, 1910.

serum, which was found in no instance to be of special value, and in some it greatly intensified the symptoms.

Our common colds are instances of local sensitization. Schittenhelm and Weichardt tell of a man who was so deeply sensitized by the inhalation of Witte's peptone that he could tell on entering the laboratory whether the peptone flask was open or closed, and some moist peptone painted on the skin caused the area covered to become red. The high degree of susceptibility to odors from the horse shown by some people has already been referred to. It seems in some instances that this susceptibility is transmitted from mother to child.

A volume might be filled with citations of cases of food and medicine idiosyncrasies. That these are, in large part at least, instances of protein sensitization has been demonstrated by rendering animals susceptible to the same food or medicine by injecting them with the serum of the susceptible individual. In other words, passive anaphylaxis has been established in the animal. In this way Brück has sensitized animals to iodoform and antipyrin with the sera of persons especially susceptible to these agents.

Thiele and Embleton<sup>1</sup> have made an extensive study of temperature variations. They employed well-fed guinea-pigs. First, they tried non-protein bodies, and with these reached the following conclusions:

1. Sodium chloride varies in its effects according to its degree of concentration when injected into feeding animals:

As normal saline it produces only a slight rise.

As 2 to 2.5 per cent. saline it produces a marked rise.

As 3 per cent. saline it produces a fall.

As 5 per cent. saline it produces a rise.

2. Calcium salts intraperitoneally produce a fall.

3. Ringer's fluid has no effect on temperature.

4. Alkalies, very dilute, produce a rise, and stronger, produce a fall.

<sup>1</sup> Zeitsch. f. Immunitätsforschung, 1913, xvi, 178.

5. Acid, same effects.

6. Lecithin injected intraperitoneally as a water emulsion has no effect on the temperature.

7. Charcoal (animal) very fine powder; in suspension intraperitoneally in large doses produces a marked fall, and in small doses a slight rise.

They notice, confirmatory of the findings of Krehl and Matthes, that fasting animals do not easily get febrile reactions with these substances. This agrees with Hirsch and Rolly, who state that glycogen-free animals cannot become febrile with sodium chloride injections. Kulz showed that injections of sodium chloride cause glycosuria. This was confirmed by Fischer, who believed the glycosuria to be due to irritation of the central nervous system, and showed that it is abolished by cutting the splanchnics, and that marked glycosuria results when direct injection into the cerebral vessels is made. Calcium salts inhibit both the glycosuria and the fever induced by sodium chloride. Freund showed that adrenalin causes glycosuria, and in large doses a fall, and in small doses, a rise in temperature. Again, calcium salts inhibit both the glycosuria and pyrexia due to adrenalin. The conclusion is reached that the fever caused by these non-protein substances is due to sympathetic irritation and consequent increased glycogen metabolism.

Turning now to the work done by Thiele and Embleton with protein bodies, we find a complete confirmation of the results obtained by previous investigators. They say: "With regard to endotoxin proper, by which we mean a toxic substance, which is specific to the bacteria or protein, there is but little evidence. The virulent and non-virulent bacteria, as well as simple egg albumen, appear to have the same temperature effects when inoculated into healthy animals in the way mentioned above, and also performed by Vaughan and Wheeler. The so-called endotoxin has been liberated from protein, from bacteria, etc., by Vaughan and Wheeler and others, also by Schittenhelm and Weichardt. In all these experiments the protein, whether simple or bacterial, has been treated for some time with caustic

alkali either in watery solution or in alcohol, so that a certain amount of protein degradation has occurred which is apparently just sufficient to form the toxic bodies which cause the acute toxic symptoms or temperature variations under discussion, according to the size of the dose. The work of Friedberger, Abderhalden, and others with anaphylatoxin production *in vitro*, and the demonstration of the presence of proteolytic degradation bodies during the process of the formation of toxic substance, is in favor of the view that the toxin (poison) is purely a degradation product of the protein simple or bacterial, as again there is no specificity in the toxins (poisons) produced from the various antigens (sensitizers) used. The reason why bacteria have a much more potent action in such small quantities would appear to be in the chemical composition of their bodies, and in the presence of normal specific ferments to them. Thus, according to Schittenhelm and Weichardt, and our own observations from the protamine injections, etc., it appears that some proteins of the normal animal body are much more toxic than others, and the toxic ones are those which have a high percentage of the diamino bases. In the experiments brought forward here the toxicity of protamine as regards producing temperature variations is almost as great as that of the tubercle bacillus. This is important in view of Ruppel's work showing that the tubercle bacillus has a large amount of diamino bases. Further, the formation of toxic substances in the case of bacteria suspended in normal saline would appear to be due to the formation of cleavage bodies from autolytic changes, just as occurs when tissues undergo autolysis. A final argument that cleavage products of protein and bacteria are the causation of temperature reactions, etc., is the observation of Matthes, who showed that in a digesting tuberculous animal, albumose injection gave rise to hyperemia of the small intestine and around the tuberculous foci just as tuberculin injection does, as we have noted in our present experiments. Here we have a protein cleavage product giving rise to the same effects as the specific antigen. Hence it would appear that the cleavage products of the

antigen are the cause of the reaction, cleavage going on continually locally at the tuberculous foci, and the addition of a little more cleavage body producing a cumulative effect, and in the intestine where cleavage is also going on in the cells during digestion, the same occurs whether the reacting dose is from simple or bacterial protein."

These investigators agree with us that it is the same substance which in larger doses causes a fall in temperature, and in small doses a rise. They say: "(1) In sensitized animals, owing to the presence of a specific enzyme, the homologous antigen undergoes more rapid degradation than in non-sensitized ones, and consequently certain degradation products are liberated in sufficient quantities from relatively small amounts of the antigen to cause temperature depression in these animals, and from still further amounts to cause fever. (2) The pyrogenic bodies are not a further stage in the degradation of the antigen, but the same degradation body or bodies cause depression or elevation according to the amounts present at any given time."

The relative effects of egg-white and tubercle protein on fresh and sensitized animals are shown by Thiele and Embleton as follows:

| Limits of                    | EGG-WHITE                |                       |
|------------------------------|--------------------------|-----------------------|
|                              | Normal animal,<br>grams. | Sensitized,<br>grams. |
| Temperature fall . . . .     | 0.05                     | 0.005                 |
| Constant temperature . . . . | 0.02                     | 0.0002 to 0.0001      |
| Temperature rise . . . .     | 0.01 to 0.001            | 0.0001 to 0.000002    |

| Limits of                    | TUBERCLE EMULSION        |                       |
|------------------------------|--------------------------|-----------------------|
|                              | Normal animal,<br>grams. | Sensitized,<br>grams. |
| Temperature fall . . . .     | 0.005 to 0.002           | 0.0005                |
| Constant temperature . . . . | 0.002 to 0.001           | 0.0001                |
| Temperature rise . . . .     | 0.001 to 0.00001         | 0.00001 to 0.000001   |

In another interesting way these investigators confirm some of our earliest work (Chapter III) when they say: "The more finely divided the bacterial protoplasm is, the more rapid are the temperature effects and the more toxic is the bacillary substance."

## CHAPTER XIV

### SPECIFIC FERMENTS OF THE CANCER CELL<sup>1</sup>

THE chief distinction between a living protein and any more or less complex chemical group or dead cell is that the living protein contains within itself different ferments or enzymes. Many of these ferments are common to several different forms of living protein or at least bear a close resemblance to each other, but it is also probable that each and every form of living cell contains within itself a specific enzyme which is distinctive for that given form of protein, and is present in no other.

While as yet ferments have not been isolated in a chemically pure state, and we have not an exact knowledge of their chemical nature, yet they are commonly regarded as albuminous, and their functions are specific and fairly well known. This at least is true of the soluble ferments such as the amylases, proteoses, and lipases. Again, there are examples of ferments of similar or nearly like constitution which are formed by more than one variety of cell (thus ptyalin formed by the salivary glands and diastase formed by the pancreas are both starch-splitting ferments).

The above examples are all of ferments which are water soluble, and which are active outside of the cell that produces them. They are ferments which are excreted by the cell and are used by the more highly developed forms of animal life for the purpose of converting complex chemical bodies into more simple forms, so that they may be of service to the whole, through their reaction with other ferments contained in other body cells.

<sup>1</sup> This chapter is contributed by J. Walter Vaughan and the work has been done by the aid of the Chase Cancer Fund in the research laboratory of Harper Hospital, Detroit, Michigan

It is well known, however, that all unicellular forms of life contain ferments which it is safe to divide into two classes. These we would designate as soluble and insoluble, or extra- and intracellular ferments; a soluble ferment being one that is excreted by the cell and which performs its ferment action without the cell, and an insoluble ferment being an enzyme that acts only within the cell structure. Obviously, with our rather limited comprehension of the nature of ferments, our knowledge is confined chiefly to the soluble variety. In this class of ferments formed by unicellular organisms might be mentioned zymase formed by the yeast cell, and the putrefactive ferments formed by many bacteria. V. C. Vaughan would also classify such substances as diphtheria toxin and tetanus toxin in this group, since it is his belief that these substances are not poisons in themselves, but liberate poisons through their ferment action.

While the known functions of most enzymes are those of decomposition, either through such processes as hydrolyzation or oxidation, yet we also know that ferment action may be one of construction; and both processes may be carried on by one and the same ferment. Thus we know that ethyl butyrate may be converted by the action of lipase into alcohol and butyric acid, while with a change in the acting masses, alcohol and butyric acid plus lipase will form the ester.

Inasmuch as we do know of the presence of constructive ferments belonging to the soluble variety, in which class we would naturally suppose the greater number to be destructive, since their function is to reduce complex proteins into simpler forms for the easier assimilation by the cell, it seems reasonable to suppose that most intracellular or insoluble ferments are of the constructive variety, and that it is through their aid that simple chemical substances are converted into the complex distinctive proteins of the cell itself.

While much of the above is theoretical, yet there is sufficient knowledge concerning some of the facts to make

the theoretical portion logical, and we have previously advanced the following theory as to the etiology of malignant disease with the foregoing in view.

Every living cell has within itself a constructive ferment whose specific action is to construct proteins of the same specific composition as the cell itself. Through the aid of this ferment a sufficient amount of cell protein is formed so that one cell may form two daughter cells and these in turn may again do likewise. As an example of this may be cited the growing of the typhoid bacillus upon an agar slant. In the original few organisms, which are spread upon the media, is contained a ferment which is capable of constructing the specific typhoid protein as long as sufficient required chemical substances are present in the agar medium. In the case of a unicellular organism like this the property of cell division and multiplication must be inherent in the cell, and the process of cellular reproduction progresses as long as suitable media are furnished. When, however, we come to consider more complex organisms, where the functions and relations of one cell are dependant upon outside cells, we have a somewhat different and more complex problem to consider. Such cells cannot continue to multiply without limit, else the cell that reproduced the fastest would soon predominate and outstrip all others. Here the growth of cells must be governed by outside circumstances to some extent, although it would appear reasonable to suppose that the property of reproduction is inherent in the cell as well as in the case of the simple unicellular organisms. This property, however, must lie dormant at times, only to be aroused by outside stimuli. A more lucid and concise way of expressing this idea would be to state that the reproductive ferment was normally stored up within the cell as a zymogen or inactive ferment, which becomes active when called upon by outside stimuli. Even in the unicellular organisms it is a well-known fact that cell division can be hastened or retarded by outside conditions, such as heat and cold or various chemical or electrical stimuli. With regard to the bearing of the fore-

going upon the cancer cell, we would state that it seems possible to conceive that a cancer cell is one which has lost its power of forcing its reproductive ferment back into an inactive stage. It is a cell whose chemical nature has become so altered that the reproductive ferment is uppermost and can no longer be influenced by outside stimuli. Just like the typhoid bacillus in the test-tube, its sole purpose is now one of active reproduction, and the reproduction will persist until the cancer cell has used up all protein that it is capable of transferring into its own specific protein, or until it itself is destroyed through the formation by the body cells of an antiferment in sufficient amount to destroy all cancer cells. How often the body itself does this it is difficult to surmise, but we do know that once such cells have multiplied so as to form a palpable tumor, the body is very seldom able to cope with it.

With the foregoing in mind the following experiments will throw some light upon the subject, especially with regard to the formation of an antiferment for the cancer cell.

As has been brought out in a previous article,<sup>1</sup> certain definite and characteristic blood changes are brought about by the injection of small amounts of dead cancer protein into a living host. Two forms of vaccine have been used in this work, cancer residue and a vaccine made of the cancer cell in its entirety.

Cancer residue is prepared by dissecting as freely as possible the cancer material from all surrounding tissues. The cancer material is next ground in a meat grinder, then is washed with water, diluted salt solution, alcohol, and lastly ether. This process removes salts, fats, wax, several protein bodies, and traces of carbohydrates. The remaining substance is then heated in a flask with a reflux condenser, with from fifteen to twenty times its weight of a 2 per cent. solution of sodium hydroxide in absolute alcohol, and by this means it is split into a toxic and a non-toxic group. The toxic portion is soluble in the alcohol, the non-toxic

<sup>1</sup> Jour. Amer. Med. Assoc., November 16, 1912.

is insoluble in alcohol, but soluble in water. This portion is dissolved in normal saline solution in sufficient quantities to form a 1 per cent. solution, and is used in the same manner as a vaccine.

Cancer-celled vaccine is prepared by grinding cancer tissue finely in a meat grinder, after which it is rubbed up as a suspension in alcohol in a sterile mortar. Next, it is rubbed through a very fine-meshed sieve, the alcohol filtered off, and the collected cell substance air-dried. This is weighed and then placed in normal saline solution, making a 2 per cent. cell suspension. To this 0.5 per cent. phenol is added for the purpose of rendering it less likely to become contaminated. In the preparation of both residues and vaccines it has been found that satisfactory blood changes can be obtained only when the tumor is of firm consistency and without necrotic or infected areas. The average injection of a 1 per cent. residue is from 5 to 20 minims; that of a 2 per cent. cancer-cell emulsion is from 5 to 10 minims.

Sheep and rabbits have been injected intravenously, intra-abdominally, and subcutaneously with both cancer residue and cancer vaccine, and frequent blood-counts made. In over 600 animals the blood changes have been practically uniform except in about 10 animals in which the vaccine used had been allowed to stand too long. The accompanying charts show that while the percentage of polymorphonuclear and small mononuclear leukocytes are not affected with any degree of regularity, the proportion of large mononuclear cells is invariably increased from 100 to 400 per cent. within from twenty-four to forty-eight hours. This increase, however, is of short duration and recedes with rapidity after reaching its height.

Fig. 24 illustrates the average blood change obtained through the injection of an active residue. The preparation used was a 1 per cent. sarcoma residue which had been made from a small round-celled sarcoma of the mediastinum. Three subcutaneous injections of 5 minims each were given at hourly intervals, the first blood count being made before the injections were commenced. The second count, made

seven hours later showed an increase in polymorphonuclear cells from 21 to 37 per cent., and a corresponding decrease in small mononuclear leukocytes. The third count made, twenty-five hours after the first, showed the characteristic increase of large mononuclear cells which were here regis-

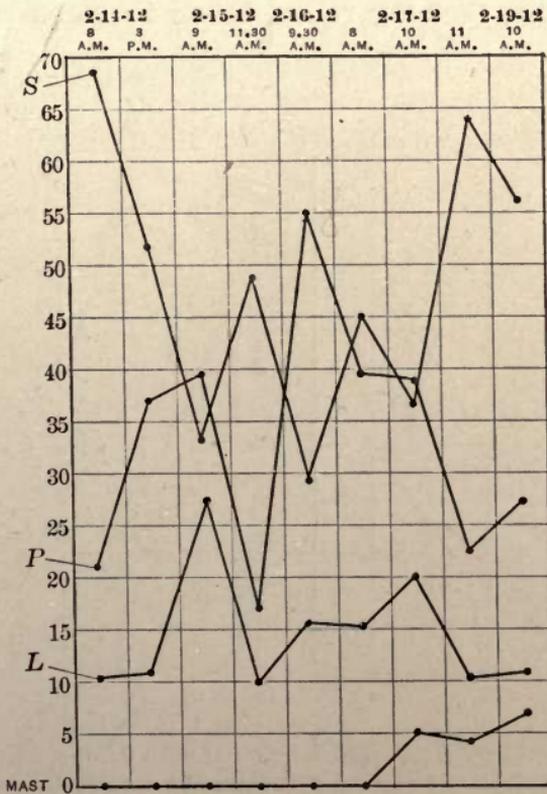


FIG. 24.—Rabbit: 5 minims of 1 per cent. sarcoma residue injected subcutaneously at 8, 9, and 10 A.M. The line designated as *P* indicates, in this and the following charts, count of polynuclears; *S* indicates small, and *L* large mononuclears.

tered at 27 per cent., with a normal at the first injection of 10 per cent. Two and one-half hours later the percentage of this form of cell had returned to normal, which was again followed by a slight increase. It is probable that the highest registration of large mononuclear cells occurred between the second and third counts.

Fig. 25 represents four rabbits which were given 0.5 c.c. each of cancer-cell vaccine by different methods. The first was given a simple subcutaneous injection of rectal adenocarcinoma. The fourth represents the same amount of the same tumor given intraperitoneally, as does also the third. The second shows the injection of vaccine made from a rapidly growing round-celled sarcoma of the neck.

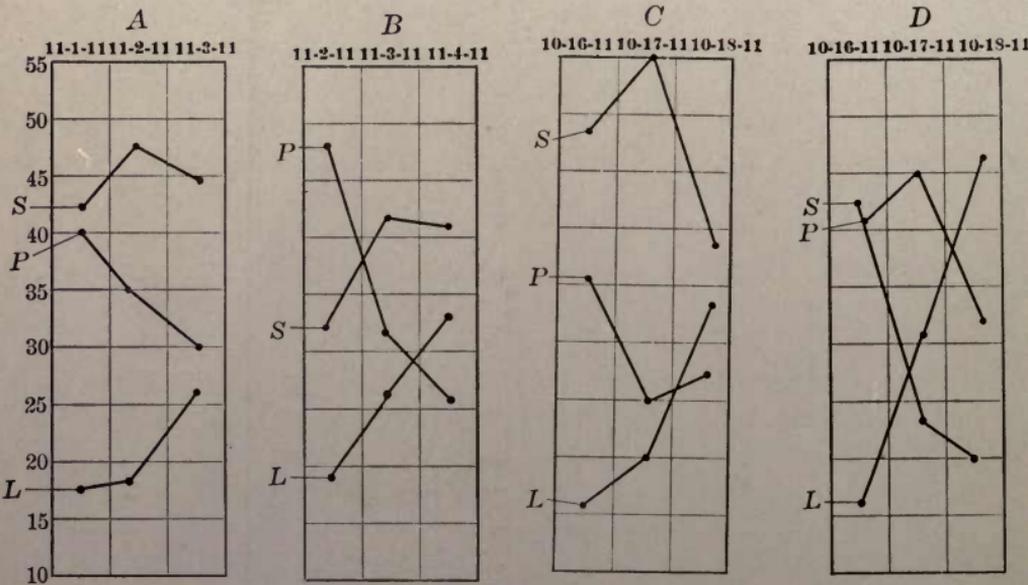


FIG. 25.—*A*, rabbit given subcutaneous injection of 0.5 c.c. rectal adenocarcinoma (Morris); *B*, rabbit injected with 0.5 c.c. small round-celled sarcoma; *C*, rabbit given intraperitoneally 0.5 c.c. rectal adenocarcinoma (Morris); *D*, rabbit given intraperitoneally 0.5 c.c. rectal adenocarcinoma (Morris).

From these charts it can be seen that the intraperitoneal method of injection gives a more rapid reaction than the subcutaneous.

Fig. 26 illustrates the blood changes occurring in a sheep following the injection of 0.5 c.c. of sarcoma residue.

Figs. 27 and 28 show more frequent differential counts following repeated subcutaneous injections.

Fig. 29 illustrates daily counts following a single intraperitoneal injection of cells from a breast adenocarcinoma. This is rather an extreme reaction, inasmuch as the proportion of large mononuclear cells is increased from 6 per cent. to 35, about 500 per cent.

Fig. 30 shows a very slight reaction following the injection of a breast-cancer residue which has almost lost its activity.

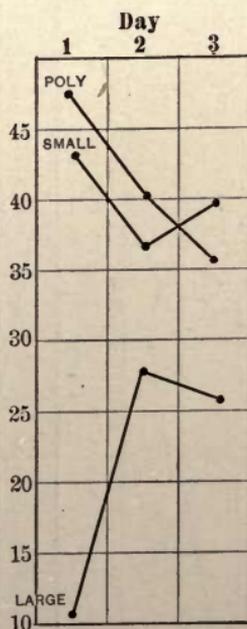


FIG. 26.—Sheep injected with 0.5 c.c. sarcoma residue.

Figs. 31, 33, and 34 illustrate rabbits giving the average reactions. Fig. 32 represents the effect of giving an intraperitoneal injection of 10 c.c. of breast-carcinoma cell-emulsion into a rabbit sensitized to sarcoma vaccine, the injection being given when the percentage of large mononuclear cells was at its highest point. Death resulted in from eight to ten hours.

In order to ascertain just what bearing this change in percentage of large mononuclear leukocytes had to the formation of a specific ferment, several rabbits were sensi-

tized to the cancer-cell and then at varying percentages 10 c.c. of cancer-cell emulsion was injected intravenously into the animals. In unsensitized rabbits there was no noticeable effect. Rabbits with a percentage of above

FIG. 27

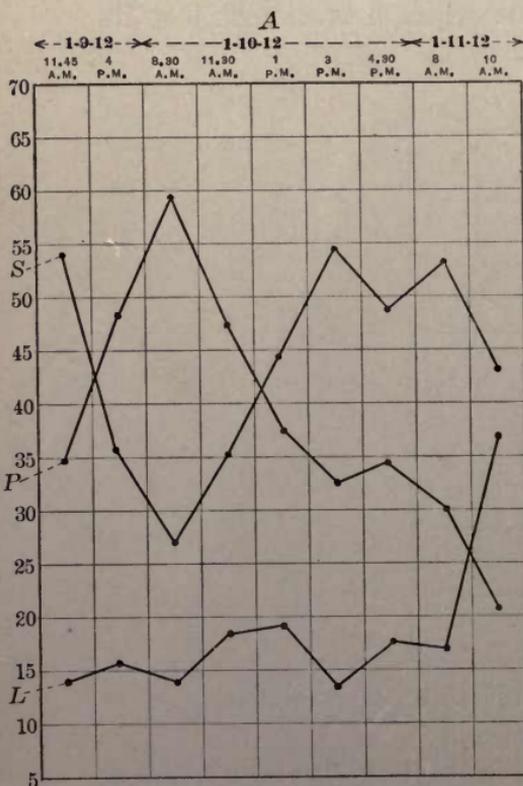
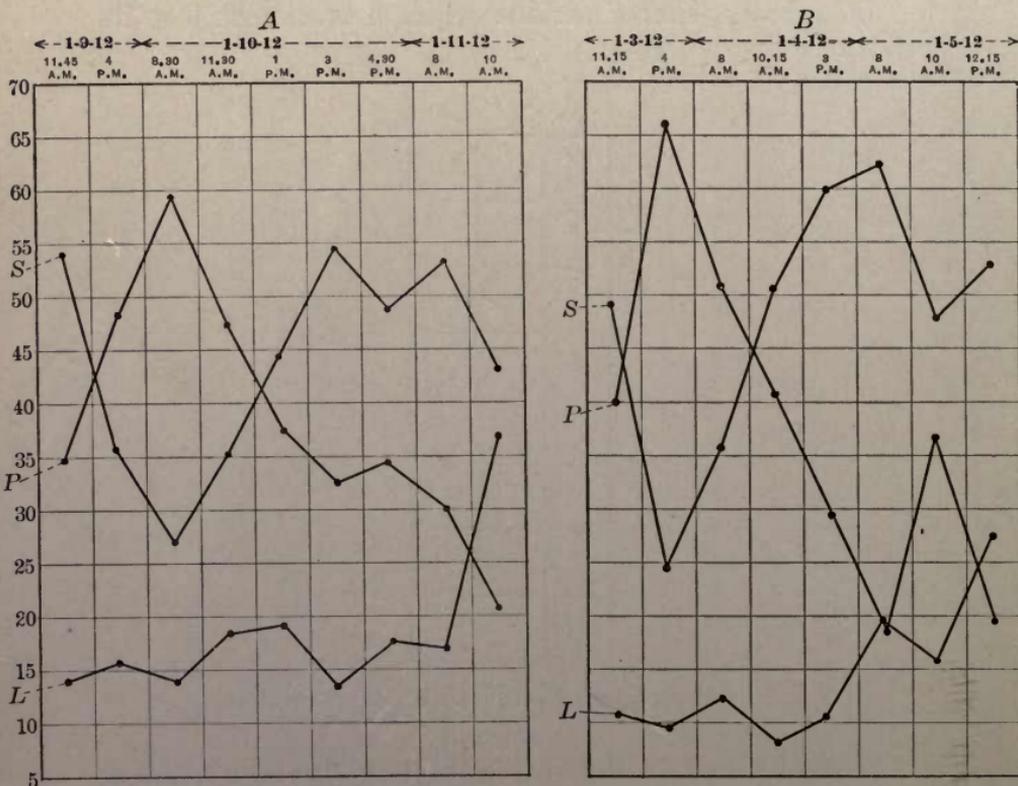


FIG. 28



FIGS. 27 and 28.—A, rabbit injected subcutaneously with 5 minims of 1 per cent. s. r. sarcoma at 12 M., 2 P.M. and 3 P.M.; B, rabbit injected subcutaneously with 0.1 c.c. of 2 per cent. mixed residue at 12.15 P.M., 2 P.M., and 4 P.M.

30 of large mononuclear cells usually died within one to three hours, and rabbits with a lower percentage, but with a marked increase, were made sick, but recovered.

Sickness is immediate after injection; the animal at once falls on its side and begins violent scratching. The respira-

tion is labored and, when death ensues, always stops before the heart-beat. Rabbits which die from a dose, after being sensitized, usually have a stage of apparent rest from one-half to three hours after the first stage of excitability, which lasts for from three to five minutes. A possible explanation

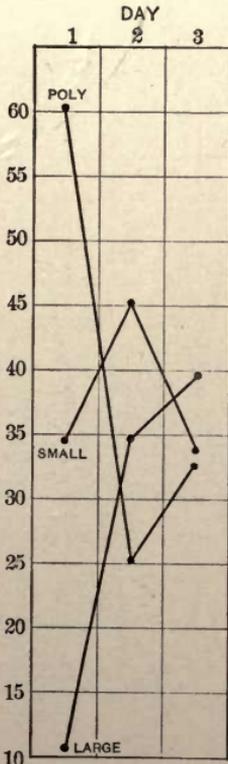


FIG. 29.—Rabbit given a single intraperitoneal injection of cells from a breast adenocarcinoma.

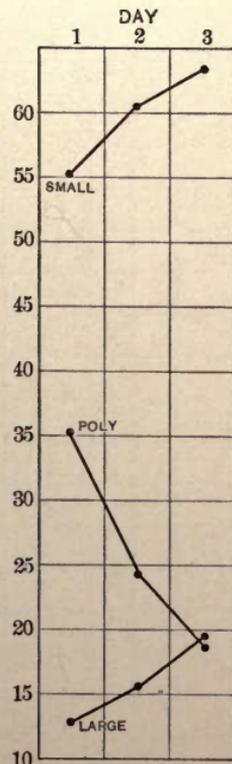
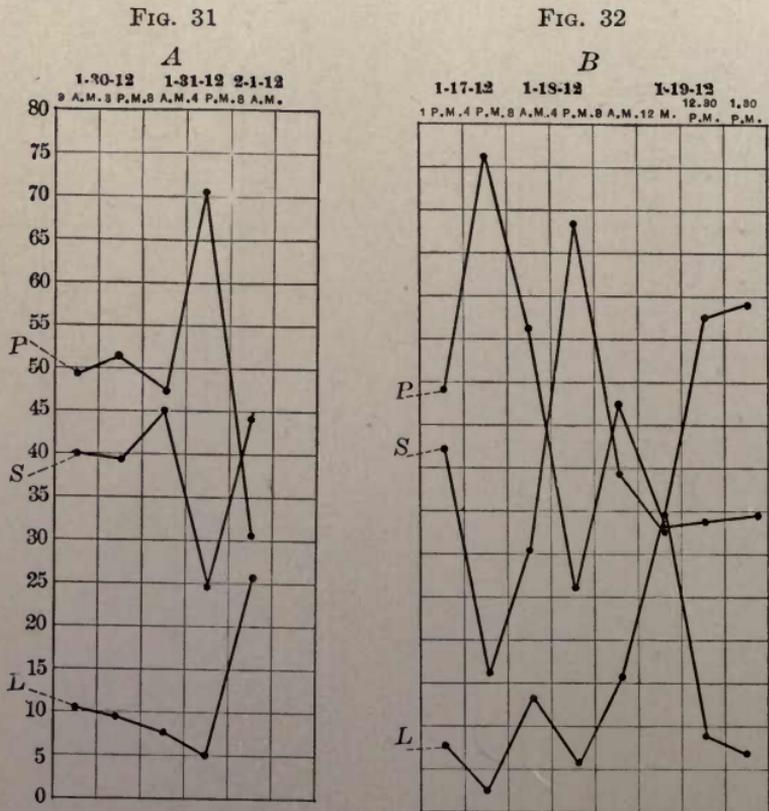


FIG. 30.—Rabbit injected with a breast-cancer residue which has almost lost its activity.

of this is that the first stage of excitement is due to the destruction of cancer cells and consequent liberation of their toxic radical by the specific ferment present in the blood serum, while the fatal result which follows later is due to the reaction between the cancer cell and the large mononuclear leukocytes.

The increased percentage of large mononuclear leukocytes is but a transitory affair, however, which usually lasts from four to ten hours, and it is impossible to produce fatal results in rabbits by intravenous injections of cancer-cell



FIGS. 31 and 32.—A, rabbit 167 W., given 5 minims of 1 per cent. sarcoma at 8.30, 9.30, and 10.30 A.M.; B, rabbit 54 T., given 5 minims of 1 per cent. sarcoma at 1, 2, and 3 P.M.; at 12.15 P.M., 10 c.c. of breast carcinoma-cell emulsion (Mel.) was injected intraperitoneally; rabbit died in eight to ten hours.

emulsion after this stage is passed. Consequently we have applied the term "transitory sensitization" to this phenomenon.

If we bleed a rabbit at the height of this transitory sensitization and obtain the serum, this, when mixed with cancer-

cell emulsion and incubated for one hour will produce marked symptoms of poisoning when injected intravenously into a normal rabbit. The severity of symptoms depends upon both the amount of cancer-cell emulsion and serum.

FIG. 33

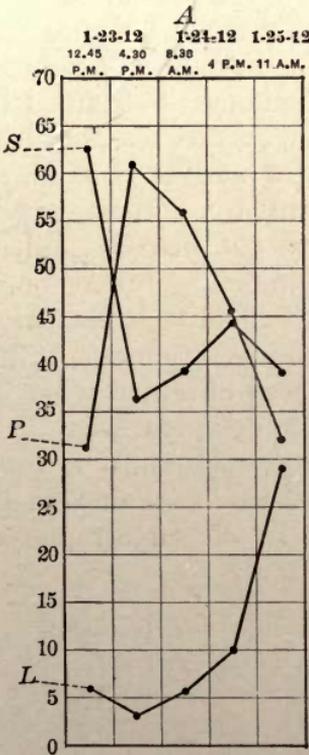
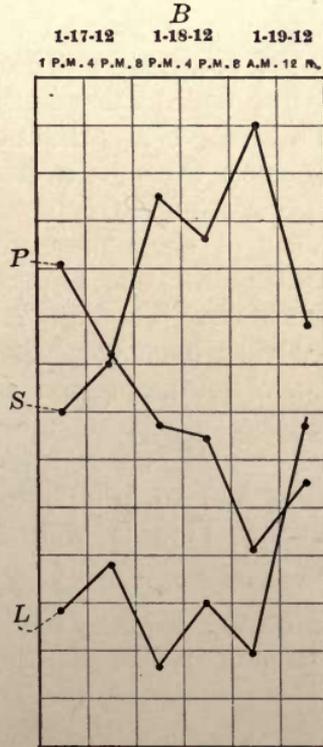


FIG. 34



FIGS. 33 and 34.—A, rabbit 54 Br., given 5 minims of 1 per cent. sarcoma at 1, 2, and 3 P.M.; B, rabbit 54 Bl., given 5 minims of 1 per cent. sarcoma at 1, 2, and 3 P.M.

Given 5 c.c. of cancer-cell emulsion and 10 c.c. of the serum, a rabbit of from 500 to 1000 grams will be sick but will invariably recover.

The next step was to ascertain whether the specific ferment could be removed from the large mononuclear leukocytes. For this purpose rabbits were sensitized to the

cancer cell and when the percentage of large mononuclear leukocytes was at its highest point, the blood was collected under sterile conditions, in 0.333 per cent. acetic acid. This was next centrifugated until the leukocytes were thrown down and the supernatant fluid decanted. The leukocytes were then placed in a sterile mortar and mixed with a sufficient quantity of sterile quartz sand to cover. This was then ground up with vigor for fifteen minutes, with the frequent addition of sterile normal salt solution, until five times the volume of the leukocytes obtained had been added. Next, this normal saline extract was separated by passing through a Berkefeld filter and tests made for the presence of the specific ferment by adding varying amounts of the leukocyte extract to cancer-cell emulsion, incubating for one hour and then injecting intravenously into rabbits. Through repeated experiments it was ascertained that 5 c.c. of 2 per cent. cancer-cell emulsion, plus 10 c.c. of leukocyte extract, which was obtained when the percentage of large mononuclear leukocytes was 25 or above, would, when injected intravenously into a rabbit of from 500 to 1000 grams, kill within from one to five minutes. This is well illustrated in the accompanying table.

TABLE XLVI.—SHOWING RESULTS OF INJECTING INTRAVENOUSLY INTO RABBITS CANCER-CELL EMULSION PLUS LEUKOCYTE EXTRACT INCUBATED ONE HOUR.

| Animal. | Weight, gm. | Rabbit leukocyte extract. | + Vaccine.   | Result.                           |
|---------|-------------|---------------------------|--------------|-----------------------------------|
| 343 G.  | 646.5       | 10 c.c. carc. 100         | 5.0 c.c. 100 | Died 1 min.                       |
| 167 G.  | 750.0       | 10 c.c. sarc. 51 res.     | 5.0 c.c. 100 | Died 1 min.                       |
| 157 W.  | 1500.0      | 10 c.c. sarc. 100         | 5.0 c.c. 100 | Very sick $\frac{1}{2}$ hr.; rec. |
| 53 Bl.  | 1050.0      | 7 c.c. sarc. 51 res.      | 5.0 c.c. 100 | Died 5 min.                       |
| 96 G.   | 1317.0      | 7 c.c. sarc. 51 res.      | 2.5 c.c. 103 | Died $4\frac{1}{2}$ hr.           |
| 251 W.  | 645.0       | 10 c.c. sarc. 51 res.     | ....         | Not sick.                         |
| 251 Br. | 842.0       | 15 c.c. carc. 100         | ....         | Not sick.                         |
| 167 W.  | 700.0       | 10 c.c. normal sal.       | 5.0 c.c. 100 | Not sick.                         |

The entire mass of leukocytes, when removed from the outside of the Berkefeld filter and mixed with 5 c.c. of

cancer-cell emulsion and incubated one hour, failed to have any effect on a rabbit when injected intravenously, thus showing that the specific ferment was soluble.

Again, 10 c.c. of leukocyte extract, prepared in the same manner from a normal rabbit which had not been sensitized, added to 5 c.c. of cancer-cell emulsion and incubated one hour, produced no effect when injected intravenously into a normal rabbit. This would show that the sensitized animal possesses some specific chemical substance which reacts with cancer tissue and which the normal rabbit does not possess. That this substance is specific for malignant cells is further shown by the fact that 10 c.c. of leukocyte extract from a sensitized animal, plus 5 c.c. of a 2 per cent. normal skin vaccine, incubated one hour, produces no effect when injected intravenously into a rabbit of 450 grams.

The same table shows also that sarcoma residue or vaccine sensitizes to carcinoma as well as sarcoma, and *vice versa*, so that the probable conclusion is that the chemical change within the cell is the same for both sarcoma and carcinoma.

Organ extracts from kidney, liver, brain, spleen, and heart, made by grinding these organs in normal saline on successive days, after sensitization to cancer protein, have no effect when mixed with cancer-cell emulsion, incubated one hour, and injected intravenously into normal rabbits.

When as small an amount as 1 c.c. or more of leukocyte extract is injected directly into the tumor of a cancer patient it may cause sudden and severe symptoms. In four cases, when this procedure was adopted, the patient has complained within from one to five minutes of difficulty of respiration. Next, he would lose consciousness, which would be accompanied by rather violent muscular twitchings and lowered pulse-rate. This stage would last from five to ten minutes, and would be followed by a period of rest, from fifteen minutes to one hour in duration, which in turn would be followed by a violent chill, and temperature ranging from  $103^{\circ}$  to  $106^{\circ}$  F. This would last from one to

six hours and would be followed by from twenty-four to forty-eight hours of extreme exhaustion. At no time has such a reaction been obtained, even with doses of 10 c.c., when given subcutaneously or intravenously away from the tumor; although a chill from one to three hours after injection has been observed. We wish now, however, to call attention only to the animal experiments, the above being mentioned simply because it is additional proof of the presence of a specific ferment.

It is of interest to note here also that a vaccine prepared from human carcinoma gives a much higher percentage of large mononuclear leukocytes when injected into a rabbit or sheep than when injected into a human being. This has been observed regardless of whether the human being had malignant disease or not. I have injected cancer vaccine into myself, and the highest resulting percentage of large mononuclear leukocytes was 15. This fact is of interest when we consider that with experimental cancer the animal injected must always be of the same family as the one that furnishes the tumor in order to obtain a "take."

It should not be understood that we consider the ferment causing reproduction in the cancer cell to be of exactly the same chemical nature as the active ferment of a reproducing normal cell, but rather that we are dealing with a chemically altered constructive ferment, a fact that we will demonstrate later.

While attempting to use leukocyte extract from animals sensitized to the cancer protein in a therapeutic way, it was found that extract prepared by filtering through a hard filter paper would upon repeated usage in the same case cause symptoms of sensitization; while the use of extract prepared by Berkefeld filtration would not be followed by these characteristic phenomena. At the same time, as previously mentioned, all of the specific ferment passes with ease through the filter.

In order to ascertain what constituents were removed from the leukocyte extract by passage through the Berkefeld

filter, definite amounts were analyzed by the Scherer and Hammersten methods for total albumins and globulins.

**Determination of Albumin and Globulin.**—25 c.c. of leukocyte extract which had been centrifugated to throw down all corpuscles and sand used in its preparation was passed through a filter paper. This was rendered acid with acetic acid, 4 grams of NaCl added, and then heated for one-half hour on a water-bath. After coagulation had occurred this was filtered through a previously dried and weighed filter and washed with hot water until the filtrate ceased to give a reaction for chlorides. Next, the residue was washed with absolute alcohol and then ether, after which it was dried at 130° to a constant weight.

|   |               |
|---|---------------|
| Paper and albumin plus globulin . . . . . | 1.66658 grams |
| Weight of paper . . . . .                 | 1.61150 grams |
|   | <hr/>         |
| Weight of albumin and globulin . . . . .  | 0.05508 gram  |

The same method was applied to 25 c.c. of the same lot of leukocyte extract after passing through a Berkefeld filter.

|  |               |
|--|---------------|
| Weight of filter plus albumin and globulin . . . . . | 1.55000 grams |
| Weight of filter . . . . .                           | 1.53125 grams |
|  | <hr/>         |
| Weight of albumin and globulin . . . . .             | 0.01875 gram  |

In order to make a separate determination of globulins and albumins the following method of Hammersten was adopted: 25 c.c. of leukocyte extract before filtration through a Berkefeld was added to 30 grams of pulverized magnesium sulphate. This was warmed to 30° with frequent stirring, and then placed in the cold for twenty-four hours. This was then filtered through a weighed filter, previously dried at 110°, and washed with magnesium sulphate until the filtrate ceased to give a reaction for albumin when heated with acetic acid. Next, the filter was dried for four hours at 110° to coagulate the globulin, after which the magnesium sulphate was washed out with

hot water. It was then washed with alcohol and ether, and dried to a constant weight at 110°.

|   |              |
|---|--------------|
| Weight of globulin and filter . . . . . | 1.5864 grams |
| Weight of filter . . . . .              | 1.5766 grams |
|   | <hr/>        |
| Weight of globulin . . . . .            | 0.0098 gram  |

25 c.c. of the same leukocyte extract after Berkefeld filtration was treated in the same manner.

|   |              |
|---|--------------|
| Weight of globulin and filter . . . . . | 1.4913 grams |
| Weight of filter . . . . .              | 1.4766 grams |
|   | <hr/>        |
| Weight of globulin . . . . .            | 0.0147 gram  |

By subtracting these globulin weights of before and after filtration from the above-given albumen plus globulin weights we arrive at the albumin weights:

|  |              |
|--|--------------|
| Albumin and globulin before filtration . . . . . | 0.05508 gram |
| Globulin before filtration . . . . .             | 0.00980 gram |
|  | <hr/>        |
| Albumin before filtration . . . . .              | 0.04528 gram |
| Albumin and globulin after filtration . . . . .  | 0.01875 gram |
| Globulin after filtration . . . . .              | 0.01470 gram |
|  | <hr/>        |
| Albumin after filtration . . . . .               | 0.00405 gram |

From the above it can be seen that we have removed a large percentage of the albumin through the passing of the extract through the Berkefeld filter. This is represented by the difference between 0.04528 gram and 0.00405 gram, which is 0.0412 gram.

The difference in the globulin weight gives an apparent increase of 5 mg. in the after-globulins, an amount so small that it can be neglected when compared with the albumin difference.

From this it can be seen that the sensitizing portion of the extract is in all probability contained within the albumin, and that the specific enzyme is not of albuminous nature.

While the globulins are filterable this does not prove that the ferment is a globulin, but only that it filters through with the globulin. The enzyme itself may be of much simpler construction.

In conclusion I may state that the most valuable deductions to be drawn from the work as so far conducted are as follows:

1. Transitory sensitization. The fact that an animal may be sensitized to certain proteins, and that such sensitization is active for only a few hours is of extreme interest and importance.

2. The active transitory ferment formed by the introduction of cancer protein into an animal is not an albumin. It is either a globulin or of simpler chemical structure.

3. Sensitization, in the use of leukocyte extract at least, is probably caused by the albumin content of the solution.

**Other Methods Used.**—In order that a more definite knowledge of the chemistry of this specific ferment might be obtained, the following experiment was made: 120 c.c. of sensitized leukocyte extract, after filtration through a Berkefeld filter, was mixed with an equal volume of saturated ammonium sulphate solution. This was allowed to stand overnight and the precipitate was then filtered off. The precipitated globulins, while still slightly moist, were removed from the filter paper and dissolved in a saturated solution of sodium chloride. This was next rendered slightly acid with 0.25 per cent. acetic acid, which again precipitated the globulins. When the precipitation was complete and had settled to the bottom, for which twelve to twenty-four hours should be allowed, the globulin was collected upon a hard filter paper. From this it was removed, while still slightly moist, to a sterile watch-glass, where it was allowed to dry. The globulin was next weighed and dissolved in normal saline solution in the proportion of 1 mg. of globulin to 1 c.c. of normal saline.

The above method is given after using many different modifications of the same. In many experiments I have precipitated the globulins with semisaturated ammonium

sulphate solution, then dissolved in water, equal in amount to the original leukocyte extract, and then precipitated again with ammonium sulphate before dissolving in saturated sodium chloride solution, but such a procedure is unnecessary when the extract has been previously filtered through a Berkefeld, inasmuch, as previously shown, four-fifths of the albumin is removed at this time.

Again, it was my custom to filter after dissolving in saturated sodium chloride solution, but this has been abandoned because of the fact that if insufficient saturated sodium chloride is used, much globulin may be retained by the filter, and, inasmuch as the excess of ammonium sulphate is all that is desired to be rid of, both time and material can be saved by not filtering. The saturated sodium chloride solution is always filtered before using.

Again, it was my custom to dialyze through parchment paper after collecting the precipitate thrown down by 0.25 per cent. acetic acid, but repeated experiment has shown that the percentage of acetate when the globulins are dissolved in the proportion of 1 mg. to 1 c.c. of normal saline is so small as to be of no moment, and it is much preferable to have a solution of normal saline than one of sterile water for injection in the patient.

It should be added that precipitation is aided by allowing hot water to pass over the outside of the flask until the temperature of the contained fluid is about 30° C.

The following experiment has been repeated many times, so that the possibility of error is slight.

(a) 10 c.c. of soluble globulins from sensitized leukocyte extract plus 5 c.c. normal saline were incubated one hour, and 10 c.c. of this injected intravenously into a small rabbit. There was no change in the animal.

(b) 10 c.c. of soluble globulins from sensitized leukocyte extract plus 5 c.c. of 2 per cent. cancer-cell emulsion were incubated one hour and then injected intravenously into a moderate-sized rabbit. The rabbit died in one-half minute.

From the above it can be seen that the specific ferment

is in all probability a part of the globulin content, and its activity is much increased by obtaining it in the more purified state.

It is now my practice to test the strength of each batch prepared by the above experiment before using it in any given case. Sensitization phenomena have been entirely lacking whenever used therapeutically, which was not true of any former preparations. The filtered leukocyte extract when used in small amount subcutaneously rarely produced sensitization phenomena, but when injected intravenously in increased dosage this symptom complex was frequently observed. The unfiltered leukocyte extract frequently produced sensitization even with small amounts given subcutaneously, so the conclusion previously arrived at; that the albumin content was responsible for these untoward symptoms is apparently confirmed. It must not be understood, however, that the globulins do not sensitize. The work so far simply shows that it is possible to remove the albumin which contains no specific ferment, and inasmuch as four-fifths of the protein has been removed, much larger doses must be given before the phenomena of sensitization can be observed.

The above substance, because of its specific ferment action and its apparent chemical nature, I would designate as anticancer globulin.

## CHAPTER XV

### THE PHENOMENA OF INFECTION

It may be of interest to go somewhat into detail concerning our ideas of the phenomena of infection. In all infections there are two principal factors—one the infecting virus and the other the body cell. In addition to these there is the environment in which the struggle for supremacy between the virus and the body cell takes place. This consists of the unorganized fluids of the body, and is of great weight in determining the result of the contest. In the first place, what do we know of the infecting virus? As we have seen, bacteria are particulate, specific proteins. Since they are particulate, we speak of them as bacterial cells. It is not, however, essential that an infecting virus be particulate in the sense that it be possessed of substance and form recognizable to our limited sense of sight even when aided by the most perfect microscope. There are many filterable viruses. Some pass through our finest porcelain filters and cannot be deposited from the fluids in which they exist even when kept for hours in the most efficient centrifuge manufactured. Theoretically, there is no reason why a virus may not exist in any degree of lability of structure. The bacteria are particulate and solid, which means that their structure is so radically different physically from the medium in which they exist that they can be recognized by our sight, aided by proper magnifying lenses, but viruses may be semi- or wholly fluid. In such instances their structure is not sufficiently differentiated from the medium that we can recognize them. According to our conception, a living protein does

not necessarily possess a form recognizable to our limited sense even when aided by the most perfect lenses.

One of the most important results of our work, in our opinion, is the demonstration that bacteria are chemically not simple, but quite complicated in structure. Morphologically, they show but little or no differentiation in structure, but chemically they are quite as complicated and complex as many of the cells of the higher animals. They contain carbohydrates, nuclein bodies, and polymers of the mono- and diamino-acids. They are glyconucleoproteins. We interpret this as signifying that functionally they are highly developed.

While an infecting virus may be solid, semisolid, gelatinous, or liquid, we will, in the further consideration of the phenomena of infection, take the particulate type, the bacterium, as an example of an infecting agent.

What are some of the capabilities of a bacterial cell? In the first place it possesses that attribute which distinguishes and characterizes all living matter—the capability of growth and reproduction. In order to grow and multiply its molecular structure must be labile—in a state of constant change. Some bacteria under certain conditions may pass into a resting state characterized by the formation of spores, but these are awakened into life when the environment becomes fit, and the spore develops into the active form when it infects. In all instances the active, infecting agent is a living protein, capable of growth and multiplication. In order to do this it must carry on a constant exchange in matter with the medium in which it exists. It must assimilate and eliminate. It must absorb groups from the molecules about it, and cast out those which it has already used. Stop this process and the continuation of life is impossible. Every living cell, be it bacterial, vegetable, or animal, must feed or cease to exist. Besides, a cell is limited in its food supply by that which lies within its reach. There must, therefore, be a certain supporting relation between the bacterial cell and the medium. The groups derived from the medium must fit into the molecular

structure of the cell, otherwise they would be of no service to it. This necessitates the cleavage of the molecules of the medium along definite lines. Many kinds of cells may live in the same or like media, but for each kind of cell the cleavage of the medium must be specific. From this it follows that the agent by which the cleavage products are secured must be supplied by the cell itself, and must be peculiar to that kind of cell. These cleavage agents which prepare foods for the cell from the medium are known as ferments, and each kind of cell has its own characteristic and specific ferments. As to the real nature of ferments, we know little or nothing, but that every kind of cell has its specific ferment or ferments, we do know. The same ferment may not be able to break up all proteins. In this respect there are great variations in the proteolytic ferments. Some digest a wide variety of proteins while others are capable of acting only on one specific protein. There must be a relation between the ferment and its substrate. As Fischer once said, the former must fit the latter as a key fits into the lock, and as there are master keys that open many doors, so there are general proteolytic ferments, and as there are special keys that fit only one lock, so there are specific proteolytic ferments. It will be observed that we have used the word "specific" in two senses in speaking of proteolytic ferments. Each kind of cell has its specific ferment, and each protein may have its specific ferment. This double use of the term "specific" should be borne in mind, since there seems to be no way to avoid it.

It follows from what has been said that a bacterium placed in a medium in which its ferment is ineffective cannot grow and multiply. A bacterium which cannot grow and multiply in the animal body cannot cause an infection. Its inability to grow and multiply in the animal body may be due to the fact that its ferment or ferments cannot digest or properly break up the proteins of the animal body. This is one of the reasons why the great majority of bacteria are non-pathogenic or are harmless. These organisms when grown on suitable media produce just as much poison

as the pathogenic bacteria, but not being able to feed upon the proteins of the body they die. This, however, is not the sole, and probably not the most important, cause of the failure of so many varieties of bacteria to do harm to the higher animals. What has been said about the production of ferments by the bacterial cell is equally true of the body cell. In fact, it is true of every living cell. The body cell has its specific ferments, and the bacterial cell being protein substance is liable to be digested by the ferments elaborated by the body cells.

In the inability of the bacterial cell to grow in the animal body either because it cannot feed upon the proteins of the body, or because it is itself destroyed by the ferments elaborated by the body cells lies the fundamental explanation of all forms of bacterial immunity either natural or acquired. Toxin immunity needs further explanation. Certain bacteria, of which the diphtheria bacillus may be taken as a type, elaborate soluble, extracellular substances known as toxins. These are probably ferments or closely allied bodies. They resemble ferments in the following particulars: (1) They are destroyed by heat. (2) They act in very dilute solution. (3) When repeatedly injected into animals in non-fatal doses they cause the body cells to elaborate antibodies which neutralize the toxin both *in vivo* and *in vitro*. (4) In the development of their effects a period of incubation is required. (5) It has been shown by Abderhalden, by optical methods, that they have a cleavage effect upon proteins. They split complex proteins into simpler bodies. In other words, they have a proteolytic action. (6) They are specific in two senses. (a) They are specific according to the cell which produces them. Diphtheria toxin is elaborated by the diphtheria bacillus and by no other organism. The toxin of snake venom is a specific product of the poisonous gland of the snake, and this is further specific inasmuch as that produced by the glands of one species is different from that elaborated in another species. (b) They are specific in the antibody elaborated in the animal body after repeated injections of

non-fatal doses. Diphtheria antitoxin protects only against diphtheria toxin, and not against that of the tetanus or dysentery bacillus, or that of snake venom.

The side-chain theory evolved by the genius of Ehrlich best explains the action of toxins and the production of antitoxins. Without subscribing to all the details of this theory, we believe that it is a biological law that when a living cell is attacked by a destructive ferment or toxin it tends to elaborate an antiferment or antibody. This is one of the ways in which the living cell may protect itself. The formation of such antibodies in multicellular animals is one of the factors in the fine adjustment essential to harmony of action between different tissues and organs. It best explains the fact that the digestive organs do not harm themselves, and the antitryptic action of blood-serum is one of the most interesting and important phases of parenteral digestion.

The number of pathogenic bacteria which produce toxins, at least in appreciable quantity, is small, and the action of toxins and antitoxins in infections due to those organisms which do not produce such bodies is of minor importance. Since all bacteria, and in fact all living cells produce ferments, and since every ferment, so far as we know, may lead cells acted upon by them to produce anti-ferments, there may be some toxin and antitoxin action in all infections, but in most bacterial infections such action is overshadowed by processes much more powerful in their effects.

In our opinion the action of the diphtheria bacillus may be stated as follows: The organism finds lodgement and the conditions for growth favorable in the upper air passages. Here it grows in mass and may kill by mechanical obstruction. It produces its soluble, diffusible toxin, which has the properties of a ferment and splits up the proteins of the body, setting free the protein poison. In case of recovery or in the production of antitoxin in animals, the body cells elaborate an antiferment or antitoxin which neutralizes the toxin and prevents its cleavage action.

The bacilli in the throat are not destroyed by natural recovery or by cure with antitoxin, but the action of the toxin is prevented by the antibody. It is not, in our opinion, the toxin itself which kills, but a cleavage product which results from the action of the toxin on the proteins of the body.

All ferments are of cellular origin. This does not mean that ultramicroscopic forms of life or non-particulate living organisms, if there be such, do not produce ferments. It would probably be better to say that all ferments are the products of living organisms and that there can be no living organism which does not produce its specific ferment. We cannot conceive of life without ferment action, because all living things must feed and food assimilation without ferment action is inconceivable. Food must be fitted for assimilation, and this is dependent upon ferment action.

Ferments are intra- and extracellular. All are formed within the cell, but some diffuse into the medium while others do not. In some instances at least cell permeation by the pabulum is essential to the feeding of the cell. In other instances it is highly probable that the ferment is accumulated on the cell surface and there acts upon the pabulum. In still other instances the ferment diffuses into the medium more or less widely from the cell which elaborates it. Many cells produce both intra- and extracellular ferments, and these are not necessarily the same. In some instances, probably in most cells, the intracellular ferment cannot be extracted from the cell or obtained in soluble form without destruction of the cell. This does not mean that it must exist in the soluble form before it can manifest its cleavage action. The pabulum may permeate the cell and in this location be split up by the intracellular ferment. We have insisted upon this as an explanation of the well-established fact that soluble proteins sensitize much more readily and completely than insoluble ones.

It will be well to illustrate what we have said about cellular ferments by a condensed sketch of the work that

has been done on the germicidal properties of the blood. As early as 1872 Lewis and D. Cunningham<sup>1</sup> demonstrated that non-pathogenic bacteria injected into the circulation soon disappear. In the blood of 12 animals thus treated bacteria could be found after six hours in only 7. Of 30 animals, bacteria were found in the blood of only 14 after twenty-four hours, and of 17 animals bacteria were found in the blood of only 2 when the examination was made from one to seven days after the injection. In 1874, Traube and Geschiedlen<sup>2</sup> found that arterial blood, taken under aseptic precautions from a rabbit into the jugular vein of which 1.5 c.c. of a fluid rich in putrefactive bacteria had been injected forty-eight hours previously, failed to undergo decomposition when kept for months. These investigators attributed the germicidal properties of blood to its ozonized oxygen. Like results were obtained by Fodor<sup>3</sup> and Wysokowicz.<sup>4</sup> The latter accounted for the disappearance of the bacteria, not through the germicidal action of the blood, but by supposing that they found lodgement in the capillaries. The first experiments made with extravascular blood were conducted by Grohmann<sup>5</sup> under the direction of A. Schmidt in his researches on the coagulation of the blood. It was found that the virulence of anthrax bacilli, as demonstrated by their effect on rabbits, was diminished by being kept in blood. He supposed that the bacilli were altered in some way by the process of coagulation. In 1887 Fodor<sup>6</sup> made a second contribution on this subject, in which he combated the retention theory of Wysokowicz. One minute after the injection of 1 c.c. of an anthrax culture into the jugular vein, in eight samples of blood, Fodor found only one colony of the bacillus. He

<sup>1</sup> Eighth Annual Report of the Sanitary Commission of the Government of India.

<sup>2</sup> Schlesische Gesellschaft f. Vaterland. Cultur.

<sup>3</sup> Archiv f. Hygiene, iv, 1886.

<sup>4</sup> Zeitsch. f. Hygiene, i, 1886.

<sup>5</sup> Ueber die Einwirkung d. Zellenfrien Blut-plasma auf einige pflanzliche Mikroorganismen, Dorpat, 1884.

<sup>6</sup> Deutsch. med. Woch.

also took blood from the heart with a sterilized pipette, and added anthrax bacilli to it. This was kept at 38°, and plates made from time to time showed rapid diminution in the number of bacteria, until after a time, when the blood having lost its germicidal properties, the number rapidly increased. In 1888 Nuttall,<sup>1</sup> working under the direction of Flügge, used defibrinated blood taken from various species of animals, rabbits, mice, pigeons, and sheep, found that the blood destroyed the bacillus anthracis, *b. subtilis*, *b. megatherium*, and staphylococcus aureus. He also confirmed the finding of Fodor that after a while the blood loses its germicidal properties and becomes a suitable culture medium. Continuing this work, Nissen<sup>2</sup> reached the following conclusions: (1) The addition of small quantities of salt solution or bouillon to the blood does not destroy its germicidal properties. (2) The bacilli of cholera and typhoid fever are easily destroyed by fresh blood. (3) For a given volume of blood there is a maximum number of bacilli that can be destroyed. (4) Blood whose coagulability has been destroyed by peptone injection is still germicidal. (5) Blood in which coagulation is prevented by the addition of 25 per cent. of magnesium sulphate has its germicidal properties decreased. (6) Filtered blood plasma from the horse is germicidal. Behring<sup>3</sup> attributed the germicidal action of the blood of the white rat on the anthrax bacillus to its great alkalinity. In 1890, Buchner and his students<sup>4</sup> published their first contribution on the germicidal properties of blood serum. At first Buchner believed that the germicidal constituent of serum is the serum albumin and the conclusions were stated as follows: (1) The germicidal action of blood is not due to the phagocytes, because it remains after destruction of the leukocytes by alternating freezing and thawing. (2) The germicidal properties of the cell-free serum must be due to its

<sup>1</sup> Zeitsch. f. Hygiene, iv, 353.

<sup>2</sup> Ibid., 1889, vi, 487.

<sup>3</sup> Ibid., 1889, vi, 1.7.

<sup>4</sup> Archiv f. Hygiene, 1890, x, 84, 101, 121, 149.

soluble constituents. (3) Neither neutralization of the serum, nor the addition of pepsin, nor the removal of carbon dioxide gas, nor treatment with oxygen has any effect upon the germicidal properties of the blood. (4) Dialysis of the serum against water destroys its activity, while dialysis against 0.75 per cent. salt solution does not. In the diffusate there is no germicidal substance. The loss by dialysis with water must be due to the withdrawal of the inorganic salts of the serum. (5) The same is shown to be the case when the serum is diluted with water, and when it is diluted with the salt solution. In the former instance the germicidal action is destroyed, while in the latter it is not. (6) The inorganic salts have in and of themselves no germicidal action. They are active only insofar as they affect the normal properties of the albuminates of the serum. The germicidal properties of the serum reside in the albuminous constituents. The difference in the effects of the active serum and that which has been heated to  $55^{\circ}$  is due to the altered condition of the albuminate. The difference may possibly be a chemical one (due to cleavage within the molecule) or it may be due to changes in mycelial structure. The albuminous bodies act upon the bacteria only when the former are in an active state.

Vaughan and McClintock<sup>1</sup> called attention to a contradiction between Buchner's work and his conclusions, in the following language: "We wish at this point to call attention to an inconsistency between the results obtained by Buchner and the conclusions that he draws: In experiment 45 he renders the serum slightly acid, and adds 0.1 gram of pepsin to each 5 c.c. of serum (showing by a side experiment that this pepsin actively digests coagulated egg albumen in neutral solution) and finds that the digestive action of the pepsin does not lessen the germicidal properties of the serum. In fact, he states this in his conclusion, but his ultimate opinion and the one held by him in his latest contribution, is that the germicidal constituent of the

<sup>1</sup> Med. News, December 23, 1893.

blood is the serum albumin. How much serum albumin remains in blood serum after it has been thoroughly digested with pepsin? He could scarcely have chosen a more positive method of demonstrating that the germicidal constituent is not serum albumin. Either his pepsin was not active and on this supposition his experiment was without value, or the active constituent of the blood-serum is a substance that is not destroyed or materially altered by peptic digestion. We know that the peptones not only have no germicidal properties, but that they belong to that class of proteins that is most favorable to the growth of bacteria. We recognize this fact when we add peptones to the various artificial media on which we cultivate bacteria." We will return to this point after proceeding farther with the chronological order in which this research has developed.

Prudden<sup>1</sup> found that ascitic and hydrocele fluids restrain the development of certain bacteria. Rovighi<sup>2</sup> reported that the germicidal action of the blood is increased in febrile conditions. Pikelharing<sup>3</sup> enclosed anthrax spores in bits of parchment and introduced these under the skin of rabbits. Thus treated the spores soon lost their virulence and finally their capability of growth. The destruction of these spores could not have been due to phagocytes which did not penetrate the parchment, but must have been caused by soluble substances. Behring and Nissen<sup>4</sup> found that the serum of the white rat, the dog, and the rabbit destroy anthrax bacilli, while serum obtained from the mouse, sheep, guinea-pig, chicken, pigeon, and frog has no such action. It will be observed that in this there is no constant relation between the germicidal action of the blood of animals of different species and their susceptibility to the infection. Thus the rabbit is highly susceptible to anthrax notwithstanding the fact that its blood destroys large numbers of this organism. On the other hand the chicken is immune to anthrax from the moment

<sup>1</sup> Medical Record, 1890.

<sup>2</sup> Atti della Accad. Med. di Roma, 1890.

<sup>3</sup> Ziegler's Beiträge, viii.

<sup>4</sup> Zeitsch. f. Hygiene, 1890, viii, 412.

when it comes from the shell, and yet the bacillus grows luxuriantly in the extravascular blood of the chick. Hankin<sup>1</sup> was one of the first to show that other cells, besides the leukocytes, contain germicidal substances. He made several contributions to the study of so-called "defensive proteins," which he believed to be globulins. This is interesting in view of the fact that ferments are often carried down with globulins on precipitation with neutral salts. Bitter<sup>2</sup> was unable to confirm Hankin's work, but it is needless to go into this because we now know that many cells elaborate germicidal substances. Christmas<sup>3</sup> prepared a germicidal substance from the spleen and other organs by the following method: The animal was killed with ether, opened under aseptic precautions, the organ removed, cut into fine pieces, covered with 50 c.c. of glycerin, and allowed to stand for twenty-four hours and then filtered. The filtrate is treated with five times its volume of alcohol and the precipitate is immediately collected and washed with absolute alcohol. Traces of alcohol are removed, so far as possible, by pressure, and the precipitate is dissolved in 25 c.c. of distilled water, and air is blown through the solution to destroy last traces of alcohol; then the fluid is filtered and its germicidal action tested. Bitter<sup>4</sup> strove hard to find fault with this agent and its method of preparation. He found it a powerful germicide, but he could not reconcile the fact that the preparation of Christmas still proved a powerful germicide after it had been heated to 65°, while blood-serum loses its germicidal effect when heated to 55°. Buchner<sup>5</sup> had the following to say on this point: "A method given by Christmas for the preparation of germicidal solutions from the organs of normal rabbits has also been tested by Bitter. Germicidal solutions were indeed obtained, which, however, differed materially from active serum, for in three experiments, notwithstanding heating to 65°, the germicidal

<sup>1</sup> Centralbl. f. Bakt., 1891, ix, 336.

<sup>2</sup> Zeitsch. f. Hygiene, 1892, xii, 328.

<sup>3</sup> Annales de l'Institut Pasteur, 1891, v, 487.

<sup>4</sup> Loc. cit.

<sup>5</sup> Archiv f. Hygiene, 1893, xvii, 112.

action remained." We have gone into this detail concerning the preparation of Christmas for the following reasons: (1) It remains to day a good method of preparing a germicidal agent from the spleen or other tissue. (2) Its method of preparation indicates that it is a ferment. (3) It is an illustration of the fact that the degree of heat borne by a ferment, without being inactivated, is dependent in part at least on the character of the solvent in which the ferment is found.

Emmerich and his students<sup>1</sup> made the following experiments: A serum was dialyzed against distilled water until its globulin was precipitated. The globulin-free serum was precipitated with alcohol, and the serum albumin thus thrown down was dissolved in 0.05 per cent. solution of potassium hydroxide. This solution was found to be markedly germicidal, and the conclusion reached was that the germicidal constituent of blood serum was an alkaline albuminate.

Vaughan and his students<sup>2</sup> published their first paper upon the germicidal properties of nuclein. In their first contribution they showed that nucleins prepared from testes, thyroid gland, and yeast cells are markedly germicidal to both pathogenic and non-pathogenic bacteria. In 1894 Kossel<sup>3</sup> quite independently announced the discovery of the germicidal action of nuclein and nucleic acid. Vaughan not only demonstrated the germicidal action of nuclein *in vitro*, but also showed (1) that it protected rabbits against subsequent inoculation with the pneumococcus; (2) it also protected a considerable percentage of rabbits against inoculation with the bacillus tuberculosis; (3) that it had a curative effect on rabbits already inoculated with tuberculosis; and (4) that it apparently benefited initial tuberculosis in man.

It now turns out that the germicidal action attributed by Vaughan and Kossel to nuclein was probably not due to

<sup>1</sup> Centralbl. f. Bakt., 1892, xii, 364.

<sup>2</sup> Medical News, May 20, 1893.

<sup>3</sup> Archiv f. Anat. u. Physiol., Physiolog. Abtheilung, 1894, 194.

this agent, but to ferments which came out with the nuclein from the cells used in its preparation. This should have been known at the time the work was done, because both of these investigators were aware of the fact that a temperature short of boiling destroyed the germicidal properties of their solutions, but we did not know so much about cell ferments then as we do now. This, of course, does not mean that all the results obtained with preparations of nuclein, such as an increase in the number of leukocytes, were due to the ferment contained in the preparations, but it is more than probable that the germicidal action was due to the ferment. The whole matter demands reinvestigation.

It should be stated that Vaughan and McClintock<sup>1</sup> demonstrated the presence of nuclein in blood-serum. This was done by precipitating a large amount of serum, obtained under aseptic conditions, with alcohol and digesting the precipitate with artificial gastric juice so long as digestion proceeded, the completion of digestion being indicated by failure to respond to the biuret test. The small amount of protein material which wholly resisted gastric digestion, and which could be only nuclein, was dissolved in 0.12 per cent. potassium hydroxide, and its germicidal action demonstrated on the bacillus of cholera, anthrax bacillus, typhoid and colon bacilli, and on various cocci. At the same time it was shown that a 0.5 per cent. solution of the alkali was without effect upon these organisms.

It then seemed that the whole question of the germicidal action of the blood was practically settled. The leukocytes contain large quantities of nuclein. The blood serum contains small quantities of the same substance. That of the serum comes from the leukocytes either in the form of a secretion or as a result of the breaking-down of the cells, and nuclein is a powerful germicide. The phagocytes destroy bacteria either by engulfing and then digesting them, or through the action of the nuclein dissolved in the

<sup>1</sup> Loc. cit.

blood. We say all this seemed clear and probably it is all right, except now it seems probable that although nuclein is abundant in the leukocytes and present in small amount in plasma and serum, it is not the germicidal agent in either. The germicidal agent in the cell and that dissolved in the plasma or serum are both most likely ferments, the one intra- and the other extracellular, and the two are not identical. Metschnikoff's phagocytic theory and Buchner's alexin theory are both in a way right, but whether the germicidal substance in the serum is a secretion of the phagocyte or a disintegration product of the cell is an interesting question.

The germicidal constituent of blood serum, studied by Buchner and named alexin by him, is inactivated by heating the serum to 55°, while the germicidal substance obtained by Kossel and Vaughan from cells, and believed by them at the time to be nucleic acid, required a temperature of 85° to render it inert. Evidently these must be two quite different bodies, or if the same substance, their behavior under the influence of temperature must be markedly affected by the conditions under which they were tested. The researches of Schattenfroth<sup>1</sup> showed further differences between the intra- and extracellular germicidal constituents of the blood. The former has no hemolytic action on the red corpuscles of other species, while the latter may have. The intracellular germicide is not affected by the salt content of the medium, retaining its activity in a salt-free menstruum, while the extracellular substance is inactivated by the removal of salt from the serum by dialysis. Daubler<sup>2</sup> came to the conclusion that the germicidal constituents of the serum and of the leukocytes are not identical, the latter remaining active after being heated to 60°. He also found that the germicidal substances obtained from the leukocytes of different species differ in measurable degree as tested upon the same bacteria. Many other investi-

<sup>1</sup> Archiv f. Hygiene, 1897, xxxi, 1; *ibid.*, 1899, xxxv, 135.

<sup>2</sup> Centralbl. f. Bakt., 1899, xxv, 129.

gators produced evidence of the fact that the intra- and extracellular germicidal constituents of the blood are not identical, but since the literature of this subject has been collected by Kling,<sup>3</sup> we will not go into detail but will content ourselves with the reproduction of the summary as given by this author. It should be stated that Petterssen designates the intracellular germicidal constituent of leukocytes and other cells as "endolysins." Kling's conclusions from the work of others and himself are stated substantially as follows: (1) The germicidal substances (endolysins) of the polymorphonuclear leukocytes may be obtained from the protoplasm by the following methods: (a) By digesting the cells for half an hour at 50° in bouillon. (b) by extracting the cells with weak acid or alkali, or (c) by alternating freezing and thawing of the cells. They cannot be obtained by digesting with bouillon at 37°, nor with physiological salt solution, nor with 5 per cent. "inactivated" serum. (2) As tested on bacillus subtilis, the endolysin bears a temperature of 65° without recognizable effect on its germicidal action, and it is not until the temperature is increased to 75° that any such effect is noticed. The endolysins can, in daylight at room temperature, and in the dark at 37°, be evaporated to dryness, and in this state they may be heated for half an hour at 100° without being destroyed. The serum alexins may be obtained in the dry state in the same manner, but when heated to this temperature they are inactivated. The endolysin as tested on the subtilis does not pass through a Pukall filter, while the serum alexin does. The endolysins as tested on the subtilis, the anthrax, and the typhoid bacillus are destroyed by the Röntgen ray, while the serum alexins are not. The endolysins cannot be extracted with ether, but are not injured by ether, while the serum alexins are destroyed by ether. (3) The activity of an inactivated extract of the leukocytes of the rabbit, as tested on the subtilis, may be restored by the addition of a small quantity of the same

<sup>3</sup> Zeitsch, f. Immunitätsforschung, 1910, vii, 1.

extract in a fresh state. Likewise, an inactivated normal serum of the rabbit or the inactive serum of the guinea-pig may be complemented by the addition of a small amount of the leukocytic extract from the rabbit or guinea-pig, respectively. Furthermore, an inactivated leukocytic extract from a guinea-pig can be activated by the addition of a small amount of the normal serum of a rabbit. (4) Extracts from the polymorphonuclear leukocytes of rabbits, guinea-pigs, and cats destroy *in vitro* the timothy bacillus, the grass bacillus II, Korn's acid-fast bacillus I, and Rubner's butter bacillus. The extract from rabbits' leukocytes has a bactericidal action on the bacillus tuberculosis of man. Extracts of rabbit, guinea-pig, and cat macrophages do not destroy these acid-fast bacilli *in vitro*. The same is true of the extracts from the thymus gland of the rabbit. Living polymorphonuclear leukocytes injected into guinea-pigs decrease the virulence of the human tuberculosis bacillus. The leukocytes of the guinea-pig do not have this effect. These experiments do not permit us to draw positive conclusions concerning the action of living macrophages and lymphocytes on tubercle bacilli, but it appears that rabbit macrophages may have a protective action against these organisms. (5) Extracts from rabbit, guinea-pig, and cat macrophages have no hemolytic effect upon the erythrocytes of chickens, goats, rabbits, or guinea-pigs.<sup>1</sup>

<sup>1</sup> This is interesting in view of the statement made by Vaughan (Med. News, December 15 and 22, 1894), from which the following quotation is taken: "On March 19, 1894, I inoculated rabbits 1, 2, 3, 4, 5, 6, *a* and *b* with a virulent culture of the (tubercle) bacillus. Animals from 1 to 6 inclusive had had previous treatments with a 1 per cent. solution of nucleinic acid as follows:

|   |     |     |     |     |    |    |    |    |
|---|-----|-----|-----|-----|----|----|----|----|
| March . . . . .                                 | 9   | 10  | 13  | 14  | 15 | 16 | 17 | 19 |
| Amount of solution in<br>cubic centimeter . . . | 0.3 | 0.5 | 0.6 | 0.7 | 1  | 1  | 1  | 1  |

*a* and *b* had had no nuclein. All of the animals were half-grown, and weighed respectively: No. 1, 714 grams; No. 2, 724 grams; No. 3, 740 grams; No. 4, 729 grams; No. 5, 647 grams; No. 6, 614 grams; *a*, 709 grams; *b*, 705 grams. On July 6, 1894, I killed No. 6, *a* and *b*. No. 6 weighed at this time 1557 grams. I found a nodule the size of a pea at the point of inoculation. In all other respects this animal was normal. I could find no

The above-mentioned facts, ascertained by experimental study, have been cited to show that the existence of both intra- and extracellular germicidal substances in the blood has been demonstrated. These substances have been called alexins, antibodies, and by other names. It seems to us that at present they should be classed as ferments. As has been said, we do not know much about ferments, but it is evident that these bodies have a lytic action. They break up complex molecules into simpler bodies. Their primary function seems to be to supply the cells which elaborate them with food. In doing this they also protect the cells, to which they belong, by the destruction of harmful bodies both particulate and formless, both animate and inanimate. The digestive ferments of our alimentary canals serve the same double purpose. Any unbroken foreign protein having found its way into the blood is a poison, but in the alimentary canal it is broken up and prepared as a food for the body cells. Every living cell has such a ferment or such ferments. Their presence and

bacilli in the nodule, which was rubbed up with beef-tea and injected into the abdominal cavity of guinea-pig No. 186, weighing 385 grams. On October 10, 1894, I killed this pig, and found a nodule the size of a pea at the point of inoculation. Three small tubercles were found in the peritoneum; the omentum and liver were filled with tuberculous nodules. One testicle was tuberculous. This is an interesting case, showing that the germ, which had not spread in the rabbit, had, when transferred to the more susceptible guinea-pig, induced a widespread tuberculosis

"Rabbit *a* weighed 1030 and *b* 1100 grams. In both, nodules as large as filberts were found at the point of inoculation, and smaller nodules in the omentum. On October 10, I killed No. 1, weight, 2134 grams. This animal was found to be wholly free from tuberculosis. On October 4, I killed No. 2, weight, 2150 grams, which was found perfectly normal. No. 3 was found dead October 2. Postmortem examination showed a pear-shaped tumor in the omentum. This was three inches long and one and one-half inches in diameter at the base. It consisted of three cysts, which contained very fetid pus, in which were found a short bacillus and a large micrococcus. There was no evidence of tuberculosis. No. 4 was killed October 10, weight 1990 grams. I found a small nodule at the point of inoculation. This was not attached to the abdominal wall, but was in the connective tissue, between the skin and the muscle. I could find no germ. In all other respects this rabbit was normal. No. 5 was killed October 10, weight 2000 grams, and found perfectly normal

"These experiments indicate that rabbits may be rendered immune to tuberculosis by previous treatment with yeast nucleinic acid "

activity distinguish living from non-living matter. We have taken the leukocyte as an illustration, but the bacterium is also supplied with its ferments, some of which are intra- while others are extracellular. We do not know that all cells elaborate both kinds of ferments, but all have at least one kind.

Before proceeding further it may be well to call special attention to some of the properties of these ferments. The extracellular ferments are diffusible. They not only pass out of the cells in which they are prepared; but they diffuse more or less widely through the medium which surrounds the cell. This suggests that in molecular structure they are relatively simple. At least some of them may pass through membranes and collodion sacs, as is shown by the fact that bacteria and other proteins enclosed in such receptacles and left in a body cavity are destroyed. The extracellular ferments are, in part at least, filterable, passing with more or less readiness through porcelain. In their activities they are easily affected by modification in the medium through which they diffuse. The alexin of the blood serum is highly sensitive to the salt content of the serum, and by variations in this the activity of the ferment may be hastened, lowered, or wholly arrested. The same is true of bacterial ferments. In one species of animal a given bacterium multiplies with great rapidity; in another it grows slowly, while in a third it cannot grow at all. There are like variations in individuals of the same species. The extracellular ferments, at least some of them, are susceptible to slight changes in temperature. It is believed that every ferment has its optimum temperature, but the range in which continued activity is possible is narrow with some and relatively wide with others.

The intracellular ferments are non-diffusible, or at least less diffusible than the extracellular. They remain in the cells in which they are elaborated. They cannot be extracted from the cell by indifferent solvents. As a rule, they can be obtained from the cell only after partial or complete destruction of the cell. Some, probably most, are best

extracted from the cell with dilute alkali, while others are best obtained by dilute acid. In either case the reagent must not be strong enough to destroy the ferment itself. They are non-filterable, or pass through filters slowly and imperfectly. We suspect that their molecular structure is relatively complex, or that they are more colloidal than the extracellular ferments. Under natural conditions the intracellular ferments act only on those bodies which are taken into the cell. The inclusion of bacteria by phagocytes is essential to the digestion of the former by the intracellular ferment of the latter. This is a phenomenon which may be seen, but cell permeation by foreign bodies is certainly necessary before such bodies can be acted upon by the intracellular ferments, and occurs with soluble proteins as well as with particulate ones. The intracellular ferment bears a wider variation in temperature, and is not so easily and delicately influenced by variations in the composition of the medium in which the cell exists. So far as we know the intracellular ferments do not diffuse from living cells. They are, however, recognizable in the fluids of abscess cavities as the leukocytes disintegrate. We are of the opinion that they are essential constituents of the chemical structure of cells. The reason for this belief will be developed later. The extracellular ferments may be regarded as secretions of cells. Much has been written about cellular and humoral theories. In our opinion every living thing has a chemical structure, which we may designate as a cell if we wish, understanding that a cell is not necessarily something that can be seen, and that it may possess widely different degrees of lability, but we are quite certain that there is no ferment which is not the product of life processes. We have been somewhat surprised to find it stated that our own theory of protein sensitization or anaphylaxis is a humoralistic doctrine.

All ferments are products of life processes, and all life processes are more or less responsive to outside influences, to change in environment. In our opinion the most valuable fact that we have learned in the study of protein sensi-

tization is that life processes manifested through ferment action are modified and may be modified at will by changes in environment. The blood-serum and organ extracts of normal guinea-pigs do not digest egg-white, but these fluids from an animal sensitized to this protein do have this action. The virus of smallpox is pathogenic to the man who has never had smallpox, and has not been vaccinated, but to the man who has had the disease or been properly vaccinated the virus of smallpox is non-pathogenic. We explain this, and in our opinion, the experiments of Pirquet have so demonstrated, that this is due to the fact that the ferments of the man's body cells have been so influenced by the disease or by vaccination that they have acquired a new function—that of digesting and thus destroying the virus of the disease. If this explanation be true, it opens up a wide field for the possible extension of the beneficial effects of preventive treatment.

There is another point of difference between intracellular and extracellular ferments, which is of the greatest importance in a study of the phenomena of infection. The extracellular ferments are comparable to those of the digestive juices of the alimentary tract in the higher animals. They roughly prepare foods for the cells. Their function is solely a lytic one. They break up complex proteins into simpler bodies, but the products thus formed are not, without further treatment, ready to be built into the structure of the cell. Proteins in the medium are rendered soluble by the extracellular ferments. They are so altered that they may be taken into the cell, but they are not so patterned that they are ready to be built into the structure. They are fitted for absorption, but are not ready for assimilation. The extracellular ferments are in a sense destructive agents. They break down complex molecules into simpler structures. The intracellular ferments are constructive. They are cell builders. They shape the material brought them and fit it into place. They build up specific proteins. They convert the raw material brought them into specific proteins, bacterial, vegetable, or animal. This does not

mean that the intracellular ferments have no cleavage action. They chip the rough stone so that it fits in at the right place. It is by virtue of their activity or through their agency that cells grow and multiply. In case of an infectious disease the intracellular ferment of the infecting organism during the period of incubation converts man's proteins into bacterial proteins, and continues to do this with more or less success during the course of the disease. This seems to be accomplished in some diseases, at least, like typhoid fever, without any marked disruption of the cells of the man's body. The bacteria multiply rapidly during the period of incubation, and at this time the man is unconscious of the fact that his body is serving as a culture flask. We must conclude from this that the conversion of human proteins into typhoid proteins in the growth of the infecting agent is not accompanied by the liberation of the poisonous group in the protein molecule. This group, probably attached to other groups, or as a constituent of a more complex group, is used in the construction process. The poisonous group is common to all proteins. The synthesis of specific proteins from other specific proteins is accomplished without the liberation of the poisonous portion. It is one of the building stones, and changes in specificity do not occur in this, but in the secondary or characteristic groups. This is, in our opinion, the explanation of the fact why incubation—a period of rapid reproduction in the infecting agent—proceeds without any recognizable disturbance in the health of the host. The typhoid bacillus therefore does not feed upon the cells of the man's body, but upon the formless, soluble proteins. Cell building is accompanied by the absorption of the poisonous group in the proteins serving as food. However, when the body cells become sensitized and elaborate a ferment which breaks down the bacterial cells, the poisonous group in the proteins of the latter is set free, and it is the effect of this poison that develops the symptom complex of the disease. The symptoms of one infectious disease differ from those of another largely according to the organ

or tissues in which the infecting agent is located. In acute miliary tuberculosis and in typhoid fever, both conditions arising from a bacteremia caused by different organisms, the symptoms are only too frequently identical, and it is only by bacteriological methods, a suggestive history, or the finding of a preëxisting tuberculous focus in some part of the body that a differential diagnosis may be reached. A cholecystitis is the same, not only in symptomatology, but frequently in gross pathology as well, whether the infecting organism be the pneumococcus, the streptococcus, the colon, or the typhoid bacillus. The most skilful diagnostician cannot tell from the symptoms alone the specific bacterial cause of a meningitis.

During the period of incubation of an infectious disease, the infecting organism supplies the ferment, the body proteins constitute the substrate, the process is essentially constructive, no poison is set free, and there are no recognizable clinical symptoms. During the active progress of an infectious disease, the body cells supply the ferment, the infecting organism constitutes the substrate, the process is essentially destructive, the protein poison is set free, the symptoms of disease appear and life is placed in jeopardy.

Our work seems to show that the body cells, when overwhelmed with a foreign protein of the blandest kind—such as egg-white—may fail to function and death may result. There is no reason for suspecting that in these cases there is any cleavage of the foreign protein or the liberation of any poison. The body cells are simply clogged with the foreign protein and fail to function. We are not sure that this phenomenon has any parallel in the infectious diseases. There is, however, something closely related to it in cholera infantum, cholera nostras, and Asiatic cholera.

We have already referred to the fact that ferments may be modified in their activities. These modifications may be so radical that it is generally believed that cells may be trained, as it were, to develop new ferments. There can be no doubt that change in environment does alter activity as manifested through the ferments. As we have stated,

it seems to be a biological law that when a living cell is brought in contact with or permeated by a foreign protein, it tends to furnish a ferment which will digest and destroy the foreign body. The ferments of the cells of man's body may be modified or new ones developed by (a) disease, (b) vaccination, and (c) sensitization. Many of the infectious diseases give immunity to subsequent exposure. In some of the chronic infectious diseases the altered behavior of the body cells to the infecting agent is evident even while the disease continues.

That the tuberculous animal behaves differently from the non-tuberculous on receiving injections of the tuberculin protein, whether it be in the form of the living bacillus, in dead cells, or in solution, has been abundantly demonstrated. Before Koch gave us tuberculin, Arloing and Courmont had come to the conclusion that the tubercle bacillus produces soluble substances which reduce the natural resistance of the body and render it more susceptible to reinfection. This corresponds closely with the first impression made by observation of the phenomena of anaphylaxis; the impression that led Richet to select this term. In 1891, Koch described a perfect example of protein sensitization as we understand it today. He stated that when a healthy guinea-pig is inoculated with the living tubercle bacillus there is no change at the site of inoculation until from ten to fourteen days later, when a hard lump forms, finally opens and ulcerates, and continues until the animal dies. On the other hand, when a tuberculous guinea-pig is inoculated with the living bacillus, on the second or third day a lump forms, soon becomes necrotic, falls out, ulcerates for a time, and finally heals without any infection of the neighboring lymph glands. In 1897 Trudeau observed that when healthy rabbits receive injections of virulent cultures in the eye, there is little to be seen for about fourteen days, when with increasing vascularity tubercles form in the iris, after which inflammation extends and the eye is practically destroyed within from six to eight weeks. Like treatment of tuberculous rabbits develops an iritis

within from two to five days, but at the end of the second or third week, at a time when the controls begin to develop destructive changes, the inflammation begins to subside. Later studies have confirmed and amplified these, and it has been found that death may be induced within twenty-four hours by injecting a large amount of a living culture into a tuberculous animal.

The same difference between healthy and tuberculous animals has been observed in their response to injections of dead cultures of the tubercle bacillus. The first observation along this line, so far as we know, was made by Strauss and Gamaleia, who found that when large numbers of dead tubercle bacilli are injected into tuberculous animals death results, while similar amounts are without immediate effect upon healthy animals.

When we come to tuberculin, every phase of its action or its failure to act is explainable on the ground that the tuberculous animal is a sensitized one. Koch found that 0.5 gram of his preparation killed tuberculous guinea-pigs, and induced no symptoms in healthy ones. A fraction of 1 mg. may cause marked symptoms in a tuberculous man, while many times this amount is borne easily by a healthy man. The inflammatory reaction about local tuberculous lesions caused by injections of tuberculin is explained by the fact of the high degree of sensitization in their localities, and the cleavage of the bacilli. The ophthalmic, cutaneous, subcutaneous, and intravenous tests with tuberculin are all typical sensitization reactions. Even in the failure to respond to tuberculin seen in advanced tuberculosis we have the condition known as anti-anaphylaxis, which simply means that the anaphylactic ferment is partially exhausted by the large amount of material supplied by the bacilli in the body.

There is a second factor in the failures of advanced cases of tuberculosis to respond to the tuberculin test, which has been generally overlooked, but to which we have already referred in our discussion of so-called anti-anaphylaxis. This is the fact that in such cases the body is saturated with

the products of the digestion of tuberculoproteins. It is a well-established fact that the accumulation of fermentation products retards and finally arrests the fermentative process. The instance in point is a perfect illustration of this law of fermentation. It must be evident from this how unscientific it is to treat advanced tuberculosis with tuberculin. It has been argued that the tuberculin reaction is not an example of sensitization, because as the treatment proceeds, larger and larger doses of tuberculin are necessary in order to induce the reaction, as shown by the development of fever. To anyone who has followed the evidence that we have given so far, the explanation must be plain. It lies in two facts, of each of which we think that we have given abundant proof. In the first place we have shown that a certain degree of tolerance for the protein poison is easily and quickly established. In the second place, the accumulation of fermentation products retards fermentation. Tuberculosis, in most instances at least, begins as a strictly local infection. This is true even when the first recognition of it has been in the acute miliary form. There has been a previous focal infection. Only those body cells in the immediate vicinity of the infection are sensitized, and only these supply a ferment capable of digesting the tuberculo-protein. It may well be that in this stage benefit may be secured by the proper use of tuberculin, which may act as a sensitizer, and develop more of the ferment to split up and destroy the tubercle bacilli. It should be always borne in mind that tuberculin contains a poison and should be used with caution.

There is another line of evidence that in tuberculosis there is a condition of specific protein sensitization. This is to be found in the fact that this disease is much more deadly in lands and among people who have recently come under its influence than it is where it has prevailed for many generations. In other words, the widespread and long-continued existence of the disease, slowly, and at the cost of much sickness, and many deaths, brings a certain degree of immunity. The readiness with which the North American

Indian has succumbed to this disease is a striking illustration, and Calmette has recently collected additional evidence on this point. He states that tuberculosis is being widely disseminated among peoples who have until recently been free from it. The world-wide wanderings of the white man are carrying the disease to every people, from the Laplander and Esquimaux of the Arctics to the negroes and Malays of the tropics. Iceland, the Faroe Islands, and the steppes of Russia are being infected, and in these new regions tuberculosis exists in its most speedily fatal forms. The same author points out that recently discovered methods for the recognition of this disease, even in latent states, shows that among Europeans not more than 7 or 8 per cent. reach more than twenty years of age without receiving the infection. Those who survive the first infection become more or less immune, and after that develop, when they do acquire the disease, the more chronic forms.

Römer<sup>1</sup> concludes that the less widely tuberculosis is distributed among a people the greater is the case mortality, and the wider the distribution the smaller is the case mortality.

Still another fact of importance is that the most speedily fatal forms of tuberculosis, such as the miliary and meningeal, are more frequent among children than among adults.

There is another matter of much importance in this connection which we must discuss. We have found the tubercle bacillus highly resistant to lytic agents, and it appears that its long experience as a parasite has led it to protect itself with deposits of wax and fat, but proteolytic enzymes digest the most firm proteins. Friedberger has found that at least some strains of this bacillus are digested by the serum of healthy guinea-pigs, and the researches of Markl, Bail, and Kraus and his students have shown that tubercle bacilli placed in the peritoneal cavity of tuberculous animals respond to Pfeiffer's reaction. Some strains are dissolved in the peritoneum of healthy guinea-pigs, but

<sup>1</sup> Beiträge z. klinik d. Tuberk., 1912, xxii, 301.

dissolution occurs more promptly and more completely in the peritoneum of a tuberculous animal. The healthy animal may have to depend upon its phagocytes to combat the invading bacillus, but the tuberculous animal supplies a specific proteolytic enzyme, and to this the fresh invader succumbs.

Nature is slowly immunizing the white man to tuberculosis, and the question arises whether or not the process employed by Nature can be aided in any way. There is before the medical profession at this time no greater question than this: Is it possible to aid in eradicating tuberculosis by vaccination? As Römer says, the problem of securing immunity to tuberculosis with a non-infective virus is of great practical importance, and recent work brings the possibility of doing this more and more to the front. What we need is a vaccine. Various methods of modifying the tubercle bacillus so that it could be used as a vaccine have been tried. The bovo vaccine of von Behring was tried, but the increased resistance given by it was found to be of short duration. Attempts to reduce its virulence by age, heat, chemicals, and by submitting it to ultraviolet and other rays and emanations have been made. What we need is a tubercle protein sensitizer. It should be soluble, and it should be free from the poisonous group in the protein molecule. In our opinion the nearest approach to this desired substance is the non-poisonous portion of the tubercle protein. So far we have not been able to secure a uniform product. Some preparations seem to fill every requirement. They sensitize animals to the unbroken bacillus, dead or alive, and in surface tuberculous lesions they cause inflammation about the tuberculous area, and we have seen the tuberculous tissue slough off and complete recovery result; but other preparations made from the same cellular substance by the same method seem inert. We have had similar difficulties with the sensitizing groups from other proteins. Some preparations from egg-white sensitize to unbroken egg-white, while others seem wholly without effect, and still all are prepared from the same material and

in the same way. Evidently the sensitizing group in the protein molecule is a highly labile body and susceptible to influences which so far we have not been able to recognize. We have no difficulty in obtaining the poisonous group uniformly, but it is otherwise with the sensitizing body. Further work along this line is needed, and if an efficient and uniformly reliable sensitizer for the tuberculous protein, free from the poisonous group, can be secured, all children should be vaccinated for tuberculosis; then with protection against natural infection the restriction of tuberculosis will be as completely under man's control as is that of smallpox. It should be clearly understood that the protection afforded by vaccination is relative and not absolute.

The studies inaugurated by Wright have demonstrated that vaccination is of service not only in prevention, but also in cure. Bacteria and protozoa are particulate, and in many diseases they are confined to limited localities. As we have seen, sensitization may also be local. No body cell is sensitized against a foreign protein until the latter comes in contact with the former and penetration of the body cell is probably essential to the most efficient sensitization. The microorganisms of acne are located in the cutaneous tissue, and being particulate and not in solution, the area sensitized by them is small, if there be any sensitization at all. By vaccine therapy the area of sensitization is greatly extended and the amount of lytic agent formed and made available is greatly increased. This being in solution and diffusible, digests and destroys the bacteria located in the skin. The same is true of the treatment of localized tuberculosis, or of any other localized infectious disease. In vaccine therapy, as in vaccination, the great need is for soluble sensitizers free from poisonous content. When these are secured, and not until then, we may develop a vaccine therapy along scientific lines, and expect to secure important results with it.<sup>1</sup>

<sup>1</sup> The following pages are taken with but little change from an article by Vaughan, Jr., in "International Clinics."

The importance of sensitization as a factor in the case of tuberculosis is evident in the widespread use of tuberculin as a diagnostic measure. The various reactions of the body to tuberculin, whether they occur as the general reaction following subcutaneous injections, or as the more local reaction following the vaccination of the skin with tuberculin, the application of a tuberculin containing ointment to the skin, or the instillation of tuberculin into the conjunctival sac, are all evidences of the sensitization of the body of the tuberculous individual to tuberculin. Thus, when a small amount of tuberculin is injected into the fluids and tissues of a normal individual, no effects are noticeable, since the enzyme which causes proteolysis of tuberculin is not present in the body. When, however, the same amount of tuberculin is injected into the tuberculous individual, it practically corresponds to a second injection of this foreign protein. The enzyme present in the body of the tuberculous individual attacks the tuberculin, liberating the poisonous cleavage products, which in turn give rise to the well-known symptom-complex designated as the tuberculin reaction. In addition to the general symptoms, such as fever, which accompany the presence of protein poisoning within the body, poisonous proteins have a decidedly irritant local effect upon the tissues with which they are brought directly in contact. This is seen in the hyperemia and inflammation of the peritoneum in cases of infection within the abdominal cavity, and is also evidenced by the local reaction of inflammatory type following the application of tuberculin to the mucous membrane or the abraded skin of the tuberculous individual.

Sensitization to tuberculin may be either local or general in type, as is quite evident to anyone who has employed the conjunctival test as a means of diagnosis in tuberculous disease. This test consists in the application of a 1 per cent. solution of specially prepared tuberculin to the conjunctival sac. The reaction following this method of applying the tuberculin test may be divided into two distinct types, the first of which we may call the reaction of general sensi-

tization, or the tuberculous reaction, in contradistinction to the second, or the reaction of local sensitization.

When a solution of tuberculin is applied to the conjunctival sac of a tuberculous individual, no changes are usually noticed for an interval varying from six to forty-eight hours. At the end of this time there is a slight smarting or gritty sensation complained of, the patient often referring to it as a sensation of sand in the eye. The examination of the conjunctiva at this time reveals a reddening and swelling of the mucous membrane of the lower lid and the caruncle. This inflammatory reaction gradually increases in intensity until from ten to fifteen hours have elapsed, at which time it has usually reached a maximum, and after which a gradual recession occurs, until at the end of from two to four days, occasionally after a longer interval, the conjunctiva has again regained its normal appearance. At the height of the reaction, and on awakening in the morning, it is not uncommon to observe a slight fibrinous or fibrinopurulent exudate accompanying the inflammatory reaction.

When a solution of tuberculin is applied to the eye of a normal individual, no reaction is obtained. If, however, a second instillation is made in the same eye after an interval of seven days, a reaction will be observed in a large proportion of cases. This reaction is quite distinct from that previously described as occurring in the eye of the tuberculous individual. The reaction is rapid in appearance, explosive in type, and subsides with great rapidity. Thus, it is not rare to find, as a result of a second instillation, within from three to four hours after the application, a highly inflamed conjunctiva associated with considerable chemosis of the lids and a profuse purulent discharge. The symptoms, however, in spite of their severity, rapidly subside. These differences in type of reaction find a satisfactory explanation if we consider the fact that in the tuberculous reaction we are dealing with what may be termed a phenomenon of general sensitization. In this case the cleavage of the tuberculin introduced within the conjunctival sac is brought about through the action of the proteolytic

enzyme which has been developed in the body of the tuberculous individual as a result of his disease. During this cleavage certain poisons are liberated which act as irritants to the conjunctival mucous membrane, and the degree of irritation present will be directly proportionate to the amount of toxic cleavage product present at a given time. However, the amount of cleavage product present at a given time will be determined by the rate of proteolysis, which depends in turn upon the quantity of proteolytic enzyme directly available. Since this enzyme is available only in such proportions as may be present in the circulating fluids of the conjunctiva, it necessarily follows that only a small amount can be operative at a given time. The result is that we have a foreign protein slowly broken up, with the liberation of a small quantity of irritant poison over a considerable interval of time. For this reason the reaction of general sensitization is slow in its development, maintained at its maximum for a considerable period, and subsides gradually.

When tuberculin is instilled into the eye of a normal individual, no apparent result is obtained, since no ferment is present in the body capable of splitting up tuberculin. However, as a result of the instillation itself, certain cells of the mucous membrane are stimulated to produce a specific ferment which will be stored up as a zymogen for future use. If subsequently a solution of tuberculin is brought in contact with these sensitized cells, the zymogen is activated, liberated in a concentrated form, and splits up at once all of the tuberculin introduced. The result is that we obtain the reaction characteristic of local sensitization, which is rapid in onset, comparatively severe in type, and disappears with great rapidity. Owing to the high grade of inflammatory reaction obtained in connection with the second instillation, it is well to use a more dilute solution of tuberculin and to ask the patient to present himself for examination within from two to four hours following the instillation. At this time, if any noticeable redness is present, the eye should be thoroughly washed out with a saturated solution of boracic acid in order to remove any

excess of tuberculin which may be present. The information obtained through the employment of the second instillation in an individual who has previously failed to react is of value in that it indicates that the body cells of the patient are capable of producing a ferment which will split up tuberculin, and consequently we should have obtained a primary reaction provided the individual was actively tuberculous. Failure of the ophthalmo-reaction occurs under the following conditions: (1) In early cases in individuals who are incapable of producing the specific ferment. Such individuals will fail to react to the second instillation. (2) Normal individuals who are not afflicted with tuberculous disease will fail to react to the primary instillation. They may, or may not, react to the second instillation, depending on whether or not their body cells are capable of producing the specific ferment. (3) Patients suffering from acute tuberculous disease or advanced cases fail to react to either the first or the second instillation. In these cases the failure of the reaction is due to the exhaustion of any specific ferment which may have been present through the overwhelming of the system with tuberculin, or to the accumulation of split products, as has been stated.

While the importance of sensitization in connection with the infectious diseases is not as yet thoroughly appreciated, later investigations have been conducted largely along these lines. Thus, sensitization is undoubtedly an important factor in the treatment of bacterial diseases through the employment of vaccine therapy. This is true whether the vaccine employed consists of the whole bacterial cell or the split products, such as those obtained after our method.

The injection of foreign proteins as such into the body always represents an abnormal condition. The symptoms of sensitization following the administration of horse serum in man may be divided into two classes, according to the interval of time elapsing between the administration of the serum and the development of symptoms. In general, it may be stated that symptoms of sensitization, provided they occur at all, show themselves either very shortly after the

administration of the serum, or, if not at this time, on the seventh to the tenth day following the injection. In instances in which effects are not noticeable until from seven to ten days following the injection, the symptoms are largely confined to those of peripheral irritation, as evidenced by urticarial lesions accompanied by intense itching. On the other hand, in cases in which a reaction follows within twenty-four hours, the symptoms of poisoning are more pronounced, and where occasionally a fatal result follows it occurs usually within an hour after the injection. In these cases, which are fortunately rare, we find that the symptoms are very similar to those obtained through the injection of the poison obtained by Vaughan through protein cleavage. Thus, Gillette<sup>1</sup> reports the case of an asthmatic fifty-two years old, to whom he gave 2000 units of antitoxin globulin, administered under the left scapula. While dressing, following the injection, the patient complained of a prickling sensation in the chest and back of the neck. He at once sat down in the chair and complained of inability to breathe. The physician felt his pulse and found it full and regular. Immediately thereafter the patient was seized with a tonic spasm, during which death ensued, the whole interval elapsing between the injection and the fatal outcome not exceeding five minutes in duration. In spite of the rapidity with which death occurred in this case, we can still recognize evidences of the three stages characteristic of fatal protein poisoning: the stage of peripheral irritation indicated by itching sensations in the skin, the stage of partial paralysis or weakening of the lower extremities, and the convulsive stage, during which death occurred.

In cases of sudden death following within a few minutes after the injection of horse serum, it is not infrequent that one of the stages is absent or ill-defined. Thus, in the instance cited above, the loss of ability to move the lower limbs was not specifically mentioned, although in other

<sup>1</sup> Jour. Amer. Med. Assoc., January 4, 1908, p. 40.

reported cases patients have before death remarked on their inability to walk.

It is quite evident from a study of the untoward results following the administration of horse serum, that the apparent differences existing between immediate manifestations and those occurring after an incubation period of from seven to ten days are of degrees of intensity rather than of character of the poisoning. Thus, in the instances, fortunately rare, in which death occurred within thirty minutes following the injection, the symptoms are due to the liberation of a fatal amount of poisonous substance at once, and in instances in which alarming but not fatal symptoms arise shortly after injection, recovery from the intoxication is usually prompt and complete. On the other hand, where symptoms appear only after an interval of from seven to ten days, and are confined to those of peripheral irritation, as evidenced by the development of urticaria, we find that complete recovery is slow and tedious.

That such differences should exist appears but natural when we consider the mechanism involved in sensitization, and the fact that immediate effects are due to the injection of the serum into a sensitized individual, whereas the remote effects are to be looked upon as a manifestation of the sensitization of the patient as the result of the injection itself. In the first instance the individual has stored up in his body cells a ferment which, liberated by the injection of the serum, splits up the foreign protein introduced at once, and sets free all of the poison contained therein immediately. The symptoms resulting therefrom are necessarily acute in character, sudden in development, and transitory in nature, since the effects of the poison rapidly disappear. In the individual developing symptoms after an incubation period of from seven to ten days, conditions are decidedly different. In this case no special ferment capable of producing proteolysis of the foreign proteins contained in the serum is present within the body at the time of injection, and as a result the foreign proteins continue to exist as such within the body for a certain length of time.

However, under their influence certain body cells are stimulated to produce a ferment which will split up the foreign substances into simpler non-specific bodies. The fact that animals injected with serum do not become hypersensitive to a second injection until after the lapse of from seven to ten days indicates clearly the length of time necessary for the new ferment to be formed in appreciable amount. Symptoms developing after an incubation period are, therefore, to be explained by the fact that the foreign proteins still existing in the tissue are acted upon by the enzyme called forth by their presence. Under these conditions, however, no large amount of ferment will be active at any given time, and consequently the amount of poison liberated through protein cleavage at any one period will be small in amount, although the cleavage itself will continue over a comparatively long interval and the resulting poisoning will be more chronic in type. This affords a plausible explanation of the fact that late manifestations of serum sickness are milder in character, being confined for the most part to those of peripheral irritation, and also of longer duration.

Provided the theory advanced above is correct, one would expect to find a difference in the time interval elapsing between the injection of the foreign serum and the subsequent appearance of symptoms of poisoning, depending on whether the individual had previously been treated with serum or not. While it is true that, in many instances in which alarming symptoms develop immediately, a history of previous treatment is unobtainable, the results quoted by Pirquet are interesting as bearing on this point. Thus it has been found that of 214 individuals who developed symptoms after the first injection of serum for therapeutic purposes, 111, or 51.8 per cent., manifested symptoms of poisoning on from the seventh to the tenth day inclusive; while in 172 patients who received a second injection, 89, or 51.7 per cent., showed signs of poisoning within the first forty-eight hours.

As has been previously mentioned, alarming symptoms

following the use of diphtheria antitoxin and other therapeutic sera are fortunately so rare that they should not be considered too seriously when indications for the use of such sera arise. However, there are certain precautions which can and should be employed, and which may aid materially in avoiding the untoward effects following the administration of these remedies. Much has been accomplished by the efforts of various pharmaceutical houses to prepare an antitoxin from which a large proportion of the foreign albumin contained in horse serum has been removed, and such products should be used exclusively whenever possible. It would, furthermore, appear to be well to make a preliminary test with regard to the sensitiveness of any given individual to the serum employed. This may be done by the injection of a very small test dose (0.05 c.c.) of the serum and watching for rapid evidences of toxic action in the patient. Alarming signs, if they occur, develop usually within an hour after treatment; and if no sign of poisoning occurs within this time, it may be safely assumed that the individual does not contain within his body the special ferment required to split up the material injected, and a second injection may be made with impunity, provided the interval of time elapsing between each is not sufficiently long to admit of the development of sensitization. In a disease such as diphtheria this is, of course, a matter which does not enter into consideration in the treatment of any given attack. The preliminary injection of atropine has been advised by Auer, who found that 18 out of 25 sensitized guinea-pigs which had been given atropine sulphate recovered from the second injection, while of 24 untreated controls, only 6 survived.

When symptoms of sensitization appear immediately or soon after the injection, the use of ether by inhalation is to be recommended, as Besredka found that in experimental sensitization animals narcotized with ether did not succumb to the second injection. When it is ascertained that a given individual is sensitive, and nevertheless the use of therapeutic sera is imperative, it may be given by

fractioning the total amount of serum, and, instead of using a single dose, give several doses at frequent intervals over a considerable period of time. In this way it may be possible to exhaust the ferment present in the body for the time being, after which further amounts may be given with impunity. A temporary exhaustion of the special ferment would explain cases of diphtheria such as are reported, in which, although alarming symptoms followed the first injection, on account of the condition of the patient, a second injection seemed advisable, and was given within a few hours without any untoward effects whatever. Experiences such as this find their analogy in experimental work in the fact that sensitized animals which have recovered from the poisoning following a second injection are not again susceptible to that particular protein until after the lapse of several days.

The idiosyncrasies which certain individuals possess with regard to certain protein articles of diet would appear to be explainable on the ground that, through some abnormal condition of the intestinal mucosa, certain protein substances are allowed to enter the body in an unchanged state. The symptoms which develop are certainly strikingly suggestive of those described as appearing in connection with sensitization. The symptom most constant in appearance is an urticaria, more or less generalized in extent and accompanied by intense itching. Thus, Bruick reported the case of a man who reacted with urticaria every time after eating pork. Smith reports the case of an individual who developed a severe urticaria within a short time after partaking of any article of diet which contained buckwheat. Numerous cases of severe urticaria accompanied by dyspnea, and occasionally incoördination of the lower extremities, have been reported as occurring in susceptible individuals after partaking of any food containing egg albumen, and one of us has recently had opportunity to observe an individual who developed a generalized urticaria accompanied by marked edema and intense itching within half an hour after partaking of peas as an article of diet.

The most striking peculiarity mentioned in connection with the above idiosyncrasies is the rapidity with which the symptoms of poisoning develop after the introduction of the attending cause within the alimentary tract. As has been mentioned, these individual peculiarities are possibly best explained by the supposition that the individual has become sensitized to certain specific proteins, the sensitization arising from the fact that the particular protein has gained entrance into the body through the intestinal mucosa in an unchanged state. When this occurs a foreign protein is present in the tissues and fluids of the body, and to counteract the abnormal condition thus produced certain body cells are called on to develop a proteolytic ferment which will have for its function the cleavage of the particular protein present in any given case. This ferment, once formed, is stored up in certain cells as a zymogen for future use. The same protein cleavage then occurs as normally takes place within the intestinal canal, with the important difference that the toxic substances formed are liberated within the body itself and consequently are capable of exerting their harmful action. That these ferments are not present in the body in inexhaustible amount is shown experimentally by the fact that animals which have recovered from the effects of sensitization following a second injection of egg albumen are not subsequently sensitive to this protein until after the lapse of several days. This is undoubtedly due to the fact that the ferment has been largely used up in bringing about the cleavage of this particular protein and time must be allowed for the body cells to produce an additional amount. In other words, it is possible in a susceptible individual to destroy their susceptibility with regard to any particular protein through an exhaustion of the special ferment present in their body. This is well illustrated clinically by the following example: A woman who was fond of strawberries, but developed an intensely disagreeable urticaria after each indulgence, was accustomed to eat this fruit two or three times during the season. Finally, being firmly convinced that the rash was simply a

nervous manifestation, she determined to eat them continuously, in order, as she said, "to break herself of the nervous habit." After the first week no unpleasant symptoms whatever were noted following the daily use of this article of diet. This appeared to the patient to be an entirely satisfactory proof of the effectiveness of Christian Science, and yet the phenomenon is explainable on a rational basis. The daily use of strawberries had led to an exhaustion of the special ferment in her body, and subsequent indulgence was consequently not followed by untoward symptoms. Whether or not the experience was repeated during the succeeding summer we have not been able to ascertain.

In conclusion, we may state that sensitization primarily represents an important phenomenon of lytic immunity. Sensitization occurs whenever a foreign protein as such gains entrance into the fluids and tissue of the body, and results from the development within the body of a special ferment which will attack the particular protein introduced. When individuals become sensitized through the introduction of dead protein substances, such as egg albumen or horse serum, the results obtained prove unfavorable to the individual. In these cases our attempt should be to bring about a desensitization of the individual through the exhaustion of the special ferment. On the other hand, sensitization occurring as a result of the entrance of bacterial cells into the body represents a beneficial process, and plays an important part in the development of active immunity to the specific infections. Since, under ordinary circumstances, pathogenic bacteria represent the only proteins which gain entrance into the body in an unchanged state, we may conclude that sensitization arises as an attempt of nature to protect the individual against bacterial disease.

# INDEX

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## A

AMINO acids, 74, 78  
Anaphylactic state, 242  
Anaphylatoxin, 296  
Anaphylaxis, 214  
    in vitro, 274  
    mechanism of, 247  
    passive, 254  
Animals, action on, 119  
Anthrax protein, 189  
Arthus phenomenon, 262  
Anti-anaphylaxis, 258

## B

BACILLUS, action of, 119  
    colon, 37  
    diphtheria, 38  
    particulate proteins, 17  
    pathogenicity of, 21  
    reducing action of, 62  
Bacterial cellular substance, 37  
 $\beta$ -iminazolyle thylamin, 291

## C

CANCER cell, specific ferments of, 416  
Cellular protein, hydrolysis of, 81  
    substance, 37  
        action of, 121  
        carbohydrates in, 57, 66  
        chemistry of, 52  
        diamino-acids of, 74  
        fats of, 60  
        immunization with, 138  
        mono-amino acids of, 78  
        nucleins in, 73  
        of pneumococcus, 205  
        proteins of, 52

Cleavage of proteins, 95  
Cultures, massive, 29

## D

DEFIBRINATED blood, the poisonous  
    action of, 334  
Diamino-acids in cellular substance,  
    74  
Diseases, infectious, 23

## E

EGG-WHITE, cleavage of, 98  
    the disposition of, when intro-  
        duced parenterally, 355  
Ergamin, 291

## F

FEVER, acute fatal, 381  
    continued, 374  
    digestive action of blood in, 395  
    followed by immunity, 397  
    intermittent, 386  
    protein, 373  
    remittent, 387

## G

GASTRIC juice, action of, 42  
Group, poisonous, 19  
    sensitizing, 234

## H

HAPTOPHOR, properties of, 112  
Histamin, 291

## I

- IMMUNITY, natural, 24  
 toxin, 27  
 with split products, 157  
 Immunization with poisonous por-  
 tion, 138  
 with residue, 144  
 Infection, the phenomena of, 436

## K

- KYRINS, the, 295

## M

- MONO-AMINO-ACIDS in cellular sub-  
 stance, 78

## N

- NUCLEUS, chemical, 20

## P

- PARENTERAL digestion, 342  
 Peptone, the fats of, 342  
 Pneumococcus, cellular substance of,  
 205  
 Poison, action of, 125  
 crude soluble, 101  
 immunization with, 138  
 physiological action of, 315  
 protein, 17, 284  
 Protein fever, 373  
 poisons, nitrogen in, 111  
 sensitization, 25, 214  
 Proteins, cleavage of, 95  
 particulate, 17  
 physiological action of, 320

## R

- RED corpuscles, production of fever  
 with, 391  
 Reinjection, the, 243  
 Residue, immunization with, 144

## S

- SENSITIZATION, cellular, 321  
 period of incubation, 241  
 protein, 214  
 symptoms of, 245  
 Sensitizers, 219  
 volatile, 231

## T

- TANKS for massive cultures, 30  
 Theories, 323  
 Theory of Friedberger, 324  
 of Nolf, 340  
 of Vaughan and Wheeler, 327  
 Toxic sera, 264  
 Toxicity of extracts from normal  
 tissue, 325  
 Toxogens, 266  
 Trypsin, action of, 43  
 Tubercle bacillus, cellular substance  
 of, 164  
 cleavage of, 165  
 poison in, 165  
 residue of, 165  
 split products of, 164  
 toxophor of, 178  
 Tuberculosis and sensitization, 181

## V

- VACCINATION, 24  
 Vaccines, 26  
 Vegetable proteins, production of  
 fever with, 403











