THE CHEMICAL NATURE OF THE ANTIGENIC SUBSTANCES IN BACILLUS COLI

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

BY

EDWARD EVERETT HALE BOYER, B. Sc., M. Sc.

The Ohio State University

1920

THE CHEMICAL NATURE OF THE ANTIGENIC SUBSTANCES IN BACILLUS COLI

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of the Ohio State University

BY

EDWARD EVERETT HALE BOYER, B. Sc., M. Sc.

The Ohio State University

1920

Publishers By Mimeograph Process
EDWARDS BROTHERS
ANN ARBOR, MICHIGAN

THE CHEMICAL NATURE OF THE ANTIGENIC SUBSTANCES IN BACILLUS COLI

Introduction.

Certain substances when taken into the body parenterally will cause the production and appearance of protective substances within the body fluids. The protective substances we call antibodies. That which calls forth the production of antibodies is known as antigen. When antigens are allowed to enter into contact with their respective antibodies, either within the animal body or in a test tube, definite changes are observed; thus we have manifestations of these changes in the phenomena of agglutination, precipitation, complement fixation and other serological reactions.

But of what do these changes consist? Are they true chemical reactions which follow the usual laws of chemistry, or are they due to alterations in surface energy and molecular attraction in a physical or physico-chemical sense? And what is the nature, chemically, of the reacting substances?

Most authorities state that all antigens are protein substances. Such statements, as will be shown later, are based to a considerable extent upon prejudice and preconceived notions which are not entirely borne out by exact experimental procedure. The substances most generally used as antigens are body fluids (blood serum), body cells, and bacterial bodies. These substances, in the dried state, are composed largely of protein and mineral salts, with a great predominance of the former. Fats and lipoidal material are often present in such small amounts as to escape detection unless large quantities of the native material are used in the analysis. Since the proteins seem to be the important constituent of substances used as antigens, and since such substances may be used with equal results after the salts have been removed by dialysis or other method, it was but natural to consider the proteins as antigenic principles.

A few investigators have endeavored to demonstrate the value of fats and lipoids in serological work. Thus, Jobling and Peterson (1) showed that when bacteria were injected into the blood stream they absorbed lipoidal material which ordinarily acts as the anti-enzyme constituent. Noguchi (2) showed that the tetanolysin fraction of the tetanus toxin was neutralized by cholesterin and alcoholic extracts of blood serum. Muller (3)

THE CHEMICAL NATURE OF THE ANTIBODIES

IN BACILLUS COLI

Introduction.

Certain substances when taken into the body parenterally will cause the production and appearance of protective substances within the body fluids. The protective substances we call antibodies. That which calls forth the production of antibodies is known as antigen. When antigens are allowed to enter into contact with their respective antibodies, either within the animal body or in a test tube, definite changes are observed; thus we have manifestations of these changes in the phenomena of agglutination, precipitation, complement fixation and other serological reactions.

But of what do these changes consist? Are they true chemical reactions which follow the usual laws of chemistry, or are they due to alterations in surface energy and molecular attraction in a physical or physico-chemical sense? And what is the nature, chemically, of the reacting substances?

Most authorities state that all antigens are protein substances. Such statements, as we will be shown later, are based to a considerable extent upon predjudice and preconceived notions which are not entirely borne out by exact experimental procedure. The substances most generally used as antigens are body fluids (blood serum), body cells, and bacterial bodies. These substances, in the dried state, are composed largely of protein and mineral salts, with a great predominance of the former. Fats and lipoidal material are often present in such small amounts as to escape detection unless large quantities of the native material are used in the analysis. Since the proteins seem to be the important constituent of substances used as antigens, and since such substances may be used with equal results after the salts have been removed by dialysis or other method, it was but natural to consider the proteins as antigenic principles.

A few investigators have endeavored to demonstrate the value of fats and lipoids in serological work. Thus, Dobling and Peterson (1) showed that when bacteria were injected into the blood stream they absorbed lipoidal material which ordinarily acts as the anti-enzyme constituent. Noguchi (2) showed that the tetanolysin fraction of the tetanus toxin was neutralized by cholesterin and alcoholic extracts of blood serum. Miller (3)

demonstrated that the action of tetanolysis (which is an antigen) has to do with lipoid substances, and that alcohol-soluble lipoids inhibit the action of tetanolysis; thus confirming Noguchi's findings. Landsteiner (4) found that ether extracts of red blood corpuscles were capable of neutralizing tetanolysin. He also showed that ether-soluble lipoidal substances were involved in serum hemolysis. Bang and Forsmann (5) found that hemolytic activity was due to lipoid substances in the stroma of the red blood cells. These lipoid substances were obtained by extraction of the cells with ether. Upon analysis they found the extracted material to be composed of lecithin, cholesterin, a phosphatid and a cerebroside. This material, when used as an antigen and injected into susceptible called forth the production of hemolytic amboceptors. Working with various serological reactions Kyes (6) (7) demonstrated that lecithin was the ingredient which played the part of complement. Sachs (8) studying hemolytic reactions, determined the importance of lecithin in hemolysis. Pick (10) obtained a precipotinogen by trypsin digestion of egg albumin, and he was unable to demonstrate protein in such an antigen. Some workers confirmed this finding, while others were unsuccessful.

The results obtained by these workers have not gone unchallenged. Nor does their work necessarily indicate anything concerning the chemical nature of antigens. It has been of value, however, in demonstrating the fact that there is a relation between fats and lipoids, and antigens. As to what this relation consists of has been quite obscure, but the evidence is strongly in favor of the theory that serological reactions are manifestations of changes in surface tension and molecular attraction, and not true chemical reactions according to Ehrlich's idea.

The chief objection to the antigenic nature of fats and lipoids has been that the investigators overlooked the possibility of a protein constituent being present. Thus the other extract, or other extracted material, might have contained protein material unsuspected by the workers. Furthermore, the antigens may have been not in a pure lipoid state, nor in a pure protein state, but rather in the condition of a complex conjugated lipoprotein which perhaps was taken up by the solvent. Thus, in their studies on anaphylaxis, Pick and Yamanouchi (11) used, as antigens, alcoholic extracts of horse serum. This extract was evaporated and redissolved until it gave a negative biuret test. They were successful in using

demonstrated that the action of tetanolysis (which is an antigen) has to do with lipoid substances, and that alcohol-soluble lipoids inhibit the action of tetanolysis; thus confirming Noguchi's findings. Landsteiner (4) found that ether extracts of red blood corpuscles were capable of neutralizing tetanolysin. He also showed that ether-soluble lipoidal substances were involved in serum hemolysis. Bang and Forssman (5) found that hemolytic activity was due to lipoid substances in the stroma of the red blood cells. These lipoid substances were obtained by extraction of the cells with ether. Upon analysis they found the extracted material to be composed of lecithin, cholesterin, a phosphatid and a cerebroside. This material, when used as an antigen and injected into susceptible called forth the production of hemolytic amboceptors. Working with various serological reactions Kyes (6) (7) demonstrated that lecithin was the ingredient which played the part of complement. Sachs (8) studying hemolytic reactions, determined the importance of lecithin in hemolysis. Pick (10) obtained a preciptogen by trypsin digestion of egg albumin, and he was unable to demonstrate protein in such an antigen. Some workers confirmed this finding, while others were unsuccessful.

The results obtained by these workers have not gone unchallenged. Nor does their work necessarily indicate anything concerning the chemical nature of antigens. It has been of value, however, in demonstrating the fact that there is a relation between fats and lipoids, and antigens. As to what this relation consists of has been quite obscure, but the evidence is strongly in favor of the theory that serological reactions are manifestations of changes in surface tension and molecular attraction, and not true chemical reactions according to Ehrlich's idea.

The chief objection to the antigenic nature of fats and lipoids has been that the investigators overlooked the possibility of a protein constituent being present. Thus the other extract, or other extracted material, might have contained protein material unsuspected by the workers. Furthermore, the antigens may have been not in a pure lipoid state, nor in a pure protein state, but rather in the condition of a complex conjugated lipo-protein which perhaps was taken up by the solvent. Thus, in their studies on anaphylaxis, Pick and Yamanouchi (11) used, as antigens, alcoholic extracts of horse serum. This extract was evaporated and redissolved until it gave a negative biuret test. They were successful in using

such an antigen to produce anaphylaxis. In a similar manner Bogomolez (12) used alcohol and ether extracts of egg yolks to produce anaphylactic phenomena. It is known, furthermore, that the solubilities of proteins and other substances may be profoundly altered by the presence of lipoids. The existence of a protein-free antigen, therefore, was not established until the recent work of Warden (to be considered further on).

There is, however, one exception, namely the protein-free antigen of Ford and Abel (9). These men, studying the poisons of Amanita phalloides, found that the toxic principle was an active glucoside. Their findings have never been refuted.

Those who favor the protein theory of the nature of antigens are prone to criticise all experiments which attribute fats and lipoids a role in antibody production. The one chief critical point lies in the fact that the lipoidal antigens have not been satisfactorily proven to be protein-free. On the other side, however, is it not fair to inquire if the so-called protein antigens are fat-free? Take practically any native antigen for use in antibody production, and we must concede that there is as definite an amount of lipoid as there is protein; and merely because of the proportionately greater quantity of protein is no reason at all to assume the role of the latter as antigen.

Numerous investigators, assuming that a given substance was an antigen, have endeavored to separate the protein material and to determine what particular fraction of the protein served in the capacity of antigen. In the beginning they find that the substance under consideration is a true antigen. They then, as a rule, proceed to separate the various protein constituents either by fractional precipitation or by enzymotic digestion. And finally they arrive at a point in the analysis beyond which antigens are not found. In reviewing this work one is surprised to find in how few experiments the fat and lipoid constituents have been eliminated. It is possible that these lipoids were sufficiently bound to the proteins as to be precipitated along with them; and due to subsequent procedure, during the separation of the various fractions, the fats were liberated and discarded, or remained in combination with higher fraction. If the antigenic principle was lodged in the fatty portion, this theory would, of course, account for the results obtained. Similarly, in analyses by means of enzymotic reactions, the fats which are present may be digested by

such an antigen to produce anaphylaxis. In a similar manner Rogomoles (12) used alcohol and other extracts of egg yolks to produce anaphylactic phenomena. It is known, furthermore, that the solubilities of proteins and other substances may be profoundly altered by the presence of lipoids. The existence of a protein-free antigen, therefore, was not established until the recent work of Warden (to be considered further on).

There is, however, one exception, namely the protein-free antigen of Ford and Abel (3). These men, studying the poisons of Amanita phalloides, found that the toxic principle was an active glucoside. Their findings have never been refuted.

Those who favor the protein theory of the nature of antigens are prone to criticise all experiments which attribute fats and lipoids a role in antibody production. The one chief critical point lies in the fact that the lipoidal antigens have not been satisfactorily proven to be protein-free. On the other side, however, is it not fair to inquire if the so-called protein antigens are fat-free? Take practically any native antigen for use in antibody production, and we must concede that there is as definite an amount of lipoid as there is protein; and merely because of the proportionately greater quantity of protein is no reason at all to assume the role of the latter as antigen.

Numerous investigators, assuming that a given substance was an antigen, have endeavored to separate the protein material and to determine what particular fraction of the protein served in the capacity of antigen. In the beginning they find that the substance under consideration is a true antigen. They then, as a rule, proceed to separate the various protein constituents either by fractional precipitation or by enzymotic digestion. And finally they arrive at a point in the analysis beyond which antigens are not found. In reviewing this work one is surprised to find in how few experiments the fat and lipoid constituents have been eliminated. It is possible that these lipoids were sufficiently bound to the proteins as to be precipitated along with them; and due to subsequent procedure, during the separation of the various fractions, the fats were liberated and discarded, or remained in combination with higher fraction. If the antigenic principle was lodged in the fatty portion, this theory would, of course, account for the results obtained. Similarly, in analyses by means of enzymotic reactions, the fats which are present may be digested by

lipase. Or the presence of catabolic materials may even produce a change in the configuration of the fat molecule. There is no doubt that some fat-free proteins are antigens, but before we eliminate fats and lipoids from the class of antigens, we must first eliminate them from the various protein substances used as antigens.

Although it would seem that some pure proteins are antigens, it would also be equally apparent that other pure proteins are not antigens. Starin (13), for instance, working with a purified gelatin, was unable to demonstrate antigenic function. It has been suggested that antigenic properties of proteins rest with the aromatic radicals attached to the amino acids. Perhaps these radicals have some affinity for fats and lipoids, so that in the absence of such radicals there is also an absence of fats; i.e., during the process of fractional analysis those portions which bear the aromatic radicals are split off with fats, and the lower fractions, being fat-free, are non-antigenic. The value and importance of the fatty acids in treatment of diseases due to acid fast bacteria have been demonstrated for several years. Thus Chaulmoogra oil, sodium salts of the fatty acids of cod-liver oil are used in treatment of tuberculosis and leprosy (14). Walker and Sweeney (15) showed that the fatty acids obtained from Chaulmoogra oil are specifically bactericidal for acid-fast micro-organisms, but not for non-acid fast micro-organisms. Similarly, Hollman and Dean (16) demonstrated the theraputic value of esters of the fatty acids of Chaulmoogra oil in the treatment of leprosy. These findings lend strength to the theory that fats are intimately concerned in immunological reactions.

The most interesting work which has been done on the antigenic nature of fats is found in the recent experimental data contributed by Warden. This investigator has been studying the problem from the experimental standpoint during a period of several years. In 1915 he suggested, as a result of his studies on the relation between bacterial fats and proteins, that some fats are in a lipoidal combination with protein and are not hydrolysed until the nitrogen portion is thoroughly broken up (17). In the same year, reporting further results, using the gonococcus as the source of his material, he found that the organic nitrogen of the gonococcus did not seem to be altogether available as antigen. The fats of the gonococcus, chemically isolated, possessed a much higher antigenic power (18). Later, (19) (20), it was found that the fats, as

lipase. Or the presence of carbolytic materials may even produce a change in the configuration of the fat molecule. There is no doubt that some fat-free proteins are antigens, but before we eliminate fats and lipoids from the class of antigens, we must first eliminate them from the various protein substances used as antigens.

Although it would seem that some pure proteins are antigens, it would also be equally apparent that other pure proteins are not antigens. Starin (13), for instance, working with a purified gelatin, was unable to demonstrate antigenic function. It has been suggested that antigenic properties of proteins rest with the aromatic radicals attached to the amino acids. Perhaps these radicals have some affinity for fats and lipoids, so that in the absence of such radicals there is also an absence of fats; i.e., during the process of fractional analysis those portions which bear the aromatic radicals are split off with fats, and the lower fractions, being fat-free, are non-antigenic. The value and importance of the fatty acids in treatment of diseases due to acid fast bacteria have been demonstrated for several years. Thus Chaulmoogra oil, sodium salts of the fatty acids of cod-liver oil are used in treatment of tuberculosis and leprosy (14). Walker and Sweeney (15) showed that the fatty acids obtained from Chaulmoogra oil are specifically bactericidal for acid-fast micro-organisms, but not for non-acid fast micro-organisms. Similarly, Hollman and Dean (16) demonstrated the therapeutic value of esters of the fatty acids of Chaulmoogra oil in the treatment of leprosy. These findings lend strength to the theory that fats are intimately concerned in immunological reactions.

The most interesting work which has been done on the antigenic nature of fats is found in the recent experimental data contributed by Warden. This investigator has been studying the problem from the experimental standpoint during a period of several years. In 1915 he suggested, as a result of his studies on the relation between bacterial fats and proteins, that some fats are in a lipoidal combination with protein and are not hydrolyzed until the nitrogen portion is thoroughly broken up (17). In the same year, reporting further results, using the gonococcus as the source of his material, he found that the organic nitrogen of the gonococcus did not seem to be altogether available as antigen. The fats of the gonococcus, chemically isolated, possessed a much higher antigenic power (18). Later, (19) (20), it was found that the fats, as

glycerol estus, did not cause as great a degree of antigen reaction when used in serological tests as did the usual gonococcus antigen; but by saponification of the fats with subsequent isolation of pure nitrogen-free fatty acids, the serological tests gave better results when such fatty acids were used as antigens. Continuing along this line of investigation, Warden (21) determined that the neutral fats of the gonococcus were of little value when used as antigens in complement-fixation tests, but very excellent and specific results were obtained by using the fatty acids, or still better, the alkaline salts of the fatty acids. Due to such findings he concluded that the important factors in such reactions were not only the empirical chemical constitutions of the respective antigens, but that the molecular configuration was as significant and as specific. It has been shown, furthermore, by the same author (22) that if the fats are added to a "solution" of colloidal cholesterol the degree of dispersion of the molecular aggregates of the antigens is greatly increased, thus presenting more surface and producing a more active and sensitive antigen. This finding, of course, adds confirmation to our ideas concerning the colloidal reactions in immunology. By similar methods the same author demonstrated the antigenic nature of the fats from Bacillus typhosus, Bacterium anthracis, pneumococci, streptococci, and red blood cells (23)(24). These fats were used not only as antigens in experiments "in vitio", but were injected into suitable animals, with subsequent production of specific antibodies. Moreover, the antibodies obtained were not only specific, but were also protective against a dose of homologous bacteria which was fatal for a control animal.

The careful work of Warden sheds a vast amount of light on the question of chemical composition of antigens. His results have not yet been confirmed, nor have they been disproved. No one will deny that certain fat-free pure proteins cannot be used as antigens. But no one, on the other hand, can maintain that all antigens are protein.

The object of this work is to study the chemical nature of the antigen or antigens, of Bacillus coli; to determine whether such antigens are protein, fats, carbohydrates, salts, or a mixture of two or more of these; and to analyse, if possible, the antigen in order that we may know of what it is composed. The experimental methods and data are set forth in the succeeding pages.

The careful work of Warden sheds a vast amount of light on the question of chemical composition of antigens. His results have not yet been confirmed, nor have they been disproved. No one will deny that certain fat-free pure proteins cannot be used as antigens. But no one, on the other hand, can maintain that all antigens are protein.

The object of this work is to study the chemical nature of the antigen or antigens of Bacillus coli; to determine whether such antigens are protein, fats, carbohydrates, salts, or a mixture of two or more of these; and to analyze, if possible, the antigen in order that we may know of what it is composed. The experimental methods and data are set forth in the succeeding pages.

EXPERIMENTAL WORK.

The strain of Bacillus coli which was selected was taken from a stock culture. To confirm the identity of the organism it was subjected to the following tests;

a. The organism was a small rod-shaped organism, with morphology, as regards the usual criteria, typical of Bacillus coli.

b. It was negative to Gram's method of staining.

c. It produced abundant acid and gas when grown in nutrient broth containing one per cent lactose. It produced acid and gas also in dextrose and sucrose broth.

d. It did not liquefy gelatin.

e. It was readily agglutinated by high dilution (up to 1:2500) of B. coli antiserum.

In order that a sufficiently large quantity of material be obtained, the cultures were grown in liter flasks. About thirty flasks were used at one time. To each flask was added ten grams of peptone, four grams of sodium chloride and one liter of tap water. This titratable acidity of such medium varied for each lot, but usually fell between 1.5 to 2.0% acid, using phenolphthalein as indicator. This comparatively strong acid reaction was not adjusted, because, as the Bacillus coli produces abundant ammonia in sugar-free protein media, the ammonia gradually neutralizes the acids present. This particular species usually thrives in a medium whose initial reaction is not greater than 2.5% acid. On the other hand, its growth ceases when the reaction reaches an alkalinity of 2.5%. It is obvious, therefore, that with a comparatively strong initial acid reaction, the longer will be the time interval before the maximum alkaline reaction is reached; and presumably, therefore, the greater will be the number of bacteria produced; and it is a large number of bacteria that is necessary for the work. The flasks were not plugged with cotton, but were capped with three thicknesses of wrapping paper. They were then sterilized in the autoclave for two hours at fifteen pounds pressure. Then they were allowed to stand at room temperature for a few days, at the end of which time were discarded any flasks which revealed the presence of bacterial growth. The paper caps were next washed with bichloride of mercury solution 1:1000. The flasks were then inoculated with a broth culture of the

EXPERIMENTAL WORK.

The strain of Bacillus coli which was selected was taken from a stock culture. To confirm the identity of the organism it was subjected to the following tests:

a. The organism was a small rod-shaped organism, with morphology, as regards the usual criteria, typical of Bacillus coli.

b. It was negative to Gram's method of staining.

c. It produced abundant acid and gas when grown in nutrient broth containing one per cent lactose. It produced acid and gas also in dextrose and sucrose broth.

d. It did not liquefy gelatin.

e. It was readily agglutinated by high dilution (up to 1:2500) of B. coli antiserum.

In order that a sufficiently large quantity of material be obtained, the cultures were grown in litre flasks. About thirty flasks were used at one time. To each flask was added ten grams of peptone, four grams of sodium chloride and one liter of tap water. This titratable acidity of such medium varied for each lot, but usually fell between 1.5 to 2.0% acid, using phenolphthalein as indicator. This comparatively strong acid reaction was not adjusted, because, as the Bacillus coli produces abundant ammonia in sugar-free protein media, the ammonia gradually neutralizes the acids present. This particular species usually thrives in a medium whose initial reaction is not greater than 2.5% acid. On the other hand, its growth ceases when the reaction reaches an alkalinity of 2.5%. It is obvious, therefore, that with a comparatively strong initial acid reaction, the longer will be the time interval before the maximum alkaline reaction is reached; and presumably, therefore, the greater will be the number of bacteria produced; and it is a large number of bacteria that is necessary for the work. The flasks were not plugged with cotton, but were capped with three thicknesses of wrapping paper. They were then sterilized in the autoclave for two hours at fifteen pounds pressure. Then they were allowed to stand at room temperature for a few days, at the end of which time were discarded any flasks which revealed the presence of bacterial growth. The paper caps were next washed with bichloride of mercury solution 1:1000. The flasks were then inoculated with a broth culture of the

Bacillus coli. The inoculations were made by the use of a hypodermic syringe and needle. The broth culture was aspirated into a sterile syringe; then, after inserting the needle diagonally through the paper caps, a few drops were forced into the flasks. This method has, to commend it, the advantage that the flasks are never opened for inoculation after having been sterilized. The inoculated flasks were then incubated at 37°C. for about ten days.

In order to separate the bacteria from the fluid, the contents of the flasks were run through a Sharples laboratory "supercentrifuge". This machine is very similar to the ordinary cream separator in construction and mechanism, but is run by steam or compressed air. Sufficient force is obtained to completely separate out all particles in suspension.

This material was washed with sterile physiological sodium chloride solution and recentrifuged. The sediment consists of bacteria with precipitated salts, sulphides, and perhaps other amorphous material. Most of these foreign substances are easily removed by filtration through cotton.

The remaining bacteria were now subjected to saponification in potassium alcoholate, at a temperature of 100°C. for one hour. The resultant material was then acidified with dilute hydrochloric acid. It was expected that this procedure would yield a definite layer of fatty acids which could be removed, but such proved not to be the case. Instead, there was a mass of solid material, including fatty acids, proteins and salts, in a state of very fine suspension, which could not be separated by gravity or by centrifugal force at three thousand revolutions per minute. It was then decided to extract the fatty acids with ether. After shaking up with ether it was found that only about ten percent of the ether could be recovered and that only when comparatively large amounts were used. Most of the ether became a part of the suspensoid mass. The entire mass was then transferred to a ten-inch porcelain evaporating dish, and evaporated at 37°C. for several days, until the moisture content was very low (too low to support the previous suspension). The residual mass was then shaken up with ether and the latter separated, removed and evaporated at room temperature. The residue consisted of a very minute amount of crystalline and amorphous material, presumably fatty acids. The amount was considered too small to work with.

Bacillus coli. The inoculations were made by the use of a hypodermic syringe and needle. The broth culture was aspirated into a sterile syringe; then, after inserting the needle diagonally through the paper caps, a few drops were forced into the flasks. This method has, to commend it, the advantage that the flasks are never opened for inoculation after having been sterilized. The inoculated flasks were then incubated at 37°C. for about ten days.

In order to separate the bacteria from the fluid, the contents of the flasks were run through a Sharples laboratory "supercentrifuge". This machine is very similar to the ordinary cream separator in construction and mechanism, but is run by steam or compressed air. Sufficient force is obtained to completely separate out all particles in suspension.

This material was washed with sterile physiological sodium chloride solution and recentrifuged. The sediment consists of bacteria with precipitated salts, sulphides, and perhaps other amorphous material. Most of these foreign substances are easily removed by filtration through cotton.

The remaining bacteria were now subjected to saponification in potassium alcoholate, at a temperature of 100°C. for one hour. The resultant material was then acidified with dilute hydrochloric acid. It was expected that this procedure would yield a definite layer of fatty acids which could be removed, but such proved not to be the case. Instead, there was a mass of solid material, including fatty acids, proteins and salts, in a state of very fine suspension, which could not be separated by gravity or by centrifugal force at three thousand revolutions per minute. It was then decided to extract the fatty acids with ether. After shaking up with ether it was found that only about ten percent of the ether could be recovered and that only when comparatively large amounts were used. Most of the ether became a part of the suspensoid mass. The entire mass was then transferred to a ten-inch porcelain evaporating dish, and evaporated at 37°C. for several days, until the moisture content was very low (too low to support the previous suspension). The residual mass was then shaken up with ether and the latter separated, removed and evaporated at room temperature. The residue consisted of a very minute amount of crystalline and amorphous material, presumably fatty acids. The amount was considered too small to work with.

Another thirty liter mass was prepared as in the above experiment. The bacteria were washed and separated by the same method, but instead of being saponified, they were treated directly with ether. This ether-bacteria mixture was allowed to stand, with frequent shaking, for six days. The ether was then pipetted off. The residue was again treated with ether in the same manner. These two ether extracts were then evaporated. The residue consisted of brownish-colored fats, some of which appeared crystalline, other amorphous. This material was washed with water, then taken up with ether, the ether removed and evaporated. The weight of the resultant fats amounted to approximately 0.4 gram. The fats were then saponified with potassium alcoholate at 100°C. After acidifying with hydrochloric acid, and cooling, a definite layer of fatty acids was obtained. The alcohol was evaporated to nearly dryness and the fatty acids extracted with ether. This ethereal extract was concentrated to dryness and the fatty acids taken up with alcohol. This was treated with sodium carbonate to convert the fatty acids into the sodium salts. The sodium salts were then drystallized out and redissolved in 100 cc. of alcohol. This constitutes the fatty antigen.

It is desirable, at this point, to examine the antigen to see if any protein material is present. Several different tests were used as follows:

a. The biuret test. To three cubic centimers of antigen was added an equal amount of strong potassium hydroxide solution. The mixture was well shaken and treated with a few drops of very dilute copper sulphate solution. Absence of color change indicated absence of protein.

b. The Kjeldahl method. 10 cc. of the antigen were mixed with 20 cc. of concentrated sulphuric acid. 0.2 gram of copper sulphate was added and the material was boiled gently for ninety minutes. It was then cooled and diluted to 250 cc. with distilled water, then neutralized with hydroxide, adding a slight excess of the alkali. The material was then distilled, the distillate being collected in 25 cc. of N/10 sulphuric acid. When one-half the liquid had passed over, the process was stopped. The acid solution was then neutralized with tenth-normal sodium hydroxide, using congo red as an indicator. An equal amount of alkali was necessary to neutralize the acid, thus indicating the absence of nitrogen in the antigen.

Another thirty liter mass was prepared as in the above experiment. The bacteria were washed and separated by the same method, but instead of being saponified, they were treated directly with ether. This ether-bacteria mixture was allowed to stand, with frequent shaking, for six days. The ether was then pipetted off. The residue was again treated with ether in the same manner. These two ether extracts were then evaporated. The residue consisted of brownish-colored fats, some of which appeared crystalline, other amorphous. This material was washed with water, then taken up with ether, the ether removed and evaporated. The weight of the resultant fats amounted to approximately 0.4 gram. The fats were then saponified with potassium alcoholate at 100°C. After acidifying with hydrochloric acid, and cooling, a definite layer of fatty acids was obtained. The alcohol was evaporated to nearly dryness and the fatty acids extracted with ether. This ethereal extract was concentrated to dryness and the fatty acids taken up with alcohol. This was treated with sodium carbonate to convert the fatty acids into the sodium salts. The sodium salts were then dryetallized out and redissolved in 100 cc. of alcohol. This constitutes the fatty antigen.

It is desirable, at this point, to examine the antigen to see if any protein material is present. Several different tests were used as follows:

a. The biuret test. To three cubic centimeters of antigen was added an equal amount of strong potassium hydroxide solution. The mixture was well shaken and treated with a few drops of very dilute copper sulphate solution. Absence of color change indicated absence of protein.

b. The Kjeldahl method. 10 cc. of the antigen were mixed with 20 cc. of concentrated sulphuric acid. 0.2 gram of copper sulphate was added and the material was boiled gently for ninety minutes. It was then cooled and diluted to 250 cc. with distilled water, then neutralized with hydroxide, adding a slight excess of the alkali. The material was then distilled, the distillate being collected in 25 cc. of N/10 sulphuric acid. When one-half the liquid had passed over, the process was stopped. The acid solution was then neutralized with tenth-normal sodium hydroxide, using congo red as an indicator. An equal amount of alkali was necessary to neutralize the acid, thus indicating the absence of nitrogen in the antigen.

c. The ninhydrin test. To ten cubic centimeters of the antigen was added 0.2 cc. of a one per cent aqueous solution of ninhydrin (triketophydridene hydrate). The mixture was boiled for exactly one minute after the appearance of the first bubbles on the side of the tube. No color change could be detected, even after cooling and standing for four hours. This indicates the absence of alplia amino acids.

d. The cyanide test. About three cubic centimeters of the antigen was evaporated, and the residue fused with metallic sodium. The fused mass was placed in a small amount of distilled water, boiled and filtered. To the filtrate was added a few drops of fenous sulphate, fenie chloride and hydrochloric acid. Absence of a blue coloration indicated an absence of nitrogen.

It is evident that we are dealing with a nitrogen-free substance.

The original bacterial cells, after being doubly extracted with ether, were then suspended in 500 cc. of sterile physiological sodium chloride solution. For preservation, sufficient carbolic acid was added to make a 0.5% solution. The greater part of the solids in this mass are supposedly bacterial proteins and for experimental purposes the mass is called the protein antigen. Obviously it is a bacterial antigen minus the fats.

For use as a control there was next prepared a suspension in physiological saline, of a twenty-four hour agar culture of the same strain of B. Coli. This was made up with 0.5% phenol and it constitutes the B. coli of antigen.

We now have three distinct antigens:

1. Sodium salts of the fatty acids of B. coli.
2. B. coli after being extracted with ether.
3. A simple suspension of B. coli.

It is the purpose now to determine whether or not these antigens are true antigens; i.e., whether or not they give proper antigenic reactions "in vitro", "in vivo", or in both. To this end the antigens were injected into suitable animals, at certain intervals, in order to obtain antibodies.

The fatty antigen was diluted with physiological salt solution, approximately one part of the alcoholic solution to five parts of the saline. This gives a very

c. The ninhydrin test. To ten cubic centimeters of the antigen was added 0.2 cc. of a one per cent aqueous solution of ninhydrin (triketohydrindene hydrate). The mixture was boiled for exactly one minute after the appearance of the first bubbles on the side of the tube. No color change could be detected, even after cooling and standing for four hours. This indicates the absence of alpha amino acids.

d. The cyanide test. About three cubic centimeters of the antigen was evaporated, and the residue fused with metallic sodium. The fused mass was placed in a small amount of distilled water, boiled and filtered. To the filtrate was added a few drops of ferrous sulphate, ferric chloride and hydrochloric acid. Absence of a blue coloration indicated an absence of nitrogen.

It is evident that we are dealing with a nitrogen-free substance.

The original bacterial cells, after being doubly extracted with ether, were then suspended in 500 cc. of sterile physiological sodium chloride solution. For preservation, sufficient carbolic acid was added to make a 0.5% solution. The greater part of the solids in this mass are supposedly bacterial proteins and for experimental purposes the mass is called the protein antigen. Obviously it is a bacterial antigen minus the fats.

For use as a control there was next prepared a suspension in physiological saline, of a twenty-four hour agar culture of the same strain of B. Coli. This was made up with 0.5% phenol and it constitutes the B. coli antigen.

We now have three distinct antigens:
1. Sodium salts of the fatty acids of B. coli.
2. B. coli after being extracted with ether.
3. A simple suspension of B. coli.

It is the purpose now to determine whether or not these antigens are true antigens; i.e., whether or not they give proper antigenic reactions "in vitro", "in vivo", or in both. To this end the antigens were injected into suitable animals, at certain intervals, in order to obtain antibodies.

The fatty antigen was diluted with physiological salt solution, approximately one part of the alcoholic solution to five parts of the saline. This gives a very

opalescent suspension. The protein antigen was used without further dilution, as was also the control B. coli antigen. The course of the immunation is given in the following proctocals:

Table #1. Rabbit injected with fat antigen.

Time interval	Dose in cc.	Weight in grams	Remarks
	0.25	1770	Prompt recovery from injection
5 days	0.5	1800	" " " "
6 days	0.75	1960	Mild signs of shock
6 days	1.00	2040	Prompt recovery
12 days	0.00	2100	Animal bled

Table #2. Rabbit injected with protein antigen.

Time interval	Dose in cc.	Weight in grams	Remarks
	0.25	1630	Moderate shock.
5 days	0.50	1635	" "
6 days	0.50	1680	Severe "
6 days	0.50	1695	Moderate "
12 days	0.00	1730	Animal bled

This animal was the third to be used for protein injections; the other two died immediately after the initial injection, thus demonstrating the possibility of a toxic fraction present in the protein mass.

Table #3. Rabbit injected with B. coli antigen

Time interval	Dose in cc.	Weight in grams	Remarks
	0.5	1690	Prompt recovery
5 days	1.0	1700	" "
6 days	1.0	1700	" "
6 days	1.0	1850	" "
12 days	0.0	1910	Animal bled

The injections were all given by the intravenous method, using the prominent veins in the ears as the sites of injection.

opalescent suspension. The protein antigen was used without further dilution, as was also the control B. coli antigen. The course of the immunization is given in the following protocols:

Table #1. Rabbit injected with fat antigen.

Time interval	Dose in cc.	Weight in grams	Remarks
	0.25	1770	Prompt recovery from injection
5 days	0.5	1800	" " " "
6 days	0.75	1960	Mild signs of shock
6 days	1.00	2040	Prompt recovery
12 days	0.00	2100	Animal bled

Table #2. Rabbit injected with protein antigen.

Time interval	Dose in cc.	Weight in grams	Remarks
	0.25	1630	Moderate shock.
5 days	0.50	1635	" "
6 days	0.50	1690	Severe "
6 days	0.50	1695	Moderate "
12 days	0.00	1730	Animal bled

This animal was the third to be used for protein injections; the other two died immediately after the initial injection, thus demonstrating the possibility of a toxic fraction present in the protein mass.

Table #3. Rabbit injected with B. coli antigen

Time interval	Dose in cc.	Weight in grams	Remarks
	0.5	1690	Prompt recovery
5 days	1.0	1700	" "
6 days	1.0	1700	" "
6 days	1.0	1850	" "
12 days	0.0	1910	Animal bled

The injections were all given by the intravenous method, using the prominent veins in the ears as the sites of injection.

Twelve days after the last injection each rabbit was anesthetized with ether and by the use of a sterile syringe and needle about forty cubic centimeters of blood were aspirated from the heart. The blood was placed in sterile glass tubes, which were then placed in the ice-box until the serum separated from the clot. The clear serum was then pipetted off into sterile tubes and an equal amount of glycerol was added for preservative.

Each of the above antisera was then tested against the three different antigens. The tests used were as follows:

a. Complement fixation
b. Agglutination
c. Precipitin
d. Anaphylaxis

a. The complement fixation test. In testing the antigens and antibodies by the method of fixation of complement, guinea-pig serum diluted with ten parts of physiological saline was used for complement. The anti-human-rabbit hemolytic system was used; i.e., the blood serum of rabbits which were immunized against human red blood corpuscles was used as hemolytic antibody. The following table sets forth the procedure for obtaining the hemolytic antibody:

Table #4. Rabbit injected with hemolytic antigen.

Time interval	Dose in cc.	Weight	Remarks
	2.0 cc.	1680	Prompt recovery
4 days	3.0 cc.	1725	" "
4 days	4.0 cc.	1755	" "
5 days	5.0 cc.	1790	" "
10 days	0.0 cc.	1835	Animal bled

The animal was bled and the serum was obtained and preserved in a manner similar to that previously described. The hemolytic system was titrated as follows: the antigens consisted of a five per cent suspension of washed blood cells; the complement was guinea pig serum diluted with ten parts of salt solution. The hemolytic antibody was then diluted with twenty parts of salt solution and titrated according to the following table:

Twelve days after the last injection each rabbit was anesthetized with ether and by the use of a sterile syringe and needle about forty cubic centimeters of blood were aspirated from the heart. The blood was placed in sterile glass tubes, which were then placed in the ice-box until the serum separated from the clot. The clear serum was then pipetted off into sterile tubes and an equal amount of glycerol was added for preservative.

Each of the above antisera was then tested against the three different antigens. The tests used were as follows:

a. Complement fixation
b. Agglutination
c. Precipitin
d. Anaphylaxis

a. The complement fixation test. In testing the antigens and antibodies by the method of fixation of complement, guinea-pig serum diluted with ten parts of physiological saline was used for complement. The anti-human-rabbit hemolytic system was used; i.e., the blood serum of rabbits which were immunized against human red blood corpuscles was used as hemolytic antibody. The following table sets forth the procedure for obtaining the hemolytic antibody:

Table #4. Rabbit injected with hemolytic antigen.

Time interval	Dose in cc.	Weight	Remarks
	2.0 cc.	1680	Prompt recovery
4 days	3.0 cc.	1725	"
4 days	4.0 cc.	1755	"
5 days	5.0 cc.	1790	"
10 days	0.0 cc.	1835	Animal bled

The animal was bled and the serum was obtained and preserved in a manner similar to that previously described. The hemolytic system was titrated as follows: the antigens consisted of a five per cent suspension of washed blood cells; the complement was guinea pig serum diluted with ten parts of salt solution. The hemolytic antibody was then diluted with twenty parts of salt solution and titrated according to the following table:

Table #5.

Tube	#1	Complement 0.2cc.	antigen 0.2 cc.	+Antibody 0.5.cc.+	No hemolysis
	#2	"	"	" 0.10	" "
	#3	"	"	" 0.15	Partial Hemolysis
	#4	"	"	" 0.20	Complete "
	#5	"	"	" 0.25	" "
	#6	"	"	" 0.30	" "
	#7	"	"	" 0.35	" "
	#8	"	"	" 0.40	" "
Controls	#9	"	"	" ——	No hemolysis
	#10	"	"	" 0.4	" "

The unit of antibody, therefore, was 0.2 cc. Using this unit of antibody and varying the amount of complement, the unit of complement was determined. Two units of complement were used in running the complement-fixation test, also two units of hemolytic antibody. Each bacterial antigen was not tested against each of the three antisera. The results of such tests are shown in the following proctocols: (The preliminary titrations of antigens and antibodies are purposely omitted because they vary with each lot and merely tend to confuse the reader.

Table #6. Protein Antigen and Hemologous Antiserum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	Antigen & Complement	+hemolytic system	Partial hemolysis
2	"	—— + "	+ +	Complete "
3	——	Antigen & "	+ +	" "

Table #5.

Tube #'s	Complement 0.2cc.	Antigen 0.2 cc.	+Antibody	No hemolysis
#2	"	"	0.6 cc.+ " 0.10	" "
#3	"	"	" 0.15	Partial
#4	"	"	" 0.20	Hemolysis Complete "
#5	"	"	" 0.25	" "
#6	"	"	" 0.30	" "
#7	"	"	" 0.35	" "
#8	"	"	" 0.40	" "
Control #9	"	"	" ———	No hemolysis
#10	"	"	" 0.4	" "

The unit of antibody, therefore, was 0.2 cc. Using this unit of antibody and varying the amount of complement, the unit of complement was determined. Two units of complement were used in running the complement-fixation test, also two units of hemolytic antibody. Each bacterial antigen was not tested against each of the three antisera. The results of such tests are shown in the following protocols: (The preliminary titrations of antigens and antibodies are purposely omitted because they vary with each lot and merely tend to confuse the reader.

Table #6. Protein Antigen and Homologous Antiserum.

Tube	56° for 30'	37° for 30'		37° for 30'	Result
1	Antiserum	Antigen &	Complement	+hemolytic system	Partial hemolysis
2	" "	———	+	+	Complete "
3	———	Antigen &	"	+	" "

Table #7. Antigen and Anti-coli Serum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Complete hemolysis
2	"	——— "	" "	" "
3				

Table #8. Protein Antigen and Anti-fat Serum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Hemolysis
2	"	——— "	" "	"

Table #9. Protein Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	+Antigen & Complement	+Hemolytic system	Hemolysis
2	"	——— "	" "	"

Table #10. B.Coli Antigen and Hemologous Antiserum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No hemolysis
2	"	——— "	" "	Complete "
3	———	" "	" "	" "

Table #12. B Coli Antigen and Anti-protein Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	Hemolytic system	Complete hemolysis
2	"	——— "	" "	" "
3	———	" "	" "	" "

Table #7. Antigen and Anti-coli Serum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Complete hemolysis
2	"	——— "	"	" "
3				

Table #8. Protein Antigen and Anti-rat Serum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Hemolysis
2	"	——— "	" "	"

Table #9. Protein Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	+Antigen & Complement	+Hemolytic system	Hemolysis
2	"	——— "	" "	"

Table #10. B.Coli Antigen and Homologous Antiserum.

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No hemolysis
2	"	——— "	" "	Complete "
3	———	"	" "	" "

Table #12. B Coli Antigen and Anti-protein Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	Hemolytic system	Complete hemolysis
2	"	——— "	" "	" "
3	———	"	" "	" "

Table #13. B Coli Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	+Antigen & Complement	+Hemolytis system	Complete hemolysis
2	"	——— "	" "	" "
3	———	" "	" "	" "

Table #14. Fat Antigen and Hemologous Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No Hemolysis
2	"	——— "	" "	Complete "
3	———	" "	" "	" "

Table #15. Fat Antigen and B. Coli Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No hemolysis
2	"	——— "	" "	Complete "
3	———	" "	" "	" "

Table #16. Fat Antigen and Protein Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Complete hemolysis
2	"	——— + "	+ " "	" "
3	———	" "	" "	" "

Table #17. Fat Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	Antigen & Complement	Hemolytic system	Complete hemolysis
2	"	——— "	" "	" "
3	———	" "	" "	" "

Table #13. B Coli Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	+Antigen & Complement	+Hemolytic system	Complete hemolysis
2	"	——— "	"	"
3	———	" "	"	"

Table #14. Rat Antigen and Homologous Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No Hemolysis
2	"	——— "	"	Complete "
3	———	" "	"	" "

Table #15. Rat Antigen and B. Coli Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	No hemolysis
2	"	——— "	"	Complete "
3	———	" "	"	" "

Table #16. Rat Antigen and Protein Antiserum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Antiserum	+Antigen & Complement	+Hemolytic system	Complete hemolysis
2	"	——— + "	+ "	"
3	———	" "	"	" "

Table #17. Rat Antigen and Normal Serum

Tube	56° for 30'	37° for 30'	37° for 30'	Result
1	Serum	Antigen & Complement	Hemolytic system	Complete hemolysis
2	"	——— "	"	"
3	———	" "	"	" "

In the preceding (16-17) protocols all tubes marked #2 are the serum controls, used to show that the serum itself will not fix complement. Similarly the #3 tubes are antigen controls.

The Bacillus coli antigen, when used with a hemologous antiserum, (table 10) gives complete fixation of complement. The same antigen when used with the anti-fat serum, (table 11) gives similar results; but when used with the anti-protein serum, (table 12) one fails to obtain a positive reaction. These findings confirm the idea that fats may act as antigens and lend proof to the specificity of the reaction. The protein antigen, when used with its homologous antiserum (table 6) gave results which were nearly completely positive, but it is worthy of note that the reaction was not as clear cut or as satisfactory as the previous tests. When used with the other antisera (tables 7-9) there was no complete fixation. Thus there is evidence that the fats are true specific antigens and that the protein material plays a minor role, if any.

B. Agglutination tests. Although the presence of agglutinins probably does not indicate protective ability, as Bordet (26) showed that horse serum clumps tetanus bacilli; yet the horse is very susceptible to tetanus, nevertheless, the experimental production of agglutinins in the serum of immunized animals is very indicative of a specific reaction.

The sera obtained from the immunized animals were tested against the B. coli antigen. Ten drops of serum, in various dilutions, were added to ten drops of antigen. The tubes were well shaken and incubated at 37°C. for one hour. The results are recorded as follows:

Complete agglutination	Partial agglutination	No agglutination

Table #18. B. Coli Antigen with Hemologous Antiserum

Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800	1:1200
Result	+	+	+	+	+	+	±	-	-

In the preceding (16-17) protocols all tubes marked #2 are the serum controls, used to show that the serum itself will not fix complement. Similarly the #3 tubes are antigen controls.

The Bacillus coli antigen, when used with a homologous antiserum, (table 10) gives complete fixation of complement. The same antigen when used with the anti-rat serum, (table 11) gives similar results; but when used with the anti-protein serum, (table 12) one fails to obtain a positive reaction. These findings confirm the idea that fats may act as antigens and lend proof to the specificity of the reaction. The protein antigen, when used with its homologous antiserum (table 6) gave results which were nearly completely positive, but it is worthy of note that the reaction was not as clear cut or as satisfactory as the previous tests. When used with the other antisera (tables 7-9) there was no complete fixation. Thus there is evidence that the fats are true specific antigens and that the protein material plays a minor role, if any.

B. Agglutination tests. Although the presence of agglutinins probably does not indicate protective ability, as Bordet (26) showed that horse serum clumps tetanus bacilli; yet the horse is very susceptible to tetanus, nevertheless, the experimental production of agglutinins in the serum of immunized animals is very indicative of a specific reaction.

The sera obtained from the immunized animals were tested against the B. coli antigen. Ten drops of serum, in various dilutions, were added to ten drops of antigen. The tubes were well shaken and incubated at 37°C. for one hour. The results are recorded as follows:

	Complete agglutination		Partial agglutination		No agglutination	
Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800	1:1200
Result	+	+	+	+	+	+	±	-	-

Table #18. B. Coli Antigen with Homologous Antiserum

Table #19. B. Coli Antigen with Antifat Serum

Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800
Result	+	+	+	+	+	±	-	-

Table #20. B. Coli Antigen with Antiprotein Serum

Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700
Result	-	-	-	-	-	-	-

Table #21. B. Coli Antigen with Normal Serum

Dilution of Serum	1:50	1:100	1:200	1:300	1:400	1:500	1:600
Result	-	-	-	-	-	-	-

A control suspension of the B. coli antigen did not show agglutination.

Comparison of the results by use of the complement fixation and agglutination tests give a very striking picture. We find that the fat antigen (table 19) gives rise to specific antibodies and that the protein antigen (table 20) is of negligible importance in this respect.

C. The Precipitin Reaction. This test has to do with the precipitation of solid matter out of solution of invisible "colloidal suspension". Obviously the B. coli and protein antigens cannot be used, but the fat antigen is suitable for the reaction. The most desirable antigen is one which is perfectly clear and transparent. Clarity, in a fat suspension, depends upon the state of the fat particles, so that the greater the degree of dispersion, the greater the clarity. A perfectly clear antigen was not obtained, but a sufficient degree of dispersion was made to run the tests. Ten drops of serum was layered under ten drops of antigen, and after the incubation at 37°C for fifteen minutes, the following results were obtained.

Table #22. Fat Antigen and Homologous Antiserum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	+	+	+	+	±	-	-

Table #19. B. Coli Antigen with Antiserum

Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800
Result	+	+	+	+	+	±	-	-

Table #20. B. Coli Antigen with Antiprotein Serum

Dilution of Serum	1:100	1:200	1:300	1:400	1:500	1:600	1:700
Result	-	-	-	-	-	-	-

Table #21. B. Coli Antigen with Normal Serum

Dilution of Serum	1:50	1:100	1:200	1:300	1:400	1:500	1:600
Result	-	-	-	-	-	-	-

A control suspension of the B. coli antigen did not show agglutination.

Comparison of the results by use of the complement fixation and agglutination tests give a very striking picture. We find that the fat antigen (table 19) gives rise to specific antibodies and that the protein antigen (table 20) is of negligible importance in this respect.

C. The Precipitin Reaction. This test has to do with the precipitation of solid matter out of solution of invisible "colloidal suspension". Obviously the B. coli and protein antigens cannot be used, but the fat antigen is suitable for the reaction. The most desirable antigen is one which is perfectly clear and transparent. Clarity, in a fat suspension, depends upon the state of the fat particles, so that the greater the degree of dispersion, the greater the clarity. A perfectly clear antigen was not obtained, but a sufficient degree of dispersion was made to run the tests. Ten drops of serum was layered under ten drops of antigen, and after the incubation at 37°C for fifteen minutes, the following results were obtained.

Table #22. Fat Antigen and Homologous Antiserum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	+	+	+	+	±	-	-

Table #23. Fat Antigen and Anti-coli Serum.

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	+	+	+	±	-	-	-

Table #24. Fat Antigen and Antiprotein Serum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	-	-	-	-	-	-	-

Table #25. Fat Antigen and Normal Serum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	-	-	-	-	-	-	-

+ = Complete precipitation
± = Partial "
- = No "

The results are very indicative of a specific precipitatin reaction, in which the fats play the most important part. Too much weight, however, should not be attached to these reactions of precipitins because in some cases (preliminary titrations) spontaneous reaction occurred. Such reactions also frequently occurred after the fifteen minute incubation period. This was probably due to the unstable dispersoid phase of the antigen, so that it was very susceptible to the reactions of the various sera.

D. Anaphylactic Reactions. It was found by trial that 1.0 cc. of a 1:10 dilution of the fatty antigen was usually fatal to guinea pigs when injected directly into the heart, but the pigs could withstand the same amount of a 1:20 dilution. This amount, therefore, was used as the "toxic" dose. The animals were sensitized by intracardiac injections of 1.0 cc. of a 1:500 dilution. They promptly recovered from the effects of the injections and gradually gained in weight. Twelve days after the sensitizing dose the pigs received the "toxic" dose. The results are recorded as follows:

Table #23. Rat Antigen and Anti-colit Serum.

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	+	+	+	±	-	-	-

Table #24. Rat Antigen and Antiprotein Serum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	-	-	-	-	-	-	-

Table #25. Rat Antigen and Normal Serum

Dilution of Serum	1:25	1:50	1:75	1:100	1:125	1:150	1:175
Result	-	-	-	-	-	-	-

+ = Complete precipitation
± = Partial "
- = No "

The results are very indicative of a specific precipitatin reaction, in which the fats play the most important part. Too much weight, however, should not be attached to these reactions of precipitins because in some cases (preliminary titrations) spontaneous reaction occurred. Such reactions also frequently occurred after the fifteen minute incubation period. This was probably due to the unstable dispersoid phase of the antigen, so that it was very susceptible to the reactions of the various sera.

D. Anaphylactic Reactions. It was found by trial that 1.0 cc. of a 1:10 dilution of the fatty antigen was usually fatal to guinea pigs when injected directly into the heart, but the pigs could withstand the same amount of a 1:20 dilution. This amount, therefore, was used as the "toxic" dose. The animals were sensitized by intracardiac injections of 1.0 cc. of a 1:500 dilution. They promptly recovered from the effects of the injections and gradually gained in weight. Twelve days after the sensitizing dose the pigs received the "toxic" dose. The results are recorded as follows:

Table #26. Anaphylactic Reactions.

	Temperature	Symptoms
Before injection	102.2°F	
5 min. after "	100.4	Slight motor paralysis of hind legs
10 " " "	100.0	Increased motor paralysis of hind legs
15 " " "	99.6	Dyspnoea
20 " " "	99.0	
25 " " "	99.8	Paralysis decreasing
30 " " "	100.4	Dyspnoea disapeared
35 " " "	101.0	Paralysis disappeared

A normal, non-sensitized animal was then injected with the "toxic" dose. The results were as follows:

Table #27

	Temperature	Symptoms
Before injection	100.8	None
5 min. after "	99.2	"
10 " " "	99.4	"
15 " " "	99.8	"
20 " " "	100.0	"
25 " " "	100.4	"
30 " " "	100.4	"
35 " " "	100.4	"

These experiments were repeated three times and the results were practically the same with each set. The sensitized animals always showed a marked sub-normal temperature, with paralysis of the hind legs. The acted as if they were trying to "huddle up" and the hair "stood out". The underlying principles of anaphylaxis are as yet very obscure, but it is very doubtful that proteins

Table #26. Anaphylactic Reactions.

	Temperature	Symptoms
Before injection	102.2°F	
5 min. after "	100.4	Slight motor paralysis of hind legs
10 " " "	100.0	Increased motor paralysis of hind legs
15 " " "	99.6	Dyspnoea
20 " " "	99.0	
25 " " "	99.8	Paralysis decreasing
30 " " "	100.4	Dyspnoea disappeared
35 " " "	101.0	Paralysis disappeared

A normal, non-sensitized animal was then injected with the "toxic" dose. The results were as follows:

Table #27

	Temperature	Symptoms
Before injection	100.8	None
5 min. after "	99.2	"
10 " " "	99.4	"
15 " " "	99.8	"
20 " " "	100.0	"
25 " " "	100.4	"
30 " " "	100.4	"
35 " " "	100.4	"

These experiments were repeated three times and the results were practically the same with each set. The sensitized animals always showed a marked sub-normal temperature, with paralysis of the hind legs. The acted as if they were trying to "muddle up" and the hair "stood out". The underlying principles of anaphylaxis are as yet very obscure, but it is very doubtful that proteins

are essential for sensitization. Either the fats may split off a molecule that is toxic, thus giving rise to the reaction according to Vaughn's idea, or the reaction may be due to variations in surface energy and molecular attraction.

So far, then, we have prepared an antigen from the fats of Bacillus coli and have demonstrated the ability of this antigen to cause the production of various serological reactions when injected into suitable animals. Our interest now turns toward the chemical composition of the antigen and the attempt is made to analyse the fatty mass.

In the previous preparation of the fatty antigen about 0.4 gram of fat was obtained from thirty litres of broth culture. In order to obtain a larger amount of material two hundred litres of broth were made and inoculated as before. The bacilli were collected in the same manner. The bacteria mass was then transferred to a litre flask and covered with ether. The flask was then fitted with a reflux condenser and heated to 50°C on an automatic electric water-bath. The heat was applied continuously for a period of forty-eight hours. At the end of this time the flask and contents were cooled and the ether was pipetted off into another flask. This latter flask was tightly stopped and placed in the icebox. The bacterial residue was extracted again in the same manner and the ether extract was added to the first lot. A total of five extractions was made. The mixed ether from the five extractions was then evaporated under reduced pressure (obtained by attaching an ordinary filter pump), at a temperature of 25°C. The remaining fatty mass was then treated with potassium alcoholate. The flask was fitted with a reflux condenser and the fats were saponified at a temperature of 90°C. The flask and contents were cooled and acidified with hydrochloric acid. The flask was then attached to the reflux condenser and the fluid was evaporated at 70°C under reduced pressure. The concentrated mass of fatty acids was then taken up with ether. The ethereal solution, containing the fatty acids, was pipetted off and the ether evaporated at room temperature under reduced pressure. The mass of residual fatty acids weighed 2.873 grams. A portion of these acids was fluid at 30°C, but the remainder were solid up to about 65°C. The acids were then subjected to distillation in steam. The distillate was treated with solid sodium chloride and then shaken up with ether. The ether layer was removed and evaporated in vacuo at room temperature. There was a very slight trace of residue, too

are essential for sensitisation. Either the fats may split off a molecule that is toxic, thus giving rise to the reaction according to Vaughn's idea, or the reaction may be due to variations in surface energy and molecular attraction.

So far, then, we have prepared an antigen from the fats of Bacillus coli and have demonstrated the ability of this antigen to cause the production of various serological reactions when injected into suitable animals. Our interest now turns toward the chemical composition of the antigen and the attempt is made to analyse the fatty mass.

In the previous preparation of the fatty antigen about 0.4 gram of fat was obtained from thirty litres of broth culture. In order to obtain a larger amount of material two hundred litres of broth were made and inoculated as before. The bacilli were collected in the same manner. The bacteria mass was then transferred to a Nitre flask and covered with ether. The flask was then fitted with a reflux condenser and heated to 50°C on an automatic electric water-bath. The heat was applied continuously for a period of forty-eight hours. At the end of this time the flask and contents were cooled and the ether was pipetted off into another flask. This latter flask was tightly stopped and placed in the icebox. The bacterial residue was extracted again in the same manner and the ether extract was added to the first lot. A total of five extractions was made. The mixed ether from the five extractions was then evaporated under reduced pressure (obtained by attaching an ordinary filter pump), at a temperature of 25°C. The remaining fatty mass was then treated with potassium alcoholate. The flask was fitted with a reflux condenser and the fats were saponified at a temperature of 90°C. The flask and contents were cooled and acidified with hydrochloric acid. The flask was then attached to the reflux condenser and the fluid was evaporated at 70°C under reduced pressure. The concentrated mass of fatty acids was then taken up with ether. The ethereal solution, containing the fatty acids, was pipetted off and the ether evaporated at room temperature under reduced pressure. The mass of residual fatty acids weighed 2.873 grams. A portion of these acids was fluid at 30°C, but the remainder were solid up to about 65°C. The acids were then subjected to distillation in steam. The distillate was treated with solid sodium chloride and then shaken up with ether. The ether layer was removed and evaporated in vacuo at room temperature. There was a very slight trace of residue, too

small to weigh with any degree of accuracy. This represents the volatile constituent.

The remaining mass was redissolved in alcohol and made slightly neutral by adding potassium alcoholate, using phenolphthalein as indicator. The solution was diluted with distilled water to about 100 cc. 30 cc. of a ten per cent solution of lead acetate was diluted to 150 cc. with water and boiled. The hot solution was run into the soap solution, constantly shaking the latter so that the lead soaps would adhere to the sides of the flask. The flask was then filled with hot water and then allowed to cool. The lead salts all adhered to the glass, leaving a clear supernatant fluid which was decanted off. The soap was then shaken up with ether at 37°C. The ethereal solution was then cooled and filtered. The filtrate contains the lead salts of the liquid fatty acids. This filtrate was then shaken with twenty per cent hydrochloric acid, to decompose the lead salts. As the fatty acids are set free from the lead salts, they are taken up by the ether. The ethereal layer was removed; washed with water until the washings were free from acid; and the ether then evaporated. The remaining liquid fatty acid weighed 1.021 gram. This acid solidified when cooled on ice and melted at 13°C. The neutralization value was then found as follows: 1.021 grams were neutralized by 34.9 cc. of N/10 KOH. This is equivalent to 34.1 cc. per gram of acid, or 191 grams KOH. The neutralized acids were now acidified with 20% hydrochloric acid and shaken out with ether. The ether was then evaporated and the iodine value was obtained as follows (Hubb's process):

The iodine solution is prepared by dissolving 13.5 grams iodine in 250 cc. of 95% alcohol, and by dissolving 15 grams of mercuric chloride in 250 cc. of 95% alcohol and mixing these two solutions. A standardized solution of sodium thiosulphate was prepared by dissolving 24 grams of the salt in one liter of water. 0.2 gram of iodine and 1.0 gram of potassium iodide are dissolved in about 50 cc. of water. This solution is titrated to neutrality by the thiosulphate solution, using starch solution as indicator. It was found that 15.4 cc. of thisulphate solution was equivalent to 0.2 gram of iodine. The 1.021 gram of fatty acid is dissolved in 10 cc. of chloroform and 50 cc. of iodine solution added. The solution was allowed to stand for fifteen hours. 20 cc. of 10% potassium iodide solution was added, and the volume made up to 500 cc. with water. This solution was then titrated with the thiosulphate solution. It was

small to weigh with any degree of accuracy. This represents the volatile constituent.

The remaining mass was readily dissolved in alcohol and made slightly neutral by adding potassium alcoholate, using phenolphthalein as indicator. The solution was diluted with distilled water to about 100 cc. 30 cc. of a ten per cent solution of lead acetate was diluted to 150 cc. with water and boiled. The hot solution was run into the soap solution, constantly shaking the latter so that the lead soaps would adhere to the sides of the flask. The flask was then filled with hot water and then allowed to cool. The lead salts all adhered to the glass, leaving a clear supernatant fluid which was decanted off. The soap was then shaken up with ether at 37°C. The ethereal solution was then cooled and filtered. The filtrate contains the lead salts of the liquid fatty acids. This filtrate was then shaken with twenty per cent hydrochloric acid, to decompose the lead salts. As the fatty acids are set free from the lead salts, they are taken up by the ether. The ethereal layer was removed; washed with water until the washings were free from acid; and the ether then evaporated. The remaining liquid fatty acid weighed 1.021 gram. This acid solidified when cooled on ice and melted at 13°C. The neutralization value was then found as follows: 1.021 grams were neutralized by 34.9 cc. of N/10 KOH. This is equivalent to 34.1 cc. per gram of acid, or 191 grams KOH. The neutralized acids were now acidified with 20% hydrochloric acid and shaken out with ether. The ether was then evaporated and the iodine value was obtained as follows (Hubb's process):

The iodine solution is prepared by dissolving 13.5 grams iodine in 250 cc. of 95% alcohol, and by dissolving 15 grams of mercuric chloride in 250 cc. of 95% alcohol and mixing these two solutions. A standardized solution of sodium thiosulphate was prepared by dissolving 24 grams of the salt in one liter of water. 0.2 gram of iodine and 1.0 gram of potassium iodide are dissolved in about 50 cc. of water. This solution is titrated to neutrality by the thiosulphate solution, using starch solution as indicator. It was found that 15.4 cc. of thiosulphate solution was equivalent to 0.2 gram of iodine. The 1.021 gram of fatty acid is dissolved in 10 cc. of chloroform and 50 cc. of iodine solution added. The solution was allowed to stand for fifteen hours. 20 cc. of 10% potassium iodide solution was added, and the volume made up to 500 cc. with water. This solution was then titrated with the thiosulphate solution. It was

found that 0.863 gram of iodine was absorbed. 100 grams of the fatty acid, therefore, would absorb 84.6 grams of iodine, the iodine value, then, being 84.6. The neutralization value of oleic acid is 198; its melting point is 14°C; and its iodine value 90. We are dealing with an unsaturated liquid fatty acid, whose melting point is 13°C; neutralization value 191; and iodine value 84.6. Oleic acid is an unsaturated liquid fatty acid with the above given constants. The unknown approaches very closely to oleic acid in these three values, and it is permissible, under the circumstances, to consider such unknown as oleic acid.

The remaining insoluble lead soaps were now decomposed with hydrochloric acid and the liberated fatty acids were extracted with ether. The ethereal portion was pipetted off, washed with water, and the ether evaporated. The residue weighed 1.84 grams. The iodine value was 2.3, due, probably, to admixture of the unsaturated acid previously described. We are dealing, therefore, with two or more saturated fatty acids, since it was found that about one-half of the mass melted at a temperature of about 32°C, while the remaining portion did not melt until heated to a temperature of 68°C. The saturated fatty acids which have melting points between 30°C and 35°C are confined to one member, capric acid. The saturated acids which melt between 65°C and 70°C are confined, likewise, to one member, stearic acid. To separate these acids, when one has such a very small amount of material, is hardly a feasible procedure. Nor can one determine the analysis by the melting points, since such acids form entectic mixtures which often have a melting point higher or lower than any of the individual constituents (27).

SUMMARY

The Bacillus coli has been divided chemically into two parts, one of a protein nature, the other fatty. The protein portion alone is not an antigen in the sense that it will give rise to the specific antibodies of the bacillus coli.

The fatty portion, on the contrary, contains the substances essential for the production of specific antigenic reactions.

found that 0.865 gram of iodine was absorbed. 100 grams of the fatty acid, therefore, would absorb 84.6 grams of iodine, the iodine value, then, being 84.6. The neutralization value of oleic acid is 198; its melting point is 14°C; and its iodine value 90. We are dealing with an unsaturated liquid fatty acid, whose melting point is 13°C; neutralization value 191; and iodine value 84.6. Oleic acid is an unsaturated liquid fatty acid with the above given constants. The unknown approaches very closely to oleic acid in these three values, and it is permissible, under the circumstances, to consider such unknown as oleic acid.

The remaining insoluble lead soaps were now decomposed with hydrochloric acid and the liberated fatty acids were extracted with ether. The ethereal portion was pipetted off, washed with water, and the ether evaporated. The residue weighed 1.84 grams. The iodine value was 2.3, due, probably, to admixture of the unsaturated acid previously described. We are dealing, therefore, with two or more saturated fatty acids, since it was found that about one-half of the mass melted at a temperature of about 32°C, while the remaining portion did not melt until heated to a temperature of 68°C. The saturated fatty acids which have melting points between 30°C and 35°C are confined to one member, capric acid. The saturated acids which melt between 65°C and 70°C are confined, likewise, to one member, stearic acid. To separate these acids, when one has such a very small amount of material, is hardly a feasible procedure. Nor can one determine the analysis by the melting points, since such acids form eutectic mixtures which often have a melting point higher or lower than any of the individual constituents (27).

SUMMARY

The Bacillus coli has been divided chemically into two parts, one of a protein nature, the other fatty. The protein portion alone is not an antigen in the sense that it will give rise to the specific antibodies of the bacillus coli.

The fatty portion, on the contrary, contains the substances essential for the production of specific antigenic reactions.

It has been analysed to show the following approximate constitution:

a. Volatile fats, trace
b. Oleic acid
c. Capric acid
d. Stearic acid

CONCLUSIONS.

The specific antigens of the Bacillus coli are chemical entities bound up in the fats of the organism.

The proteins of the organism are not concerned in the specificity of the antigens.

The fats of the antigens consist of volatile, saturated and unsaturated acids.

It has been analyzed to show the following approximate constitution:

a. Volatile fats, trace
b. Oleic acid
c. Capric acid
d. Stearic acid

CONCLUSIONS.

The specific antigens of the Bacillus coli are chemical entities bound up in the fats of the organism.

The proteins of the organism are not concerned in the specificity of the antigens.

The fats of the antigens consist of volatile, saturated and unsaturated acids.

BIBLIOGRAPHY.

1. Jobling & Peterson, Quotes from Zinsser, "Infection and Resistance", 1918, p. 39.
2. Noguchi, H., Univ. Pa. Med. Bull., Nov. 1902.
3. Muller, Centrabbl. f. Bakt., 34, 1903, p. 562.
4. Landsteiner, " " " , 39, 1905, p. 318.
5. Bang & Forsmann " " " , 40, 1906, p. 151.
6. Kyes, Berl, klin. Wchnschi, 1902, pp. 866 & 918.
7. " " " " 1904, p. 494.
8. Sachs, Wien, " " 1905, p. 901.
9. Ford & Abel, Jour. Biol. Chem., 1902, 2, p. 273.
10. Pick, E., Kolle u. Wassermann Handbuch Vol. 2.
11. " u. Yamanouchi, Zeitche. f. Gumuntatschforch, 1, 1909.
12. Bogomolez, " " " 5, 1910.
13. Starin, W., Jour. Infec. Dis., 1918, 23, p. 139.
14. Editorial, Jour. Am. Med. Assoc. 1919, 73, p. 609.
15. Walker & Sweeney, Jour. Infec. Dis., 1920, p. 256.
16. Hollmann & Dean, Jour. Cut. Dis., 1919, 37, p. 367.
17. Warden, C.C., Jour. Infec. Dis., 1915, 16, p. 426.
18. " " " Jour. Am. Med. Assoc., 1915, 65, p.2080.
19. " " " " " " 1917, 68, p. 432.
20. " " " & Schmidt, L.E., Jour. Lab. & Clin. Med., 1916, 1, p.333.
21. " " " Jour. Infec. Dis., 1918, 22, p. 133.
22. " " " " " " 1918, 23, p. 504.
23. " " " " " " 1919, 24, p. 285.
24. " " " " " " 1919, 25, p. 399.
25. Vaughn, V., Protein Split Products, Lea & Febiger, 1912.
26. Bordet, J., Am. de l'inst. Pasteur, 1896, 10, p. 193.
27. Lewkowitsch, Chemical Technology of Oils, Fats & Waxes. 3rd ed. vol. 1.

AUTOBIOGRAPHY

I, Edward Everett Hale Boyer, was born in Lynn, Massachusetts, February 15, 1893. I received all of my secondary school education in the public and high schools of the City of Lynn; my undergraduate education at the Massachusetts Agricultural College, from which I obtained the Degree of Bachelor of Science in 1916. I pursued graduate studies at the Ohio State University, from which I obtained the Degree of Master of Science in 1917 and the Degree of Doctor of Philosophy in 1920.

BIBLIOGRAPHY.

1. Jobling & Peterson, Cuvoes from Zimmer, "Infection and Resistance", 1918, p. 39.
2. Noguchi, H., Univ. Pa. Med. Bull., Nov. 1902.
3. Muller, Centralbbl. f. Bakt., 34, 1903, p. 562.
4. Landsteiner, " " " 32, 1905, p. 318.
5. Bang & Forsmann " " " 10, 1906, p. 151.
6. Kyes, Bonl, Klin. Wchnschft, 1902, pp. 856 & 918.
7. " " " " 1904, p. 494.
8. Sachs, Wien, " " 1905, p. 901.
9. Ford & Abel, Jour. Biol. Chem., 1902, 2, p. 273.
10. Pick, E., Kolle u. Wassermann Handbuch Vol. 2.
11. " u. Yamamouchi, Zeitschr. f. Immunitatsforsch., 1, 1909.
12. Bogomolez, " " " " 5, 1910.
13. Starin, W., Jour. Infec. Dis., 1918, 23, p. 139.
14. Editorial, Jour. Am. Med. Assoc. 1919, 73, p. 609.
15. Walker & Sweeney, Jour. Infec. Dis., 1920, p. 258.
16. Hollmann & Dean, Jour. Cut. Dis., 1919, 37, p. 367.
17. Warden, C.C., Jour. Infec. Dis., 1915, 16, p. 426.
18. " " " " Jour. Am. Med. Assoc., 1915, 65, p. 2060.
19. " " " " " " " " 1917, 68, p. 432.
20. " " & Schmidt, L.E., Jour. Lab. & Clin. Med., 1916, 1, p. 353.
21. " " " Jour. Infec. Dis., 1918, 22, p. 133.
22. " " " " " " 1918, 23, p. 504.
23. " " " " " " 1919, 24, p. 285.
24. " " " " " " 1919, 25, p. 399.
25. Vaughn, V., Protein Split Products, Lea & Febiger, 1913.
26. Bordet, J., Am. de l'Inst. Pasteur, 1895, 10, p. 193.
27. Lewkowitsch, Chemical Technology of Oils, Fats & Waxes. 3rd ed. vol. 1.

AUTOBIOGRAPHY

I, Edward Everett Hale Boyer, was born in Lynn, Massachusetts, February 15, 1893. I received all of my secondary school education in the public and high schools of the City of Lynn; my undergraduate education at the Massachusetts Agricultural College, from which I obtained the Degree of Bachelor of Science in 1916. I pursued graduate studies at the Ohio State University, from which I obtained the Degree of Master of Science in 1917 and the Degree of Doctor of Philosophy in 1920.