STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

X. Immunological Distinctions between the Hemotoxin and the "Protein Fraction" of the Pneumococcus Cell.

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INTRODUCTION.

It is known (1, 2) that the hemotoxin and the "protein fraction"[1] of the pneumococcus cell possess the following properties in common: (1) both are cellular constituents of Pneumococcus and are present in solutions prepared from pneumococcus cells; (2) both react with species-specific, rather than type-specific antibodies; (3) antibodies (antihemotoxin and precipitins) reactive with both of them are present in immune serum produced by injection of solutions of pneumococcus cells. The object of the present paper is to determine whether or not they represent distinct and separate antigenic constituents of Pneu-

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[1] The term "protein" or "protein fraction" of the pneumococcus cell is limited in this paper to the protein substances which react with the species-specific antiprotein precipitins which are present in immune serum produced by immunization with solutions of pneumococcus cells (3, 4). These substances, as pointed out by Avery and Heidelberger (5, 6), include the bacterial proteins (mainly nucleoprotein and mucoid) which are precipitated in the cold by acetic acid, and thus may include a number of separate but unrecognized antigens which give rise to individual antiprotein precipitins. For clearness of expression in this paper, it is desirable to use the term "protein" in the inclusive sense of Avery and Heidelberger (6) although it is probable that the hemotoxin itself, like all true antigens, is also protein in nature; as a matter of fact, it is also precipitated, at least in part, by the acetic acid treatment since the reversible oxidation products of the hemotoxin are demonstrable in some solutions of pneumococcus "protein" (7).

mococcus, which are immunologically reactive with distinct and separate antibodies.

The presence of protein precipitins in immune sera is a factor always to be considered in connection with the neutralization of primarily toxic antigens, and a distinction between the antibody which neutralizes the hemotoxin and other antibodies present in the serum is particularly important in view of the fact that the antihemotoxin can be produced by immunization with hemolytically inactive forms of the hemotoxin (2, 8). Hence, the immediate object of this study is to establish pneumococcus hemotoxin as an integral antigenic substance in order to show that the preceding reports (2, 8, 9) had dealt with a true antigen. However, proof of the individuality of the hemotoxin is also of interest from a more general point of view since it adds another definite antigen to the list of constituents included in the "antigen mosaic" of the pneumococcus cell.

EXPERIMENTAL.

Methods.—The methods were essentially the same as described in preceding papers (2, 8, 9).

Differences in the Effect of Previous Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

The following experiment dealt with the difference in the effect of previous exposure to 55°C. upon the antibody-invoking properties of the hemotoxin and of the "protein" fraction of Pneumococcus. One series of rabbits was immunized with bacterial extract which had been heated at 55°C. for 10 minutes, a treatment which previous experiments (8) had indicated was sufficient to destroy the antibody-invoking property of the hemotoxin; a control series was immunized with the unheated extract. In order to make the comparison more convincing, different amounts of pneumococcus cell solution were injected into corresponding animals in the two series and the immunization of some animals was carried over a long period. Bleedings were made after each course of injections, and the sera tested for antihemotoxin and antiprotein precipitin. The objects of the experiment are satisfied by presenting in Table I the results obtained with the animals immunized with 0.2 cc. doses of the pneumococcus solution.

The results (Table I) of this experiment reveal a distinct difference in the effect of short exposure to 55°C. upon the antibody-invoking

properties of the hemotoxin and the "protein fraction" of Pneumococcus. The heating treatment only slightly weakened the effective-

TABLE I.

Influence of Exposure to 55°C. upon the Antibody-Invoking Properties of Pneumococcus Hemotoxin and of Pneumococcus "Protein."

Animals immunized with pneumococcus cell solution	Serum	Antihemtoxin[1]		Antiprotein precipitins[2]	
		Hemolysis by 5 "units" of hemotoxin, which had been incubated in presence of serum before addition of blood cells		Precipitation of protein from solutions of a heterologous type of pneumococci	
		Amount of serum, *cc.*		Dilution of antigen	
		0.05	0.01	1/10	1/50
		Hemolysis	Hemolysis	Precipitation	Precipitation
Unheated	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	0	*	++	+
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	0	0	+++	+++
Heated 10 min. at 55°C.	Normal serum; before immunization	****	****	0	0
	Immune serum; after 1 course of 6 injections of 0.2 cc. of the bacterial solution	****	****	+	0
	Immune serum; after 6 courses of 6 injections of 0.2 cc. of the bacterial solution	****	****	+++	+++

[1]0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.
[2]0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.

ness of the antigen (or antigens) related to the antiprotein precipitin; but completely destroyed the antibody-invoking property of the hemo-

toxin. A quantitative loss in the antigenic capacity of the heated "protein" was evident throughout the experiment; *i.e.*, either a larger amount of the bacterial solution, or a more prolonged immunization was always required with the heated than with the unheated bacterial solution in order to obtain a serum of the same antiprotein precipitin content. However, these quantitative differences became less as the immunization was continued, and (as shown in Table I) after several courses of injections the sera obtained by use of the heated (55°C. for 10 minutes) bacterial extract were equal in antiprotein precipitating power to those obtained by injection of the unheated material.

Quite different relations obtained with the hemotoxin. In agreement with the results of a previous investigation (8) the heating treatment seemed to result in an absolute loss of its antibody-invoking property, for even after prolonged immunization, no antihemotoxin could be detected in the serum of animals injected with the heated pneumococcus solutions. This distinction between the effect of heat upon the antibody-invoking properties of the hemotoxin and the "protein fraction" is particularly convincing in view of the quantitative control of the amount of material employed in the immunizations.

Differences in the Influence of Previous Exposure to 55°C. upon the Antibody-Combining Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

That the hemotoxin completely loses its antibody-combining property when heated at 55°C. for 10 minutes has been shown in a previous paper,[2] no antihemotoxin being combined when heated pneumococcus hemotoxin was added to immune serum. A number of experiments were made to determine whether or not the same heating treatment affects the antibody-combining property of the protein fraction. The pneumococcus cell solution was heated for 10 minutes at 55°C. without causing any visible clouding or precipitation in the clear solution; as far as we could determine this heated solution (in which the antibody-combining property of the hemotoxin had been destroyed) was precipitated by antiprotein serum to exactly the same degree as was unheated pneumococcus solution in parallel titrations against the same immune serum.

From the results of the above experiment, it is evident that exposure of a solution containing both hemotoxin and "protein" to a tempera-

[2] Neill, Fleming, and Gaspari (8), Table IV.

ture of 55°C. for 10 minutes inactivates the antibody-combining property as well as the antibody-invoking property of the hemotoxin, but destroys neither of these properties of the "protein." Since these two properties are the fundamental properties of all true antigens, the loss of both of them by the hemotoxin and the loss of neither by the "protein" indicate that although contained in the same bacterial solution, the hemotoxin represents an antigenic constituent distinct from those which give rise to the antiprotein precipitins.

Differences in the Influence of Treatment with High Concentrations of H_2O_2 upon the Antigenic Properties of Pneumococcus Hemotoxin and of Pneumococcus Protein.

A distinction between the hemotoxin and the protein fraction was also made upon the basis of the effect of treatment with high concentrations of H_2O_2 upon their antigenic properties. It was found that treatment with this oxidizing agent which destroyed the antigenic properties of the hemotoxin (8) did not inactivate the protein fraction; the treated solutions, although containing no antigenically effective hemotoxin, still retained both the *in vivo* property of invoking the production of antiprotein precipitins and the *in vitro* property of reacting with antiprotein precipitins.

Production of Immune Serum Containing Antiprotein Precipitins but No Antihemotoxin by Immunization with Pneumococcus Cell Solutions from Which the Hemotoxin Had Been Removed by Absorption with Red Blood Cells.

The experiments consisted of a comparison of the antihemotoxin and antiprotein precipitin content of the immune serum obtained from two series of rabbits: (1) the first series, immunized with "unabsorbed" pneumococcus cell solution containing both the hemotoxin and the "protein fraction;" (2) the second series, immunized with the same amounts of pneumococcus cell solution from which the hemotoxin had been removed by previous absorption.

Immunization.—Each animal in the two series received six daily subcutaneous injections of 0.1 cc. of the "absorbed" or "unabsorbed" pneumococcus solution. To avoid the possibility of producing isohemolysins or isoagglutinins in the series immunized with the "absorbed" material, the solution used for the immunization of each rabbit was kept separate and absorbed with the cells from its own blood. The blood cells were washed before each absorption from a stock of the normal

defibrinated blood of each animal obtained before immunization. The absorptions were carried out as described for previous experiments. The material for one course of injections was absorbed with blood cells at the beginning of the experiment; a second absorption was carried out every other day on quantities of the previously absorbed solution sufficient for two injections. The animals were bled after each course of injections, and the immune serum titrated for antihemotoxin and antiprotein precipitins by the previous methods.

TABLE II.

Antihemotoxin and Antiprotein Precipitins in Serum of Animals Immunized with "Absorbed" and "Unabsorbed" Pneumococcus Cell Solutions.

Immunization material	Rabbit	Antihemotoxin titration[1] (hemolysis by 5 units of hemotoxin which had been incubated in presence of serum before addition of blood cells)		Antiprotein precipitin[2] titration	
		Amount of serum, *cc.*		Dilution of antigen	
Pneumococcus cell solution		0.06	0.01	1/2	1/10
		Hemolysis	Hemolysis	Precipitation	Precipitation
"Absorbed" with erythrocytes	1	***	****	++	+
	2	****	****	++	+
Not "absorbed" with erythrocytes	3	0	*	++	+
	4	0	*	++	+

[1]0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.
[2]0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.

The results of the tests of the sera after one course of injections are presented in Table II.

The results (Table II) of this experiment show that the absorption of pneumococcus cell solution with erythrocytes removed the antigen responsible for antihemotoxin production, but was without effect upon

the antigens responsible for the production of the antiprotein precipitins. Immunization with pneumococcus cell solutions which had been absorbed with red blood cells yielded the same type of serum as did immunization with pneumococcus solutions which had been heated at 55°C.; *i.e.*, an immune serum containing antiprotein precipitins but no antihemotoxin.[3] The fact that it is possible to obtain an immune serum containing only one of the antibodies (the antiprotein precipitin) is due in both cases to the loss of the other antigen (hemotoxin) which is effective in the original pneumococcus solution, the hemotoxin antigen being lost in the first instance (Table I) by heat inactivation and in the second instance (Table II) by removal through combination with erythrocytes.

Effect of Removal of the Hemotoxin upon the Protein Precipitation Reaction of Pneumococcus Cell Solutions with Antiprotein Immune Serum.

This experiment dealt with the effect of the removal of the hemotoxin upon the protein precipitation reaction of pneumococcus cell solutions with antiprotein immune serum. Parallel antiprotein precipitin titrations were made with immune antiprotein serum, (1) against "unabsorbed" pneumococcus cell solution (containing both the hemotoxin and the "protein fraction"); and (2) against "absorbed" pneumococcus cell solution (from which the hemotoxin had been removed by previous absorption with red blood cells).

The hemotoxin was absorbed from the pneumococcus solution by the procedure employed in previous experiments. The "absorbed" bacterial solution and "unabsorbed" bacterial solution were then tested against immune serum (obtained by immunization with heated pneumococcus solution) which contained antiprotein precipitins but no antihemotoxin. The protein precipitin tests were made with a constant dilution of serum (2/5) against two dilutions (1/10 and 1/20)

[3] The separation of the hemotoxin by absorption with erythrocytes is apparently not so absolute as that obtained by heating the bacterial solutions. With pneumococcus solutions in which the hemotoxin has been inactivated by heat, no trace of antihemotoxin was produced even after prolonged immunization. In our experiments with "absorbed" solutions, slight traces of antihemotoxin were produced by one of the animals when the immunization was continued. We believe, however, that this was probably due to traces of hemotoxin remaining in the "absorbed" solution which, while insufficient to induce a definite antigenic response to the one course of injections reported in Table III, were sufficient to cause a weak response when a large number of injections were given.

of the bacterial solution. Controls of the bacterial solutions were prepared with normal serum instead of salt solution, since the "absorbed" bacterial extract became cloudy if too greatly diluted with salt solution. The protocol is presented in Table III.

The results (Table III) show that removal of the hemotoxin had no detectable effect upon the protein precipitation reaction of pneumococcus cell solutions when tested against antiprotein immune serum. While this fact in itself is not proof that the hemotoxin is a distinct

TABLE III.

Effect of Removal of the Hemotoxin upon the Protein Precipitation Reaction of Pneumococcus Cell Solutions with Antiprotein Immune Serum.

Pneumococcus solution	Protein reactive with antiprotein immune serum[1]		Hemotoxin[2]	
	Dilution of antigen		Amount of bacterial solution, *cc.*	
	1/10	1/20	0.10	0.01
	Precipitation	Precipitation	Hemolysis	Hemolysis
"Absorbed" with red blood cells	++	+	0	0
"Unabsorbed"	++	+	****	****

[1]0 = no detectable reaction.
\+ = faint cloudiness.
++ = moderate cloudiness.
+++ = heavy cloud, with large amount of precipitate in bottom of tube.
[2]0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

antigenic entity, it is evidence that the hemotoxin in "unabsorbed" solutions, if it reacts at all with the antiprotein precipitin, does not constitute a significant portion of the material precipitated by the precipitins in antiprotein immune serum.

The Occurrence of Antihemotoxin and Antiprotein Precipitins in Antipneumococcus Diagnostic and Therapeutic Serum from Immune Horses.

The antipneumococcus serum usually employed for typing and for therapeutic purposes is obtained from horses instead of from rabbits. It was of interest,

therefore, to determine whether or not the relations established with rabbit immune serum hold true for serum obtained from immune horses. Horses are usually immunized with suspensions of the bacterial cells instead of with the filtered solutions employed in the previous experiments. However, as Avery and Heidelberger (6) have pointed out, these pneumococcus suspensions consist of a mixture of the type-specific antigen and of the species-specific protein which gives rise to the antiprotein precipitin. Hence, the usual antipneumococcus serum from immune horses contains in addition to the type-specific antibody, varying amounts of the species-specific antiprotein precipitin.

The antihemotoxin, like the antiprotein precipitin, is produced in response to an endocellular antigen, and hence, is likewise present in the usual antipneumococcus horse serum. Provided the strain employed in the immunization was not a poor hemotoxin producer, one would expect a certain parallelism between the antiprotein precipitins and antihemotoxin, since the production of both of them would be enhanced by the presence of a large number of autolyzed pneumococci and the continued injection of large amounts of material. As a matter of fact, most antipneumococcus horse serum does contain much more antihemotoxin and antiprotein precipitins than do the usual immune rabbit sera. It seemed that this fact might be due not only to the large amount of material injected and the prolonged immunization, but also to the common use of live pneumococci in the final stages of the immunization.

In connection with experiments on the production of antipneumococcus serum at the Massachusetts Antitoxin and Vaccine Laboratory by Dr. Benjamin White and Dr. Elliott S. Robinson, one series of horses was immunized with heated pneumococcus suspensions alone, and another series with the unheated filtrate of a frozen and thawed suspension of pneumococci in addition to the heated bacteria. Samples of different bleedings of these two sorts of antipneumococcus sera furnished to us by Dr. White and Dr. Robinson, were employed in the experiments. In addition to the sera from the Massachusetts State Laboratory, two antipneumococcus horse sera from other laboratories which employ live bacteria were included.

The experiments consisted of comparisons of the antihemotoxin content of antipneumococcus horse serum produced by immunization with three sorts of material: (1) heated suspensions of pneumococci; (2) heated pneumococci plus unheated filtrate of dissolved pneumococci; (3) heated pneumococci followed by injections of live pneumococci. The sera included Type I serum prepared for therapeutic purposes, and Type III serum prepared for diagnostic typing; no Type II sera were used because of the type-specific carbohydrate precipitation which would have occurred in the (Type II) pneumococcus solution used as the source of the hemotoxin in the experiment proper. (The antihemotoxin is not type-specific and the use of a heterologous type of pneumococcus solution in the antihemotoxin test of the serum is to be preferred.)

A summarized protocol is presented in Table IV.

The results (Table IV) of the tests of the antipneumococcus serum from horses are in agreement with the previous results with immune rabbit serum. Like the rabbit serum obtained after immunization with heated pneumococcus solution, the horse serum obtained by immunization with heated pneumococci alone contained no antihemotoxin; the slight inhibition by the largest amount of serum (0.05 cc.) is no more than that exhibited by normal horse serum and is undoubtedly due to the non-specific action of the lipoid constituents which are unrelated to the immunological antihemotoxin (2). In contrast, the horse sera obtained by immunization with either unheated solutions of pneumococci or with the live bacteria, contained large amounts of the specific antihemotoxin.

TABLE IV.

Antihemotoxin in Serum of Horses Immunized with Heated and Unheated Pneumococci.

Antipneumococcus serum from immune horses	Material employed in immunization	Antihemotoxin titrations[1]			
		Hemolysis by 5 units of hemotoxin which had been incubated in the presence of serum before addition of the blood cells			
		Amount of serum, *cc.*			
		0.05	0.01	0.007	0.003
Type I (therapeutic)	Heated pneumococcus cells (vaccine)	***	****	****	****
Type I (therapeutic)	" " " "	***	****	****	****
Type I (therapeutic)	" " " plus unheated pneumococcus solution	0	0	0	0
Type I (therapeutic)	Heated pneumococcus cells plus unheated pneumococcus cells	0	0	0	0
Type III (diagnostic)	" "	0	*	**	****

[1] 0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

All the same sera also contained significant amounts of antiprotein

precipitins, the relative amounts of the precipitins in each serum being roughly parallel to its content of hemotoxin. A certain relation between the content of antihemotoxin and antiprotein precipitins is to be expected in antipneumococcus serum, since the heating treatment which causes a complete loss of the effectiveness of the hemotoxin antigen also causes a quantitative diminution in the antigenic effectiveness of the protein fraction. (A more complete study of the effect of heat upon the antigenic effectiveness of pneumococcus protein will be presented in a later paper.) However, there is the same evidence of the separation of the hemotoxin from the protein fraction in the serum from horses immunized with heated pneumococci as in the serum from rabbits immunized with heated solutions of the bacterial substances, each of them being devoid of antihemotoxin although containing easily demonstrable antiprotein precipitins.

Separation of the Hemotoxin from the Toxic Substance in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Since the present paper deals with the hemotoxin as an integral antigenic constituent of the pneumococcus cell, it is desirable to present evidence that the hemotoxin is distinct from the toxic substances ("endotoxin" (10)) contained in the same pneumococcus solutions. That the presence of active hemotoxin itself was not essential to the toxicity of pneumococcus solutions was indicated by the fact that the hemolytic activity of the solutions could be destroyed either by heat (55°C. for 5 minutes) or by oxidation, without loss in their toxicity. However, it was desired to obtain more direct evidence of the distinction between the hemotoxin and the toxic substances concerned in the acute death of rabbits. The experiments consisted of a comparison of the toxicity of pneumococcus solutions containing the hemotoxin, with the toxicity of the same solutions after removal of the hemotoxin by selective absorption with red blood cells. The comparison was made more valid by using two different doses of each pneumococcus solution: (1) a dose of 0.25 cc. which was not over twice the minimum dose required for the invariable production of acute death in rabbits; (2) a dose of 0.025 cc. which served as a control that the first dose was not an excessive one.

Two series of four rabbits of equal weight were injected intravenously with the "absorbed" and "unabsorbed" pneumococcus solutions. The rabbits which were killed by the injections were autopsied at once by Dr. Arthur Wright of the Department of Pathology. The pathological findings were the same in the animals injected with the "absorbed" solutions (containing no hemotoxin) as in those injected with the "unabsorbed" solutions. The heart was still beating; the lungs were collapsed and not congested; there was marked congestion in the small in-

testine; the bladder wall was completely collapsed. There were never more than a few, if any, slight focal hemorrhages in the lung. The relative absence of hemorrhage or hemolysis was equally evident in the animals injected with the unabsorbed and the absorbed solutions, and was due in all probability to the small doses of bacterial solution employed.

A protocol showing the toxicity and hemolytic activity of the two solutions is given in Table V.

TABLE V.

Separation of the Hemotoxin from the Toxic Substances in Pneumococcus Solutions Which Cause the Acute Death of Rabbits.

Pneumococcus solution	Toxic action (Effects of intravenous injections into rabbits)		Hemolytic action[1] (Hemolysis of 2.0 cc. of 1 per cent red blood cells)	
	Amount of pneumococcus solution, *cc.*		Amount of pneumococcus solution, *cc.*	
	0.25	0.025	0.10	0.01
"Absorbed" with red blood cells	Rabbit 1—died 1 hr. 50 min. Rabbit 2—died 1 hr. 10 min.	Rabbit 5—no visible effect Rabbit 6—no visible effect	0	0
"Unabsorbed"	Rabbit 3—died 1 hr. 30 min. Rabbit 4—died 1 hr. 10 min.	Rabbit 7—no visible effect Rabbit 8—no visible effect	****	****

[1] 0 = no hemolysis.
* = hemolysis approximately one-fourth complete.
** = hemolysis approximately one-half complete.
*** = hemolysis approximately three-fourths complete.
**** = hemolysis complete.

The results (Table V) of these experiments present evidence that different constituents are involved in the hemolytic and toxic activities of solutions of dissolved pneumococci. The solution from which all the hemotoxin had been removed by test-tube absorption with erythrocytes possessed the same degree of toxicity and caused the same gross pathological changes when injected intravenously into rabbits, as did the "unabsorbed" solution which contained the hemotoxin. Since the test doses employed were sufficiently small to detect a significant quantitative diminution in the toxicity of the solutions, one can con-

clude that the hemotoxin is a substance quite distinct from the pneumococcus substances ("endotoxin") which cause the acute, anaphylactoid death of rabbits. Probably the hemotoxin is not involved at all in the toxic action of moderate doses of the usual pneumococcus solutions, for unless excessive doses were injected, the inhibiting action of lipoid constituents of normal serum would neutralize a large amount of the hemotoxin.

DISCUSSION.

In a previous paper (2) the true antigenic nature of pneumococcus hemotoxin was indicated by the specificity of its neutralization by an antibody (antihemotoxin) invoked by immunization with solutions of pneumococcus cells; the immunological neutralization was differentiated from the non-specific inhibitory effect of normal serum by evidence that the antibodies in the immune serum did not affect the hemolytic activity of digitonin nor of the hemotoxins of other bacteria. While these facts seemed to establish the hemotoxin as a true antigen which invokes the production of a specific neutralizing antibody, it was desired to differentiate the hemotoxin from the other antigenic substances contained in pneumococcus solutions, and to distinguish the antihemotoxin from the other antibodies contained in the serum produced by immunization with the same bacterial solutions. The most important of the other antigenically effective substances contained in pneumococcus solutions are the protein constituents which give rise to antiprotein precipitins. Proof of the hemotoxin as an individual antigenic constituent of Pneumococcus, therefore, requires a satisfactory differentiation of the hemotoxin and the "protein fraction," and a like differentiation of the antihemotoxin and the antiprotein precipitins.

The present investigation has differentiated the two antigens on the basis of the following differences in their properties: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) Removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity

to react with antiprotein immune serum. Consequently, immunization with pneumococcus solutions containing both hemotoxin and the "protein fraction" yielded a serum containing both antihemotoxin and antiprotein precipitins, but immunization with solutions which either had received certain heating or oxidation treatment or had been absorbed with red blood cells, yielded a serum containing only antiprotein precipitins and no antihemotoxin. If one accepts the fundamental principle that one antigen gives rise to one antibody, the production of an immune serum containing the usual titre of antiprotein precipitins but no antihemotoxin is in itself evidence that the hemotoxin is an integral entity which gives rise to a specific antibody (antihemotoxin) distinct from the antiprotein precipitin invoked by other antigenic constituents of the pneumococcus cell.

The evidence obtained in a study of different antipneumococcus sera from immune horses agreed with that obtained from experiments on rabbits, and indicated that the injection of either unheated solutions of pneumococci or of unheated suspensions of pneumococcus cells will always yield an immune serum containing relatively large amounts of the antihemotoxin; while the injection of heated solutions or heated suspensions will yield a serum containing little, if any, of the antihemotoxin antibody.[4]

The establishment of the hemotoxin as a distinct antigen is of further interest as an additional example of the variety of separate and distinct antigenic substances contained within a single bacterial cell. It is now recognized that the presence of a number of different antibodies contained in an antibacterial serum is due to the presence of a number of different antigenic substances in the material injected into the animals, each antigen in all probability giving rise to its own antibody. From Avery and Heidelberger's work (6) the presence of the two most important antibodies (the type-specific anticarbohydrate precipitin and the species-specific antiprotein precipitin) in antipneumococcus serum can be assigned to the presence of two distinct antigens in the

[4] While in our experiments the heating treatment of the immunization material seemed to be the most important factor, the antihemotoxin content of the immune serum would also be affected by variations in the hemotoxin-producing capacities of different strains of pneumococci, amount of material injected, duration of immunization, and differences in the response of individual animals.

pneumococcus cell. Due to the above differentiation of the antihemotoxin from the antiprotein precipitin, it is now possible to assign a third type of antibody (antihemotoxin) which is present in most antipneumococcus sera, to another separate and distinct constituent (hemotoxin) of the pneumococcus cell.

It is desirable to point out, however, that the evidence has simply established the hemotoxin as an individual antigenic entity; and in spite of the described differences in the effect of certain heating and oxidative treatments upon their antigenic properties, the hemotoxin and the "protein fraction" may possess other immunological properties in common. From one point of view (8), there is no real proof that the hemotoxin is not also a "precipitinogen" which possesses an active hemolytic property demonstrable in higher dilutions than can be detected by serological precipitation. The "protein fraction" of the pneumococcus cell is known to include a number of different proteins, which are difficult to separate. Each of these proteins, if sufficiently different in chemical structure can be expected to give rise to its own antibody; but none of these different antigens can be distinguished immunologically in the absence of strikingly specific properties comparable to the specific property of the hemotoxin. For example, in spite of the fact that it is an individual antigenic entity, the removal of the hemotoxin from the "protein" was detected only by virtue of its hemolytic property; and neither its removal nor that of any other of the "protein" constituents would be detected by the precipitin reaction if it constituted too small a proportion of the total reactive protein.

SUMMARY.

The investigation deals with the immunological differentiation of the hemotoxin and the "protein fraction" of Pneumococcus, and with a like differentiation of the antihemotoxin and the antiprotein precipitins. The distinction is made upon the basis of the following evidence: (1) The antigenic (antibody-invoking and antibody-combining) properties of the hemotoxin were destroyed by heat and oxidation treatments which did not cause the loss of the antigenic effectiveness of the "protein fraction." (2) The removal of the hemotoxin from pneumococcus solutions by combination with erythrocytes caused no loss in

the capacity of the solution to invoke the production of antiprotein precipitins nor in its capacity to react with antiprotein immune serum.

Titrations of the antihemotoxin content of antipneumococcus horse serum (both diagnostic and therapeutic) indicated that the heating treatment of the immunization material is the most important factor in determining the antihemotoxin content of the immune sera obtained from horses, as well as of that from rabbits.

A distinction was also made between the hemotoxin and the toxic substances ("endotoxin") which cause the acute anaphylactoid death of rabbits.

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