STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

VIII. The Antigenic Properties of Hemolytically Active and Hemolytically Inactive Modifications of Pneumococcus Hemotoxin.

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INTRODUCTION.

The present paper reports a comparison of the antigenic (antibody-invoking and antibody-combining) properties of the hemolytically active (reduced) form of pneumococcus hemotoxin with the antigenic properties of the inactive modifications derived from the original antigen (hemotoxin) by oxidation and by heat. The following inactive modifications were studied: (1) the reversible inactive oxidation product which can be reconverted to the active substance by test-tube reduction; (2) the irreversible, inactive products formed by treatment with strong oxidizing agents (exposure to strong concentrations of H_2O_2 and to sunlight); (3) the irreversible inactive products formed by heat.

The bacterial hemotoxins, as a class, are of immunological interest since they belong to the same large group of antigens ("antitoxinogens") as the important true toxins. Previous papers (1–4) in this series established certain relations between the hemolytic activity and the oxidation-reduction state of several bacterial hemotoxins. The present study of pneumococcus hemotoxin deals with the effect of oxidation and of heat upon the following properties which are possessed by the "true" toxins as well as by hemotoxins: (1) the cell-injuring property of the hemotoxin (including the cell combination reaction which precedes the injury), (2) its antibody-invoking property *in vivo*, and (3) its antibody-combining

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property *in vitro*. The results are reported in two papers; the present paper reporting the study of the antigenic properties, and the following paper reporting the study of the hemolytic or "cell injury" property.

EXPERIMENTAL.

General Methods.

Source of the Hemotoxin.—Pneumococcus extract (prepared by previously described methods (5)) supplied the hemotoxin used in the experiments. Enrichment of the broth culture medium by the addition of Avery's yeast extract (6) resulted in a particularly strong preparation, not only in hemotoxin concentration but also in oxidizing and reducing properties.

Titration of Antihemotoxin in Immune Serum.—The antihemotoxin potency was determined by adding different amounts of serum to a constant dose of hemotoxin; the hemotoxin-serum mixtures were shaken, and then incubated for 45 minutes at 25°C. to allow time for combination of hemotoxin and its neutralizing antibody; a constant amount of blood cells was then added and the final test systems incubated at 38°C. for 1 hour to determine the presence of free or unneutralized hemotoxin. 5 "units" (five times the amount of extract required to hemolyze completely 2.5 cc. of a 1 per cent suspension of washed rabbit cells) were used as the constant "dose" of hemotoxin instead of the 3 units employed in a previous study (7). This has the advantage of obscuring the inhibitory effect of normal serum, which is due to non-specific lipoid constituents unrelated to the true and specific neutralizing antibody.

The previously described (7) controls were included to prove that none of the apparent neutralization was due to oxidative inactivation during the incubation of the hemotoxin-serum mixtures previous to the introduction of the blood cells. It is also important that there was no visible protein precipitation in our hemotoxin-serum mixtures, since this phenomenon, if it had occurred, might have introduced errors by the mechanical removal of active hemotoxin.

Hemotoxin Inhibition by Normal Serum.—Although it is well known that normal serum contains constituents that inhibit the usual bacterial hemotoxins, it is desirable to emphasize the fact that these substances are not related at all to the true immune neutralizing antibody. The immune pneumococcus antihemotoxin is species-specific and does not inhibit the hemotoxins of tetanus or of the Welch bacillus (7) while the normal serum constituents are non-specific, and inhibit to some extent almost all the bacterial hemotoxins.

In our experiments, the normal serum of each animal, obtained before immunization, was included as a control in the antihemotoxin titration of the immune serum. The small amount of immune serum required for hemotoxin neutralization is of an entirely different order of magnitude than the relatively large amount required for the comparable inhibition of hemolysis by normal serum. Hence, while a certain part of the apparent neutralizing capacity of

immune serum is always due to normal constituents of serum, there is little chance for confusion between the non-specific inhibition and the specific neutralization by immune serum, if the dose of hemotoxin and the amount of serum are properly chosen.

Toxicity of the Extracts Employed in the Immunization.—The pneumococcus solution used to supply the hemotoxin contained, in addition to the hemotoxin and other pneumococcus constituents, the endocellular toxic products described by Cole (8). While we believe that these toxic substances are distinct from the hemotoxin, it was necessary to consider them a possible source of complication in the immunization of the animals.

The results of tests of the toxicity of the extract employed in this study showed that intravenous injections must be limited to relatively small doses, while amounts as large as 0.4 cc., if desired, could be injected by the subcutaneous route without injury to rabbits.

Preliminary Experiments on the Influence of Size of Dose of Antigen (Hemotoxin) upon the Antibody (Antihemotoxin) Response.

Comparisons of the effectiveness of different modifications of any antigen should consider the factor of the size of dose employed in the immunization. This becomes especially important in comparisons of the effectiveness of toxoid solutions containing traces of the active substance with the effectiveness of solutions of the active substance itself, for if there were no relation at all between dosage and response, the traces of active substance in the toxoid solution might be responsible for the antibody production. Hence, it was considered an essential step in the present investigation to determine whether or not there was a direct relationship between the dosage of antigen (hemotoxin) and the antibody (antihemotoxin) response; and if such a relation existed, in what zone of antigen dosage it held valid. It was assumed that the quantitative relation, if it existed at all, could be demonstrated best by keeping the amount of antigen well below that required for the maximum immunity response, for if the amounts of antigen were so large that even the smallest test doses were sufficient to invoke the maximum response, any quantitative relation would be obscured.

Since it was sufficient for the objects of the subsequent major experiments to show immunity differences resulting from large differences in magnitude of the antigen dosage, the following experiments were limited to two sizes of dose of antigen, one of which was ten times as great as the other.

Four male rabbits of approximately the same age and weight were selected. The test doses of antigen were chosen to be 0.2 cc. and 0.02 cc. of the hemotoxin solution given subcutaneously in order to keep even the larger amount somewhat below that required for the maximum immunity response. Two rabbits were immunized with the 0.2 cc. dose and two others with the 0.02 cc. dose. Injections were made daily for 6 successive days with a test bleeding 8 days later. A second

course of injections, begun immediately after the first bleeding, consisted of five injections on successive days with a test bleeding after a rest period of 9 days. The results are given in Table I.

The results of this experiment (Table I) show that the amount of antigen (hemotoxin) injected does affect the antibody (antihemotoxin) response, provided the dose of antigen is kept within proper limits (that is, when the larger doses are not excessive, and when the smaller doses are near the minimum required for the stimulation of a detect-

TABLE I.

Influence of Size of Dose of Antigen (Hemotoxin) upon Antibody (Antihemotoxin) Response.

Immunization		Response (antihemotoxin titrations of serum)							
		Hemolysis by 5 "units" of hemotoxin which had been incubated in presence of serum for 45 min. at 25°C., before addition of red blood cells							
Rabbit	Size of dose; amount of bacterial extract in each injection	Serum obtained before immunization	Serum obtained after first course of injections		Serum obtained after second course of injections				
		0.05 cc.	0.05 cc.	0.013 cc.	0.05 cc.	0.013 cc.	0.006 cc.	0.003 cc.	0.002 cc.
	cc.								
1	0.2	++++	0	++	0	0	0	0	+
2	0.2	++++	0	++	0	0	0	±	++
3	0.02	++++	++++	++++	0	++	++++	++++	++++
4	0.02	++++	++++	++++	0	+++	++++	++++	++++

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

able response). In view of the well known differences in the immunity response of different individual animals, one cannot expect too much, but the relationship between dosage and response was sufficiently definite to be utilized in the following major experiments which compare the antigenic properties of different modifications of pneumococcus hemotoxin.

Comparison of the Antibody-Invoking Properties of the Reduced (Active) and the Reversible Oxidized (Inactive) Forms of Pneumococcus Hemotoxin.

This experiment consisted of a comparison of the antibody-invoking properties of the hemolytically active (reduced) hemotoxin and of its inactive, reversible oxidation products. In the "unoxidized" solution, the hemotoxin was preserved

TABLE II.

Proportion of the Hemolytically Active (Reduced) and Hemolytically Inactive (Reversibly Oxidized) Modifications of the Hemotoxin Contained in the Solutions Employed in the Immunizations.

Amount of hemotoxin solution	Unoxidized hemotoxin solution		Oxidized hemotoxin solution	
	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversible oxidized form; hemolytic activity of solution after treatment with reducing agent	Measurement of active hemotoxin; hemolytic activity of solution before treatment with reducing agent	Measurement of active hemotoxin plus the reversibly oxidized form; hemolytic activity of solution after treatment with reducing agent
cc.				
0.03	++++	++++	+++	++++
0.02	++++	++++	±	++++
0.01	++++	++++	0	++++
0.005	++++	++++	0	++++
0.001	+++	+++	0	++++
0.0005	+	+	0	+
0.0002	0	0	0	0

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

in its original active or reduced form by the reducing properties of the system in the absence of air. In the "oxidized" pneumococcus extract (prepared by exposing the extract to air for about 24 hours at 37°C.), the reducing properties of the system had been destroyed and the oxidizing agents (peroxides) had converted the hemotoxin to hemolytically inactive oxidation products; these inactive oxidation products were reversible, and could be reconverted to the original active form of the hemotoxin by biological or chemical reducing agents.

All the material employed in the immunization was analyzed in order to deter-

mine what proportion of the total amount of antigen was in the active (reduced) state and what proportion was present in the inactive form; this was particularly important in the case of the "oxidized" or "inactive" material which (like most toxoid material) did contain residual traces of the active hemotoxin. The dosage employed in the immunization was kept low in order to stay within the zone in which there is a direct relation between the amount of antigen and the degree of immunity response. These two conditions were essential: (1) knowledge of the nature of the material used in immunization, (2) control of antigen dosage, since the crux of the experiment consisted not only in a comparison of the antibody response to the equal amounts of the unoxidized and of the oxidized solutions, but also in determining whether or not the residual traces of active hemotoxin persisting in the oxidized solution were in themselves sufficient to invoke a significant response.

1. Analysis of the Material Utilized in the Immunization.—One series of animals was immunized with "unoxidized" hemotoxin solution, and another series with the solution which had been oxidized by exposure to air for 24 hours at 37°C. The two solutions were examined by methods (1–4) which measure both the reduced and reversibly oxidized forms of the hemotoxin.

The results of these tests (Table II) show that in the unoxidized solution, all the hemotoxin was in the reduced or active form. In contrast to the unoxidized solution, almost all the hemotoxin in the oxidized solution was present in the reversibly oxidized form, as indicated by the great increase in the hemolytic activity of this solution when treated with the reducing agent. It is important to note that residual traces of the active (reduced) form did persist in the "oxidized" solution; the amount of active substance, however, was small, since 0.02 cc. of the oxidized extract gave less hemolysis than did 0.0005 cc. of the unoxidized material.

The total antigen (hemotoxin) in the two extracts was the same, since their hemolytic activity was approximately identical when measured after the reduction treatment. The unoxidized extract can be assumed to have contained 100 per cent of the total antigen in the active or reduced state. In the oxidized solution, it is sufficient for the present purpose to estimate that more than 90 per cent of the total antigen was present in the oxidized (inactive) state, and that the traces of the reduced (active) form represented less than 10 per cent of the total hemotoxin. (This estimate of 10 per cent is purposely generous; 0.03 cc. of the oxidized solution possessed no more hemolytic activity than 0.001 cc. of the same solution after reduction treatment.)

2. Control of Dosage in the Immunization.—Eight rabbits, all obtained from the same breeder, and all of approximately the same weight, were selected; four were immunized with the unoxidized and four with the oxidized material.

Two doses were employed in the injection of the animals of each series; the larger dose (0.2 cc.) of the unoxidized and oxidized solutions for the respective series was somewhat less than that required for the maximum response, and the smaller dose (0.02 cc.) was near the minimum required for any detectable response.

These two doses, chosen from the results of previous experiments (Table I), presented a convenient basis for a valid evaluation of the antibody-invoking properties of the oxidized (inactive) and reduced (active) forms of the hemotoxin, the quantitative relations between them being so arranged that the traces of reduced (active) hemotoxin contained in the larger test dose of the oxidized material were quantitatively less than the amount of the reduced substance contained in the smaller test dose of the unoxidized material.

The routine of the immunization was the same as previously described; the antibody response was determined by measurements of the antihemotoxin content of the immune sera after one and two courses of injections. Since the quantitative relations between dosage and antibody response were more valid in the sera obtained after the first course of injections than in those obtained after the second course, the objects of the experiment are satisfied by presenting in Table III a summary of the results after the first course of the immunization.

TABLE III.

Comparison of the Antibody-Invoking Properties of the Reduced (Hemolytically Active) and of the Reversibly Oxidized (Hemolytically Inactive) Forms of Pneumococcus Hemotoxin.

Condition of the antigen (hemotoxin) used in the immunization	Immunity response (5 "units" of hemotoxin tested against 0.05 cc. of the immune serum obtained after 1 course of injections)	
	Immunization with 0.02 cc. doses	Immunization with 0.2 cc. doses
	Neutralization	Neutralization
100 per cent of the total antigen in reduced (hemolytically active) state	0	+
Over 90 per cent of the total antigen in the reversibly oxidized (hemolytically inactive) state; less than 10 per cent of antigen in the reduced (active) state	0	+

Table III presents two important facts. First, one course of injections of 0.2 cc. of either the unoxidized or oxidized material invoked the production of significant amounts of antihemotoxin; second, the same number of injections of 0.02 cc. (one-tenth the size of the first dose), proved insufficient to cause a significant immunity response. In spite of the fact that 100 per cent of the total antigen (hemotoxin) was in the reduced state in the one solution and 90 per cent was in the oxidized state in the other solution, like immunity responses were invoked in all instances by equal doses of the unoxidized and oxidized

material. The lack of any essential difference in the degree of response to the larger or effective dose (0.2 cc.) of the two solutions containing such widely different proportions of the two modifications of the antigen, indicates that there is no essential difference in the antibody-invoking properties of the reduced and of the reversibly oxidized forms of the hemotoxin. Since it was known that less than one-tenth of the total hemotoxin (reduced plus oxidized forms) was in the reduced state in the oxidized solution, the fact that the same number of injections of 0.02 cc. of the unoxidized solution (or one-tenth the larger dose of the solution containing 100 per cent active hemotoxin) did not invoke antibody production, serves to prove that the residual traces of reduced substance remaining in the oxidized materials were by themselves insufficient to account for the antigenic effectiveness of the larger dose (0.2 cc.) of the oxidized hemotoxin solution. Although the possibility has not been eliminated that the effectiveness of the oxidized form may be increased by the presence of traces of the reduced form, the above evidence indicates that the antibody-invoking properties of the reduced and reversibly oxidized forms of the antigen are identical; and that the loss of the hemolytic property is not necessarily accompanied by any loss in the capacity to invoke antibody production.

Experiments on the Antibody-Invoking Property of Inactive, Irreversible Modifications of the Antigen (Hemotoxin) Derived by Means of Strong Oxidizing Agents.

The preceding experiment showed that the loss of hemolytic activity by the reversibly oxidized modification of the hemotoxin was not accompanied by any detectable change in its antigenic effectiveness. The reversibly oxidized form is the product obtained when the hemotoxin is oxidized by the peroxides produced when this type (9) of bacterial solution is exposed to air. However, other modifications of the hemotoxin are formed when the bacterial solutions are treated with strong oxidizing agents; these products agree with the reversibly oxidized form in that they have lost the original hemolytic property, but differ in that they cannot be reconverted to the original form by test-tube reduction. The irreversible, inactive modifications are not so easily produced from pneumococcus hemotoxin as are the similar products derived from the hemotoxins of tetanus and Welch bacilli (2, 3). (With the latter substances, the irreversible products are formed so readily that it is difficult to induce the formation of the reversible product without at the same time causing the formation of some of the irreversible products.)

Experiments were made to determine whether the changes in pneumococcus hemotoxin which are involved in the formation of the irreversible products cause a loss of the antibody-invoking property. The hemotoxin solution was exposed to 0.5 molar H_2O_2 ("Dioxogen" diluted in phosphate solution) for 7 days at 37°C. It was desired to eliminate the H_2O_2 at the end of this period of exposure so that it would not interfere with the reduction tests to be made for the presence of the reversible product of the hemotoxin. In order to accelerate the "spontaneous" deterioration of the H_2O_2, the mixture was exposed for 3 days to intense summer sunlight in addition to the 7 days storage at 37°C. in the dark. After this treatment, all the hemotoxin had been converted to irreversible modifications and the H_2O_2 destroyed.

Four rabbits were immunized with the doses used in the preceding experiment, but none of them developed any detectable antihemotoxin.

The results showed that the irreversible products formed by treatment with strong H_2O_2 were devoid of the antibody-invoking property of the original hemotoxin. While there is a striking contrast between the antigenicity of the reversibly oxidized modification and the non-antigenicity of the irreversible products studied in this particular experiment, it is possible that other irreversible products which retain antigenicity are formed by other agents than those employed in our experiments. It must be remembered that drastic treatment was employed to convert the first formed reversible modification to the irreversible modification. Treatment of any antigenic substance with high concentrations of H_2O_2 is likely to cause profound changes in the molecule,[1] changes indeed which frequently involve hydrolysis or splitting of the mo ecule as a whole in distinction to oxidation of individual groupings without profound changes in other parts of the molecule. Hemoglobin, for example, when treated with the proper oxidizing agent, is converted quantitatively to methemoglobin (also a reversible product), but if treated with a high concentration of H_2O_2, a part of the hemoglobin (or more properly, a part of the methemoglobin) is split to globin and hematin (10). One must distinguish

[1] While the exposure to 0.5 molar H_2O_2 which results in loss of antigenicity of the hemotoxin would also partially convert hemoglobin to (reputedly) antigenically ineffective products, it was not an unreasonably drastic treatment with which to treat the hemotoxin. It will be shown in a later paper that the same treatment does not result in a comparable loss of the antigenicity of certain other protein substances which give rise to anti-pneumococcus-protein precipitins.

between these two types of changes in the treatment of antigens with different oxidizing agents. Pneumococcus hemotoxin, for example, is converted to a hemolytically inactive but antigenically effective modification when the oxidizing agents are properly chosen, as shown by the above results, but treatment with other oxidizing agents yields a product which is not only hemolytically inactive, but also antigenically ineffective.

It is important not to emphasize any apparent correlation between the reversibility of the hemolytic property of hemotoxoids and their retention of antigenicity, for while the two products differ from each other from the standpoint of hemolytic activity, in that one is reversibly inactive and the other is irreversibly inactive, this is probably by no means the only difference between them. Indeed, from a fundamental point of view, the reversibility of the one product and the irreversibility of the second are important simply as an index that the first product has suffered less profound molecular changes, and hence is more likely to retain the antigenic properties of the original substance. The dangers entailed in the assignment of antigenic effects to prominent toxic properties are evident from analogy with the antigenic properties of hemoglobin, methemoglobin, and globin (11, 12).

Although methemoglobin has lost the most prominent property of the original hemoglobin, the loss of the oxygen-combining property caused by the change in the valency of the iron is not accompanied by changes in the antigenically effective groups of the complex protein molecule. Since the oxygen-combining property is just as important a property to hemoglobin as is hemolysis to the reduced hemotoxin, it is no more remarkable for the reversibly oxidized hemotoxin to undergo changes affecting the hemolytic property without alteration of its antigenically active groupings. Similarly, the loss of antigenicity by the irreversible products of the hemotoxin is probably due not to the fact that it does not regain hemolytic activity upon reduction, but simply to the fact that the hemotoxin molecule as a whole has undergone profound changes which include alteration of the antigenically effective groupings; just as the reputed lack of antigenicity on the part of globin is due not to the fact that it cannot regain its oxygen-combining activity, but to the fact that it is a split product of the original antigenic hemoglobin.

Experiments on the Antibody-Invoking Properties of Heat-Inactivated Modifications of the Hemotoxin.

Two rabbits were immunized with hemotoxin solution which had been inactivated by exposure to 55°C. for 6 minutes and two others with solution boiled for 10 minutes. One animal in each series was given two courses of subcutaneous injections of 0.2 cc.; this was equivalent to the maximum immunization employed in previous experiments. The immunization of the other animal in each series was continued for 7 months, and consisted of eight courses of five or six injections of 0.2 cc. with one rest period of 2 months in addition to the usual periods of 8 or 9 days between each course.

None of the animals immunized with the heat-inactivated modifications developed any detectable antihemotoxin. The failure to respond to the prolonged immunization indicated that the loss of antigenicity was absolute. While a quantitative decrease in the effectiveness of heated antigens is common experience, the complete loss of antigenicity after short periods of exposure to 55°C. is an unusual example of the effect of heat upon bacterial substances.

Antibody-Combining Properties of the Different Modifications of Pneumococcus Hemotoxin.

That the reduced (hemolytically active) form of the antigen possesses the property of combining with the antibody, can be regarded as an established fact, since its neutralization by combination with the antihemotoxin was the basis of the tests of the antihemotoxin content of the immune serum studied in the preceding experiments. The object of the following experiments was to determine whether or not the different modifications of the hemotoxin (antigen) which have lost the hemolytic property of the original antigen, still retain the property of combining with the antibody.

The experiments consisted of a comparison of the amount of immune serum required to neutralize a constant dose of active hemotoxin under the following four sets of conditions: (1) test mixtures containing the constant dose of active hemotoxin but no inactive derivatives of the hemotoxin; (2) test mixtures containing an equivalent amount of the reversibly oxidized form of hemotoxin in addition to the dose of active hemotoxin; (3) test mixtures containing an equal amount of the irreversibly "oxidized" products in addition to the active hemotoxin; (4) test mixtures containing an equal amount of the heat-inactivated products in addition to the active hemotoxin.

Four solutions were prepared, 1 cc. of which contained, respectively: Solution

I: 5 "units" active hemotoxin; Solution II: 5 "units" of active hemotoxin plus 5 "units" of the reversibly oxidized form; Solution III: 5 "units" of active hemotoxin plus 5 "units" of the irreversibly oxidized products; Solution IV: 5 "units" of active hemotoxin plus 5 "units" of heat-inactivated products. 1 cc. amounts of the four solutions were put into different series of tubes. Dilutions of immune serum (containing antihemotoxin) were prepared so that 1.0 cc. of the respective dilutions contained the following increments of serum: 0.10, 0.067, 0.050, 0.040, 0.030, 0.020, 0.016, 0.010 cc. 1 cc. of each of the serum dilutions was then added to the four series of tubes which contained, respectively, Hemotoxin Solutions I, II, III, IV. The rest of the procedure was identical with that in previous experiments, the four series of hemotoxin-serum mixtures being incubated for 45 minutes at 25°C. before the addition of the red blood cells.

Controls of Active Hemotoxin Content of the Solutions Utilized in the Neutralization Tests.—It was essential to prove that the different hemotoxin solutions did not differ significantly in their content of active hemotoxin solution when tested in the absence of immune serum. This control was especially important in the case of Hemotoxin Solution II since there frequently are significant traces of the active or reduced form persisting in the oxidized extracts containing the reversibly oxidized form of the hemotoxin. (The oxidized extract itself was examined for the presence of the active or reduced hemotoxin, and was chosen for use in this experiment because of the almost complete absence of active lysin, 0.20 cc. of the undiluted extract causing only traces of hemolysis in 2.0 cc. of 1 per cent red blood cells; the amount (0.015 cc.) used in the experiment proper was entirely without hemolytic effect.)

The results are condensed in Table IV.

Several experiments of another type were also made in which the active hemotoxin was added to previously incubated mixtures of serum and inactive hemotoxin, instead of incubating the immune serum with mixtures of active and inactive hemotoxin. From analogy with the Danysz phenomenon which occurs in the fractional addition of toxin in toxin-antitoxin mixtures, one would expect this second set of conditions to emphasize the difference between the amount of serum required to neutralize the active hemotoxin alone and the amount required for the active hemotoxin plus inactive forms which unite with the antihemotoxin. Our results in the latter type of experiments, however, were not significantly different from those obtained when the active hemotoxin was added to the serum mixture at the same time as the hemotoxoid. Since the results were practically the same as those shown in Table IV, no protocol need be presented.

Table IV shows that when the reversibly oxidized products were present in the serum mixture during the period allowed for neutralization of the active hemotoxin, a larger amount of immune serum was required for the neutralization than when an equal dose of the active form of the hemotoxin was incubated with serum alone; but that the

presence of either one of the other two hemolytically inactive modifications was without effect upon the neutralizing dose of the immune serum. These results indicate that the reversible oxidation products

TABLE IV.

Antibody-Combining Properties of Different Modifications of Pneumococcus Hemotoxin; Effect of the Presence of Inactive Modifications of the Hemotoxin upon the Amount of Immune Serum Required for the Neutralization of a Constant Dose of the Active Hemotoxin.

Hemotoxin solutions	Tests of removal of effective antibody (antihemotoxin) by combination with inactive forms of the antigen (hemotoxin)				Controls; to prove that all test solutions contained approximately the same amount of the hemolytically active form of the hemotoxin		
	Test dose employed in the titrations against immune serum	Hemolysis by constant dose of the hemotoxin solution when incubated in presence of different amounts of immune serum, before addition of red blood cells			Hemolysis by different amounts of the hemotoxin solutions, tested in absence of immune serum, against 2.5 cc. of 1 per cent red blood cells		
		Amount of serum, *cc.*			Amount of hemotoxin solution, *cc.*		
		0.067	0.030	0.016	0.24	0.12	0.08
Solution I	5 units of active hemotoxin alone	0	0	+++	+++	+	0
Solution II	5 units of active hemotoxin plus an equivalent amount of its inactive, reversibly oxidized products	+	++++	++++	+++	+	0
Solution III	5 units of active hemotoxin plus an equivalent amount of its inactive, irreversibly oxidized products	0	0	+++	+++	+	0
Solution IV	5 units of active hemotoxin plus an equivalent amount of its heat-inactivated products	0	0	+++	+++	+	0

0 = no hemolysis.
± = trace of hemolysis.
\+ = hemolysis approximately one-fourth complete.
++ = hemolysis approximately one-half complete.
+++ = hemolysis approximately three-fourths complete.
++++ = complete hemolysis.

of the hemotoxin, although they have lost the cell injury (hemolysis) property, retain the property of uniting with the antibody; and that the other "inactive" modifications of the hemotoxin have lost not only the hemolytic property but also the property of combining with the antibody.

It is significant that the same two modifications of the hemotoxin, the hemolytically active reduced form and the hemolytically inactive, reversibly oxidized form, which possess the property of invoking antibodies *in vivo*, are also the ones which combine with the antibody *in vitro;* and that the other two modifications, which are devoid of the antibody-invoking property, are likewise devoid of the antibody-combining property. The same correlation between the two properties of antigens seems to be established for diphtheria toxin since it is the basis of the Ramon flocculation method for measurement of the total antigenically effective products (toxin plus effective toxoid).

It is important to note that the "constant dose" of active hemotoxin in the four hemotoxin solutions in Table IV is based upon measurements which detect only the active form of the hemotoxin. Thus, while all the solutions contain the same amount of the hemolytically active form, the amount of total hemotoxin (*i.e.*, active plus inactive modifications) is in fact only half as great in Hemotoxin Solution I (where all the "total hemotoxin" is active) as in Hemotoxin Solutions II, III, IV (where only half of the "total hemotoxin" is hemolytically active). With the true toxins, when the toxin content is based upon M.L.D., the conditions are much the same (*i.e.*, the M.L.D. detect only the part of the "total toxin" which is still in the "active" form); and the amount of immune serum required for the neutralization of a "constant dose" of the "active" antigen is likewise increased by the presence of its non-toxic but antigenically effective modifications.

DISCUSSION.

The following modifications of the antigen (hemotoxin) were studied: (1) the original, active substance; (2) the inactive, reversible oxidation product formed by exposure of hemotoxin solutions to air; (3) the inactive, irreversible products formed by prolonged treatment with high concentrations of H_2O_2; (4) the inactive products formed by heat.

The first experiments compared the antibody-invoking property of the hemolytically active (reduced) form of the antigen with that of the hemolytically inactive, reversible oxidation products. Two series of

animals were immunized; one series, with unoxidized hemotoxin solutions in which 100 per cent of the total antigen was present in the reduced state; a second series, with oxidized solutions in which more than 90 per cent of the total antigen was present in the reversibly oxidized state. Equal doses of the oxidized and unoxidized solutions invoked like immunity responses, and the traces of the reduced hemotoxin contained in the larger doses of the oxidized solution were shown to be quantitatively insufficient to account for the antibody production. Although it is possible that the antigenic effectiveness of the oxidized form was increased by the presence of traces of the reduced form, the evidence indicated that the antibody-invoking properties of the reduced and reversibly oxidized forms of pneumococcus hemotoxin are identical, and that the hemolytic property of the antigen is not essential to the stimulation of the neutralizing antibody.

In contrast to the apparent identity of the antibody-invoking properties of the reduced and reversibly oxidized forms of the hemotoxin, the irreversible products formed by treatment with strong H_2O_2 proved devoid of antigenic properties; but the failure of antibody response to the type of strongly oxidized material used in these experiments does not constitute evidence that the reversibility of the inactive products is correlated with the antigenic function, for other irreversible products possessing antigenic properties might be formed by other agents. The important fact is that the products yielded by one oxidation treatment (that induced by the peroxides formed in the aerated bacterial extract) were hemolytically inactive but antigenically effective, and that the products yielded by more drastic treatment were devoid of antigenic properties. Thus, unless the treatment be properly chosen, the antibody-invoking properties may be destroyed by the processes employed to destroy the toxic properties of the original antigen. This fact is of interest in connection with the mechanism of toxoid immunization. In many instances when toxoids are used for immunization, the response is most effective when traces of the original active substance are present in the material injected into the animals; and it is sometimes suggested that these traces of active substance, although by themselves quantitatively insufficient to invoke antibody production, may accelerate the response to the nontoxic toxoid. However, from analogy with the differences in anti-

genicity of the different modifications of pneumococcus hemotoxin, it is probable that the less effectiveness of toxoid fluids which contain no traces of active toxin is due simply to the fact that a considerable portion of the antigenically effective toxoids are always transformed to ineffective products whenever the treatment is violent enough to inactivate all traces of active toxin.

The results of the comparison of the different modifications of hemotoxin from the standpoint of the antibody-combining property were similar to the results of the comparison of their antibody-invoking properties. The same modifications of the hemotoxin (the reduced and the reversibly oxidized forms) which possessed the antibody-invoking property also possessed the antibody-combining property; and the other two modifications which were devoid of antibody-invoking properties *in vivo* were likewise devoid of the antibody-combining property *in vitro*. The fact that one of the modifications which possessed the two antigenic properties was hemolytically inactive is evidence that the groups of the hemotoxin molecule in which the antibody-combining property and the antibody-invoking property are resident are not necessarily altered by processes which inactivate the molecular groupings responsible for the cell injury function of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes in the molecule which affect antigenically effective groupings. In this sense, from a purely immunological aspect, the reversibility of the hemolytic activity of the antigenically effective oxidation product of pneumococcus hemotoxin is important, simply as an index that the change of the antigen molecu'e has not been a profound one.

The fact that some of the non-toxic modifications of antitoxinogens can retain the antigenic properties of the original toxic antigen raises the question of whether or not there is any essential *immunological* difference between the antitoxinogen or primarily toxic type of antigen and the sensitizing or non-toxic type of antigen.[2] The most

[2] Zinsser's (13) separation of the antitoxinogens from other antigens serves its intended purpose as a convenient basis for the inclusive treatment of the sensitizing antigens in a single group, but it should not be misinterpreted to

fundamental distinction between them lies in the fact that the antitoxinogens alone have the property of combining with and injuring specific cells, which is a pharmacological rather than an immunological property. All antigens must possess the fundamental property of stimulating the animal body to produce a specific antibody, and the method of stimulation may be the same for all.

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indicate essential differences between the two groups of antigens from the standpoint of antibody stimulation, nature or mode of antigen-antibody reaction, or other immunological reactions. The distinction is based almost entirely upon the specific cell-injuring properties of the one group and the absence of such properties in the other—a criterion not intended to separate the antitoxinogens from other antigens regardless of the possibility of more fundamental chemical and immunological relationships.

The conception of the "essential identity" of sensitizing antibodies, derived from one antigen but detected in different systems, can in many respects be projected to indicate fundamental likenesses between all antibodies. The usual distinction between the two types of antibodies (antitoxins and sensitizing antibodies) depends upon differences between the properties of the antigen-antibody compounds and the properties of the specific antigens. With the toxin-antitoxin compounds, the *prominent* change is in pharmacological properties; with the other antigen-antibody compounds, it is usually a change in physical properties. But the physical properties of toxin-antitoxin can also differ from those of the toxin as exemplified in the Ramon flocculation; and some hypersensitive phenomena might be interpreted as due to the acquisition by the sensitizing antigen-antibody compounds of pharmacological properties not possessed by the antigens. In a certain sense, "neutralization" as well as "agglutination" can be considered as a phenomenon secondary to the actual combination of antigen and antibody. The lack of toxicity of toxin-antitoxin compounds is essentially a fortunate circumstance employed to detect the combination of these antigens with their antibodies, just as the change in physical properties is a convenient means of observing the union of agglutinogen with agglutinin. If differences in the test systems frequently obscure the identity of the sensitizing antibodies in spite of the essential likeness of their antigen-antibody combinations, differences in the test systems may also obscure fundamental likenesses between the antitoxinogens and other antigens.

While the quantitative relations between antitoxinogens and antitoxins are usually more distinct than with other antigens, the application of the rule of "neutralization by multiple proportions" to the explanation of many phases of toxin-antitoxin titration curves or to the Danysz phenomenon is just as difficult as the explanation of the quantitative relationships between the other antigens and their antibodies.

Although no definite evidence is available, it is highly doubtful, at least in the case of some antitoxinogens, whether the particular cells for which they are specifically toxic have anything to do with antibody production. Hemotoxins, for example, are considered to belong in the antitoxinogen class of antigens because they have the specific property of combining with and injuring erythrocytes, but from their nature it is unlikely that red blood cells can be the agents of antibody production. If the mechanism of antibody production with antitoxinogens is essentially the same as with sensitizing antigens (*i.e.*, a similar response to stimulation by specific antigenic groupings of a foreign protein rather than a defensive response to stimulation by the groups responsible for the toxic property), then the antigenicity of modifications of toxic antigens would be determined by the same conditions that determine the antigenicity of derivatives of all antigens. While the specific toxicity is the most prominent property of all antitoxinogens, the loss of one prominent and characteristic property without loss of antigenicity is a set of conditions which also occurs among the sensitizing antigens. For example, the oxygen-combining property is just as important and prominent a property of the hemoglobin molecule as is the specific cell injury property of toxin molecules; and yet, the loss of the oxygen-combining property by methemoglobin is not accompanied by loss of the antigenic properties of hemoglobin. While it cannot be said that the pharmacological action of toxins has no more to do with the production of antitoxin than the oxygen-combining property of hemoglobin has to do with the production of antihemoglobin precipitins, the prominence of any one particular property of a complex molecule implies no relation to its antigenicity. Fundamentally, the antigenicity of toxoids requires no other explanation than that for methemoglobin, *i.e.*, that any substance can lose any one of its properties without loss of antigenicity provided the alteration does not include changes in the antigenically effective groupings nor render it insoluble in the body. From this standpoint, the antigenic effectiveness of the reversibly oxidized modification of the hemotoxin requires no assumption of the possibility of its reconversion to the hemolytically active form by *in vivo* reduction.

On the other hand, one cannot dismiss the possibility that the cells of certain tissues might be able to reconvert the inactive derivatives

to the original active substance. While evidence is lacking that the reversibility of toxoids is related at all to antigenicity, the different cells and different tissues of the body present a variety of systems which are known to differ in acidity and other factors, and which consequently might differ in their capacity to reconvert inactive toxoids to the original form of the antigen. It does not seem at all impossible for at least some of the cells or tissues responsible for antibody production to present the requisite conditions for the reversal of toxoids to the original toxin, and yet for the particular cells with which the toxin is pharmacologically reactive to be devoid of the conditions required for the reconversion. However, conjectures from this aspect are futile in view of the almost complete lack of knowledge of the site of antibody production.

SUMMARY.

The following modifications of the antigen (pneumococcus hemotoxin) were studied: (1) the hemolytically active (reduced) substance; (2) the hemolytically inactive, reversible oxidation product; (3) the inactive irreversible products formed by treatment with high concentrations of H_2O_2; (4) the inactive products formed by heat. The antibody-invoking property of the reversibly oxidized form seemed to be identical with that of the original, hemolytically active or reduced form; neither of the other two hemolytically inactive products invoked antibody production. The same modifications of the antigen which exhibited the antibody-invoking property *in vivo* possessed the antibody-combining property *in vitro;* and the modifications which lacked the one property also lacked the other. Evidence is presented that the groups of the hemotoxin molecule in which the true antigenic properties are resident are not necessarily altered by processes which inactivate the groupings responsible for the toxic (hemolytic) action of the original antigen. The lack of antigenic properties on the part of the other two hemolytically inactive modifications is evidence that the treatment employed to alter the toxic property of the molecule must be properly chosen to avoid profound changes which affect the antigenically effective groupings. From an immunological point of view, the reversibility of the antigenically effective oxidation product of pneumococcus hemotoxin is important as an index that the loss of

toxicity (hemolysis) was accomplished without a profound change in the molecule.

The theoretical significance of the antigenicity of non-toxic modifications of toxic antigens is discussed.

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