STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

I. Pneumococcus Hemotoxin.*

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INTRODUCTION.

Certain general principles which were established in previous papers (1, 2) on the biological oxidation-reduction of hemoglobin are reviewed below since they are to be utilized in the present series of studies.

Methemoglobin may be considered as the "inactive" form of hemoglobin, in that it no longer combines with oxygen or carbon monoxide. The essential difference between hemoglobin, the "active" blood pigment, and methemoglobin, its "inactive" oxidation product, is the change of the ferrous iron of the molecule to the ferric state. The conversion of hemoglobin to methemoglobin thus, is a "true" oxidation in the electronic sense (3) and must be distinguished from the process of "oxygenation" involved in the formation of oxyhemoglobin.

The oxidation to methemoglobin may be brought about by biological oxidizing agents, and is a reversible process. Both the oxidation ("inactivation") and the reduction ("reactivation") seem to be induced by the same system; the substances or biological systems which in the presence of molecular oxygen, bring about the oxidation of the hemoglobin, induce the reverse process if air is excluded. Apparently these substances, if not disturbed by the presence of oxygen, are essentially reducing agents, but when oxygen is present, they are changed to oxidizing agents (peroxides or "activated oxygen") which bring about the oxidation of other more difficultly oxidized substances such as hemoglobin. All of the hemoglobin oxidations of this type proceed most rapidly at an oxygen tension of approximately 20 mm., which permits the formation of the oxidizing agents but which also permits

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over half of the total hemoglobin to exist in the reduced (deoxygenated) state. The "spontaneous deterioration" of hemoglobin to methemoglobin is also an oxidation process and it is influenced by the tension of molecular oxygen in exactly the same way. This gradual deterioration of Hb (so called "spontaneous MetHb formation") which always occurs when blood or Hb solutions are stored under the usual conditions outside the body, can be inhibited or wholly prevented if the hemoglobin is stored in a sealed system in the presence of a biological reducing agent. Thus, when air is excluded, the stability of the active blood pigment is actually increased by the presence of the same biological substances which rapidly oxidize it in the presence of air.

A study has now been undertaken in which it is attempted to apply these principles, as outlined for hemoglobin, to the oxidation and reduction of substances of importance in immunology. It seemed that such a study might well be begun with experiments on bacterial hemotoxins. These substances, which apparently are true antigens, although themselves of relatively little importance, have proved valuable objects of study in the historical establishment of many of the general principles of immunology. The hemotoxin of Pneumococcus was the particular one chosen as the subject of the first investigation because the mechanism of the oxidation of this bacterial substance had been established in an earlier paper (3).

The term bacterial *hemotoxin* has been proposed by Přibram (5) to distinguish the bacterial lysins which exhibit antigenic properties from other substances of bacterial origin which although possessing hemolytic properties are not antigenic. It is also important to avoid confusing the hemotoxins with the so called immune hemolysins. The hemotoxins are substances which are specifically (or primarily) toxic for red blood cells, just as diphtheria toxin and tetanus toxin are, respectively, selective for other tissue cells. Again, like the "true" toxins, the hemotoxins are *antigenic*, or more properly speaking "antitoxinogenic," in that when injected into animals they induce the formation of a neutralizing antihemotoxin (analogous to antitoxin). The hemolysins, on the other hand, are "sensitizing" *antibodies* induced by the injection of a primarily non-toxic antigen (red blood cells), and, unlike the hemotoxins, their species-specific hemolytic action is dependent upon the cooperative mechanism of the sensitizing hemolysin and alexin (complement).

Pneumococcus hemotoxin has been described by a number of workers (6, 7) but its first complete study was made by Cole (8) in 1914. It is a true antigen and when injected into animals causes the formation of an antihemotoxin, an antibody which neutralizes the hemotoxin (8). Unlike most bacterial hemotoxins, the hemotoxin of Pneumococcus is an endocellular substance liberated into the culture fluid only upon disintegration of the cells. As might be expected, the

detection of active hemotoxin in filtrates of pneumococcus cultures depends not only upon the liberation of the lysin by cell autolysis, but also upon its protection from air (9). There seems to be no relation between the virulence of the strain of Pneumococcus and its hemotoxin-producing capacity. By comparisons of the strains in the collection at the Hospital of The Rockefeller Institute, we have found that the most virulent strains frequently are weak lysin producers, and that avirulent strains as often as not produce larger amounts of the hemotoxin.

It is evident from the foregoing that in pneumococcus hemotoxin we are dealing with a specific antigenic constituent of the bacterial cell.

EXPERIMENTAL.

Methods.—Sterile pneumococcus extracts (10) supplied the hemotoxin. The term "reduced" extract, as used in this paper, refers to pneumococcus extracts which have been protected from oxidation. The term "oxidized" extract denotes extracts in which the hemotoxin has been oxidized by exposure to air in unagitated Erlenmeyer flasks. The pneumococcus extracts employed throughout the present study consisted of the filtered extract of a concentrated suspension of pneumococcus cells which had been disrupted by repeated freezings and thawings. This type of extract (the "complete system" type) (10–13) contains easily oxidized substances which upon exposure to air form oxidizing agents which inactivate the hemotoxin. The differences between this type of pneumococcus extract and the so called "incomplete system" type of extract have already been described (11). The hemolysis tests were made in salt suspensions of well washed sheep or horse erythrocytes. The extracts which were used in most of the experiments were prepared by Dr. Louis A. Julianelle, of the Hospital of The Rockefeller Institute for Medical Research.

"Reactivation" of Oxidized Hemotoxin by the Reducing Activity of Bacteria.

In a preceding paper (14) it was shown that the methemoglobin formed by the oxidizing action of pneumococci or of sterile pneumococcus extracts, could, in the absence of air, be reconverted to hemoglobin by the reducing action of the bacteria. Hence, experiments were made to determine if the same biological reducing agents can reconvert the hemolytically inactive oxidation product of pneumococcus hemotoxin to the original active form. Accordingly, bacteria were added to pneumococcus extracts in which the hemotoxin had previously been inactivated by oxidation. The mixtures were sealed

and incubated for several hours; after the period allowed for reduction, the bacterial cells were removed by centrifugation, and the bacteria-free supernatant of the mixture was titrated for its hemotoxin content. Controls were included to prove that the bacteria used as reducing agents were devoid of hemolytic activity.

Pneumococcus hemotoxin inactivated by oxidation was obtained by exposing an active pneumococcus extract (Type III) to air for 3 hours at 38°C.

Cultures of *B. coli* and of Anaerobic Bacillus *T* (an anaerobic organism isolated from a wound and morphologically resembling *B. tetani*) were grown in 50 cc. centrifuge tubes under vaseline seal; these cultures were centrifuged and the bacterial cells were suspended in 1.0 cc. of the supernatant broth.

TABLE I.

"Reactivation" of Oxidized Pneumococcus Hemotoxin by the Anaerobic Action of Bacteria.

Amount of pneumococcus extract.	Original (reduced) lysin.	Oxidized lysin.	Oxidized lysin after reduction by *B. coli*.	Oxidized lysin after reduction by anaerobic bacilli.	Controls on bacterial reducing agents.
cc.					
0.10	++++	−	++++	++++	−
0.05	++++	−	++++	++++	−
0.04	++++	−	++++	++++	−
0.02	++++	−	++++	++++	−
0.01	++++	−	++++	++++	−

The following test mixtures were prepared:

(1) 0.5 cc. oxidized pneumococcus extract + 0.5 cc. broth.
(2) 0.5 " " " " + 0.5 " suspension of *B. coli*.
(3) 0.5 " " " " + 0.5 " " " Anaerobic Bacillus T.
(4) 0.5 " broth + 0.5 cc. suspension of *B. coli*.
(5) 0.5 " " + 0.5 " " " Anaerobic Bacillus T.

These mixtures were sealed with vaseline and incubated at 38°C. for 1½ hours, after which time they were centrifuged at high speed. The hemolytic activity of the supernatants of these mixtures was then titrated. Mixtures (4) and (5), comprising controls on the bacterial reducing agents employed, were always negative whether or not the bacterial cells were removed from the test samples added to the erythrocyte suspensions. The results of the titrations of these mixtures ((4) and (5)) are presented jointly in the protocol under the heading "Controls on bacterial reducing agents."

The protocol of a typical experiment is presented in Table I.

The results (Table I) of experiments such as that described indicate that the inactive, oxidation product of pneumococcus hemotoxin may be reconverted to an actively hemolytic substance by the action of certain bacteria which are not themselves hemolytic. A sufficient number of control tests have been made to prove that the hemolytic substance produced by the action of these bacterial agents upon oxidized pneumococcus extract is not yielded by the action of the same agents upon the broth medium.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by Reduction.

Experiments were designed to prove that the hemolytic substance yielded by the action of bacterial reducing agents upon oxidized pneumococcus extracts was identical with the original hemotoxin present in the "reduced" pneumococcus extract. These experiments included comparisons of the heat lability of the lysins and tests of their immunological neutralization.

A. Comparison of Heat Lability.—Although bacterial hemotoxins, as a class, are relatively heat-labile, certain degrees of difference in lability exist between the lysins of different bacterial origin, as will be shown in subsequent papers. The hemotoxin of Pneumococcus stands out as an exceptionally heat-labile substance. When heated in narrow, thin walled tubes, it is entirely inactivated by 90 seconds exposure to 55°C. This is a considerably greater heat lability than that of most other hemolytic substances. It is conceivable that if the lysin yielded in the preceding experiment differed from the one present in the original reduced extract, it might be found to possess a higher degree of heat resistance than the original pneumococcus hemotoxin.

Comparative heating tests were made on 0.6 cc. of reduced pneumococcus extract and 0.6 cc. of the supernatant of the reduced mixtures of oxidized extract and bacterial cells (mixtures analogous to (2) and (3) in the preceding experiments). The test liquids were placed in narrow thin walled tubes, sealed with vaseline, and heated at 55°C. for 90 seconds in an agitated water bath.

B. Specific Immunological Neutralization.—Tests of the neutralization of the hemolytic substance by the antihemotoxin in pneumococcus immune serum were made as follows: 3 hemolytic units of the original hemotoxin, and the same amount of the hemolytic substance produced by the action of the bacteria upon the oxidized extract, were diluted to 1.5 cc. with salt solution; 0.005 cc. of normal horse serum and 0.005 cc. of the serum of a horse immunized against Type I Pneumococcus were added to separate tubes of each of the lysins; after 45 minutes incubation of the mixtures, 0.5 cc. of 20 per cent sheep cells was added to each tube. (The

extracts used as source of hemotoxin were of Type III pneumococci. Since the antihemotoxin is not "type-specific" a heterologous immune serum was used to avoid the specific precipitation of the Type III "S" substance also present in the extracts.)

The results of both of these experiments are given in Table II.

As shown in Table II, the hemolytic substance produced by the anaerobic action of the bacteria upon oxidized pneumococcus hemotoxin possesses heat lability comparable to that of the original reduced hemotoxin. This fact merely suggests that the two are identical. More convincing proof is furnished by the immunological neutralization of the "reactivated" hemotoxin by the antihemotoxin present in pneumo-

TABLE II.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by the Anaerobic Action of Bacteria.

	Heat lability.		Neutralization by anti-pneumococcus serum of heterologous type.	
			Hemolysis by 3 hemolytic units previously incubated with 0.005 cc. serum.	
	Hemolysis by 0.01 cc. unheated extract.	Hemolysis by 0.1 cc. extract heated 90 sec. at 55°C.	Normal serum.	Immune serum.
Original active hemotoxin in reduced extract	++++	−	++++	−
Hemotoxin inactivated by oxidation and "reactivated" by reduction	++++	−	++++	−

coccus immune serum. This antibody is specific and does not neutralize the lytic substances produced by other bacteria.[1]

Failure to "Reactivate" Hemotoxin Which Has Been Inactivated by Heat.

Experiments were next made to determine if the inactive product obtained by heating pneumococcus hemotoxin can be "reactivated" by the reducing action of bacterial cells. Obviously, the lysin should be heated as little as possible, else it may be so changed chemically that it cannot be reactivated. It seemed probable from the studies made of heated hemoglobin (1) that "reduced" pneumococcus hemo-

[1] Unpublished experiments to be presented in a subsequent paper.

toxin heated anaerobically may yield inactive products which cannot be "reactivated" by the reducing agents which serve to "reactivate" the oxidation product of the hemotoxin.

"Reduced" (active) hemotoxin was heated anaerobically for a period just sufficient to destroy its activity. The heating was carried out exactly as described in the preceding experiment. Another portion of the same extract was inactivated by exposure to air. The reduction tests were made by use of mixtures analogous to those described in the experiments reported in Table I. The results are presented in Table III.

From Table III, it is obvious that the biological reducing agents which serve to "reactivate" the oxidation product of pneumococcus

TABLE III.

Attempt to "Reactivate" Heat-Inactivated Hemotoxin.

Amount extract.	Hemotoxin in reduced pneumococcus extract.		Hemotoxin in unheated oxidized extract (before action of bacteria).	Hemotoxin in unheated oxidized extract (after action of bacteria).	Hemotoxin in heated oxidized extract (after action of bacteria).
	Unheated.	Heated.			
cc.					
0.06	++++	−	−	++++	−
0.04	++++	−	−	++++	−
0.03	++++	−	−	++++	−
0.02	++++	−	−	++++	−
0.01	++	−	−	+	−

hemotoxin, fail entirely to "reactivate" the inactive products formed in the anaerobic heating of the reduced hemotoxin. These results serve as a valuable check on the preceding experiments, and indicate, moreover, that the "inactive" products formed in the oxidation of the hemotoxin are quite different from those formed when the hemotoxin is destroyed by heat. Since different products are formed, the reactions involved in the inactivation of the hemotoxin by oxidation and by heat must be of different nature, the inactivation by oxidation apparently being similar in nature to the reversible change of hemoglobin to methemoglobin, while the inactivation by heat is comparable to the destruction of hemoglobin to irreversible blood pigment derivatives.

"Reactivation" of Oxidized Pnemococcus Hemotoxin by Treatment with Sodium Hydrosulfite.

It seemed probable from the preceding experiments, that the "reactivation" of the oxidized hemotoxin by the anaerobic action of bacterial cells represented a reduction process. More definite evidence was obtained by the following tests which are similar to those previously described, with the exception that a chemical reducing agent, sodium hydrosulfite, was substituted for the biological reducing agents previously employed.

Weighed amounts of sodium hydrosulfite ($Na_2S_2O_4$) were placed in large test-tubes. Solutions of the required strength were made in measured amounts of 0.1 M, pH 7.5 phosphate buffer mixtures. The solutions were made up fresh for each test and were used within less than 5 minutes after their preparation.

Preliminary tests were made of the possible hemolytic action of the hydrosulfite itself. If solutions of hydrosulfite were added to broth in sufficient strength to give a concentration of 5 per cent in the reduction mixture, the addition of from 0.02 to 0.10 cc. of the mixtures to 1 cc. of blood cell suspension was not without effect. Upon incubation of the blood cell suspensions containing this amount of hydrosulfite, a part of the blood pigment was converted to methemoglobin. This is in conformity with the reports of Conant and Fieser (15) on the action of the oxidation products of the hydrosulfite. As in the case of the biological reducing agents described in previous papers (1, 2), the hydrosulfite, which essentially is a reducing agent, forms oxidation products in the presence of air which oxidize the blood pigment. This phenomenon is important in tests such as are to be described, from two points of view. First, the "methemoglobinized" red blood cell may be much more difficultly hemolyzed than the unaltered cell. (Reports to this effect in the literature have been partially confirmed by incomplete experiments of our own.) Second, there is also a possibility that the same oxidizing agents which oxidize the hemoglobin in the presence of air, may also oxidize the hemotoxin if the reduction mixture is freely exposed to air *before* its addition to the blood cells.

However, both of these difficulties are readily overcome. Amounts of hydrosulfite too small to injure the blood cell suffice to reduce the oxidized hemotoxin.

The possible injurious action of the aerobic oxidation products of hydrosulfite upon the hemotoxin can be avoided by adding the samples of the reduced mixtures to the red blood cells with a minimum exposure of the reduced mixture to air. Probably the small samples of the mixtures taken for the hemolysis tests include insufficient hydrosulfite to injure the hemotoxin, or possibly the hemotoxin after combination with the red blood cell is difficult to destroy (in analogy with the relative stability exhibited toward oxidizing agents by hemoglobin after combination with oxygen or carbon monoxide) (2). If sufficiently large amounts of the reduction mixtures are added to the blood cells, enough hydrosulfite is included to reduce the oxyhemoglobin. This, however, does not seem to interfere significantly with the hemolytic titres.

In the experiments tabulated in the protocol to be presented in Table IV the following detailed procedure was used.

0.2 cc. of pneumococcus extract previously inactivated by oxidation, diluted to 1.0 cc. with 0.1 M phosphate solution (pH 7.5), was placed in a series of tubes.

TABLE IV.

"Reactivation" of Unheated Oxidized Pneumococcus Hemotoxin by Reduction with Sodium Hydrosulfite.

	Hemotoxin titrations.		
	0.03 cc.	0.02 cc.	0.01 cc.
Oxidized extract untreated	−	−	−
Oxidized extract treated with $Na_2S_2O_4$	++++	++++	++
Heated extract treated with $Na_2S_2O_4$	−	−	−

Another series of tubes containing 0.2 cc. of extract inactivated by heat and similarly diluted, and a third series containing broth instead of pneumococcus extract were prepared.

To these tubes was added 0.2 cc. of various dilutions of sodium hydrosulfite dissolved in 0.1 M phosphate solution (pH 7.5). These mixtures were sealed with vaseline. After allowing time for reduction, varying from 5 minutes in the case of the higher concentrations of the reducing agent to 30 minutes in the case of the lower concentrations, the hemolytic activity of the reduced mixtures was titrated. The samples were added to the red blood cells immediately after the removal of the seal. The reduction, of course, is dependent upon the pH, the temperature, and the time, as well as the concentration of hydrosulfite. The pH was maintained at 7.5, the temperature was approximately 25°C., and the time allowed for reduction was never over 30 minutes. The concentrations of $Na_2S_2O_4$ in the reduction mixtures reported in the table ranged from 1.0 to 0.008 per cent.

The hemolytic test mixtures were incubated for 1 hour at 38°C. The test mixtures were then centrifuged and the hemolytic titres recorded.

The results are given in Table IV.

The results (Table IV) show that the inactive product formed by the oxidation of pneumococcus hemotoxin may be "reactivated" by chemical reducing agents as well as by the reducing action of bacteria. Reducing agents convert the inactive products formed during exposure of the hemotoxin to air, to the original active hemotoxin, but they do not "reactivate" the products formed by the anaerobic heating of the active hemotoxin.

Attempts to "Reactivate" Oxidized Pneumococcus Hemotoxin by the Reducing Action of Sterile Animal Tissues.

In previous studies (12) it has been shown that methemoglobin is formed by action upon hemoglobin by the same agents responsible for the oxidation of the hemotoxin. The oxidized, "inactive" blood pigment can subsequently be converted to "active" hemoglobin by the reducing action of sterile animal tissues *in vitro* (14). It seemed probable that the reducing action of animal tissues would likewise "reactivate" the oxidation product of the hemotoxin. The importance of such an action of the tissues is an obvious one, since the hemotoxin is an antigen of bacterial origin.

A limited number of tests have been made by adding sterile, rabbit testicle to oxidized pneumococcus hemotoxin. In no case was an "active" lysin obtained. The negative results of these experiments, however, do not rule out a reducing action of animal tissue to "reactivate" the oxidized hemotoxin. It is quite possible that the hemotoxin was actually reduced but was combined with the lipoids of the tissues. The literature furnishes many reports of the "inactivation" or neutralization of bacterial hemotoxins by tissues *in vitro;* and the inhibitory action of lipoids upon the hemotoxins has been demonstrated for pneumococcus lysin by Cole (8).

Reduction of Inactive Oxidized Hemotoxin Present in Solutions of the "Protein Fraction" of Pneumococcus Cells.

The hemotoxin of Pneumococcus, unlike most bacterial hemotoxins, is endocellular. It is apparently protein in nature, possesses the power of inducing antibody formation, and is destroyed by trypsin digestion (Cole (8)). All this being true, it seemed probable that the

hemotoxin might be included in the "protein" fraction of the Pneumococcus which is precipitated by acetic acid in the cold (16). A solution of the "pneumococcus protein" furnished by Dr. Avery was used in the following experiment.

This solution had been prepared from Strain F, a Type IV Pneumococcus, by the method described by Avery and Heidelberger (16). It contained 2.8 mg. of protein in 1 cc., had not been protected from air, and was several weeks old when used.

Preliminary tests showed that the solution was non-hemolytic when an amount as large as 0.3 cc. was added to 0.5 cc. of red blood cells. This indicated that if the hemotoxin had been precipitated by the acid, it was present in the inactive form.

Reduction tests similar to those described in experiments with the oxidized "pneumococcus extracts" were made. Two portions of 0.5 cc. of the protein solution were placed in separate tubes; to one of the tubes, 0.5 cc. of 0.1M phosphate solution was added; 0.5 cc. of 0.4 per cent solution of hydrosulfite was added to the other tube. Both tubes were sealed. At the end of the 15 minute period which was allowed for reduction, portions of the "reduced" solution and of the phosphate-diluted control were added to 0.5 cc. of red blood cells. The volume of each hemolysis test mixture was adjusted to 1.0 cc. by the addition of salt solution. After 1 hour's incubation at 38°C., the tests were centrifuged and observations made of the hemolysis.

Samples as large as 0.6 cc. of the phosphate-diluted control gave no hemolysis, which confirmed the previous observation of the absence of active lysin in the protein solution. On the other hand, in the case of the solution treated with the reducing agent, samples as small as 0.03 cc. gave complete hemolysis.

The results of this experiment indicate that the pneumococcus hemotoxin is precipitated by acetic acid in the cold, and is included in solutions of the "protein fraction" of the cell. Although the hemotoxin is not oxidized rapidly in solutions of washed cells in the absence of other easily oxidized substances (4), it is probably slowly oxidized during the preparation and storage of the protein solutions. There is every reason to believe that the inactive lysin which was "reactivated" in the above experiment is identical with the reversible oxidation product of pneumococcus hemotoxin which has been reported in the preceding experiments with sterile pneumococcus extracts.

Reversibility of the Oxidation-Reduction of Pneumococcus Hemotoxin.

The endocellular nature of pneumococcus hemotoxin, which is not liberated into the culture fluid until disruption of the cell membrane, made possible the fol-

lowing experiment which would be difficult to attempt with "exocellular" bacterial lysins set free during the stage of active growth of the culture.

The object of the experiment was to determine whether both the oxidation and reduction processes can be induced by bacterial cells of the sort from which the hemotoxin was originally derived. Pneumococci possess both oxidizing and reducing powers (1, 10, 13), the reaction induced being dependent upon the presence or absence of air. Hence, pneumococci can be employed as the agents for both oxidation and reduction processes, by admitting or excluding air. If only young, unautolyzed cells are used, it is possible to remove the bacteria by centrifugation and obtain a supernatant solution which includes no hemolytic substances introduced by the microorganisms used as oxidizing and reducing agents. This point was carefully controlled by parallel tests carried out with mixtures of broth and pneumococci at the time of each reduction and oxidation treatment.

The experiment consisted in several successive reversible oxidations and reductions of the hemotoxin by young, unautolyzed pneumococci. The oxidation in each instance was effected by aeration of the mixture of hemotoxin solution and bacterial cells. After allowing time for the oxidation of the hemotoxin, the bacteria were removed by centrifugation, and the supernatant solution was "titrated" for active hemotoxin. The oxidized solution was then again reduced by adding a fresh suspension of young pneumococci and sealing the reduction mixture from air. When sufficient time had elapsed for reduction, the mixture was centrifuged again to remove the bacteria, and the supernatant of the reduced solution was titrated for active hemotoxin content.

This procedure was repeated with the same original solution of hemotoxin for three successive series of alternate oxidations and reductions. The sequence of conversions of the same solution of hemotoxin from the active reduced form to the inactive oxidized form, and back again, is outlined in Table V.

Solution I represents the original active hemotoxin present in the reduced extract at the beginning of the experiment. The first oxidation of the hemotoxin was effected by the oxidizing agents formed in the extract itself when exposed to air (4). The oxidized extract, owing to the inactivation of the labile cellular components of its oxidation-reduction systems, is no longer able to oxidize hemoglobin or reduce methylene blue and methemoglobin (11). Since the extract itself is rendered devoid of oxidizing and reducing activity after the first oxidation treatment, Solutions II, IV, and VI can be regarded simply as solutions of oxidized hemotoxin. Thus, the successive oxidations (inactivations) and reductions (reactivations) in Solutions II to VI can be referred directly to the oxidizing or reducing action of the young pneumococci added to the respective mixtures.

TABLE V.

The Reversible Oxidation and Reduction of Pneumococcus Hemotoxin by Living Pneumococcus Cells.

Oxidation or reduction mixture.	Treatment.	Oxidizing or reducing agent.	Period of oxidation or reduction.	Hemolytic activity. Amounts of mixture expressed in terms of original extract.				
				0.06 cc.	0.03 cc.	0.02 cc.	0.01 cc.	0.006 cc.
Solution I	Original solution.	Sterile pneumococcus extract sealed from air.		++++	++++	++++	++++	+++
Solution II	Solution I after oxidation.	Sterile pneumococcus extract in the presence of air.	1.5 hrs.	−	−	−	−	−
Solution III	Solution II after reduction.	Young, intact pneumococci in the absence of air.	2.5 "	++++	++++	+++	+	−
Solution IV	Solution III after oxidation.	Young, intact pneumococci in the presence of air.	1.0 hr.	−	−	−	−	−
Solution V	Solution IV after reduction.	Young, intact pneumococci in the absence of air.	3.0 hrs.	++++	+++	++	±	−
Solution VI	Solution V after oxidation.	Young, intact pneumococci in the presence of air.	0.5 hr.	−	−	−	−	−
Solution VII	Solution VI after reduction.	$Na_2S_2O_4$ in the absence of air.	10 min.	++++	++++	++++	++	+
Supernatant of controls (broth plus young pneumococci) for each reduction and oxidation mixture.				−	−	−	−	−

Control Tests with Mixtures of Broth and Pneumococci.—Broth was treated in exactly the same manner as the solutions of the hemotoxin. Upon removal of the bacteria by centrifugation after each oxidation and reduction period, the supernatant of these mixtures was tested for hemolytic activity. They never contained a trace of hemolytic substances, a fact which can be taken as proof that no hemotoxin was liberated by the cells used in the experiment proper.

The results of this experiment (Table V) illustrate the reversible oxidation-reduction of pneumococcus hemotoxin by pneumococcus cells. This experiment is analogous to one upon the reversible oxidation-reduction of blood pigment by pneumococci (Table II, in a previous paper (14)). Here, too, longer periods of time were required for the anaerobic reduction of the hemotoxin than for its oxidation in the presence of air.

COMMENT.

Pneumococcus hemotoxin is an actual constituent of the bacterial cell. In the reduced condition it possesses certain properties which are lost upon oxidation. As it exists in the cell it is protected by the reducing activity of the living bacteria. If the cells are sufficiently exposed to air, oxidizing agents are formed which oxidize the hemotoxin. If, however, the exposure to air is not too prolonged, nor sufficiently drastic to inactivate completely the reducing activity of the cells, this cellular constituent can be reconverted to the original reduced substance whenever the tension of molecular oxygen tension does not mask the native reducing powers of the bacterial cells. The failure of the reduction systems present in the sterile filtered extracts to "reactivate" subsequently the oxidized hemotoxin in aerated extracts is analogous to the loss of their ability to reduce methylene blue and methemoglobin. As previously shown, the loss in reducing power is due to the inactivation of the thermolabile constituent of the oxidation-reduction system (11). Apparently, these thermolabile constituents are more readily inactivated or "overtaxed" in the sterile, filtered, cell extracts than in the living intact bacterial cell.

While the hemotoxin itself may be of no physiological importance, it is highly probable that the reversible oxidation-reduction of some other thermolabile cellular constituents is involved in the maintenance of the physiological activity of bacterial cells.

SUMMARY.

Pneumococcus hemotoxin, an antigenic substance of bacterial origin, is converted by oxidation to a product devoid of hemolytic action. The oxidation product of the hemotoxin may be converted to the original hemolytic substance by reduction, by the anaerobic action of certain bacteria, or by sodium hydrosulfite. The active lysin, or hemotoxin, produced by the reduction of the inactive oxidized extracts is identical with the original, active, reduced hemotoxin; it possesses the same degree of thermolability and is neutralized by the same specific antibody. The inactive products formed by heating the hemotoxin anaerobically cannot be "reactivated" by reducing agents. The immunological significance of these relations will be discussed in a subsequent paper.

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