STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

IV. Oxidation of Hemotoxin in Sterile Extracts of Pneumococcus.

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INTRODUCTION.

In 1912 Cole (1) described the presence of a hemotoxin in pneumococcus extracts and later (1914) (2) defined many of the biological properties of this substance. Pneumococcus hemotoxin is intracellular in nature, exists preformed in the bacterial bodies, and is liberated only after rupture and dissolution of the cells. The earlier study was carried out on bile or cholate solutions of pneumococci. The hemotoxin in these bacterial solutions which produced acute death in guinea pigs was found to cause rapid hemolysis of red blood corpuscles. In the later experiments, Cole confirmed these observations and brought convincing evidence that this hemolysin is an intracellular constituent of pneumococcus, and is unassociated with the action of the bile salts previously used, since he found it present in bacterial autolysates and in saline extracts prepared from washed, frozen, and ground pneumococci. The substance in pneumococcus extracts upon which this hemolytic property depends was found to be labile; its activity was destroyed in extracts heated to 55°C.; greatly decreased or completely lost by passage through a Berkefeld filter; destroyed by digestion with trypsin, and markedly inhibited by normal rabbit serum. Cole also observed that the hemolytic power of autolytic extracts was markedly diminished after 18 hours and completely absent after 48 hours exposure at 37°C.

In a preceding paper of this series on oxidation and reduction by pneumococcus, it has been demonstrated that sterile extracts of pneumococci are markedly reactive with molecular oxygen and that

peroxide is formed as a result of this union. These studies have afforded an opportunity to inquire whether these oxidative reactions have any effect upon the presence and potency of the hemotoxin contained in pneumococcus extracts.

EXPERIMENTAL.

Method.

Sterile Extracts of Pneumococcus.—The pneumococcus extracts were prepared by the methods described in the preceding papers (3, 4). They were of two types: (1) unwashed pneumococci extracted in broth; (2) washed pneumococci extracted in phosphate solution. In most instances these extracts were filtered through Berkefeld candles before use. All extracts employed in these tests were proved sterile by cultural and animal methods.

Hemolysis.—The hemolytic reaction of the extracts was tested as follows: The red blood corpuscles were removed by centrifugation from sterile, freshly drawn, defibrinated rabbit blood, washed three times, and finally suspended in salt solution. To 2.5 cc. of the 1 per cent suspension of red cells 0.1 cc. of the hemolytic extract was added. The mixture of red blood corpuscles and extract was incubated 3 hours at 37°C. The degree of hemolysis was recorded after incubation and final readings were made at the end of 12 hours in ice box.

Experiment 1. To Determine the Hemolytic Power of Pneumococcus Extract.—The extract used in the following experiment was prepared from Pneumococcus Type II. The extraction was carried out in the supernatant broth in concentration such that 1 cc. of the extract represented the extractable material from the bacterial cells in 35 cc. of the original broth culture. The bacteria were grown anaerobically. During the process of extraction the preparation was subjected to the minimal exposure to air, to prevent oxidation reactions, which will later be shown to affect materially the potency of the hemotoxin. This extract was passed through a Berkefeld filter to remove all formed elements, filtration being carried out in a nitrogen atmosphere. The final extract was preserved in the reduced state under heavy vaseline seal. The hemolytic titer of this extract was determined by finding the minimum amount of extract which would cause lysis of a constant suspension of red blood corpuscles.

From Table I it is apparent that as little as 0.001 cc. of the filtered, reduced extract caused marked but incomplete hemolysis of the red cells of rabbit blood and that 0.01 cc. of extract brought about complete cell solution. The minimal lytic amount of extract effecting complete hemolysis of a standard suspension of blood corpuscles represents, in this particular instance, the hemolytic substance extractable from the bacterial cells in 0.35 cc. of original culture. Con-

siderable loss of the hemolytic property of these extracts occurs during the procedures incident to their preparation. As pointed out by Cole the hemolytic effect of an extract is markedly diminished by Berkefeld filtration.

Experiment 2. To Determine the Effect of Heat on the Hemolytic Action of Pneumococcus Extract.—An unfiltered portion of the extract used in Experiment 1 was employed in this test. Equal quantities of the unfiltered, reduced extract were placed in a series of small narrow tubes and immediately sealed with vaseline. The various fractions of the extract were heated by immersing the sealed tubes for 10 minutes in water baths at 45°, 50°, 55°, and 65°C., respectively. Titrations of the hemolytic power of the heated and unheated extract were made by the method already described.

TABLE I.

Hemolytic Activity of Filtered, Reduced Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis.
cc.	
0.10	++++*
0.05	++++
0.01	++++
0.005	+++
0.001	++

* ++++ = complete hemolysis.
+++ = almost complete hemolysis.
++ = incomplete hemolysis.
+ = slight "
− = no "

The effect of heating a sterile broth extract of pneumocccous for 10 minutes at temperatures ranging from 45°–65°C. is shown in Table II. Exposure for 10 minutes to 55°C. completely destroyed the hemolytic activity of the extract. This heat sensitiveness of the substance upon which hemolysis depends, is identical with that described by Cole for pneumococcus hemotoxin. The destruction of the reactive substance at 55°C. is evidence of its thermolability. A rapid increase in the rate of destruction occurs between 50° and 55°C. In a preceding paper it is pointed out that a similar acceleration in the rate of destruction of the peroxide-forming activity of pneumococcus extracts occurs between 60° and 65°C.

In previous communications it was shown that sterile broth extracts of pneumococci which have been preserved in the reduced state rapidly form peroxide when exposed to molecular oxygen. Moreover, with the accumulation of this compound the oxidized extract suffers a decrease and final loss of both its peroxide-forming and methylene blue-reducing activity. It seemed of interest, therefore, to study the effect of oxidation upon the hemolyzing action of a broth extract. Accordingly the following experiment was carried out.

Experiment 3. To Determine the Influence of Oxidation upon the Hemolytic Activity of a Broth Extract of Pneumococcus.—A comparison of the hemolytic activity of the same extract in the oxidized and reduced state was made in the following manner.

TABLE II.

The Effect of Heat upon the Hemolytic Activity of a Sterile Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis by				
	Unheated extract.	Extract heated 10 min. at			
		45°C.	50°C.	55°C.	65°C.
cc.					
0.2	++++	++++	++++	−	−
0.1	++++	++++	++++	−	−
0.05	++++	++++	++++	−	−
0.01	++++	++++	++++	−	−
0.005	++++	+++	++	−	−

A sterile broth extract, which when kept in the reduced state exhibited marked hemolytic properties, was divided into three portions. One part was placed in a shallow layer in an Erlenmeyer flask and exposed to air at 37°C. in the dark. A second fraction was similarly treated except that it was exposed to air at 2°C. The third portion was transferred to a narrow tube, immediately sealed with vaseline, and held at 37°C. At the end of 1, 2, and 3 days, given amounts were removed from each of the three portions, and the hemolytic power of the oxidized and reduced fractions compared (see Table III).

The hemolytic power of a broth extract of pneumococcus differs, depending upon whether the extract is in the reduced or oxidized form. In Table III it is seen that if an extract is exposed to air it suffers loss of its hemolytic potency. On the other hand this property

may be conserved for considerable periods of time if the extract is kept in the reduced state unexposed to molecular oxygen. The reduced extract, after having been stored for 3 days at 37°C., caused prompt and complete lysis of the red blood cells in amounts of 0.01 cc. On the other hand, this amount of the same extract, after having been oxidized by exposure to air at 37°C. for 24 hours, failed to show any evidence of hemolysis when subsequently added to blood corpuscles. Active broth extracts of pneumococcus have been shown to unite with or accept oxygen when exposed to air. During and probably as a result of this oxidative reaction, peroxide is formed; on continued exposure to air, however, the extract loses its peroxide-

TABLE III.

Comparison of Hemolytic Activity of Reduced and Oxidized Sterile Broth Extract of Pneumococcus.

Amount of extract.	Hemolysis by								
	Reduced extract at 37°C. for days			Extract oxidized by exposure to air					
				At 37°C. for days			At 2°C. for days		
	1	2	3	1	2	3	1	2	3
cc.									
0.01	++++	++++	++++	−	−	−	−	−	−
0.005	+++	+++	+++	−	−	−	−	−	−
0.001	++	++	++	−	−	−	−	−	−

forming activity. This loss is the direct result and natural sequence of the oxidation of the extract. However, since the mechanism of hemolysis itself is not so easily referable to oxidation-reduction processes, it seems reasonable to assume that the hemolytic substance is indirectly destroyed by active products which are formed during the oxidation of other constituents of the extract. Since peroxide is known to accumulate during oxidation of a broth extract of pneumococcus, the effect of adding hydrogen peroxide directly to a sterile extract unexposed to air was determined in the following experiment.

Experiment 4. To Determine the Influence of Hydrogen Peroxide on the Hemolytic Activity of Sterile Broth Extracts of Pneumococcus.— The destruction of diphtheria and tetanus toxins by hydrogen peroxide

has been reported by Löwenstein (5) and by Sieber (6). Tests of the action of hydrogen peroxide upon pneumococcus hemotoxin seemed pertinent to a study of the nature of the reaction by which the hemolytic substance is destroyed, not only because peroxide is formed during oxidation of the extract, but because hydrogen peroxide is itself an active, chemical oxidizing agent.

M/3 and M/20 dilutions of titrated "Dioxygen" were prepared in sterile phosphate solution, pH 7.5. To 0.6 cc. of reduced broth extract of pneumococcus an equal amount of diluted hydrogen peroxide was added. The tubes containing the peroxide and extract mixture were immediately sealed with vaseline. The extract was exposed to the action of the hydrogen peroxide in final concentrations of 0.08 and 0.5 per cent for 4 hours at room temperature. At the end of this period the hemolytic titer of the peroxide-treated extract was compared with that of the untreated extract. As control, hydrogen peroxide in similar concentration was added to plain broth, and treated in the same manner as the extract to determine any possible action of the peroxide upon the red blood cells used in hemolysis test. These tests proved entirely negative and, therefore, are not included in the protocol.

The data presented in Table IV indicate clearly that reagent hydrogen peroxide destroys the hemolytic property of sterile broth extracts of pneumococcus. The untreated extract in amounts of 0.005 cc. caused complete hemolysis. Exposure of this same extract to 0.08 per cent of hydrogen peroxide for 4 hours so reduced the hemolytic activity that it required 0.1 cc. of the treated extract to effect an equal degree of hemolysis. In concentration of 0.5 per cent hydrogen peroxide destroyed completely the hemolyzing action of the extract. This is suggestive though not conclusive evidence that the destruction of the hemolytic substance, which occurs on exposing extracts of this type to the air, is related to the action of the peroxide which is formed during the oxidation of certain reactive constituents of the extract. Although the concentration of hydrogen peroxide added in this experiment is greatly in excess of the peroxide actually formed during oxidation of pneumococcus extract, it is well to remember that a great variety of higher organic peroxides may be formed as intermediate products in autoxidation, and that these substances are known to possess powerful oxidizing properties.

In the studies on oxidation and reduction by pneumococcus, the bacterial extracts employed were found to exhibit marked differences

in activity depending on whether or not they were prepared from washed or unwashed cells. If by the process of repeated freezing and thawing unwashed pneumococci are extracted in broth, the resulting extract possesses the properties of actively reducing methylene blue and of combining directly with molecular oxygen to form peroxide. It must be borne in mind that it is in extracts of this type that destruction of pneumococcus hemotoxin has been found to occur. Under these circumstances the actual destruction of the hemolytic substance may itself be the result of an oxidation process induced by oxidizing agents (peroxides) which are formed by the union of molecular oxygen with the autoxizable substances in the cell extracts.

TABLE IV.

Destruction of the Hemolytic Activity of Sterile Broth Extracts of Pneumococcus by Hydrogen Peroxide.

Amount of extract.	Hemolysis by		
	Untreated reduced extract.	Same extract after 4 hrs. exposure to H_2O_2.	
		0.08 per cent.	0.5 per cent.
cc.			
0.1	++++	++++	−
0.05	++++	+++	−
0.01	++++	+	−
0.005	++++	−	−

On the other hand, preparations obtained by extracting washed pneumococci in phosphate solution have been found incapable of inducing oxidation or reduction unless activated by the addition of cell washings, yeast extract, or broth. Since these saline extracts of washed pneumococci are by themselves not reactive with molecular oxygen, slight or no oxidative reactions occur in them on exposure to air. Pneumococcus hemotoxin in extracts of this type might be expected, therefore, to be more stable in the presence of molecular oxygen. To ascertain whether this is actually the case the following experiment was carried out.

Experiment 5. Comparison of the Effect of Exposure to Air on the Hemolytic Activity of Broth Extracts of Unwashed Pneumococci, and Saline Extracts of Washed Cells.—Unwashed cell extract: prepared as previously described (3) by extraction

of unwashed pneumococci in broth. This extract reduced methylene blue and formed peroxide on exposure to air.

Washed cell extract: prepared as previously described (3) by extraction of washed pneumococci in phosphate solution. This extract did not reduce methylene blue or form peroxide until activated by the addition of yeast extract or broth.

Exposure to air: each preparation was exposed to the air in shallow layer in an Erlenmeyer flask at 37°C.

Titration of hemolysis: at stated intervals portions of each extract were removed and the hemolytic activity determined by the method described. The results are given in Table V and presented graphically in Text-fig. 1. In the latter the logarithms of the number of "hemolytic units" in 1 cc. of extract are plotted against the hours of exposure to air.

TABLE V.

The Effect of Exposure to Air upon the Hemolytic Activity of Extracts of Unwashed and Washed Pneumococci.

1. Broth extract of unwashed pneumococci.

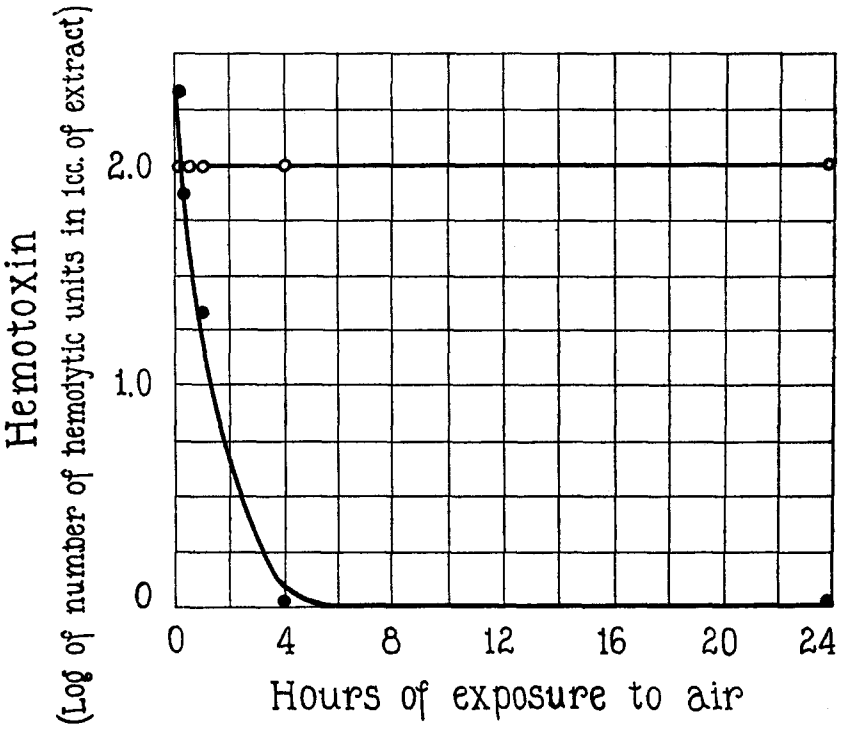
Amount of extract.	Hemolysis by					
	Extract not exposed to molecular oxygen.	Extract after exposure to molecular oxygen at 37°C. for				
		30 min.	1 hr.	4 hrs.	24 hrs.	96 hrs.
cc.						
0.05	++++	++++	+++	±	−	−
0.01	++++	+++	+	−	−	−
0.005	++++	±	+	−	−	−

2. Saline extracts of washed pneumococci.

cc.						
0.05	++++	++++	++++	++++	++++	+++
0.01	+++	+++	+++	+++	+++	+
0.005	+	+	+	+	±	−

Pneumotoxin is an intracellular substance which is liberated from the bacterial bodies whenever the cell undergoes dissolution. This hemolytic agent is present in pneumococcus extracts prepared both from washed and unwashed cells. The occurrence of an easily detectable hemolysin in these two kinds of extract, one type of which is reactive with molecular oxygen and the other wholly unaffected by exposure to air, afforded an opportunity to determine the effect of oxidation upon the stability of this hemotoxin. The results of an experiment of this nature are recorded in Table V. It is evident

that in the absence of air the hemotoxin is remarkably stable in both types of extract. In the presence of molecular oxygen, however, the stability of the hemolytic substance is different in each type of extract. In broth extracts of unwashed cells, which contain oxygen-reactive substances, and in which the oxidation processes



● = unwashed cell extract.
○ = washed cell extract.

TEXT-FIG. 1. Effect of exposure to air upon the hemolytic activity of extracts of unwashed and washed pneumococci.

result in the formation of peroxide, the hemotoxin rapidly disappears when the extract is exposed to air. On the other hand, in saline extracts of washed cells which by themselves are not reactive with molecular oxygen, this hemolytic substance remains practically unchanged in activity even after prolonged exposure to air.

This latter fact is evidence that the hemolytic substance as such is not affected by oxygen, even in the presence of the enzymes in washed cell extracts. It is evident that pneumococcus hemotoxin, a substance not reactive with molecular oxygen, is destroyed in extracts only in the presence of other constituents which are capable of undergoing autoxidation.

SUMMARY.

In the present work on oxidation and reduction by sterile extracts of pneumococcus, the preparations employed contain among other constituents, a hemolytic substance the properties of which have been described by Cole (1, 2) in his studies on pneumococcus hemotoxin. Pneumococcus extracts prepared by the methods described are actively hemolytic, 0.005 cc. of extract causing complete lysis of 2.5 cc. of a 1 per cent suspension of red cells from rabbit blood. This hemolytic property of pneumococcus extracts is destroyed by 10 minutes exposure to 55°C. When pneumotoxin-containing extracts are protected from the action of molecular oxygen, their hemolytic activity remains unimpaired for considerable periods of time. In the presence of air, on the other hand, the stability of the hemolytic substance depends upon whether the particular type of extract contains a "complete" or "incomplete" oxidation-reduction system. Sterile broth extracts of unwashed pneumococci are reactive with molecular oxygen, and as a result of this union peroxide is formed whenever these extracts are exposed to air. The hemolytic activity of "complete" extracts of this type is rapidly decreased and finally destroyed in the presence of molecular oxygen. On the other hand, the "incomplete" type of extract prepared by saline extraction of washed pneumococci may be exposed to air with little or no loss of hemolytic power. This "incomplete" washed cell extract, unless reactivated, does not undergo autoxidation in the presence of air; under these circumstances peroxide is not formed and the hemolytic activity of this type of extract is not impaired by exposure to air. The stability of the hemolytic agent in the "incomplete" type of extract is evidence that this substance is itself not reactive with or affected by molecular oxygen, even in the presence of the cell enzymes. The destruction of the same hemolytic substance in extracts capable

of undergoing autoxidation may be ascribed to the action of some peroxide formed by the union of molecular oxygen with easily oxidized or autoxidizable substances of the extract. It is now known that a peroxide, having the reactions of hydrogen peroxide, accumulates in sterile pneumococcus extracts during oxidation. It has been shown in the present study that the addition of preformed hydrogen peroxide destroys the hemolytic activity of pneumococcus extracts, although higher concentrations were required than were detected in oxidized extracts themselves. These facts and the known action of superoxides in analogous types of reaction make it seem not unlikely that the active agent in the destruction of pneumotoxin in oxidized cell extracts may be a peroxide; either hydrogen peroxide or some higher organic peroxide formed during autoxidation of the extract.

CONCLUSIONS.

When pneumococcus extracts are exposed to air, the hemotoxin is destroyed only in those extracts capable of undergoing autoxidation. The active agent responsible for this destruction is probably a peroxide formed by the union of molecular oxygen with some other, easily oxidizable constituents of the extract.

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