STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

V. The Destruction of Oxyhemoglobin by Sterile Extracts of Pneumococcus.

By JAMES M. NEILL, Ph.D., and OSWALD T. AVERY, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, December 12, 1923.)

In the first of this series of papers suspensions of living pneumococci, which during growth had been protected from the action of air by anaerobic cultivation, were shown to possess the property of uniting with molecular oxygen to form peroxide when exposed to air under conditions unfavorable for cell multiplication (1). The three succeeding papers have dealt with the formation of peroxide (2), the reduction of methylene blue (3), and the oxidation of hemotoxin (4) by sterile extracts of pneumococci under conditions excluding the participation of living or intact cells in these oxidative reactions.

The present paper is concerned with another activity of reduced extracts of pneumococci: the destruction of oxyhemoglobin and the formation of methemoglobin. The principal object of this study is the attempt to obtain more knowledge concerning the mechanism of the destruction of oxyhemoglobin by pneumococcus, and to correlate, if possible, the nature of this phenomenon with the other known oxidation-reduction activities of pneumococcus extracts.

EXPERIMENTAL.

Methods.

Bacteriological.—The sterile extracts of pneumococci were prepared by the methods described in a preceding paper (2). Sterile broth extracts of unwashed pneumococci were used in the following experiments unless otherwise stated. All of the extracts used were proved sterile by cultural and animal tests. These extracts were prepared from anaerobically grown pneumococci and during their preparation were protected from the action of air by being kept under a heavy vaseline seal. Extracts which have been stored in sealed tubes protected from air are referred to in the text as reduced extracts.

Chemical.—The crystalline hemoglobin was prepared from horse blood by Heidelberger's method (5). The hemoglobin crystals were dissolved in 0.1 M phosphate solution (pH 7.5) and the solutions passed through Berkefeld filters. The oxyhemoglobin solutions from laked washed red cells were prepared without crystallization from sterile blood. The spectroscopic examinations were made with a prism spectroscope. The oxyhemoglobin analyses were made by the oxygen capacity method of Van Slyke (6), with the apparatus and technique described by Van Slyke and Neill (7). In determining the "total hemoglobin" the colorimetric method of Stadie (8) was used.

Destruction of Oxyhemoglobin by Sterile Extracts of Pneumococci.

Although a few investigators have demonstrated the production of methemoglobin by sterile extracts and culture filtrates of pneumococci, others have been unable to obtain this reaction in the absence of the living organism. The literature on this subject has been reviewed by Butterfield and Peabody (9), Cole (10), Stadie (11), Schnabel (12), and others.

In the present study, sterile reduced extracts of unwashed pneumococci prepared as described, have been found to induce marked and rapid pigment changes when added to blood or to hemoglobin solutions. Methemoglobin production is evident in 30 minutes in tests in which 0.05 cc. of the extract is used. The greenish, discolored areas formed by pneumococci growing on blood agar plates can also be produced in the complete absence of living cells by placing a small portion of the sterile extract in a depression cut in a fresh blood agar plate.

Quantitative measurements show that the amount of oxyhemoglobin destroyed by these sterile extracts is quite significant. The addition of 0.5 cc. of the extract which has not previously been exposed to air, to 5 cc. of rabbit or human blood, causes a destruction of between 200 and 300 mg. of oxyhemoglobin within a few hours at 37°C.

Destruction of Oxyhemoglobin to Products beyond Methemoglobin.

The intensity of the reaction and the marked pigment changes induced by increasing amounts of active extract indicated that in many instances the destructive process may yield degradation products still lower than methemoglobin. To obtain quantitative evidence of this action, measurements were made of the "total hemo-

globin" (oxyhemoglobin, hemoglobin, and methemoglobin) in solutions to which large and small amounts of active extract had been added.

The data obtained in a number of experiments furnished quantitative evidence that sterile extracts of pneumococci, if present in high concentration, not only convert oxyhemoglobin to methemoglobin, but destroy these blood pigments to still lower degradation products. In low concentration the action of these same extracts is limited to the conversion of oxyhemoglobin to methemoglobin.

Oxyhemoglobin Destruction by Hydrogen Peroxide in the Presence and Absence of Catalase.

Hydrogen peroxide is a chemical agent which is known to effect many oxidations in much the same manner as do living cells. As the destruction of oxyhemoglobin by sterile pneumococcus extract is in all probability an oxidation reaction, it seemed of interest to compare the effect on hemoglobin of the reaction brought about by this chemical agent with that induced by the sterile bacterial preparation. This comparison acquires especial interest from the fact that a peroxide, having the reactions of hydrogen peroxide, is known to accumulate in media during the aerobic growth of pneumococcus. However, the following experiments are introduced here, not to emphasize the importance of hydrogen peroxide as such, but rather to compare the action of an active extract with that of a known oxidizing agent in the presence and absence of blood catalase.

Influence of Catalase in Protecting Oxyhemoglobin from the Action of Hydrogen Peroxide.—The crystalline hemoglobin used in these experiments was almost entirely devoid of catalase. On the other hand, the solutions of hemoglobin prepared directly from laked corpuscles were rich in blood catalase. From these two sources, therefore, it was possible to prepare solutions of equal hemoglobin concentration, which differed widely in their content of blood catalase. In previous experiments the presence or absence of catalase was found to have little or no effect on the oxyhemoglobin-destroying power of the reduced extract of pneumococcus, since practically identical results were obtained with solutions of oxyhemoglobin from laked cells containing abundant catalase and with solutions of crystalline hemoglobin containing only traces of catalase. Quite different results, however, might be expected if preformed hydrogen peroxide ("Dioxogen") were added directly to these two types of hemoglobin, since catalase is known to destroy hydrogen peroxide.

Experiments were made to contrast the hemoglobin-sparing action of catalase in the presence of hydrogen peroxide with the lack of this protection in the presence of reduced extract of pneumococcus; and

to compare the type of change in oxyhemoglobin brought about by the action of hydrogen peroxide with that induced by pneumococcus extract. Because of lack of space the detailed protocols of these experiments are omitted.

As might be expected, the presence of catalase greatly inhibits the action of hydrogen peroxide upon hemoglobin. It was found that hemoglobin, prepared from the crystalline product containing only traces of catalase, was entirely destroyed to colorless products by amounts of hydrogen peroxide which failed to produce any detectable change in solutions of the same concentration of hemoglobin having abundant catalase. The influence of catalase in protecting oxyhemoglobin from the action of hydrogen peroxide is in striking contrast to the almost complete indifference to catalase of the oxyhemoglobin-destroying power of reduced extracts of pneumococcus. However, in spite of this marked protective action of catalase, large amounts of hydrogen peroxide can destroy small amounts of hemoglobin with the production of methemoglobin.

Comparing the type of change in blood pigment effected by this chemical agent with that induced by bacterial extracts, reactions of a quite similar nature were found to occur in both instances. Increasingly large amounts of pneumococcus extracts caused an increasing and progressive degradation of hemoglobin to products beyond methemoglobin. Similar relations have been found to hold true for hydrogen peroxide. Moreover, in the presence of an excess of preformed hydrogen peroxide, the successive phases through which the reaction proceeds simulate most closely those brought about by the action of the bacterial extract upon hemoglobin.

These relations may be interpreted as evidence that the active substance involved in hemoglobin destruction by pneumococcus extract is an oxidizing agent which induces the same general type of reaction as does hydrogen peroxide. However, in the presence of blood catalase, very large amounts of preformed hydrogen peroxide are required; while the hemoglobin-destroying agent in pneumococcus extract is apparently indifferent to the action of catalase.

Influence of Heat on the Hemoglobin-Destroying Activity of Sterile Extracts of Pneumococcus.

The peroxide-forming and methylene blue-reducing activity of a sterile broth extract of unwashed pneumococci is completely destroyed by heating the extract at 65°C. for 10 minutes (3). In a number of experiments the heat stability of the methemoglobin-forming function of pneumococcus extract has been tested and compared with the heat sensitiveness of the system responsible for the formation of peroxide in the same extract.

If an extract is heated at a constant temperature (55°C.) for gradually increasing periods of time, from 10 to 60 minutes, there results a gradual and progressive loss in what may be termed the total oxidizing and reducing power of the extract; less peroxide is formed, methylene blue is more slowly and less completely decolorized, and the rate and degree of hemoglobin destruction decrease. Moreover, not only is the activity of these three functions destroyed at the same rate at 55°C. but the destruction of each shows the same marked acceleration between 60° and 65°C. These relationships, together with the nature of the processes induced, indicate that these properties are functions of the same or closely related systems in the extract.

Activation of the Hemoglobin-Destroying Power of Sterile Extracts of Washed Pneumococci.

In the experiments thus far recorded in this paper, sterile broth extracts of unwashed pneumococci have been used exclusively. The results recorded are sufficient evidence of the marked hemoglobin-destroying activity of extracts of this type. It will be recalled that extracts of washed cells in phosphate solutions have been found to contain potential but incomplete oxidation-reduction systems, and that extracts of this type form peroxide (2) and reduce methylene blue (3) only if they are activated or completed by the addition of cell washings, meat infusion, or yeast extract. In view of the close relationships which seem to exist between these various functions, it might be expected that hemoglobin destruction by washed cell extracts would also be dependent upon the presence of these complementing substances.

Analysis of Table I reveals the interesting fact that an extract of washed pneumococci is unable by itself to destroy hemoglobin, and that this same extract when activated or completed by the addition of certain complementary substances rapidly converts the oxyhemoglobin to methemoglobin. It is evident that yeast extract and muscle infusion complete some otherwise deficient system in the washed cell extract and thus activate the function upon which oxyhemoglobin destruction depends. These relations are analogous to the completion in the same extracts of the peroxide-forming and methylene blue-reducing systems. In an earlier study on the formation of methemoglobin by pneumococcus extracts Avery and Cullen[1] found that an acetone-ether extract of muscle infusion or serum contained substances which served to activate the methemoglobin-forming system of washed bacterial extracts. Although in the present study no attempt has been made to identify further the nature of these substances, it is possible that they may be related to the acetone-ether soluble substances which Meyerhof (13) has shown to be important in the autoxidation processes of tissue cells. However, it is not unlikely that meat infusion broth and yeast extract contain other types of autoxidizable substances.

TABLE I.

Activation of the Oxyhemoglobin-Destroying Power of Sterile Extracts of Washed Pneumococci.

Saline extract of washed pneumococci.	PO_4 solution.	Meat infusion.	Yeast extract.	HbO_2 solution.	HbO_2 destruction and MetHb formation after 2 hrs. at 37°C.
cc.	*cc.*	*cc.*	*cc.*	*cc.*	
0.6	0.6	—	—	0.6	—
0.6	—	0.6	—	0.6	+
0.6	—	—	0.6	0.6	+
—	—	0.6	—	0.6	—
—	—	—	0.6	0.6	—

[1]Unpublished observation.

Comparison of the Amount of Oxyhemoglobin Destroyed by Hydrogen Peroxide and by Reduced and Oxidized Extracts of Pneumococcus.

Throughout these studies the extracts used have been prepared from anaerobically grown cultures of pneumococci and care has been taken to exclude air during the processes of extraction. Sterile broth extracts of unwashed cells prepared under these precautions and preserved under seal are referred to as reduced extracts. Whenever a reduced extract of this type is exposed to air, oxidation products are formed which in turn react upon other constituents of the extract. Extracts in which these changes have been induced by exposure to air are spoken of as oxidized. In preceding papers (3, 4) marked differences have been shown to exist in the activity of the same extract in the reduced and oxidized form.

It seemed of interest to determine the effect of oxidation upon the hemoglobin-destroying activity of an extract. For purposes of comparison, the amount of oxyhemoglobin destroyed by an excess of preformed hydrogen peroxide is contrasted with the amount of blood destruction induced by the pneumococcus extract. This comparison is pertinent since peroxide is formed whenever an extract passes from the reduced to the oxidized form. Moreover, it furnishes quantitative proof of the fact that while blood catalase seriously impairs the action of hydrogen peroxide on hemoglobin, it exerts little or no effect on the hemoglobin-destroying activity of reduced extracts of pneumococcus.

Sterile broth extract of pneumococcus which had been preserved in the reduced form was divided; an aliquot portion of the reduced extract was exposed to the air at 20°C. for 7 hours. This oxidized extract contained demonstrable amounts of peroxide, which had formed during the process of oxidation. The hydrogen peroxide consisted of titrated dilutions of "Dioxogen" in concentration of 1.12 M and 0.28 M solutions. Sterile, freshly defibrinated rabbit blood in which the red cells were hemolyzed by freezing and thawing was added in 2 cc. amounts to six tubes; to each of the first two, 3 cc. of a known dilution of hydrogen peroxide were added; in the next two, 0.4 cc. of reduced and oxidized extract was placed respectively. The last two served as controls. In each instance the total volume was made up to 6 cc. by the addition of broth, phosphate solution, or water as required. All tubes were incubated at 37°C. for 6 hours, and then the oxyhemoglobin content of the series was determined. One of the control tubes containing diluted blood was held at 2°C. until the end of the experiment as a control of the

initial HbO_2. The loss of HbO_2 in the control during incubation at 37°C. was subtracted from the total loss of HbO_2 in all samples. The results are given in Table II.

In terms of the amount of hemoglobin destroyed, Table II presents two striking contrasts; first, the disparity of action between reduced pneumococcus extract and preformed hydrogen peroxide, and second, the extreme difference in the activity of the reduced and oxidized extract. The first contrast is strikingly brought out by assuming, for the purposes of comparison, that the hydrogen peroxide formed during oxidation of the reduced extract is actually concerned *per se* in the destruction of the blood pigment. If the

TABLE II.

Comparison of the Amount of Oxyhemoglobin Destroyed by Hydrogen Peroxide and by Reduced and Oxidized Extracts of Pneumococcus.

Sterile defibrinated rabbit blood.	Amount of hydrogen peroxide (Dioxogen).	Pneumococcus extract.		Amount of oxyhemoglobin destroyed in 6 cc.	
		Reduced.	Oxidized.		
cc.	*millimols*	*cc.*	*cc.*	*millimols*	*mg.*
2	3.36	—	—	0.015	25
2	0.84	—	—	0.008	14
2	—	0.4	—	0.072	121
2	—	—	0.4	0.003	5
2	—	—	—	—	—

production of one molecule of H_2O_2 requires the utilization of one molecule of O_2, then it would be necessary for 0.4 cc. of the reduced bacterial extract to utilize 75,300 c.mm. of oxygen to produce 3.36 millimols of hydrogen peroxide, an amount equal to that added in the experiment. However, the addition of this quantity of hydrogen peroxide resulted in the destruction of only 25 mg. of oxyhemoglobin. On the other hand, 0.4 cc. of the reduced extract destroyed 121 mg. of hemoglobin, approximately five times as much as that destroyed by 3.36 millimols of hydrogen peroxide. These figures do not exclude the possibility that hydrogen peroxide as such may function as the active agent in the destruction of hemoglobin by pneumococcus. However, if oxyhemoglobin destruction under these conditions is attributable to the action of the hydrogen peroxide formed by the

extract, then these figures furnish a striking example of the greater activity of this agent at the moment of its formation than when subsequently added to the system in a preformed state.

The second striking contrast revealed in Table II is the extraordinarily marked difference in activity exhibited by the same extract in the reduced and oxidized form. The reduced extract in amounts of 0.4 cc. destroyed 121 mg. of hemoglobin, while a like quantity of the oxidized extract destroyed only 5 mg., an amount so slight that it may be ignored, being not more than twice the experimental error involved in the analytical technique.

When a reduced extract of pneumococcus undergoes oxidation, its hemoglobin-destroying activity is rapidly lost. After 30 minutes aeration the extract contains demonstrable amounts of peroxide and shows a certain decrease in the rate of methemoglobin formation when added to blood. The loss in activity of the methemoglobin-forming system is progressive with increasing oxidation until at the end of 2 to 3 hours exposure to air, complete and final loss of the methemoglobin-forming power of the extract has occurred. During oxidation, peroxide accumulates in the extract but the peroxide formed in the oxidized extract if subsequently added to blood is unable by itself to effect any change in the pigment in the presence of blood catalase.

The destruction of hemoglobin, with the resulting formation of methemoglobin by sterile extracts of pneumococcus, is apparently a function of the oxidation-reduction systems contained in the extracts. An extract in which oxidation has occurred is subsequently incapable of converting oxyhemoglobin to methemoglobin.

On the other hand, an extract in which the active substances are protected from oxidation retains its potency for months, even if stored at 37°C. These facts indicate that methemoglobin formation is a process which accompanies and is dependent upon the initial oxidation of some constituent of the extract. Moreover, in the formation of methemoglobin, it is necessary that the oxyhemoglobin be present in the system during the oxidation of the extract; if an extract is completely oxidized before the addition of oxyhemoglobin the reaction does not occur in the presence of catalase.

Comparison of the Action of Reduced and Oxidized Extract of Pneumococcus on Oxyhemoglobin in the Presence and Absence of Blood Catalase.

In a preceding experiment it was shown that when hydrogen peroxide (reagent) is added to oxyhemoglobin, the pigment changes which result are determined by and dependent upon the balance between the concentration of the chemical agent and the amount and activity of the blood catalase present. In solutions of crystalline oxyhemoglobin containing little or no catalase, methemoglobin was rapidly formed by amounts of hydrogen peroxide which produced no change in catalase-containing solutions of red cells. In the latter instance the catalase in decomposing hydrogen peroxide served to protect the oxyhemoglobin, while in the former case the absence of catalase exposed the blood pigment to the direct action of hydrogen peroxide itself. On the other hand, reduced pneumococcus extract effects the transformation of oxyhemoglobin to methemoglobin in the presence as well as in the absence of catalase. This difference of behavior suggests that the mechanism of the latter reaction is more complex than the simple interaction between hydrogen peroxide and oxyhemoglobin. This assumption would be further strengthened if it could be shown that the peroxide which accumulates as a final product of the oxidation of pneumococcus extract were quite as readily destroyed by the catalase of the blood as is hydrogen peroxide when added directly as a chemical reagent. In order to test the validity of this point the following experiment was carried out.

Oxyhemoglobin containing only faint traces of catalase was obtained by preparing 0.4 mM solutions of the crystalline product in phosphate solution (pH 7.5). Oxyhemoglobin containing abundant catalase was obtained by the use of defibrinated blood undiluted or diluted tenfold in phosphate solution. A portion of the same extract as that used in the reduced state was exposed to air for 12 hours at 20°C. The oxidized extract at the end of this period and before oxyhemoglobin was added, gave a strong peroxide reaction. Portions of the oxidized and reduced extract were heated 10 minutes at 75°C. to inactivate the labile constituents of the extract. The oxidized extract after heating still gave a marked peroxide reaction. 0.1 cc. portions of heated and unheated extract, both reduced and oxidized, were added to 0.9 cc. of the catalase-free and catalase-rich solutions of oxyhemoglobin in the order presented in Table III. Spectroscopic examinations were made after 4 hours at 37°C.

This experiment shows that when reduced extract is added to defibrinated blood methemoglobin is formed in spite of the presence of catalase. Furthermore, the same pneumococcus extract if allowed to oxidize with the consequent production of hydrogen peroxide fails to induce methemoglobin formation when subsequently added to blood because of the capacity of the catalase present to destroy this

agent. That the hydrogen peroxide formed in the oxidized extract is active in the absence of catalase is easily shown by the facility with which it destroys crystalline hemoglobin. Moreover, to insure against the participation in this reaction of any active agent other than the hydrogen peroxide, the oxidized extract was completely inactivated by exposure to 75°C. for 10 minutes, without any effect upon its action on crystalline hemoglobin. Oxidized extract in which the final products of oxidation have accumulated behaves toward oxyhemoglobin, under these circumstances, precisely like a dilute

TABLE III.

Comparison of the Action of Reduced and Oxidized Extract of Pneumococcus upon Oxyhemoglobin in the Presence and Absence of Blood Catalase.

Pneumococcus extract.		Presence of peroxide in extract before adding HbO_2.	Methemoglobin formation.	
			Oxyhemoglobin from laked blood (abundant catalase).	Solutions of crystalline oxyhemoglobin (no catalase).
Reduced.	Unheated.	—	+	+
	Heated 10 min. at 75°C.	—	—	—
Oxidized.	Unheated.	+	—	+
	Heated 10 min. at 75°C.	+	—	+

solution of hydrogen peroxide; that is, it is unable to form methemoglobin in the presence of blood catalase and is no more heat-labile than is hydrogen peroxide itself.

Obviously, catalase can protect hemoglobin against the action of oxidized extract containing the final product, H_2O_2; but it offers no protection when the hemoglobin is present in the system *during* the oxidation of the extract. A probable explanation of the difference in activity under these two sets of circumstances is that during the process of oxidation of a pneumococcus extract certain intermediary products are formed which like hydrogen peroxide are strong oxidizing agents, but unlike this substance are not destroyed by catalase. If hemoglobin is present during the formation of these intermediary bodies, it is in turn oxidized by these substances. Presumably when the process of oxidation of pneumococcus extract has been completed,

these higher peroxides are converted into simple hydrogen peroxide the action of which, when subsequently added to oxyhemoglobin, is conditioned by the presence or absence of blood catalase.

Influence of Hydrogen Peroxide on the Oxyhemoglobin-Destroying Activity of Pneumococcus Extract.

When a reduced extract of pneumococcus is exposed to the air it suffers a rapid and progressive loss of its ability to form methemoglobin. During this oxidation hydrogen peroxide is formed and it was previously suggested that part of the loss of activity may be due to the destructive action of this oxidation product upon the extract. Evidence of the probable importance of the accumulated peroxide as a factor in the destruction of the methemoglobin-forming system in oxidized extracts has been furnished by a number of quantitative experiments. Treatment of active extracts with reagent hydrogen peroxide resulted in a complete loss of ability of the treated extract to destroy oxyhemoglobin.

Influence of the Oxygen Tension upon Methemoglobin Formation by Sterile Extracts of Pneumococci.

In an earlier investigation (1914) Cole (10) observed that the degree of oxygenation of the blood was an important factor in the formation of methemoglobin by living pneumococci. To obtain further evidence of the influence of the degree of oxygenation of hemoglobin upon methemoglobin formation it seemed desirable to repeat Cole's experiments with sterile extracts instead of living bacteria. In the following experiment, therefore, a sterile pneumococcus extract was added to and allowed to act upon hemoglobin in various degrees of oxygenation.

4.5 cc. of sterile defibrinated rabbit blood and 0.5 cc. of 0.1 M phosphate solution (pH 7.5) were placed in each of three tonometers of 300 cc. capacity. One of these samples of blood was then deoxygenated by evacuation and by preliminary saturation with hydrogen. Another was saturated with oxygen at 20 mm. tension and the third was saturated with oxygen at 710 mm. As a control 5 cc. of blood similarly diluted to which phosphate solution instead of extract was added served as control at atmospheric tension. After the preliminary saturations, sterile reduced extract in amounts of 0.8 cc. was added to each specimen of blood, precautions being taken to prevent any oxidation of the extract or any change in the composition of the gas during the manipulation.

The tonometers containing mixtures of sterile pneumococcus extract and of blood at different degrees of oxygenation, were then rotated in a water bath at

38°C. for 2 hours. The formation of methemoglobin in the different samples was then determined by measurements of the loss of oxygen capacity of the blood. The results are given in Table IV.

Table IV shows that when a mixture of reduced extract of pneumococcus and deoxygenated blood was exposed to an oxygen tension of less than 3 mm., little or no methemoglobin was formed. The actual figures in the table are not more than twice the experimental error and indicate that if the preliminary deoxygenation had been complete the hemoglobin would have suffered no change under

TABLE IV.

Influence of Oxygen Tension and Degree of Oxygenation of Hemoglobin upon the Formation of Methemoglobin by Sterile Extracts of Pneumococcus.

Oxygen tension in gas phase.	Degree of oxygenation of hemoglobin $\left(\text{Ratio } \frac{Hb}{Hb + HbO_2}\right)$.		Action of reduced extract of pneumococcus.	
	Hb	HbO_2	Hemoglobin content after 2 hrs. at 37°C. (oxygen capacity).	Hemoglobin destroyed (methemoglobin formed).
*mm.**	*per cent**	*per cent**	*mM*	*mg. in 5.8 cc.*
0	100	0	5.35	15
20	50	50	3.90	168
710	0	100	5.24	27
Control.			5.50	

* Approximate figures.

these conditions. When the reaction was carried out at high oxygen tensions, 710 mm., that is under conditions such that the concentration of deoxygenated hemoglobin was practically negligible, only a very small amount of methemoglobin was formed. At an oxygen tension of 20 mm., both oxyhemoglobin and deoxygenated hemoglobin were present in approximately equal amounts. When the extract acted upon blood under these conditions significant amounts of methemoglobin were formed as shown by the marked decrease in oxygen capacity. This oxygen tension was chosen at random and although serving the purpose of the experiment it may not represent the optimum for the reaction.

From the results of this experiment it may be concluded that the methemoglobin-forming reaction of pneumococcus is influenced by

oxygen tension in two ways. Thus, while the presence of oxygen is necessary for the formation of the oxidizing agent which oxidized hemoglobin to methemoglobin, the reaction does not proceed to advantage at high oxygen tensions which exclude the presence of hemoglobin in the deoxygenated form. As previously observed by Cole (10), the formation of methemoglobin by pneumococcus apparently involves a preliminary deoxygenation of hemoglobin. The present study suggests that this process of deoxygenation may have

TABLE V.

Oxygen Consumed in the Destruction of Oxyhemoglobin by Pneumococcus Extract.

	Oxygen capacity.			Oxygen content.		
	0	1 hr.	2 hrs.	0	1 hr.	2 hrs.
	mM	mM	mM	mM	mM	mM
Control.	6.5	6.5	6.5	6.5	6.4	6.3
Extract.	6.5	5.1	4.5	6.5	3.2	1.5
	Hemoglobin destroyed by extract.			Oxygen consumed by extract.		
	0	1.4	2.0	0	3.2	4.8

a twofold function; it affords a source of oxygen to the peroxide-forming system of the cell and in turn it leaves the hemoglobin in a form more readily oxidized to methemoglobin by the peroxide thus formed.

The Consumption of Oxygen during the Destruction of Oxyhemoglobin by Sterile Pneumococcus Extract.

From the results of the preceding experiments it was inferred that the oxygen united with some constituent of the extract to form an oxidizing agent which *oxidized* deoxygenated hemoglobin to methemoglobin. In view of these relations, experiments were conducted to correlate the amount of oxygen consumed with the amount of hemoglobin destroyed.

From the results given in Table V it appears that during the reaction between pneumococcus extract and oxygenated blood, the molecular amount of oxygen consumed is greater than that of hemo-

globin destroyed. From the previous experiment it was inferred that deoxygenated hemoglobin represents an intermediary product in the formation of methemoglobin. Although it is frequently impossible to demonstrate the presence of intermediary substances in biological oxidations, it is interesting to observe that deoxygenated hemoglobin accumulates in significant concentrations during this reaction, at least in systems in which oxyhemoglobin is the source of molecular oxygen.

DISCUSSION.

Sterile broth extracts of unwashed pneumococci actively destroy oxyhemoglobin with the resulting formation of methemoglobin. The rate and degree of destruction are largely dependent upon the concentration of the reagents used. Larger amounts of extract not only transform oxyhemoglobin to methemoglobin but may destroy the blood pigment to products beyond methemoglobin. This process seems to be a function of the same system or systems responsible for the formation of peroxide and the reduction of methylene blue by pneumococcus extract (3, 4). This fact is further evidenced by the heat sensitiveness of the mechanism involved, for all three of these activities are destroyed when an extract is heated at 65°C. Moreover, an extract prepared from washed pneumococci, that is the "incomplete" type of cell extract which by itself exhibits none of these reactions, may be activated in each instance by the addition of the same substances, meat infusion or yeast extract.

As already pointed out in the first paper of this series (1), the peroxide-forming activity varies with different strains of pneumococci. These differences are reflected in the activity of the bacterial extracts not only in the peroxide-forming function, but also in the other oxidation-reduction processes described (2–4), including the hemoglobin destruction discussed in this paper. The fact that the sterile extracts possessing the most marked peroxide-forming activity cause the most marked destruction of hemoglobin may be interpreted as further evidence that the same system is involved in these two functions of the extracts.

In the destruction of oxyhemoglobin, as in the oxidation of pneumococcus hemotoxin by sterile extracts, the type of reaction induced

represents one in which a substance, not itself easily oxidized by molecular oxygen, is oxidized through the agency of some product formed by the union of molecular oxygen with the reactive substance in the extract. It may be recalled that an active pneumococcus extract prepared from unwashed bacterial cells, consists of two factors, each of which is separably inactive. One of these factors represents certain constituents of the bacterial cell which are heat-labile and are present in saline extracts of washed pneumococci. Extracts of this type are "incomplete" and non-reactive with molecular oxygen. The other constituents which are essential to the complete oxidation-reduction system may be furnished from sources other than those of bacterial origin. Meat infusion and yeast extract contain thermostable substances which, although themselves inactive, serve to complement the potentially active but otherwise deficient system of the washed cell. With these distinctions in mind, the process involving the change of oxyhemoglobin by an active reduced extract of pneumococcus may be schematically represented as consisting of the following intermediary reactions. If C be allowed to represent the thermolabile cell constituents including the bacterial enzymes and RH the easily oxidizable thermostable substances, then the active system in the broth extract in the reduced state would consist of [C + RH]; and

$$[C + RH] + HbO_2 \longrightarrow ROOH + Hb$$

$$ROOH + Hb \longrightarrow MetHb + \text{other oxidation products.}$$

The first intermediary step involves the deoxygenation of the oxyhemoglobin.[2] That a source of oxygen in the system is essential

[2]It is equally in keeping with the experimental data to interpret the methemoglobin-forming reaction in a manner somewhat more analogous to Conant's interpretation of the action of potassium ferricyanide on hemoglobin solutions. Reduced hemoglobin, oxyhemoglobin, and molecular oxygen are in equilibrium in the system. Upon the addition of reduced pneumococcus extract the molecular oxygen unites with constituents of the extract. The oxidizing agent thus formed oxidizes reduced hemoglobin to methemoglobin. This reaction, by removing either (or both) reduced hemoglobin or molecular oxygen, causes more oxyhemoglobin to dissociate until there are established the final equilibria between the oxidizing agent, reduced hemoglobin, and methemoglobin, and between oxyhemoglobin, reduced hemoglobin, and molecular oxygen.

for the initiation of the reactions is clearly shown by the fact that reduced extract in the presence of reduced hemoglobin does not form methemoglobin. The oxygen furnished by the oxyhemoglobin unites with the reactive substance of the extract to form a peroxide (ROOH). This peroxide in turn oxidizes the reduced hemoglobin to methemoglobin. The necessity of oxygen for the initiation of the reaction and the demonstration of hydrogen peroxide in extracts which under the influence of ordinary oxygen have changed from the reduced to the oxidized form, suggest that hydrogen peroxide as such may be the active agent in the oxidation of hemoglobin. However, the fact that hydrogen peroxide may be detected as an end-product in oxidized pneumococcus extract does not necessarily imply that other active, but transient products may not be formed in the earlier stages of the reaction. Indeed, there are reasons to believe that the actual agent in the oxidation of hemoglobin under these conditions may be a higher, organic peroxide, a precursor of hydrogen peroxide. One reason for this view is the fact that if blood is present during the oxidation of a reduced extract, the active substance that is formed is wholly unaffected by blood catalase, transforming hemoglobin in the presence as well as in the absence of this enzyme. On the other hand, if pneumococcus extract is completely oxidized before it is added to oxyhemoglobin, methemoglobin is formed only in the absence of catalase. In this respect an oxidized extract behaves toward hemoglobin merely as does a dilute solution of hydrogen peroxide. These differences are brought out in those experiments in which the comparative action of a reduced and oxidized extract on oxyhemoglobin was studied in the presence and absence of catalase.

The conception that methemoglobin formation by pneumococcus involves the preliminary deoxygenation of oxyhemoglobin was suggested by Cole (10). The present study supports this interpretation and is in accord with the recently published evidence (Conant (14)) that the transformation of hemoglobin to methemoglobin is a true oxidation in the electronic sense, while the conversion of hemoglobin to oxyhemoglobin is merely an oxygenation. Conant interprets the transformation of oxyhemoglobin to methemoglobin by the action of ferricyanide as a reaction involving the *oxidation* of previously

deoxygenated hemoglobin. The actual oxidation process, which gives methemoglobin, Conant believes to consist in the oxidation of the ferrous iron of deoxygenated hemoglobin to the ferric form. This reaction may be brought about by a number of oxidizing agents. The present study suggests that the oxidizing agent in the case of pneumococcus is a peroxide of bacterial origin.

From the experimental evidence available, it is inferred that in the formation of methemoglobin by pneumococcus, the initial reaction involves the deoxygenation of oxyhemoglobin and the union of the free oxygen with some autoxidizable substance of the cell and that a peroxide thus formed serves as the actual oxidizing agent in the conversion of the hemoglobin to methemoglobin.

SUMMARY.

1. Sterile broth extracts of unwashed pneumococci actively destroy hemoglobin to methemoglobin and lower degradation products.

2. Sterile saline extracts of washed pneumococci do not by themselves form methemoglobin; extracts of this type may be completed or activated by the addition of certain complementary substances such as meat infusion and yeast extract.

3. The hemoglobin-destroying activity of pneumococcus extract is lost by exposure to 65°C. for 10 minutes.

4. The properties of an extract upon which these blood changes depend are related to other known oxidation-reduction functions of the same extract.

5. Oxyhemoglobin is converted to methemoglobin only by cell extracts in the reduced form; completely oxidized extracts are inactive in the presence of blood. The action of hydrogen peroxide and the influence of blood catalase on these reactions are discussed.

6. During the reaction between oxyhemoglobin and pneumococcus extract oxygen is consumed.

7. The mechanism of methemoglobin formation by pneumococcus is interpreted as an oxidation process in which deoxygenation of oxyhemoglobin and peroxide formation occur as intermediary reactions. The active agent of the oxidation of hemoglobin is considered to be a peroxide of bacterial origin.

BIBLIOGRAPHY.

1. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 347.
2. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 357.
3. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 543.
4. Avery, O. T., and Neill, J. M., *J. Exp. Med.*, 1924, xxxix, 745.
5. Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.
6. Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127.
7. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924 (in press).
8. Stadie, W. C., *J. Biol. Chem.*, 1920, xli, 237.
9. Butterfield, E. E., and Peabody, F. W., *J. Exp. Med.*, 1913, xvii, 587.
10. Cole, R., *J. Exp. Med.*, 1914, xx, 363.
11. Stadie, W. C., *J. Exp. Med.*, 1921, xxxiii, 627.
12. Schnabel, A., *Z. Hyg. u. Infectionskrankh.*, 1921, xciii, 175.
13. Meyerhof, O., *The Harvey Lectures*, 1923.
14. Conant, J. B., *J. Biol. Chem.*, 1923, lvii, 401.
15. M'Leod, J. W., and Gordon, J., *J. Path. and Bact.*, 1922, xxv, 139.