# THE PRODUCTION OF METHEMOGLOBIN BY PNEUMOCOCCI.*

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When pneumococci are grown in media containing blood or hemoglobin, the red color of the latter is changed to a greenish brown, as may be well seen in blood agar plates on which pneumococci are growing. This change is undoubtedly due to the formation of methemoglobin. Butterfield and Peabody[1] have demonstrated that methemoglobin is formed when pneumococci are cultivated in media containing blood.

A similar change in color, though not so pronounced, is seen in the blood of animals dying of acute pneumococcus septicemia, and to a still less extent in the blood of patients severely ill and dying of pneumonia. Peabody[2] has shown that during the terminal stages of fatal cases of pneumonia there occurs a progressive decrease in the oxygen content and the oxygen-combining capacity of the blood. This is evidently due to the transformation of oxyhemoglobin into methemoglobin. A similar change occurs in the blood of rabbits severely infected with pneumococci.[3] While the presence of methemoglobin in artificial culture media may be readily demonstrated by spectroscopic methods, this is more difficult in the blood of patients and infected animals, since a considerable concentration of methemoglobin is necessary for spectroscopic demonstration.

It has seemed of importance to learn more of the nature of the reaction during which methemoglobin is formed by pneumococci, and the present paper gives a report of this study, as far as it has been carried out.

Peabody stated that the transformation of oxyhemoglobin into

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[1] Butterfield, E. E., and Peabody, F. W., *Jour. Exper. Med.*, 1913, xvii, 587.

[2] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 7.

[3] Peabody, F. W., *Jour. Exper. Med.*, 1913, xviii, 1.

methemoglobin is brought about by the filtrates of pneumococcus cultures as well as by the cultures containing bacteria; but our experiments, which have been repeated many times, make it evident that in the experiments on which this statement was based faulty filters were employed. If the bacteria are all removed from a culture fluid, either by centrifugalization or by filtration through a Berkefeld filter, and then blood be added to the filtrate, no formation of methemoglobin occurs. The reaction therefore cannot depend merely upon the production of an acid reaction in the fluid in which the bacteria grow. This is also shown by careful neutralization of the culture before the addition of blood, when the rate of reaction is not changed unless to be increased.

Boiling a broth culture, or even heating it to 56° C. for one half hour, before addition of blood prevents the reaction from occurring. That this inhibition of the reaction by heat depends upon the destruction of the bacteria is rendered probable by the following experiment. A series of tubes containing broth cultures of different races of pneumococci were heated one half hour at 45° C.,—about the thermal death point for most races of pneumococci. Blood corpuscles were then added to each tube and transplantations were made from each tube on fresh media. In the cultures from which growth in the transplantation was obtained the formation of methemoglobin occurred. In the cultures, on the other hand, from which no growth occurred and in which the bacteria were therefore all killed, no formation of methemoglobin took place.

While extracts of pneumococcal bodies cause lysis of red blood corpuscles,[4] they never cause the formation of methemoglobin, unless they contain living pneumococci. The fact that small amounts of sodium cholate added to broth cultures of pneumococci inhibit the formation of methemoglobin might explain the failure of extracts of pneumococci in cholate solution to produce methemoglobin, but a similar failure to form methemoglobin is seen when the extracts are prepared by freezing and grinding the bacteria without the addition of cholate.

These experiments indicate that for the formation of methemoglobin the presence of living bacteria is necessary.

[4] Cole, R., *Jour. Exper. Med.*, 1914, xx, 346.

TABLE I.

| Tube No. | Experiments. | Methemoglobin formation. | |
|---|---|---|---|
| | | 1 hr. at 37° C. | 1 hr. at 37° C. and 24 hrs. on ice. |
| 1 | 1 c.c. broth culture A8/0/6 + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 2 | 1 c.c. broth culture A8/0/6 (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 3 | 1 c.c. broth culture A8/0/6 (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 4 | 1 c.c. broth culture A8/0/6 (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 5 | 1 c.c. broth culture A8/0/6 (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 6 | 1 c.c. emulsion A + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 7 | 1 c.c. emulsion A (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + in 20 min. | + |
| 8 | 1 c.c. emulsion A (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + | + |
| 9 | 1 c.c. emulsion A (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | + | + |
| 10 | 1 c.c. emulsion A (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 11 | 1 c.c. emulsion B + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 12 | 1 c.c. emulsion B (1 : 2) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 13 | 1 c.c. emulsion B (1 : 4) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 14 | 1 c.c. emulsion B (1 : 8) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |
| 15 | 1 c.c. emulsion B (1 : 16) + 1 c.c. sodium chloride solution + 0.5 c.c. emulsion sheep corpuscles | 0 | 0 |

It was now important to learn whether or not washed pneumococci in salt solution could produce the reaction or whether the presence of nutrient medium also is required. That the latter is necessary is shown by the protocol of an experiment which follows. Two portions of a twenty-hour broth culture of pneumococcus A8/o/6 were centrifugalized, the supernatant fluid was removed, and each sediment thoroughly washed in sodium chloride solution and again centrifugalized. One portion of the sediment was now made up to the original volume in broth (emulsion A), and the other portion was made up to the original volume in sodium chloride solution (emulsion B) and tests were made to determine the methemoglobin-producing power (table I) of the two solutions in varying dilutions, compared with the methemoglobin-producing power of an untreated broth culture.

It was suggested that the failure of the reaction to occur in salt solution might be due to the toxicity of sodium chloride in the absence of other inorganic salts. To test this, experiments were carried out with organisms suspended in Ringer solution. No reaction, however, occurred. It is evident, therefore, that some organic constituents of the broth are necessary for the reaction.

Experiments were then made to determine which class of organic substances in broth is essential. The following is a protocol of one such experiment (table II).

While experiments like those in table II showed that the reaction occurs when either sugar, peptone, or protein is present, further studies have shown that while protein substances must be present in quite high concentration in order that the reaction may occur, at least one part of a 10 per cent. solution of crystallized egg albumin to twenty parts of salt solution being required, the reaction occurs with great rapidity when sugar in very great dilution is present, even in dilutions as great as one part of a 5 per cent. dextrose solution to 10,000 parts of salt solution. The presence of traces of sugar in peptone solution and even in solutions of egg albumin can only with great difficulty be excluded, and it is possible that upon the presence of such traces of sugar the availability of these solutions in this reaction depends, since only extremely small amounts of sugar are required.

Various kinds of sugar were next tested to determine whether

TABLE II.

| Tube No. | Experiments of December 13, 1913 | Methemoglobin formation. | |
|---|---|---|---|
| | | 1 hr. at 37° C. | 24 hrs. on ice. |
| 1 | 1.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | 0 | 0 |
| 2 | 1 c.c. sterile broth + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 3 | 1 c.c. sterile broth (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 4 | 1 c.c. sterile broth (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 5 | 1 c.c. sterile broth (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 6 | 1 c.c. sterile broth (1 ; 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 7 | 1 c.c. 5% dextrose + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 8 | 1 c.c. 5% dextrose (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 9 | 1 c.c. 5% dextrose (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 10 | 1 c.c. 5% dextrose (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 11 | 1 c.c. 5% dextrose (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 12 | 1 c.c. 5% peptone + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 13 | 1 c.c. 5% peptone (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 14 | 1 c.c. 5% peptone (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 15 | 1 c.c. 5% peptone (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 16 | 1 c.c. 5% peptone (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 17 | 1 c.c. 4% egg albumin + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 18 | 1 c.c. 4% egg albumin (1 : 2) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 19 | 1 c.c. 4% egg albumin (1 : 4) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 20 | 1 c.c. 4% egg albumin (1 : 8) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 21 | 1 c.c. 4% egg albumin (1 : 16) + 0.5 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution + 0.5 c.c. emulsion A8 | + in 10 min. | + |
| 22 | 1 c.c. sterile broth + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution | 0 | 0 |
| 23 | 1 c.c. 5% dextrose + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution | 0 | 0 |
| 24 | 1 c.c. 5% peptone + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution | 0 | 0 |
| 25 | 1 c.c. 4% egg albumin + 1 c.c. sodium chloride solution + 0.5 c.c. hemoglobin solution | 0 | 0 |

The hemoglobin solution = 2 c.c. washed sheep corpuscles plus 8 c.c. distilled water plus 30 c.c. 0.85 per cent. sodium chloride solution.

Dextrose = 5 per cent. solution of chemically pure dextrose in distilled water, autoclaved for 20 minutes.

Peptone = 5 per cent. solution of Witte's peptone in distilled water, autoclaved for 20 minutes.

Egg albumin = 4 per cent. solution of crystallized egg albumin in distilled water.

Bacterial emulsion = emulsion in sodium chloride solution of washed sediment of 20-hour broth culture A8. The bacteria were in twice as great concentration as in the culture.

pneumococci cause the formation of methemoglobin only in the presence of dextrose or whether this sugar may be replaced by others in which the molecular configuration is different. Several races of pneumococci as well as streptococci were employed and the results are given in table III.

In another experiment *d*-xylose and *d*-arabinose were also tested. The results are given in table IV.

While in the experiments given no reaction occurred in the presence of inulin, in several other experiments a slight reaction occurred in solutions containing 2 per cent. of this sugar. In these and many other experiments as well, however, no reaction ever occurred in the solutions containing ribose. It is therefore apparent that while the reaction occurs in the presence of most sugars, the configuration of the molecule makes some difference. This is also seen in the effect of different sugars on the rate of reaction. The reaction always occurred more slowly with saccharose and arabinose than with the other sugars. Since the reaction occurs when pneumococci are placed in solutions containing traces of sugar, and it is known that pneumococci cause breaking down of many sugars, the possibility suggested itself that the reaction is due to some substance formed during the decomposition of the sugar molecule. The reaction cannot, however, be due simply to the formation of carbon dioxide. If carbon dioxide be passed through a solution of oxyhemoglobin the solution becomes cherry red, and if oxygen now be passed through it again becomes bright red. Such changes never occur in solutions of hemoglobin acted upon by pneumococci. A similar change is well seen when yeast is added to solutions containing blood corpuscles. In this case the blood takes on a magenta color which on shaking becomes bright red. It would appear possible, however, that the change might be due to some intermediate product of sugar metabolism. The exact transformation which the sugar molecule undergoes during the process of changing into carbon dioxide and water is still obscure, but there are several substances which are thought to represent intermediate stages. A series of such substances which are thought to be intermediate products in the metabolism of sugar and also certain ones that are known to be end products under certain conditions were tested. These substances

TABLE III.

| Experiments. | Methemoglobin formation. | | | |
|---|---|---|---|---|
| | A18 | P1.77 | M1.77 | Streptococcus. |
| 1 c.c. 2% ribose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution..... | 0 | 0 | 0 | 0 |
| 1 c.c. 2% arabinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.... | + in 1 hr. | + in 10 min. | + in 1 hr. | 0 |
| 1 c.c. 2% xylose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution..... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% maltose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution..... | + in 1 hr. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% lactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution..... | + in 1 hr. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% saccharose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution ... | + in 1 hr. | + in 1 hr. | + in 1 hr. | 0 |
| 1 c.c. 2% dextrose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% levulose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% mannose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% galactose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution.... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |
| 1 c.c. 2% inulin solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution..... | 0 | 0 | 0 | 0 |
| 1 c.c. 2% raffinose solution + 1 c.c. bacterial emulsion + 1 c.c. hemoglobin solution .... | + in 10 min. | + in 10 min. | + in 10 min. | 0 |

The tubes were all kept at 37° C.

A18, P1.77, and M1.77 are pneumococci of type I. The streptococcus is a virulent hemolytic streptococcus.

TABLE IV.

| Experiments. | Methemoglobin formation. | | |
|---|---|---|---|
| | A 19/5/7 | A 26/9/3 | 1/73 |
| 1 c.c. ribose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | 0 | 0 | 0 |
| 1 c.c. *l*-arabinose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | + in 15 min. | + in 15 min. | + in 15 min. |
| 1 c.c. *d*-arabinose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | 0 | 0 | 0 |
| 1 c.c. *l*-xylose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | + in 15 min. | + in 15 min. | + in 15 min. |
| 1 c.c. *d*-xylose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | 0 | 0 | 0 |
| 1 c.c. dextrose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | + in 15 min. | + in 15 min. | + in 15 min. |
| 1 c.c. mannose . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | + in 15 min. | + in 15 min. | + in 15 min. |
| 1 c.c. inulin . . . . . 2 % solution + 1 c.c. bacterial emulsion + 0.5 c.c. hemoglobin solution | 0 | 0 | 0 |

The tubes were kept for 2 hours at 37° C.

were kindly supplied by Dr. G. M. Meyer. They were methylglyoxal, acetaldehyde, pyruvic acid, formaldehyde, formic acid, and acetic acid. Methylglyoxal, acetaldehyde, formaldehyde, and pyruvic acid produced methemoglobin only when very concentrated solutions were employed. The presence of bacteria did not increase their effect. Since such extremely small amounts of sugar are required in the formation of methemoglobin by bacteria, it is not possible that under these conditions any of the substances are present in a concentration sufficient to bring about the reaction, unless such substances are much more active in a nascent state than they are in solution.

The tests with formic acid showed that this substance caused the formation of methemoglobin only when it was used undiluted or but slightly diluted. Even under these conditions the reaction is due to the acidity of the solution, since, after neutralization and the addition of a mixture of primary and secondary potassium phosphates, in order to keep the mixture neutral, no reaction occurred, even with the undiluted acid. When a solution of formic acid, not in itself able to produce methemoglobin, is added to the bacterial emulsion plus hemoglobin solution, no formation of methemoglobin occurs, while the addition of a very dilute solution of dextrose under similar conditions causes the reaction to occur within a few minutes.

Similar results were obtained when acetic acid was used.

These studies, therefore, did not lead to the detection of any substance formed during sugar metabolism or fermentation to which the change of oxyhemoglobin into methemoglobin could be ascribed.

Other substances, such as alcohols, were also tested as to their effect in the formation of methemoglobin. The following results were obtained (table V).

None of these substances, except glycerin, when employed in low dilutions are able to replace dextrose in the mixtures in which methemoglobin is formed. When, however, glycerin, even in a dilution of 1 to 10,000, is added, the formation of methemoglobin occurs rapidly.

A large number of quantitative studies with these and similar substances were made, in the hope of finding some relationship in chemical structure between those substances which by their presence per-

TABLE V.

| Experiments. | Results. |
|---|---|
| 2 c.c. glycerin (10 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 2 c.c. mannite (10 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 2 c.c. methyl alcohol (10 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 2 c.c. ethyl alcohol (10 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. glycerin (1 : 10,000) + 0.5 c.c. hemoglobin solution | + in 10 min. |
| 1 c.c. bacterial emulsion + 1 c.c. mannite (10 % solution) + 0.5 c.c. hemoglobin solution | + in 3 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. methyl alcohol (10 % solution) + 0.5 c.c. hemoglobin solution | + in 3 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. ethyl alcohol (10 % solution) + 0.5 c.c. hemoglobin solution | + in 2 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. dextrose (5 % solution) + 0.5 c.c. hemoglobin solution | + in 10 min. |
| 1 c.c. bacterial emulsion + 1 c.c. starch (1 % solution) + 0.5 c.c. hemoglobin solution | + in 1 hr. |
| 1 c.c. bacterial emulsion + 1 c.c. urea (2 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. ethyl acetate (0.2 % solution) + 0.5 c.c. hemoglobin solution | + in 1 hr. |
| 1 c.c. bacterial emulsion + 1 c.c. leucin (2 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |
| 1 c.c. bacterial emulsion + 1 c.c. cholesterin (1 % solution) + 0.5 c.c. hemoglobin solution | No change in 24 hrs. |

mit pneumococci to form methemoglobin. So large a number of substances are suitable for the purpose that it has not been possible to show that any form of molecular configuration or grouping is essential.

It seems probable, judging from these studies, that the formation of methemoglobin occurs whenever pneumococci in contact with hemoglobin are able to carry on metabolic and functional activities, probably including multiplication, and that this happens when traces of sugar and also when large amounts of other organic substances are present. While in the absence of those substances death of the bacteria does not necessarily occur, it is probable that their metabolic activities are reduced to a minimum.

Cellular metabolic processes occur through the agency of ferments contained within the cells, and it would therefore seem probable, if the reaction under consideration were due to the active processes of oxidation occurring in the medium immediately surrounding the bacterial cells, that similar reactions would appear when bacterial extracts are used instead of the living bacteria. As Warburg[5] has pointed out, however, the reactions which occur in the living cell are not identical, at least in intensity, with those induced by the ferments when removed from the cell by extraction or other means. The activity of zymase, for instance, is markedly less than that of a corresponding number of living yeast cells. The importance of structure in cellular activity cannot be neglected, and from the fact that the reaction under consideration is not induced by the presence of bacterial extracts, it does not necessarily follow that the reaction is not due to the functional activities of the bacterial cells.

Studies concerning the formation of methemoglobin from oxyhemoglobin by means of chemicals indicate that the reaction is of the nature of an oxidation. The exact chemical nature of oxyhemoglobin as well as that of methemoglobin has not been absolutely determined. It is not even definitely known whether the methemoglobin molecule contains an amount of oxygen equal to or less than that of the oxyhemoglobin molecule. There is considerable evidence,[6] however, for the view that while in oxyhemoglobin the

[5] Warburg, O., Ueber die Wirkung der Struktur auf chemische Vorgänge in Zellen, Jena, 1913.

[6] von Reinhold, B., *Ztschr. f. physiol. Chem.*, 1913, lxxxv, 250.

oxygen is loosely combined as in an oxide, the formula being usually written $Hb\langle^{O}_{O}$, in methemoglobin the oxygen is as in an hydroxide. Whether the molecule contains one hydroxyl group as in Hb–OH, or two hydroxyl groups as in $Hb\langle^{O\ H}_{O\ H}$, is not determined, though there is experimental evidence for both points of view. In either case, however, methemoglobin would represent a lower stage of oxidation than oxyhemoglobin, and the transformation of oxyhemoglobin into methemoglobin would therefore be of the nature of a reduction.

On the other hand evidence has been presented by Heubner[7] to show that the formation of methemoglobin is always an oxidation process. As is well known, substances which are known to be oxidizing agents and also those considered to be reducing agents may bring about the transformation of oxyhemoglobin into methemoglobin. An explanation of this fact, which Heubner presents, is that the reducing agents are first oxidized, this oxidation occurring better in the presence of oxyhemoglobin, and then reduced, giving up their oxygen to form methemoglobin. It is of great interest that certain substances, such as aminophenol, $NH_2\langle\bigcirc\rangle OH$, are able to convert much more hemoglobin into methemoglobin than could occur if the reaction were a simple molecular one. One molecule of aminophenol may apparently transform at least fifty molecules of hemoglobin into methemoglobin. The aminophenol must therefore repeatedly react with the hemoglobin. Heubner's view is that the aminophenol is first oxidized to a quinone, (quinonimine, $NH\langle\bigcirc\rangle O$) and then again reduced to aminophenol, in this reaction the hemoglobin being changed to methemoglobin. The reaction may be repeated many times, the aminophenol therefore having a catalytic-like action.

If this be the true explanation of the kind of reaction occurring in the transformation of oxyhemoglobin into methemoglobin by organic chemical substances, it is possible that the mode of action of pneumococci in causing the formation of methemoglobin may fol-

[7] Heubner, B., *Arch. f. exper. Path. u. Pharmakol.*, 1913, lxxii, 239.

low similar lines. The metabolic activities of cells consist largely of oxidative and reduction processes. When oxyhemoglobin is brought into contact with pneumococci it may be changed into methemoglobin, because it is then exposed to active oxidative and reduction processes occurring in the neighborhood of the bacterial cells. These processes must be of a special type in the case of pneumococci, however, since otherwise all living cells would cause the transformation.

That this change goes on in the neighborhood of the cells and not necessarily in the cells themselves is shown by the fact that while the reaction occurs more readily when the hemoglobin is in solution in the medium surrounding the bacteria, it may also occur when the hemoglobin is contained within red blood corpuscles, the blood cells and bacteria being merely in intimate contact. If, however, the bacteria and blood cells are separated by a membrane of any kind, even a very thin layer of oil, the reaction does not occur.

If the change of hemoglobin into methemoglobin taking place in the neighborhood of pneumococcal cells is due to oxidative processes occurring there, it was thought that evidence for this fact would be obtained by studying the effect which the presence or absence of free oxygen would have on the reaction. This was done by testing the rate of reaction under the following conditions: To remove oxygen from a solution, a stream of hydrogen was passed through it for ten minutes, the entrance tube passing completely to the bottom of the tube containing the solution. The solution was then covered by a layer of paraffin oil. Passing hydrogen in this way through a solution of hemoglobin causes the solution to take on a dark color due to the formation of reduced hemoglobin. In mixing two solutions so treated, a pipette was passed through the paraffin layer of one to the bottom of the tube and the fluid was drawn into the pipette and added to the second solution, by plunging the tip of the pipette through the oil on the surface. Care was taken not to empty the pipette completely, as in this way air would be admitted. While complete absence of oxygen could not be obtained by this method, it was sufficiently excluded for the purpose intended.

It was found that if an emulsion of red blood cells, so treated with hydrogen, was added to a broth culture of pneumococci, also treated with hydrogen, no formation of methemoglobin occurred for some hours. When the tubes stood over night there usually

occurred some change into methemoglobin, usually only at the surface under the layer of oil. If, moreover, such a mixture has stood for some time without the formation of methemoglobin, and oxygen now be bubbled through, the change into methemoglobin occurs with great readiness. It is evident, therefore, that the presence of oxygen is necessary for the reaction.

On the other hand, an excess of free oxygen somewhat delays the reaction. If two tubes are prepared, each containing a mixture of an emulsion of red blood cells and an emulsion of pneumococci, and if oxygen be bubbled through one tube, while the other is simply allowed to stand exposed to the air, the two tubes being kept at the same temperature, the change into methemoglobin occurs more slowly in the tube through which oxygen is passing. To control the possibility that the mechanical disturbance due to the bubbling gas may account for the difference, air was passed through the second tube. The reaction was again delayed in the tube through which oxygen was passing.

Two tubes were now prepared, one tube containing a mixture of an emulsion of red blood cells and an emulsion of bacteria, each of which had been previously saturated with hydrogen, and the other containing an identical mixture except that no hydrogen had been passed through. Oxygen was now bubbled through both tubes, which were kept at the same temperature and under identical conditions. In the tube containing the mixture previously saturated with hydrogen the reaction occurred more rapidly than it did in the other tube.

The above results are briefly shown in the following protocol of one experiment (table VI).

It is now easy to interpret these experiments in the light of

TABLE VI.

| Tube No. | Experiments. | | Methemoglobin formation. |
|---|---|---|---|
| 1 | 4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution..... | | + in 7 min. |
| 2 | 4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen | | 0 |
| 3 | 4 c.c. broth culture A82/1/12 + 2 c.c. hemoglobin solution | Oxygen bubbled through | + in 28 min. |
| 4 | 4 c.c. broth culture A82/1/12 saturated with hydrogen + 2 c.c. hemoglobin solution saturated with hydrogen | Oxygen bubbled through | + in 17 min. |

The tubes were all kept at 37° C.

what is known concerning the mode of production of methemoglobin by substances like aminophenol. According to this interpretation the formation of methemoglobin by pneumococci occurs as the result of reduction and oxidative processes occurring in the neighborhood of the bacteria. The oxyhemoglobin is first reduced and if this is inhibited by an excess of free oxygen the reaction is delayed. On the other hand, after reduction has occurred a free supply of oxygen accelerates the reaction. If oxygen be excluded no reaction whatever can occur.

The writer realizes that with the present knowledge it is impossible to conclude that precisely this mode of reaction occurs; but it seems to be the best explanation at present available of the observed experimental facts. It may be objected that the effect of the presence of hydrogen and oxygen is to inhibit or accelerate the metabolic activities of the bacteria rather than to cut off or to increase the supply of oxygen required for the chemical changes. The fact that an excess of oxygen delays the reaction is against this interpretation, though it is known that an excess of oxygen may inhibit cellular action or even be directly toxic to cells.

Experiments were also undertaken to determine whether, in the absence of free oxygen, the oxygen required for the reaction could be obtained from methylene blue, if this be added to the mixture. It was found that the reaction proceeds under these circumstances, but more slowly and less completely than in the presence of free oxygen.

It is believed that the experiments concerning the production of methemoglobin by pneumococci are important not only because they may possibly explain a reaction which probably occurs in every animal severely infected with pneumococci, but they are also important because they suggest a possible explanation for the pathological action of those bacteria which apparently do not produce an active toxin. Since bacteria may injure red blood corpuscles by merely changing oxidative processes in their vicinity, and without producing substances capable of isolation, it is possible that bacteria may injure other tissue cells in a similar manner. Therefore, the pathological effects of bacteria are not necessarily due to the action of a definite poison, but may be due to disturbances in oxidation in the immediate neighborhood of the bacteria.

## CONCLUSIONS.

1. Pneumococci in contact with hemoglobin transform this into methemoglobin. This reaction occurs only when the pneumococci are living; it is not induced by the culture fluid or by extracts of the bacteria.

2. The reaction does not occur when hemoglobin is added to an emulsion of washed pneumococci in salt solution. However, if minute traces of dextrose be added to such a mixture, the reaction quickly occurs. The dextrose may be replaced by any one of a number of other sugars, and also by certain other organic substances, if the latter are added in large amounts. Certain other organic substances are not able to replace dextrose, but it has been impossible to determine any special molecular configuration on which this property depends.

3. The formation of methemoglobin by pneumococci probably resembles the formation of methemoglobin by certain chemical substances, such as aminophenol.

4. From the work of others it is probable that the formation of methemoglobin is always a reaction of oxidation. In the formation of methemoglobin by reducing agents, the latter are first oxidized, this occurring better in the presence of oxyhemoglobin. In certain instances an alternate oxidative and reduction of the transforming agent occurs, so that the reaction is continuous.

The effect which the presence or absence of free oxygen has on the reaction with pneumococci suggests that this follows similar lines.

5. The reaction does not occur in the absence of oxygen. If the free oxygen be first removed, and then replaced, the reaction occurs more rapidly than if the oxygen had not been removed. The presence of free oxygen in excess slightly delays the reaction, possibly because of the inhibition of the reduction process which forms the first part of the reaction.

6. The explanation of this phenomenon of methemoglobin production is not only of importance so far as this special reaction is concerned, but also because it suggests an explanation for the manner in which pathological effects are produced by those bacteria which apparently produce no soluble toxin.