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The Greenish Discoloration Produced on Blood Agar by the Growth of Pneumococcus.

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The fact that the pneumococcus, when grown on media containing blood pigment, produces a greenish disintegration product of hemoglobin, has long been utilized as one of the means of identifying this organism. Jensen[1] states, "Pneumococci and certain streptococci produce a green pigment when grown in blood agar, and this pigment is termed methemoglobin in all text books on bacteriology. Unfortunately the literature of blood pigments is in a state far from clarified. Many European investigators do not attempt to name the particular form of blood pigment, but refer to it simply as 'a green discoloration'."

Since the work of Conant[2] and Conant and Feiser[3] methemoglobin has been characterized as oxidized or monodelectronated reduced hemoglobin. It differs from hemoglobin only in having its iron in ferric form. Its color is definitely brownish without any element of green, and in that manner differs from the color of green hemolysis. Because of this discrepancy, an investigation was undertaken to determine the exact status of the green pigment.

Experimental. The green portions produced in pneumococcus cultures on blood agar were excised and tested for methemoglobin spectroscopically, by ammonium hydroxide, and by sodium cyanide. The differences in the effect produced by the green pigment and by methemoglobin are tabulated:

Methemoglobin	Green Pigment
Characteristic absorption band in the red end of the spectrum	No definite absorption bands
Ammonium hydroxide caused the appearance of reddish alkaline methemoglobin	Caused the appearance of a lemon yellow color
The addition of sodium cyanide solution (0.1%) led to the formation of orange cyanmethemoglobin	Produced a coloration which did not differ from that following the use of ammonium hydroxide (probable alkali effect)

It appears therefore that the green pigment differs radically from

[1] Jensen, L. B., *Bull. Inst. Am. Meat Packers*, 1929, 4, Univ. of Chicago.

[2] Conant, J. B., *J. Biol. Chem.*, 1923, **57**, 401.

[3] Conant, J. B., and Feiser, L., *J. Biol. Chem.*, 1924, **62**, 595.

methemoglobin. Although Neill and Hastings[4] studied the formation of methemoglobin from hemoglobin by the action of soluble extracts of pneumococcus, this does not seem to be a feature of their growth in culture. For this reason, it was decided to determine why methemoglobin production does not take place under these circumstances. It was decided to utilize the observations of McLeod and Gordon[5] that pneumococcus in culture produces hydrogen peroxide. The effects of hydrogen peroxide on the blood pigment were studied.

The addition of hydrogen peroxide to a solution of oxyhemoglobin (prepared by laking human red blood cells with distilled water) which is at, or near its isoelectric point decolorizes it, turning it first brown and then a yellowish white. Is this brownish intermediary methemoglobin? A consideration of the relationship submitted by Conant[3] would lead us to believe not. Methemoglobin is formed only by oxidation of reduced hemoglobin (Conant[3]). In the presence of hydrogen peroxide, which constantly liberates molecular oxygen, little if any of the methemoglobin precursor could exist as such.

Addition of ammonium hydroxide to the reaction mixture did not cause the formation of alkaline methemoglobin nor did sodium cyanide give the characteristic color reaction that it develops with methemoglobin. Examination of the reaction spectroscopically reveals that the bands of oxyhemoglobin gradually fade and finally disappear, without the appearance of new absorption bands in the visible spectrum.

To test further the possibility of methemoglobin being an intermediary in the oxidation of blood pigment by peroxide, hydrogen peroxide was added to a solution of methemoglobin prepared by the action of ferricyanide on hemoglobin. A preliminary formation of oxyhemoglobin took place and the reaction proceeded as above. Two other samples of methemoglobin, one prepared by the action of quinone on hemoglobin and one prepared by allowing a solution of oxyhemoglobin to undergo spontaneous denaturation at room temperature, gave the same results.

(The oxyhemoglobin prepared from methemoglobin by the action of hydrogen peroxide shows a definite catalase activity.)

From the foregoing, it is evident that provided pneumococcus produces hydrogen peroxide in culture, there is no possibility for the production of methemoglobin, inasmuch as the latter cannot exist in the presence of the former.

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[4] Neill, J. M., and Hastings, A. B., *J. Biol. Chem.*, 1925, **63**, 487.

[5] McLeod, J. W., and Gordon, J., *J. Path. Bact.*, 1923, **26**, 326.

The action of hydrogen peroxide on hemoglobin produces a substance which may be the end product of alpha hemolysis. It is assumed to be an oxidation of the unsaturated pyrrols in the prosthetic group. Such an action would furnish an interpretation of the disappearance of the absorption band without detachment of iron from the molecule (free iron could not be demonstrated in the reaction product of peroxide on hemoglobin) the pyrrol nucleus, itself, being chromatophoric as in hematoporphyrin.

That methemoglobin should be unable to exist in solutions containing hydrogen peroxide may be deduced from certain considerations. As demonstrated by Ray and Isaacs[6] the change from methemoglobin to oxyhemoglobin, brought about by tissue juices or by ferrous ion, in the presence of oxygen, is a consideration following the exposition of the reversible oxidation reduction system:

$$\text{hemoglobin} - e^- = \text{methemoglobin}$$

The transformation is rendered clear if we formulate this system

$$E_1 = E_o - \frac{RT}{nF} \log_e \frac{(Hb^{+++})}{(Hb^{++})} \qquad (1)$$

in the presence of oxygen (Hb^{++}) is governed by the equation

$$(Hb^{++}) = \frac{K_{HbO_2}}{(O_2)} \qquad (2)$$

substituting:

$$E_1 - E_o - \frac{RT}{nF} \log_e \frac{(Hb^{+++})\,(O_2)}{K_{HbO_2}} \qquad (3)$$

Since at atmospheric pressures, hemoglobin is practically completely saturated with oxygen, and since at any E_1 some quantity of reduced hemoglobin must exist in solutions of methemoglobin, the equilibrium is disturbed by saturation of the solution with oxygen. In this manner the conversion of methemoglobin to ferrous hemoglobin is accomplished by reducing agents, which we would ordinarily term 'weak' ones, provided oxygen is present.

The oxidation reduction potential of the system

$$2H_2O_2 \leftrightarrow 2\,O + 2H_2O$$

lies in the vicinity of 0.150 millivolts on the saturated calomel scale (unpublished results). The activity of nascent oxygen, as a reducing agent, is evinced only below this potential. However, a side reaction, which participates in the reduction of methemoglobin by nascent oxygen

$$Hb + O_2 \leftrightarrow Hb\,O_2$$

[6] Ray, G. B., and Isaacs, L., *Am. J. Physiol.*, 1930, **91**, 377.

apparently serves to raise the equilibrium potential of the methemoglobin-hemoglobin system above this point.

Hydrogen peroxide slowly decolorizes cyanhemoglobin, whereas, when allowed to act on nitrite hemoglobin, the end product is green. This greenish color and its striking resemblance to the pneumococcus discoloration of blood, led us to investigate the nature of this substance.

Pneumococcus cultures were therefore tested for nitrite by the phenylbenzidine reaction with negative results. However, nitrite is not the only compound which, along with peroxide and hemoglobin gives the green color. Nitrites, hydroxylamine and hydrazine were also found capable of converting hemoglobin to this modification. In all probability, some oxide of nitrogen is concerned, the probability being strengthened by the fact that the reaction will not develop in alkaline solutions.

Next we attempted to elicit whether the reaction was due to an action of this nitrogen and peroxide compound on the hematin or the globin portions of the hemoglobin molecule. Experiments with pure hemin were negative. Other proteins, however, gave definite reactions, among these being casein, egg albumin and serum albumin. The fact that in these cases the color was slightly more yellowish than the reaction with hemoglobin but that on alkaliniation they would turn the same lemon color, leads us to believe that we are here dealing with an xanthoproteic reaction and that this reaction is dependent (as is the xanthoproteic reaction utilizing nitric acid) on the type of protein employed. In fact the xanthoproteic reaction of hemoglobin and nitric acid has a definitely greenish cast.

Summary and Conclusions. 1. Methemoglobin will not exist in the presence of hydrogen peroxide. Therefore the possibility that the green disintegration product of hemoglobin, produced by the growth of pneumococcus is highly remote. 2. A green pigment, identical in all respects with that produced by the growth of pneumococcus on hemoglobin, has been prepared by the action of peroxide and certain nitrogen compounds on the blood pigment. 3. A chemical study of this artificial green pigment indicates that it is not methemoglobin but an xanthoproteic compound.