STUDIES OF METHEMOGLOBIN FORMATION.

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Conant (1923) and Conant and Fieser (1925) have shown that it is characteristic of methemoglobin that by proper reducing agents it is transformed into reduced hemoglobin, with restoration of ability to combine with oxygen and carbon monoxide. The ability to be changed by reduction from a substance incapable of binding molecular O₂ or CO into reduced hemoglobin appears to be the most specific chemical characteristic of methemoglobin that we know. Determinations of methemoglobin based on it may therefore be considered to be more certain to measure that substance, and exclude other hemoglobin derivatives, than may qualitative spectroscopic examinations or quantitative estimation of loss of oxygen capacity, uncontrolled by reduction to reduced hemoglobin. The methods of Conant and Fieser (1925), of Nicloux and Fontès (1924), and the modification of the latter in the preceding paper by Van Slyke (1925), include this control. It appears desirable to review experimentally with such methods the effects on hemoglobin of some of the substances that have been considered to transform hemoglobin into methemoglobin.

We have, accordingly, with the technique described in the preceding paper, studied the effects on hemoglobin of aniline, nitrobenzene, ferricyanide, and nitrite, which represent four different types of supposed methemoglobin formers.

Aniline.

Time Reaction of Aniline and Hemoglobin in Blood.

To 100 cc. of horse blood containing 11.3 millimols of hemoglobin per liter (by CO capacity) 4 mols of aniline were added per mol of hemoglobin (the amount of aniline calculated for 4 mols per mol of Hb is 93 × 11.3...
The amount added was 0.10 cc. The aniline and blood were mixed and agitated in a water bath in cylinders filled with air at 38°. A similar experiment was performed with half as much aniline. At varying periods samples were withdrawn and used for determination of total hemoglobin and methemoglobin as described by Van Slyke (1925). The blood was not hemolyzed to a significant extent. The results are indicated in Fig. 1.

It is evident that the kinetics of methemoglobin formation by aniline are not simple. The outstanding qualitative facts are that:

1. The reaction is a slow process. It is increased in rate by increasing aniline concentrations. The quantitative relationship between aniline concentration and rate of methemoglobin formation does not appear to be one of simple direct proportion, as is evidenced also by the data in Fig. 2.

2. The reaction has a peculiar lag. No methemoglobin at all was formed by 4 mols of aniline at 38° until after 2 hours, and none by 2 mols until after 5 hours. The lag is even more marked if the reaction is carried out at room temperature, as shown by Table I.

In the experiment there recorded varying amounts of aniline were mixed with oxygenated blood and permitted to stand at
room temperature. (The total hemoglobin contents in the different samples vary somewhat, apparently because of sedimentation of corpuscles in the stock of oxalated horse blood from which the material was taken.)

![Graph showing the effect of variation in aniline concentration on methemoglobin formation at 25°.](image)

**FIG. 2.** Effect of variation in aniline concentration on methemoglobin formation at 25°.

**TABLE I.**

<table>
<thead>
<tr>
<th>Mols aniline per mol Hb</th>
<th>Time of reaction</th>
<th>Total Hb.</th>
<th>Active Hb.</th>
<th>Methemoglobin.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hrs.</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>9.5</td>
<td>9.5</td>
<td>0.0</td>
</tr>
<tr>
<td>2.0</td>
<td>2</td>
<td>9.3</td>
<td>9.3</td>
<td>0.0</td>
</tr>
<tr>
<td>4.0</td>
<td>4</td>
<td>9.5</td>
<td>9.5</td>
<td>0.0</td>
</tr>
<tr>
<td>8.0</td>
<td>4</td>
<td>11.3</td>
<td>11.3</td>
<td>0.0</td>
</tr>
<tr>
<td>8.0</td>
<td>16</td>
<td>8.8</td>
<td>6.4</td>
<td>2.4</td>
</tr>
</tbody>
</table>

The lag suggests that the methemoglobin formation is caused not by the aniline itself, but by some product into which it is changed by the blood. Ellinger (1920) obtained evidence that acetanilide *in vivo* is converted into acetyl-phenyl-hydroxylamine by change of the $\text{C}_6\text{H}_4$-$\text{NH}$ group to $\text{C}_6\text{H}_5$-$\text{N(OH)}$. It is possible
that aniline is changed similarly. From the chemical structure of aniline itself it would be difficult to imagine how it would act either as an oxidizing agent or a "hydrogen acceptor." Heubner and Rohde (1923) found that phenyl-hydroxylamine is in fact a former of methemoglobin. Lipschitz and Weber (1924) found that \(\text{C}_6\text{H}_5\text{NH}_2\) and \(\text{C}_6\text{H}_5\text{NH(OH)}\) have no effect on reduced hemoglobin, but form methemoglobin in the presence of oxygen.

![Graph](image)

**Fig. 3.** Approximate formation of 1 mol of methemoglobin per 1 mol of nitrite at 25°.

A possible explanation for the formation of methemoglobin in blood by aniline is that the latter is converted into phenyl-hydroxylamine, which acts as a catalyst to accelerate the oxidation of reduced hemoglobin by molecular oxygen to methemoglobin. The scope of the present work has prevented further testing of this hypothesis.

We have attempted to produce methemoglobin formation in rabbits *in vivo* by painting the shaven abdomen with aniline to simulate the conditions under which human aniline intoxication
usually happens. As in Stadie's (1921) attempts to produce methemoglobinemia in rabbits, however, the result was only anemia. Presumably methemoglobin is formed, but does not stay in the blood. The results of an experiment, in which the painting was repeated daily for 4 days, are given in Table II. The skin at the end of the experiment was necrotic.

That the aniline penetrates the blood cells and forms methemoglobin within them in vitro is indicated by the following experiment.

Horse blood was mixed with 2 mols of aniline per mol of hemoglobin, and left overnight at room temperature. The cells were then centrifuged and washed several times. The saline suspension was dark brown and gave the following CO capacities:

After treatment with Na₂S₂O₄: 6.62 mM per liter.
Without “ “ “: 4.48 “ “ “
Methemoglobin: 2.14 “ “ “

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>Before painting</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>After “ “ “</td>
<td>5.5</td>
<td>5.5</td>
</tr>
</tbody>
</table>

The fact that aniline in vivo causes a disappearance of hemoglobin (presumably after transformation to methemoglobin) instead of the appearance of methemoglobin indicates an effect of factors, presumably hemolytic, which do not act in vitro.

The above results demonstrate (1) that the product formed from the action of aniline on hemoglobin is genuine methemoglobin, so far as we can characterize it at present, (2) that the process of formation is a slow one, with a peculiar latent period during which no formation at all occurs, and (3) that the process can occur in the cells, which therefore appear permeable either to aniline or to whatever product of it produces the change to methemoglobin.

Nitrobenzene.

Nitrobenzene is one of the substances usually listed as methemoglobin formers. It is certain that it can reduce the oxygen-
combining capacity of blood \textit{in vivo}, but whether the inactive substance into which the hemoglobin is changed is methemoglobin or some other derivative appears less certain. Loeb, Bock, and Fitz (1921) in two men with nitrobenzene poisoning found the oxygen capacity of the blood reduced to 6.2 and 8.9 volumes per cent respectively. The red cell count and total hemoglobin by Stadie’s method were normal, corresponding to about 20 volumes per cent of oxygen capacity. The differences between total hemoglobin and oxygen capacity would indicate that over half the hemoglobin was inactivated in each case. Yet no methemoglobin could be detected by the spectroscope.

We have attempted to simulate human nitrobenzene poisoning in rabbits by painting the skin with the substance, and by administering it with a stomach tube. The effect seems quite different from that in man. Relatively much more severe treatment appears required to produce intoxication in the rabbit, and the effect on the blood is to produce not inactive hemoglobin or methemoglobin, but anemia. Thus, an animal killed by 5 days painting of the abdomen showed at death the oxygen capacity reduced to 4.5 volumes per cent, but there was no evidence of methemoglobin. The spleen was filled with dark pigment.

If nitrobenzene and blood are mixed \textit{in vitro} the mixture exhibits a peculiar behavior. The nitrobenzene produces no immediate decrease in the CO-combining capacity of the blood untreated with hydrosulfite. When the blood is treated with hydrosulfite, however, an actual decrease occurs in the CO capacity, as though the hydrosulfite, instead of changing methemoglobin back to

\begin{table}[h]
\centering
\caption{Effect of Nitrobenzene on Carbon Monoxide Capacity of Blood Determined with and without Hydrosulfite.}
\begin{tabular}{|c|c|c|c|c|}
\hline
Blood. & Nitrobenzene. & \multicolumn{3}{c|}{CO capacity of blood.} \\
& & Without & With & With \\
& & nitrobenzene & nitrobenzene & nitrobenzene \\
& & or & without & without \\
& & hydrosulfite. & hydrosulfite. & hydrosulfite. \\
(cc.) & (cc.) & (mM) & (mM) & (mM) \\
\hline
5 & 0.05 & 10 & 8.4 & 8.3 & 7.9 \\
10 & 0.006 & 0.5 & 9.6 & 9.5 & 8.9 \\
\hline
\end{tabular}
\end{table}
reduced hemoglobin, had the reverse effect (see Table III). It is evident that the hydrosulfite-CO methemoglobin method is not suitable for determination of the product formed by the action of nitrobenzene on hemoglobin.

**Ferricyanide.**

Potassium ferricyanide, as shown by common experience in blood oxygen determinations, and by Conant’s (1923) electrometric titrations, reacts almost instantly with dissolved hemoglobin to form methemoglobin. The ferricyanide seems quite unable to penetrate the cells, however. Even after some hours standing with equimolar amounts of ferricyanide we have found blood with intact cells to show but a trace of methemoglobin formation. The presence of ferricyanide in the amounts used did not interfere with the hydrosulfite-CO method.

The following experiment demonstrates the impermeability of the cells to ferricyanide.

10 cc. of a suspension of horse erythrocytes in 0.9 per cent NaCl solution (hemoglobin content = 4.7 mM) were treated with 0.5 mol of potassium ferricyanide per mol of hemoglobin and kept 4 hours at 38°. The cells were washed several times with saline solution, and were finally suspended in saline solution and analyzed for methemoglobin.

<table>
<thead>
<tr>
<th>mM</th>
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</thead>
<tbody>
<tr>
<td>CO capacity after hydrosulfite</td>
</tr>
<tr>
<td>“ “ without “</td>
</tr>
<tr>
<td>Methemoglobin</td>
</tr>
</tbody>
</table>

As a control, blood hemolyzed with saponin was treated with 0.5 mol of ferricyanide per mol of hemoglobin.

<table>
<thead>
<tr>
<th>mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO capacity after hydrosulfite</td>
</tr>
<tr>
<td>“ “ without “</td>
</tr>
<tr>
<td>Methemoglobin</td>
</tr>
</tbody>
</table>

Stadie (1921) found that relatively large amounts of ferricyanide were necessary to cause disappearance of hemoglobin from rabbit blood, and that the rate of disappearance was much slower than after nitrite injection. Presumably the impermeability of the cells to ferricyanide is responsible for the difference. It may also be responsible for the relatively slight toxicity of ferricyanide administered orally. It is furthermore the impermeability of the cells for ferricyanide that necessitates complete laking of blood.
Studies of Methemoglobin Formation

before the oxygen content can be determined by the ferricyanide method.

Nitrites.

Nitrites, like ferricyanide, quickly change active hemoglobin to a form which does not bind O₂ or CO. That nitrite hemoglobin is the same methemoglobin as that formed by the action of ferricyanide or spontaneous oxidation of partially oxygenated blood appears uncertain from the results of Hartridge (1920). The product does, nevertheless, react with hydrosulfite like methemoglobin, with regeneration of reduced hemoglobin.

10 cc. of a cell solution containing 4.3 mM of Hb by oxygen capacity measurement were treated with 10 mg. of NaNO₂ (3 mols per 1 mol Hb). After a few minutes the solution was divided into two parts, and the CO capacities were determined with and without hydrosulfite, as described for methemoglobin determination.

<table>
<thead>
<tr>
<th>mM</th>
<th>CO capacity with hydrosulfite</th>
<th>CO capacity without hydrosulfite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Methemoglobin ............................................. 4.1

Unlike ferricyanide, nitrite penetrates the red cells, as shown by the following.

A suspension of washed horse cells in saline had oxygen capacity of 8.1 mM. Two portions were treated, one with 5 mols of potassium ferricyanide per mol of hemoglobin, the other with 5 mols of sodium nitrite. After 30 minutes both suspensions were centrifugated, washed, made up to original volume, and the oxygen capacities were redetermined.

<table>
<thead>
<tr>
<th>mM</th>
<th>O₂ capacity with nitrite</th>
<th>O₂ capacity with ferricyanide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.89</td>
<td>8.15</td>
</tr>
</tbody>
</table>

The rapidity of the action of nitrite is indicated by the following experiment.

A hemoglobin solution was prepared by taking 5 cc. of horse erythrocytes in 10 cc. of water. The oxygen capacity of the solution was 6.70 mM. To 10 cc. of the solution 5 mg. of NaNO₂ were added (1.07 mols nitrite per mol hemoglobin) and the oxygen capacities were determined at intervals. (See Table IV.)

The fact that approximately 1 mol of nitrite reacts with 1 mol of hemoglobin is shown by the following experiment.
A solution of cells containing 7.30 mM of hemoglobin, by oxygen capacity measurement, was divided into 5 cc. portions. To four such portions were added respectively 0.05, 0.1, 0.2, and 0.4 cc. portions of 1 per cent NaNO₂ solution, containing 0.1, 0.2, 0.4, and 0.8 mols of nitrite per mol of hemoglobin. (The NaNO₂ stock solution was standardized by permanganate titration.) After 30 minutes at room temperature the oxygen capacities were redetermined. The results are given in Fig. 3.

The permeability of the cells for nitrite is presumably the cause for the facts noted by Stadie (1921), that the effect of nitrite on the blood of rabbits in vivo occurs in a few minutes, that intravenously injected nitrite causes the formation of about equimolar amounts of methemoglobin, and that the methemoglobin remains in the cells.

### TABLE IV.

Rate of Reaction of Nitrite and Hemoglobin.

<table>
<thead>
<tr>
<th>Time after addition of nitrite.</th>
<th>Oxygen capacity.</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>mM</td>
</tr>
<tr>
<td>0</td>
<td>6.7</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
</tr>
<tr>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>40</td>
<td>0.4</td>
</tr>
<tr>
<td>60</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**SUMMARY.**

Nitrobenzene acting on blood in vitro did not yield a product determinable as methemoglobin by the hydrosulfite-CO method. When absorbed by rabbits nitrobenzene caused anemia, without methemoglobinemia.

The actions of aniline, ferricyanide, and nitrite on hemoglobin in the presence of air yielded products which were identical with methemoglobin in that they could not bind molecular O₂ or CO until converted by reduction with hydrosulfite into reduced hemoglobin.

The action of aniline showed a latent period at the beginning, no methemoglobin being formed for a time that might extend for some hours. After methemoglobin formation began it proceeded slowly, and several mols of aniline per mol of hemoglobin were required to complete it. The behavior accords with the possi-
Studies of Methemoglobin Formation

ability, indicated by previous writers, that a product of aniline rather than aniline itself causes the methemoglobin formation. Aniline forms methemoglobin in the cells of unlaked blood; consequently either aniline or its product penetrates the cells.

Nitrite and ferricyanide alike react almost instantly in laked blood to form methemoglobin, and 1 mol of each forms approximately 1 mol of methemoglobin.

Nitrite and ferricyanide differ in that nitrite penetrates the cells instantly and forms methemoglobin within them. The cells, on the contrary, appear entirely impermeable to ferricyanide anion, and methemoglobin formation by ferricyanide in vitro occurs only after they are laked.

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