THE REACTION OF HEMOGLOBIN WITH NITRITE

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(Received for publication, September 10, 1943)

Although nitrite has been extensively used as an agent for the formation of methemoglobin, the quantitative relations of the reaction appear to be uncertain; three widely different values have been reported for the amount of methemoglobin formed to nitrite utilized. The first statement of a quantitative relationship was made by Barcroft and Müller (1) in 1911. In a preliminary report containing no experimental data or equations, and for which none was supplied in subsequent publications, they indicated the molar ratio of nitrite utilized to methemoglobin formed as 2; that is, 2 molecules of nitrite react with 1 molecule of hemoglobin to produce 1 molecule of methemoglobin. This relation was accepted by Stadie (2) in 1921 in his investigations on methemoglobin formation. From experimental determinations, Van Slyke and Vollmund (3) in 1925, Meier (4) in 1925, and von Issekutz (5) in 1939 reported that 1 molecule of nitrite was used in forming 1 molecule of methemoglobin; i.e., a molar ratio of 1. Austin and Drabkin (6) in 1935 reported a ratio of 0.5 to 0.7 and one of approximately 0.5 can be calculated from data published in 1942 by Darling and Roughton (7) dealing with the effect of methemoglobin on the oxygen dissociation curve.

The reasons for some of these discrepancies will be dealt with in detail in subsequent discussion. An obvious source of error in several of the investigations was in the assumption that all nitrite added to blood or solutions of hemoglobin was quickly and completely utilized in methemoglobin formation. Analyses were not made for residual nitrite. In the investigation reported here, this source of error was avoided. The amount of nitrite reacting with hemoglobin was determined and the influence of temperature, concentration of nitrite, and of hydrogen ion concentration on the reaction was studied.

EXPERIMENTAL

Adult white rats were used for experiments in vivo and freshly shed heparinized1 rat blood for experiments in vitro. Hemoglobin and methemoglobin were determined by the method of Evelyn and Malloy (8). The concentration of nitrite in the blood was determined by a modification of

1 Crystalline liquaemin was kindly supplied by the Roche-Organon Corporation.
the method of Stieglitz and Palmer (9) with sulfanilic acid instead of 2-naphthylamine-6,8-disulfonic acid.

To three 2 cc. portions of whole blood containing 8.20 mM of hemoglobin per liter there were added 0.02, 0.04, and 0.06 cc. of a 1.0 per cent solution of sodium nitrite yielding 1.45, 2.90, and 4.35 mM of nitrite per liter in the respective portions. The blood was maintained at 20°. At the end of 30 minutes, 1 hour, and each hour thereafter, determinations were made for methemoglobin and nitrite. The findings are recorded in Fig. 1. The residual nitrite, as mM per liter, is shown by the small figure at each point plotted.

The curves express the relation between the rate of formation of methemoglobin from the gradually disappearing nitrite and the rate of the continuous reduction of methemoglobin. At the time intervals used, the maximum formation of methemoglobin was found for the two smaller amounts of nitrite at 30 minutes and for the largest at 1 hour. At these times, 9 to 17 per cent of the nitrite was still present; it did not disappear.
until the 2nd or 3rd hour. The rate of disappearance of methemoglobin increased as the nitrite concentration approached zero. At the low concentrations of methemoglobin reached by the time all, or nearly all, of the nitrite had disappeared, the rate of reduction slowed, as has been indicated by Gelinsky (10) and Cox and Wendel (11). On the assumption that, except at these low levels, the rate of reduction is at a uniform rate (11), an approximation can be made of the total methemoglobin formation from the nitrite by extending the curve for the maximum rate of reduction back to zero time. These extensions are shown by the dotted lines in Fig. 1; the amounts of methemoglobin indicated by these extrapolations are 2.5, 5.0, and 7.2 mM per liter for 1.45, 2.9, and 4.35 mM of nitrite respectively. The corresponding ratios of nitrite utilized to methemoglobin formed are 0.58, 0.55, and 0.59. In another series of experiments, ratios of 0.55, 0.52, and 0.53 were obtained. The possible sources of error in this extrapolation would tend toward a high rather than a low ratio: (a) during the period of maximum rate of reduction of methemoglobin a small amount of nitrite was still present; (b) the rate of reduction from which the extrapolation was made occurred when the reduction was more than half complete and if, as reported by Gelinsky (10), but contrary to Cox and Wendel (11), the rate of reduction varies with the concentration, the slope of the curve expresses a rate less than the average.

In the second series of experiments, laked blood was employed in order to eliminate the reduction of methemoglobin. To each of two portions of red cells laked with distilled water and containing 6.65 mM of hemoglobin per liter, 2.84 mM per liter of sodium nitrite were added. One portion was kept at 20° and the other at 37°. At 30 minutes, 1 hour, and each hour thereafter, methemoglobin was determined, and at 5 hours, the residual nitrite. The findings are given in Fig. 2. The rate of methemoglobin formation was more rapid at 37° than at 20°; at 5 hours the respective concentrations were 4.5 and 2.75 mM. The residual nitrates were 0.57 and 1.50 mM per liter. The molar ratios of methemoglobin formed to nitrite utilized were 0.49 and 0.51. Thus, although the rate of methemoglobin formation in laked blood is markedly influenced by temperature, the molar ratio of the reaction of hemoglobin and nitrite is not affected.

In a third series of experiments, sodium nitrite was added to each of six portions of red cells laked by water or blood laked by saponin in amounts sufficient to give molar ratios of 0.5 to 1.05 with the hemoglobin present. The temperature was 20°. At 1 and 3 hours, determinations were made of methemoglobin and residual nitrite. The molar ratios of methemoglobin formed to nitrite used, Table I, at both times were between 0.48 and 0.57; they were not influenced by the concentration of nitrite present.

In a fourth series of experiments, nitrite was added to blood laked by
saponin in quantities to give molar ratios of 0.15 to 1.16 with the hemoglobin present; potassium dihydrogen phosphate was then added to make the blood acid and the concentrations of methemoglobin and nitrite were determined 3 and 5 minutes later. These short intervals were employed to avoid appreciable autoxidation of hemoglobin in the acid medium (12) and because the reaction between nitrite and hemoglobin proceeds rapidly in such a medium. As seen from the data of Table II, even within these short periods all the nitrite had disappeared when the hemoglobin was in excess and all the hemoglobin had reacted when the nitrite was in excess.

![Graph](http://example.com/graph.png)

**Fig. 2.** Methemoglobin and residual nitrite in laked blood after addition of 2.84 mM of sodium nitrite per liter.

Acidification had no influence upon the molar relation of methemoglobin formed to nitrite utilized; the ratios as found ranged from 0.49 to 0.54.

In a final series of experiments, the methemoglobin formed from nitrite was determined in the living animal to test the validity of the findings of von Issekutz (5). He injected sodium nitrite in doses of 8 to 30 mg. per kilo (0.116 to 0.435 mM per kilo) and from the maximum concentration of methemoglobin in the blood, which developed in 30 minutes to 1 hour, he calculated an average nitrite-methemoglobin molar ratio of nearly 1. In the experiments here, rats were given, by intraperitoneal injection, 0.218 and 0.435 mM per kilo of sodium nitrite. At 30 minutes, 1 hour, and each
hour thereafter, to a total of 4 hours, the methemoglobin was determined
and, in contrast to the work of von Issekutz (5), the concentration of nitrite.
With a dose of 0.218 mM per kilo the methemoglobin concentration reached
a maximum of 1.84 mM per liter at 30 minutes; the corresponding nitrite
value was 0.218 mM per liter. With a dose of 0.435 mM per kilo the concen-
tration of methemoglobin reached a maximum of 3.85 mM per liter in 1
hour; that of nitrite was then 0.333 mM. In both experiments there were

TABLE I
Formation of Methemoglobin in Laked Blood As Determined from Nitrite Added and
Nitrite Utilized

<table>
<thead>
<tr>
<th>Red cells laked with</th>
<th>Moles nitrite added</th>
<th>1 hr, moles nitrite calculated from</th>
<th>3 hrs, moles nitrite calculated from</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moles Hb</td>
<td>Nitrite added</td>
<td>Nitrite utilized</td>
</tr>
<tr>
<td>Water</td>
<td>0.5</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.03</td>
<td>2.65</td>
<td>0.50</td>
</tr>
<tr>
<td>Saponin</td>
<td>0.52</td>
<td>2.03</td>
<td>0.48</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.03</td>
<td>2.65</td>
<td>0.53</td>
</tr>
<tr>
<td>&quot;</td>
<td>1.05</td>
<td>2.70</td>
<td>0.57</td>
</tr>
</tbody>
</table>

TABLE II
Formation of Methemoglobin in Acidified Blood

<table>
<thead>
<tr>
<th>Moles nitrite added</th>
<th>MHB formed</th>
<th>Nitrite utilized</th>
<th>Nitrite remaining</th>
<th>Moles nitrite utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moles Hb</td>
<td>mm per l.</td>
<td>mm per l.</td>
<td>mm per l.</td>
<td>Moles MHB</td>
</tr>
<tr>
<td>0.15</td>
<td>2.11</td>
<td>1.03</td>
<td>0.00</td>
<td>0.49</td>
</tr>
<tr>
<td>0.24</td>
<td>2.62</td>
<td>1.46</td>
<td>0.00</td>
<td>0.52</td>
</tr>
<tr>
<td>0.30</td>
<td>3.91</td>
<td>2.05</td>
<td>0.00</td>
<td>0.52</td>
</tr>
<tr>
<td>0.50</td>
<td>5.46</td>
<td>2.75</td>
<td>0.00</td>
<td>0.50</td>
</tr>
<tr>
<td>0.59</td>
<td>6.10</td>
<td>3.23</td>
<td>0.87</td>
<td>0.53</td>
</tr>
<tr>
<td>1.16</td>
<td>7.10</td>
<td>3.86</td>
<td>0.35</td>
<td>0.54</td>
</tr>
</tbody>
</table>

still appreciable amounts of nitrite in the blood at the time the maximum
amount of methemoglobin was present. In these experiments, and also
in those of von Issekutz (5), no allowance was made for the elimination of
nitrite in the urine. Thus for two reasons it cannot be assumed that the
maximum amount of methemoglobin reached in the blood expresses the
reaction of hemoglobin with the total amount of nitrite given. In addi-
tion, the maximum amount of methemoglobin observed is the algebraic
sum of the amount produced and the amount reduced; this point has already
been discussed. The only conclusion justified from such experiments is
that the molar ratio of nitrite utilized to methemoglobin formed is less than 1.

DISCUSSION

Barcroft and Müller (1) define the relation of methemoglobin formation to nitrite utilization in the statement: "Methemoglobin is formed quantitatively when potassium nitrite is added to blood, the amount of hemoglobin converted containing an amount of dissociable oxygen equivalent to that necessary to convert the nitrite to nitrate." Since each molecule of hemoglobin contains 1 molecule of dissociable oxygen for each atom of iron, it follows from this statement that 2 molecules of nitrite react with 1 molecule of hemoglobin to form 1 molecule of methemoglobin. From present knowledge of methemoglobin as a ferric compound (13) expressed by the formula Hb₅O (or HbOH), it is impossible to write an equation fulfilling the conditions in the statement of Barcroft and Müller (1). Thus the equation

$$4\text{NO}_3^- + 2\text{HbO}_2 \rightarrow \text{Hb}_5\text{O} + 4\text{NO}_3^-$$

cannot be balanced. In any possible reaction between nitrite and hemoglobin to form methemoglobin and nitrate the amount of hemoglobin converted must always contain an amount of dissociable oxygen greater than that necessary to convert the nitrite to nitrate.⁵ One may thus write the equation

$$3\text{NO}_3^- + 2\text{HbO}_2 \rightarrow \text{Hb}_5\text{O} + 3\text{NO}_3^-$$

The nitrite-methemoglobin molar ratio in this reaction is 1.5. Other equations may be written in which free oxygen is liberated as a product of the reaction,

$$\text{NO}_3^- + 2\text{HbO}_2 \rightarrow \text{NO}_3^- + \text{Hb}_5\text{O} + \text{O}_2$$

or

$$4\text{NO}_3^- + 4\text{HbO}_2 \rightarrow 4\text{NO}_3^- + 2\text{Hb}_5\text{O} + \text{O}_2$$

In the first of these equations, the nitrite-methemoglobin molar ratio is 0.5; in the second, it is 1.0.

In the experimental work of Van Slyke and Vollmund (3) from which

\(^2\) Since 2 molecules of methemoglobin are formed by each molecule of nitrite, 0.5 mole of oxygen should be liberated per molecule of hemoglobin reacting, if the nitrite goes only to nitrate. We have found in four determinations, using no excess of nitrite, a liberation of 0.80, 0.77, 0.79, and 0.80 mole of oxygen per mole of hemoglobin. The determinations were made by the micro gasometric method of Roughton and Echolander (14). These data would indicate that products other than nitrate are produced in the reaction.
they reported a nitrite-methemoglobin molar ratio of 1, laked blood was employed. To 5 cc. portions containing 7.30 mM of hemoglobin per liter there were added 0.05, 0.1, 0.2, and 0.4 cc. of a 1.0 per cent solution of sodium nitrite. These investigators calculated these amounts as representing 0.1, 0.2, 0.4, and 0.8 mole of nitrite per mole of hemoglobin. An arithmetical error appears in this calculation; the amounts of nitrite used are correctly 0.2, 0.4, 0.8, and 1.6 moles per mole of hemoglobin—twice the amounts they state. This recalculation results in a corresponding change in the nitrite-methemoglobin molar ratio from 1, which they report, to 2.

The explanation for this high ratio obtained after correction of the calculations of Van Slyke and Vollmund may be in the facts that the determinations of methemoglobin were made at the end of 30 minutes at room temperature and that none was made for residual nitrite. From a preliminary experiment in which they found virtually all of the hemoglobin converted to methemoglobin in 20 minutes at room temperature, they concluded that 30 minutes were adequate for the reaction. This conclusion would be justified were it known that all of the nitrite added had been utilized. If, as shown here, 1 mole of nitrite reacts with 2 moles of hemoglobin, nitrite was present in considerable excess in their experiment. The rate of reaction of nitrite and hemoglobin depends upon their relative concentrations (15). Thus with an excess of nitrite its relative concentration would become greater as the hemoglobin disappeared and the reaction would proceed at an undiminishing rate. If, however, the hemoglobin were in excess, the relative concentration of nitrite would become progressively smaller with a correspondingly diminishing rate of reaction. The results of the present investigation have, in fact, shown that except in acidified hemoglobin solutions, or when the nitrite is in excess, the reaction is far from complete after 30 minutes, particularly at room temperature. This is in agreement with the observations of Austin and Drabkin (6) who found that upon addition of small amounts of nitrite to hemoglobin solutions the maximum formation of methemoglobin did not occur for many hours.

Meier (4), in estimating a nitrite-methemoglobin ratio of 1, made no determinations of methemoglobin but measured the amount of free oxygen liberated in the reaction. When he added large excesses of nitrite to acid blood, oxygen was taken up. This he explained by the oxidation of nitrite to nitrate which he showed would occur in the presence of acid, in air, without hemoglobin.

\[ 2\text{HNO}_2 + \text{O}_2 \rightarrow 2\text{HNO}_3 \]  

At a concentration of nitrite, however, which was just sufficient to convert all of the hemoglobin to methemoglobin Meier found that an amount of
oxygen was liberated equal to one-fourth of the amount in oxyhemoglobin. For this reaction, he therefore wrote the equation

$$4\text{HNO}_2 + 4\text{Hb}_2\text{O}_2 \rightarrow 4\text{HNO}_3 + 2\text{Hb}_2\text{O} + \text{O}_2$$

in which 1 mole of nitrite forms 1 mole of methemoglobin. The error made by Meier was that he arbitrarily chose 1 mole of nitrite per mole of hemoglobin as the amount just sufficient to convert all of the hemoglobin to methemoglobin. Had he used 0.5 mole of nitrite per mole of hemoglobin, he would have found that this too was enough to convert all of the hemoglobin. The equation for this reaction is

$$\text{HNO}_2 + 2\text{Hb}_2\text{O}_2 \rightarrow \text{HNO}_3 + \text{Hb}_2\text{O} + \text{O}_2$$

in which one-half of the oxygen in the oxyhemoglobin is liberated instead of one-fourth of it, as found by Meier. Since he added twice as much nitrite as is necessary, only half of it reacted with the hemoglobin according to Equation 3; the other half reacted with oxygen according to Equation 1, utilizing half of the oxygen liberated in the formation of methemoglobin and resulting in a sum of reactions which is expressed by Equation 2. Meier gave no evidence that all of the nitrite he added reacted exclusively with hemoglobin.

The determination of the nitrite-methemoglobin ratio for the living animal by the method used by von Issekutz (5) is, as pointed out, impossible, since (a) the maximum concentration of methemoglobin occurs at a time when there is considerable residual nitrite, (b) before the maximum is reached, there is reduction of methemoglobin, (c) part of the nitrite is excreted in the urine (16).

Since the nitrite-methemoglobin ratio in vitro does not appear to be dependent upon temperature, concentration, or pH and appears to be constant for the reaction between nitrite and hemoglobin, it is probably the same in the living animal as in vitro.

**SUMMARY**

1. In the reaction between nitrite and hemoglobin, in vitro, 1 molecule of nitrite reacts with 2 molecules of hemoglobin to form 2 molecules of methemoglobin.

2. Temperature, concentration of nitrite, and pH are without influence upon the ratio of the nitrite utilized to methemoglobin formed.

3. In an acid medium the reaction of nitrite with hemoglobin is complete in a short time.

4. In a neutral or slightly alkaline medium, the reaction may take many hours for completion, depending upon the concentration of nitrite.

5. The amount of methemoglobin formed per mole of nitrite utilized
cannot be determined directly in the living animal but is probably the same as it is in vitro.

Acknowledgment is made of the technical assistance of Evelyn Shukovsky.

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