Relation of Methemoglobin to Hemolysis

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It is well known that a number of chemical agents, which under certain circumstances cause intravascular hemolysis and the development of hemolytic anemia, also produce methemoglobinemia. Some examples of these agents are sulfanilamide, phenylhydrazine, aniline, acetanilid, p-aminophenol, plasmochin, etc. Such a correlation may lead to the inference that intracorpuscular methemoglobin is related in some way to the process of hemolysis. It has been observed that the dosage level of acetanilid and acetophenetidin causing significant anemia also exceeds the threshold for methemoglobin formation. The data of Rimington and Hemmings might also be interpreted to lend some additional support to the view that methemoglobin plays some positive role. In studies on drugs related to sulfanilamide, they observed a close correlation between the action of aromatic amino compounds to increase porphyrin excretion and to form methemoglobin. Since the increased porphyrin excretion in rodents was due largely to coproporphyrin III, it has been suggested that this represents an abnormal pathway for the degradation of hemoglobin possibly through methemoglobin.

However, Rimington and Hemmings were not certain as to the role of the methemoglobin in either the increased erythrocyte destruction or the increased porphyrin excretion since the latter was not observed after sodium nitrite administration, which readily produces methemoglobin. Fox and Ottenberg studying the hemolytic anemias from sulfonamides in man observed a greater percentage of methemoglobin in the serum than in the erythrocytes and suggest that it was the cells containing methemoglobin which underwent hemolysis. This discrepancy between serum and cells could be readily accounted for by the presence in serum of oxidants with low penetrability into the erythrocytes, or by different equilibrium conditions, as in fact the high percentage of methemoglobin observed in the urine suggests may have been the case.

On the other hand, there are some reasons to doubt that methemoglobin per se favors hemolysis. Hemolytic anemias have not been reported as a feature of nitrite toxicity, and as will be seen, we have not observed it in experiments designed to test this point. Also Keilin and Mann state that intracorpuscular hemoglobin may be readily changed to methemoglobin with sodium nitrite without altering the integrity of the cell membrane. It is well known that certain drugs may produce a hemolytic anemia without the formation of methemoglobin. Furthermore, the alteration of hemoglobin to form carbon monoxide hemoglobin has been shown not to alter the osmotic fragility of erythrocytes.

However, these are insufficient reasons to decide whether methemoglobin...
plays any definitive role in the process of hemolysis. There is a paucity of any quantitative data on the subject. Therefore a comprehensive study has been made in order to provide quantitative data with highly sensitive techniques. A comparison has been made in vitro of the resistance of oxyhemoglobin and methemoglobin containing erythrocytes to hemolysis by saponin, hypotonic saline, bile salts and lyssolecithin. Also included are observations on the hemolytic effects in vivo of chronic methemoglobinemia produced by sodium nitrite.

**Experimental Methods**

In order to carry out these experiments, it was necessary to use some chemical agent to produce intracorpuscular methemoglobin. The requirements for such an agent are (1) that it forms methemoglobin in vitro as well as in vivo, (2) that it can readily penetrate the erythrocyte membrane and (3) that it produces no injury to the membrane in the concentrations required to form relatively high concentrations of methemoglobin. These requirements eliminate many aromatic compounds, some of which at least act as simple lysins or accelerate certain lysins. After numerous preliminary experiments sodium nitrite was chosen as the most suitable agent available. The nitrite readily penetrates the erythrocyte and forms methemoglobin rapidly at low concentrations. In the procedure of preparing the erythrocyte suspensions, there was no gross evidence of hemolysis associated with the presence of the nitrite; after repeated washings with saline no trace of nitrite could be detected. Furthermore, the intentional addition of low concentrations of sodium nitrite to the lytic system (under those conditions in which oxyhemoglobin and methemoglobin cells hemolyzed in the same time) did not significantly alter the time required for hemolysis, indicating that this agent did not act to an important degree as an accelerator or inhibitor of the lysins used. Keilin and Mann have also presented some evidence that similar treatment of erythrocytes does not modify their permeability.

**Erythrocyte suspensions.** Dog erythrocytes were employed for all of the data to be reported. However, human erythrocytes were also employed in a sufficient number of experiments to establish the qualitative similarity of the results. The standard cell suspensions were prepared in the following way. A sample of blood, about 4 cc., was drawn into a centrifuge tube containing 25 cc. of 0.85 per cent sodium chloride and 250 mg. of sodium citrate. This saline-citrate suspension was divided into two portions; one portion was treated with sodium nitrite, and 10 mg. of blood was allowed to stand for ten minutes during which time about 80 to 90 per cent of the hemoglobin was converted to methemoglobin. When lower percentages of methemoglobin were desired proportionately smaller amounts of sodium nitrite were added. The cells of both portions were then washed three times by centrifugation and resuspension in 30 cc. of saline per cc. of packed cells. The suspensions were carefully standardized in the final dilution with saline to contain 0.8 Gm. of total hemoglobin per 100 cc., which corresponded approximately to 250,000 cells per cu.mm. These two cell suspensions will be referred to as “oxyhemoglobin cells” and “methemoglobin cells.”

All experiments except those with hypotonic saline were carried out in buffered saline. Sorensen's M/15 phosphate mixtures were used in proportion of 1 part in 10 of 0.85 per cent saline to obtain the desired pH indicated in the experiments; the initial pH before hemolysis for a sufficient number of experiments was checked by the glass electrode.

**Saponin.** The time required for complete hemolysis was determined for a suspension of washed erythrocytes and saponin in a saline-phosphate buffer with the tubes in a constant temperature water bath according to the general method described by Ponder. The hemolytic system consisted of 2 cc. of buffered saline, 2 cc. of the saponin dilution in buffered saline and 1 cc. of the standard cell suspension in soft glass test tubes 12 X 100 mm. A series of dilutions of saponin were employed to obtain time-dilution curves. The dilutions of saponin indicated in the figures are the final dilutions in the hemolytic system.

**Hypotonic Saline.** Percentage hemolysis curves were obtained with a series of dilutions of hypotonic saline using the general technic described by Hunter. The erythrocyte suspensions were prepared as described above except that the final dilution was that of the
original volume of blood. One-tenth cc. of such a suspension was added to 10 cc. of each
dilution of hypotonic saline. After standing for one hour at room temperature, the series
of tubes were centrifuged and the liberated hemoglobin determined in the supernatant.

Bile Salts. The same general technic for 100 per cent hemolysis as described for saponin
was used. Both sodium glycocholate and taurocholate were tested and yielded qualita-
tively similar results, but only the data on glycocholate will be presented.

Lyssolecithin. The lyssolecithin used was prepared by the action of snake venom* on egg
yolk, as described by King and Dolan. The material obtained was probably somewhat
impure, since no attempt was made to separate out the lysocephalin, but nevertheless pro-
vided a preparation of constant activity. For use a stock solution of lyssolecithin was pre-
pared each day containing 10 mg. per cc. in absolute methyl alcohol from which appropriate
dilutions were made with saline and kept in paraffined test tubes. The standard suspen-
sions of erythrocytes were prepared in the same way as described above.

The time required for 50 per cent hemolysis was used as the end point for this lysin;
this could be estimated accurately from the time per cent hemolysis curves which were
obtained by an adaptation of the photoelectric method described by Wilbur and Collier. The
hemolytic system consisted of 8 cc. of buffered saline, 1 cc. of the standard cell sus-
pension and 1 cc. of the lyssolecithin dilution in saline. The dilutions indicated in the data
are the final dilutions of the lysin in the colorimeter tube. The per cent hemolysis curves
were obtained using the Evelyn colorimeter with filter 660. The galvanometer was set at
100 for the one hundred per cent hemolysis blank containing the above system of the cor-
responding type of cells which were completely hemolyzed with saponin. The initial opac-
ity reading for zero per cent hemolysis was obtained for both kinds of cells with the above
system in which the lyssolecithin solution was replaced by saline. Such control tubes were
prepared for each pH and temperature used. (In most cases it was impractical to obtain
an initial opacity reading following the addition of the lyssolecithin because of the initial
rapid rate of hemolysis with concentrations of lysin yielding fifty per cent hemolysis in a
reasonable time.) In the experimental tubes following the addition of lyssolecithin, galva-
nometer readings were recorded at appropriate time intervals. The per cent of hemolysis
was read directly from semi-log graph paper, using the opacity reading of the unhemolyzed
control tube to establish the point for zero hemolysis.

RESULTS

Saponin. Time-dilution curves for 100 per cent hemolysis were determined
with saponin in parallel experiments on both oxyhemoglobin and methemo-
globin cells. The average results in 16 parallel experiments at pH 7.3 and 38 C.
are shown in figure 1A, and indicate that the presence of methemoglobin in the
erthrocytes has no effect on their resistance to saponin lysis. Qualitatively
similar results were obtained at pH 6.2 and 38 C, and also at 25 C, pH 6.2 and
7.3, and with human erythrocytes. The addition of free oxyhemoglobin and met-
hemoglobin initially to the system had a small but equal inhibitory effect on the
subsequent saponin hemolysis.

Another series of experiments, using both the methemoglobin and oxyhemo-
globin cells, were performed in an identical manner except that chemical agents
known to accelerate saponin lysis were included in equal concentrations in both
systems. Thus, each experiment consisted of four time-dilution curves; that is,
oxymhemoglobin and methemoglobin cells each in the presence and absence of
the accelerator. The results are illustrated in figure 1B, C, and D. The accelerators
varied from the highly active quinine to the relatively weak acetonilid. The
curves obtained for the oxyhemoglobin and methemoglobin cells were es-

* Crotalus atrox venom kindly supplied by Sharpe and Dohme.
sententially identical. Similar data were obtained with several other accelerators, such as aniline, acetophenetidin, and p-aminophenol; the latter is the only agent listed which forms methemoglobin in vitro under the conditions of these experiments. (Sulfanilamide was found to be a weak inhibitor and to affect both types of cells to the same degree.) The results clearly indicate that the presence of methemoglobin does not alter the time required for hemolysis of erythrocytes by saponin alone or combined with an accelerator.

Fig. 1.—Effect of methemoglobin on saponin hemolysis at pH 7.3 and 38 C.

O Oxyhemoglobin cells
● Methemoglobin cells
△ Oxyhemoglobin cells plus accelerator
▲ Methemoglobin cells plus accelerator

Hypotonic saline. Percentage hemolysis curves were obtained for a series of dilutions of hypotonic saline at room temperature (about 25 C.). Ten parallel experiments on both oxyhemoglobin and methemoglobin cells were made, and the averaged percentage of hemolysis for each concentration of saline appears in figure 2. These data indicate that the presence of methemoglobin does not alter the resistance of the erythrocytes to hemolysis by hypotonic saline.

In a second series of experiments a sub-lytic concentration of quinine (i.e., a concentration producing no hemolysis in 0.85 per cent saline in one hour) was included in the hypotonic saline before the addition of the erythrocytes. As shown in figure 2, the presence of quinine increases the percentage of hemolysis.
for a given concentration of saline, but again no difference is evident in the percentage of hemolysis of the oxyhemoglobin and methemoglobin cells.

**Bile salts.** The experiments with the bile salts were carried out using the same technic for 100 per cent hemolysis as was described for saponin. Most of the experiments were made at 38 C. with sodium glycocholate. In preliminary experiments, it was found that the relative rates of hemolysis of the oxyhemoglobin and methemoglobin cells differed according to the pH of the system. This relationship is illustrated in figure 3, which represents times for hemolysis for the two types of cells by 1:2000 glycocholate over a range of pH values. Below pH 6.6, hemolysis of the methemoglobin cells takes place at a slightly increased rate. On the other hand, above pH 7.0 the time required for the hemolysis of the methemoglobin cells becomes excessively long as compared to the oxyhemoglobin cells. The two curves cross at approximately pH 6.7. The general relationship evident at pH 6.2 and 7.3 was also observed to hold for a series of dilutions of both glycocholate and taurocholate. The inhibition of methemoglobin cells at pH 7.3 and their slight acceleration at pH 6.2 was found to be roughly proportional to the methemoglobin concentration in the cells.

In view of the reversal of the rates of hemolysis of the two types of cells with respect to pH, it seemed advisable to determine whether solutions of free oxyhemoglobin and methemoglobin differed in their inhibitory activity. A portion of the buffered saline in the hemolytic system was replaced by buffered hemo-
Fig. 3.—Effect of methemoglobin on glycocholate hemolysis as influenced by pH concentration of glycocholate 1:2,000 at 38 C.

- Oxyhemoglobin cells
- Methemoglobin cells

Fig. 4.—Relative inhibitory effect of free oxyhemoglobin and methemoglobin on hemolysis by glycocholate at pH 7.3 and 38 C.

- Oxyhemoglobin cells alone
- Oxyhemoglobin cells plus free oxyhemoglobin
- Oxyhemoglobin cells plus free methemoglobin
globin solutions equivalent to 10 per cent of the hemoglobin contained in the cells in the system. It was found as shown in figure 4 that at pH 7.3, where the methemoglobin cells were apparently resistant to hemolysis, that the addition of a solution of methemoglobin to the oxyhemoglobin cells caused a much greater inhibition than the addition of oxyhemoglobin in a similar system. At pH 6.2 the two types of hemoglobin had very little inhibitory effect. The same qualitative results for pH 6.2 and 7.3 were also obtained at 25 C. It therefore seems probable that the apparent increased resistance of the methemoglobin contain-

![Image of graph](attachment:image.jpg)

**Fig. 5.**—Effect of methemoglobin on lysolecithin hemolysis as influenced by pH and temperature.
- ○ Oxyhemoglobin cells at pH 7.3
- Δ Oxyhemoglobin cells at pH 6.2
- ● Methemoglobin cells at pH 7.3
- ▲ Methemoglobin cells at pH 6.2

ing cells at pH 7.3 is due to the greater inhibitory effect of the initially released methemoglobin on further hemolysis.

*Lysolecithin.* In these experiments, the time required for fifty per cent hemolysis was determined as described in the section on methods. The results with lysolecithin were found to be influenced in a rather complex way by pH and temperature, with these factors determining whether the oxyhemoglobin and methemoglobin cells hemolyzed in the same or different times.

The influence of varying the pH from 6.0 to 8.0 at 25 and 38 C. was determined for a 1:200,000 dilution of lysolecithin. At 25 C. and between pH 6.8 to 7.6
the oxyhemoglobin and methemoglobin cells hemolyzed at the same times. However, at pH 6.0 to 6.8 at 25 C. and at all pHs tested at 38 C. the methemoglobin cells consistently hemolyzed more rapidly than the oxyhemoglobin cells. From this data pH 6.2 and 7.3 were selected for a further study of the effect of temperature; an example of the results obtained with a 1:200,000 dilution of lyssolecithin are illustrated in figure 5. From the above data and figure 5, it may be said that around pH 7.3 and 25 C. both the oxyhemoglobin and methemoglobin cells hemolyzed in approximately the same time, and more extensive data show that there is no significant difference. However, under several other conditions of pH and temperature, the methemoglobin cells hemolyzed more rapidly.

In other experiments these differences in the hemolysis times of oxyhemoglobin and methemoglobin cells were found to be proportional to the methemoglobin concentration. As might have been predicted, at pH 7.3 and 25 C., varying the methemoglobin concentration resulted in no significant difference in the hemolysis time. However, for the other conditions tested (pH 6.2 at 25 C. and pH 6.2 and 7.3 at 38 C.), increasing the methemoglobin concentration caused a proportional acceleration of hemolysis.

An attempt to explain these varying results suggested the possibility that free oxyhemoglobin and methemoglobin initially released might cause differing degrees of inhibition of subsequent hemolysis, especially since an example of this had already been found in the case of the bile salts. This possibility was tested by comparing the relative inhibitory effects produced by replacing a portion of the buffered saline in the system by buffered hemoglobin solutions equivalent to 20 per cent of the hemoglobin contained in the cells in the system. Representative results are shown in table 1. It is evident that at 25 C. and pH 7.3, where the oxyhemoglobin and methemoglobin cells hemolyze at approximately the same rate, the addition of free oxyhemoglobin and methemoglobin caused the same degree of inhibition. However, for the other conditions under which the methemoglobin cells hemolyzed more rapidly, free oxyhemoglobin caused a much greater degree of inhibition than did free methemoglobin; in fact, the latter at times caused some acceleration, especially at 25 C. and pH 6.2. These differences in the inhibitory action of the two kinds of hemoglobin were more easily demonstrated using oxyhemoglobin cells, although they were evident in a certain number of the experiments using methemoglobin cells. These data suggest that the observed differences in the inhibitory action of the two kinds of hemoglobin

### Table 1: Effect of Free Hemoglobin on Lyssolecithin Hemolysis (Lyssolecithin dilution 1:200,000)

<table>
<thead>
<tr>
<th>System</th>
<th>25°C</th>
<th>38°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6.2</td>
<td>pH 7.3</td>
</tr>
<tr>
<td>Hbo₂ Cells</td>
<td>77</td>
<td>24</td>
</tr>
<tr>
<td>Hbo₂ Cells + Hbo₂</td>
<td>120</td>
<td>35</td>
</tr>
<tr>
<td>Hbo₂ Cells + Mhb</td>
<td>65</td>
<td>35</td>
</tr>
<tr>
<td>Mhb Cells</td>
<td>22</td>
<td>28</td>
</tr>
</tbody>
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Time in seconds for 50 per cent hemolysis.
may account for the difference noted in the rates of hemolysis of the oxyhemoglobin and methemoglobin cells.

Some experiments were also made with lysolecithin prepared from dog blood as described by Singer using the method we have outlined for "synthetic" lysolecithin. In a general sense the results were similar, but the conditions under which they occurred were different. At 38 C. and pH 6.2 and 7.3, there was no definite difference in the rate of hemolysis of oxyhemoglobin and methemoglobin cells. However, at 25 C. and both pH 6.2 and 7.3, the methemoglobin cells hemolyzed more rapidly than the oxyhemoglobin cells.

Hematologic effects of methemoglobinemia in vivo. Chronic methemoglobinemia was maintained in 5 dogs over a period of thirty-five days by the administration of sodium nitrite subcutaneously as a 5% solution once daily. The hemoglobin pigments were measured by the method of Evelyn and Malloy. The dosage of nitrite was adjusted to obtain a maximal methemoglobinemia ranging between 50 and 70% each day. These maximal values which occurred about two hours after the administration of nitrite are represented in figure 6;
thereafter, the methemoglobinemia progressively decreased so that none was present the following morning when the blood samples were obtained for the other observations recorded. The data summarized in figure 6 indicate that no significant alterations occurred in the values for total hemoglobin, erythrocytes, and leukocytes. There was a small but significant increase in reticulocytes, and in some cases a small increase in cell volume. The reticulocytosis indicates an increased erythropoiesis that may be a response to increased erythrocyte destruction. If so, it must have been so small as to be covered by an increased erythrocyte production, for there is no evidence of anemia. An alternative explanation for the reticulocytosis appears more likely. It has been previously observed in this laboratory that this degree of methemoglobinemia causes some reduction in the venous oxygen tension, and this could act as a stimulus to the bone marrow. This view is strengthened by the observation that an additional increase in both total hemoglobin and reticulocytes occurs shortly after the nitrite administration when the methemoglobinemia is greatest. Furthermore, Rimington and Hemmings have observed no increased porphyrin excretion following nitrite administration. Therefore, these data indicate that chronic methemoglobinemia maintained by nitrite cause no significant anemia, and suggest that no significant decrease in erythrocyte resistance to the natural in vivo hemolysins occurred.

Discussion

The principal purpose of this paper was to determine whether the change in the composition of intracorpuscular hemoglobin to methemoglobin may influence the response of the erythrocyte to hemolysins. As outlined in the introduction, the reasons for assuming that methemoglobin may have some influence are chiefly circumstantial; it was also indicated that there were insufficient data on which to base a decision.

The in vitro experiments with saponin and hypotonic saline indicate that intracorpuscular methemoglobin does not influence hemolysis by these lysins. Neither did methemoglobin appear to alter the susceptibility of the erythrocytes to the combined action of these lysins plus chemical accelerators.

The in vitro results with bile salts and lysolecithin hemolysis were more complex; it was found that the presence of methemoglobin either did or did not influence hemolysis depending upon the experimental conditions of pH and temperature. It seems significant, however, that for both lysins there are conditions (pH and temperature) under which the times required for hemolysis of oxyhemoglobin and methemoglobin containing erythrocytes are the same. Also significant are the findings that for those conditions under which the hemolysis times were different, free oxyhemoglobin and methemoglobin (extracorpuscular hemoglobin added initially to the hemolytic system) had different inhibitory effects on subsequent hemolysis, and that these differences were directly correlated with the differences in the behavior of the oxyhemoglobin and methemoglobin cells. It is difficult to quantitate these effects. However, qualitatively such results do permit the reasonable assumption that the difference in hemolysis of the oxyhemoglobin and methemoglobin containing erythrocytes may be explained on the basis of the different degrees to which the originally released oxyhemoglobin and methemoglobin inhibits subsequent hemolysis. If this is an
adequate explanation, then the observed data with bile salts and lysolecithin could be accounted for; and the interpretation is permitted that intracorpuscular methemoglobin has not altered the resistance of the erythrocytes to hemolysis by bile salts and lysolecithin. A full explanation of the different degrees of inhibitory effect of oxyhemoglobin and methemoglobin for these lysins under certain experimental conditions cannot be given at present.

The in vitro results with saponin and hypotonic saline seem to indicate very clearly that intracorpuscular methemoglobin does not alter the resistance of erythrocytes to hemolysis by these lysins. The results with bile salts and lysolecithin are capable of a similar interpretation for the reasons outlined in the preceding paragraph, although admittedly the results are more complex, and there may be other interpretations, but the one given seems the most reasonable from a consideration of the experimental data as a whole. The in vivo experiments with chronic methemoglobinemia produced by the daily administration of sodium nitrite did not produce a significant anemia, and this together with the reported absence of increased porphyrin excretion suggests that no significant decrease in erythrocyte resistance to the intravascular lysins occurred.

These experiments present strong prima-facie evidence that intracorpuscular methemoglobinemia per se is not primarily concerned in the process of intravascular hemolysis. It is suggested that in those cases where methemoglobin formation is associated with the action of chemical agents producing hemolysis or acceleration of lysins that methemoglobin formation is not an integral part of the hemolytic action, but is an independent effect indicating the presence of oxidants capable of penetrating the erythrocyte. Several such substances have been studied in this laboratory and found to be active accelerators of various lysins; however, under specified conditions there was no difference in the degree of action on erythrocytes containing principally oxyhemoglobin as compared to erythrocytes containing 80 to 90 per cent methemoglobin.

**SUMMARY**

Experiments are described which attempt to define the relation of methemoglobin to hemolysis. A comparison was made in vitro of the resistance of erythrocytes containing chiefly oxyhemoglobin or methemoglobin to hemolysis by saponin, hypotonic saline, bile salts and lysolecithin. These data are interpreted to indicate that intracorpuscular methemoglobin does not alter the resistance of erythrocytes to hemolysis by these lysins, although there are certain peculiarities in the results with bile salts and lysolecithin.

In the case of bile salts and lysolecithin, oxyhemoglobin or methemoglobin may have an equal or different inhibitory effect on hemolysis depending upon the conditions of pH and temperature.

Chronic methemoglobinemia in dogs maintained by the administration of sodium nitrite does not produce an anemia.

These data suggest that the formation of methemoglobin is an independent effect and not a part of the hemolytic action of certain chemical agents.

**REFERENCES**

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Relation of Methemoglobin to Hemolysis
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