Studies on the Formation of Heinz Bodies. I. Methemoglobin Production and Oxyhemoglobin Destruction

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The concept that the formation of methemoglobin is a significant feature of those drug-induced hemolytic anemias associated with the appearance of Heinz bodies in the erythrocytes is not new. After a single subcutaneous injection of nitroglycol in cats, Gross et al.1 noticed transient but marked methemoglobin production in the first 3 to 5 hours, followed by Heinz body formation and erythrocyte destruction. More recently Magos and Sziza2 found a similar phenomenon following the administration of a single large dose of nitrobenzene to rats.

There has been no agreement that methemoglobinemia is produced by other drugs which cause Heinz body formation and erythrocyte destruction. In a recent review, Beutler3 said that, "Some potently hemolytic aniline derivatives to which these [ primaquine-sensitive cells] are sensitive, such as phenylhydrazine, cause little or no methemoglobin formation." Dacie4 stated that neither sulfhemoglobin nor methemoglobin is formed as a result of the action of phenylhydrazine or acetyl phenylhydrazine on red cells.

However, in some preliminary experiments we were impressed by the obvious darkening of erythrocytes incubated in vitro with acetyl phenylhydrazine. With the method of Evelyn and Malloy,2 high levels of methemoglobin were found in these cells. In view of the suggestion that methemoglobin formation is an essential step in Heinz body production,2 these studies were extended to other drugs capable of inducing Heinz body formation in erythrocytes to determine if methemoglobin is a constant feature of Heinz body production. Thus, modifications of the methods of Michel and Harrist and Evelyn and Malloy5 were used to determine the oxyhemoglobin and methemoglobin concentrations respectively of erythrocytes incubated with the following compounds: acetyl phenylhydrazine, phenylhydrazine hydrochloride, $\beta$-naphthol, vitamin K1, menadione sodium bisulphite, menadiol sodium diphosphate, 4-amino-2-methyl-1-naphthol hydrochloride, nitrofurantoin, potassium chlorate, phenacetin, acetanilide, sulfonilamide, sulfisoxazole diethanolamine, novobiocin sodium, serotonin (5-hydroxy-tryptamine creatinine sulfate), nitrobenzol, sulfonate sodium, and primaquine phosphate. The trade, pharmaceutical and chemical names of the vitamin K preparations that were used are given in table 1.

As the primary object of this investigation was to study the changes in oxyhemoglobin and methemoglobin, no attempt was made to analyze or quantify the late products of hemoglobin breakdown.

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Table 1.—The Trade, Pharmaceutical and Chemical Names of the Preparations of Vitamin K1 and of Vitamin K Used in the Experimental Studies

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Pharmaceutical Name</th>
<th>Chemical Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mephyton™</td>
<td>Vitamin K1</td>
<td>2-methyl-3-phytyl-1, 4-naphthoquinone</td>
</tr>
<tr>
<td>Hykinone™</td>
<td>Menadione sodium bisulfite</td>
<td>2-methyl-1, 4-naphthoquinone sodium bisulfite</td>
</tr>
<tr>
<td>Synkayvite®</td>
<td>Sodium diphosphate</td>
<td>The hexahydrate of the tetrasodium salt of 2-methyl-1, 4-naphthalenediol diphosphate</td>
</tr>
<tr>
<td>Synkamin®</td>
<td>4-amino-2-methyl-1-naphthol hydrochloride</td>
<td></td>
</tr>
</tbody>
</table>

**Materials and Methods**

Heparinized venous blood from healthy white adults was used as a source of erythrocytes for all in vitro experiments. The blood was centrifuged for 10 minutes at 2100 G, and the supernatant plasma discarded. The erythrocytes were washed three times in four volumes of an isotonic buffer at pH 7.4. The buffer was prepared by mixing 137 ml. of 0.154 molar KH2PO4 and 863 ml. of 0.1027 molar Na2HPO4. After the third wash the erythrocytes were suspended in an approximately equal volume of the buffer. The hemoglobin content of this cell suspension was determined in millimols per liter by the method of Drabkin and Austin.7'8'9

Spectrophotometric studies were done with a Beckman Model B spectrophotometer with flat cuvettes of 1 cm. width and 1 cm. depth. A Beckman Model G pH meter was used for pH measurements. Determinations of packed cell volume were done by the method of Strumia, Sample and Hart.10

**Incubation Mixtures and Sampling**

The incubation mixtures were prepared in the following way. A weighed amount of the test material was dissolved in isotonic buffer and the final solution made isomolar by the addition of distilled water. When necessary, the material was first buffered to pH 7.4 by the addition of the appropriate isotonic phosphate solution.

One milliliter of the erythrocyte suspension was then added to such a volume of the buffered test solution as would provide the desired molar ratio of test material to hemoglobin in the incubation mixture. The initial concentration of hemoglobin in the incubation mixtures was from 0.45 to 0.50 millimols per liter. A control incubation mixture was prepared by adding 1 ml of the erythrocyte suspension to a volume of buffer equal to that of the test solution. The incubation mixtures always were prepared within an hour of drawing the blood and of dissolving the test material.

In some experiments, test mixtures were also prepared containing added glucose in the molar ratio to hemoglobin of 24:1. In those experiments an equal amount of glucose was added to the control incubation mixture.

The mixtures were incubated in unstoppered Erlenmeyer flasks in a shaker-incubator at 37 C., with the shaker oscillating at a rate of 80 per minute. At intervals from zero to four hours during incubation, 1 ml. aliquots from the test and control incubation mixtures were transferred to 12 ml. calibrated graduated centrifuge tubes and the cells washed once with isotonic buffer. The supernatant solution was discarded and the erythrocytes hemolyzed by adding one drop of a 1 per cent saponin solution, followed by distilled water to a total of 9 ml. The contents of the tube were then thoroughly mixed. After 10 minutes, 0.0667 molar phosphate buffer at pH 7.4, prepared by mixing 808 ml. of 0.0667 molar Na2HPO4 and 192 ml. of 0.0667 molar KH2PO4, was added to make 12 ml. The contents of the tube were mixed, and then centrifuged for 10 minutes at 2100 G. The supernatant
solution was decanted into a test tube and saved for determinations of oxyhemoglobin and methemoglobin concentrations.

**Spectrophotometric Examination of Hemolysates**

The concentration of oxyhemoglobin in the hemolysates was determined by a modification of the method of Michel and Harris. Approximately 3 ml of the hemolysate were added to a flat 1 x 1 cm. cuvette and the optical density at wave length 620 mµ determined. Water was used as a blank solution for all of the determinations. One drop of 0.15 molar potassium ferricyanide was then added from a dropper delivering 25 drops per milliliter. The cuvette was sealed with Parafilm® and the contents mixed by inverting gently five times. Five minutes after mixing, the optical density at wave length 620 mµ was again determined. The oxyhemoglobin concentration of the hemolysate was derived from the change in optical density resulting from the addition of potassium ferricyanide.

The concentration of methemoglobin in the hemolysates was determined by a modification of the method of Evelyn and Malloy. Approximately 3 ml of hemolysate were added to a flat 1 x 1 cuvette and the optical density determined at wave length 620 mµ. From a dropper which delivered 25 drops per milliliter was added one drop of buffered 0.25 molar sodium cyanide, prepared within one hour by mixing equal parts of solutions of 0.5 molar sodium cyanide and 0.5 molar acetic acid. The cuvette was then sealed with Parafilm® and inverted gently five times. One minute after mixing, the optical density at wave length 620 mµ was again determined. The methemoglobin concentration of the hemolysate was derived from the change in optical density resulting from the addition of sodium cyanide.

The determinations were performed at wave length 620 mµ because of its proximity to the isobestic point of methemoglobin so that alterations in the optical density of methemoglobin from small changes in pH of the solutions would be reduced.

The term "intact" hemoglobin was adopted to describe the total hemoglobin present as oxyhemoglobin plus methemoglobin to distinguish forms of hemoglobin actually or potentially capable of carrying oxygen from degradation products no longer directly convertible into oxygen-carrying pigments. Thus the "intact" hemoglobin concentration of a mixture was defined as the sum of the oxyhemoglobin and methemoglobin concentrations.

In all in vitro experiments, the concentrations of oxyhemoglobin, methemoglobin, and "intact" hemoglobin in samples from the test mixtures were expressed as a per cent of the concentration of hemoglobin present in an aliquot simultaneously obtained from the control mixture. In this way, a correction was made for the progressive increase in hemoglobin concentrations resulting from the evaporation of water from the mixtures.

The assumption was made that "intact" hemoglobin no longer detectable in a test mixture had been converted to later degradation products. The per cent "intact" hemoglobin thus destroyed was determined by subtracting the per cent "intact" hemoglobin remaining from 100.

**Animal Experiments**

Full grown mongrel dogs and rabbits were used as subjects. Blood samples were obtained and test substances injected through a polyethylene catheter placed in the external jugular vein under light Nembutal® anesthesia. The catheter was filled with heparin saline between samples.

Blood samples were taken before and at various intervals after the injection of a test substance. One-tenth milliliter of the heparinized blood sample was added to approximately 10 ml of isotonic buffer and thoroughly mixed. The mixture was centrifuged for 10 minutes at 2100 G. and the supernatant fluid discarded. The erythrocytes were then hemolyzed and the hemolysate examined spectrophotometrically as described above. Oxyhemoglobin, methemoglobin and "intact" hemoglobin concentrations were calculated in millimols per liter. To convert the hemoglobin concentrations from millimols per liter to grams per cent, the values were multiplied by 1.652.11
Results

In Vitro Experiments

The changes demonstrated in the hemoglobin of erythrocytes incubated for four hours with each of the test substances are shown in table 2. In each case the molar ratio of test substance to hemoglobin was 4:1. Methemoglobin formation was demonstrated in the erythrocytes incubated with the following compounds: acetyl phenylhydrazine, phenylhydrazine, β-naphthol, vitamin K1, menadione sodium bisulfite, menadiol sodium diphosphate, 4-amino-2-methyl-1-naphthol, nitrofurantoin and primaquine. A significant degree of destruction of “intact” hemoglobin was produced by acetyl phenylhydrazine, phenylhydrazine, β-naphthol, menadione sodium bisulfite, 4-amino-2-methyl-1-naphthol and primaquine.

Macroscopic changes were also noticed in those incubation mixtures in which changes in hemoglobin were demonstrated spectrophotometrically. In contrast to the control mixtures which retained their original red color throughout the incubation period, the changing mixtures became in turn darker red, reddish brown, chocolate brown and then finally almost black.

Similarly, macroscopic changes were seen in hemolysates prepared from these changing mixtures. Hemolysates from the control mixtures were pink and clear throughout the incubation period. However, hemolysates prepared from changing mixtures first had a brownish tinge, and then as the incubation time increased, turbidity of the solution was noticed and a green precipitate was found after centrifugation. Centrifugation after hemolysis of erythrocytes exposed to prolonged incubation with high concentrations of these substances produced a clear greenish supernate and large amounts of greenish precipitate.

The addition of glucose to those incubation mixtures in which significant changes were demonstrated reduced the amount of methemoglobin formed and either reduced or eliminated the destruction of “intact” hemoglobin in each case (table 2).

The above substances were all tested at molar ratios to hemoglobin of 4:1. To assess the effect of varying the molar ratio of test substance to hemoglobin, the following studies were designed.

Erythrocytes, with and without added glucose, were incubated with acetyl phenylhydrazine in molar ratios of test substance to hemoglobin of 2:1, 4:1, 8:1 and 16:1. The results of this study are shown in figure 1. At the end of one hour of incubation, the concentration of methemoglobin in the mixture increased with increasing concentrations of acetyl phenylhydrazine. Thereafter, the degree of destruction of “intact” hemoglobin was likewise related to the concentration of acetyl phenylhydrazine. At all molar ratios, the addition of glucose to the mixture protected the hemoglobin from the destructive effects of the drug.

The effect of varying the molar ratios of other test substances to hemoglobin in the incubation mixtures is shown in figure 2. Erythrocytes were incubated without added glucose with phenylhydrazine, β-naphthol, menadione sodium bisulfite, and 4-amino-2-methyl-1-naphthol in molar ratios to hemoglobin of
Table 2.—Methemoglobin, Oxyhemoglobin, and "Intact" Hemoglobin Concentrations of Erythrocytes from Normal White Adults after 4 Hours Incubation with Certain Test Substances

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>No Glucose Added</th>
<th>Glucose Added</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxyhemoglobin</td>
<td>Methemoglobin</td>
</tr>
<tr>
<td></td>
<td>Per Cent</td>
<td>Per Cent</td>
</tr>
<tr>
<td>Acetyl phenylhydrazine</td>
<td>63</td>
<td>29</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>β-naphthol</td>
<td>33</td>
<td>57</td>
</tr>
<tr>
<td>Vitamin K₁</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>Menadione sodium bisulfite</td>
<td>19</td>
<td>70</td>
</tr>
<tr>
<td>Menadione sodium diphosphate</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>4-amino-2-methyl-1-naphthol</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>Primaquine</td>
<td>80</td>
<td>16</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>94</td>
<td>6</td>
</tr>
<tr>
<td>Scrtonin</td>
<td>98</td>
<td>2</td>
</tr>
<tr>
<td>Potassium chlorate</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Acetanilide</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Sulfonilamide</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Sulfinosalidol</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Novobiocin</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Nitrobenezol</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>Sulfoxone</td>
<td>99</td>
<td>1</td>
</tr>
</tbody>
</table>

The initial molar ratio of test substance to hemoglobin was 4:1. When glucose was added, the initial molar ratio of added glucose to hemoglobin was 24:1. All concentrations are expressed as a per cent of the initial total hemoglobin concentration.

0.25:1, 0.5:1, 1:1, and 2:1. Differences were noticed among these four drugs, both as to the effect on hemoglobin at the same molar ratio and the degree of change occurring with increasing molar ratios.

The effect of varying the molar ratio of phenylhydrazine to hemoglobin was of particular interest. As the molar ratio increased to 1:1, increasing concentrations of methemoglobin were formed with minimal destruction of "intact" hemoglobin. However, at a molar ratio of 2:1, approximately 50 per cent of "intact" hemoglobin was destroyed, and at a molar ratio of 4:1, 82 per cent of the "intact" hemoglobin was destroyed after four hours incubation (table 2).

Changes in the hemoglobin of erythrocytes incubated with four vitamin K derivatives are shown in figure 3. Vitamin K₁, menadione sodium diphosphate, menadione sodium bisulfite, and 4-amino-2-methyl-1-naphthol were incubated, with and without added glucose, with erythrocytes in the molar ratio to hemoglobin of 4:1. Again, differences were noticed among these four compounds as to the concentration of methemoglobin and the degree of "intact" hemoglobin destruction induced by incubation of these drugs with intact erythrocytes. As before, the addition of glucose to the incubation mixtures decreased the changes in hemoglobin.
Some of the substances tested did not result in methemoglobin production or "intact" hemoglobin destruction when incubated for four hours with erythrocytes in a molar ratio to hemoglobin of 4:1 (table 2). However, certain of these substances when incubated in greater concentration or for longer periods of time did result in changes in hemoglobin. As shown in table 3, serotonin in a molar ratio to hemoglobin of 10:1 produced a significant amount of methemoglobin without destruction of "intact" hemoglobin. Potassium chlorate produced high concentrations of methemoglobin with some destruction of "intact" hemoglobin in molar ratios of 16:1 and higher. Although sulfisoxazole in a molar ratio of 32:1 produced a small amount of methemoglobin, sulfanilamide at the same molar ratio failed to induce either methemoglobin production or "intact" hemoglobin destruction.

**Animal Experiments**

The following experiments were designed to study the effect of some of these compounds on hemoglobin in vivo. In the first experiment, a single intravenous
Fig. 2.—Changes in the hemoglobin when erythrocytes from normal white adults were incubated with different concentrations of the following test substances: phenylhydrazine, \(\beta\)-naphthol, menadione sodium bisulfite, and 4-amino-2-methyl-1-naphthol. Glucose was not added to the incubation mixtures.

Injection of buffered phenylhydrazine hydrochloride in a dose of 50 mg. per kilogram of body weight was administered to an adult mongrel dog. Blood samples were obtained before the injection and at intervals thereafter. The hematocrit was determined and the concentrations of oxyhemoglobin, methemoglobin, and "intact" hemoglobin were measured in each blood sample. The results of these studies are shown in figure 4.

Cyanosis of the animal and marked darkening of the blood were noticed almost immediately after the injection of phenylhydrazine. Significant degrees of methemoglobin production and "intact" hemoglobin destruction were detected in a blood sample obtained 5 minutes after the injection. A maximal methemoglobin concentration of 4.5 Gm. per cent was reached 30 minutes after the injection and then the concentration of methemoglobin in the blood gradually decreased to 0.5 Gm. per cent 8 hours after injection. The "intact" hemoglobin concentration of the blood decreased rapidly after the injection. The hematocrit also decreased rapidly but proportionately to a lesser extent than did the "intact" hemoglobin concentration.
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Fig. 3.—Changes in the hemoglobin when erythrocytes from normal white adults were incubated with various test substances, both with and without added glucose. The initial molar ratio of test substance to hemoglobin was 4:1, and the initial molar ratio of added glucose to hemoglobin was 24:1. The following test substances were used: vitamin K₁, menadiol sodium diphosphate, menadione sodium bisulfite, and 4-amino-2-methyl-1-naphthol.

Buffered phenylhydrazine hydrochloride was also administered as a subcutaneous injection to an adult rabbit in a dose of 50 mg. per kilogram of body weight. Cyanosis of the animal was noticed within 20 minutes and the maximal concentration of methemoglobin was found to be 2.0 Gm. per cent 90 minutes after the injection. The methemoglobin concentration had decreased to 0.1 Gm. per cent 6 hours after the injection. The “intact” hemoglobin concentration decreased from a preinjection level of 15.4 Gm. per cent to 11.1 Gm. per cent after 30 minutes. Six hours after the injection this “intact” hemoglobin concentration was 8.4 Gm. per cent.

A single intravenous injection of menadione sodium bisulfite in a dose of 50 mg. per kilogram of body weight was administered to an adult rabbit. Methemoglobin reached a maximal concentration in the blood of 0.8 Gm. per
Table 3.—Methemoglobin, Oxyhemoglobin, and “Intact” Hemoglobin Concentrations of Erythrocytes from Normal White Adults after Incubation with Certain Test Substances

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Molar Ratio Test Substance: Hemoglobin</th>
<th>Hours of Incubation</th>
<th>Oxyhemoglobin Per Cent</th>
<th>Methemoglobin Per Cent</th>
<th>“Intact” Hemoglobin Per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>10:1</td>
<td>4</td>
<td>91</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>86</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>79</td>
<td>21</td>
<td>100</td>
</tr>
<tr>
<td>Potassium chlorate</td>
<td>16:1</td>
<td>4</td>
<td>77</td>
<td>19</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32:1</td>
<td>0</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64:1</td>
<td>2</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td>Sulfisoxazole</td>
<td>4:1</td>
<td>4</td>
<td>90</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8:1</td>
<td>90</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16:1</td>
<td>90</td>
<td>1</td>
<td>100</td>
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<tr>
<td></td>
<td></td>
<td>32:1</td>
<td>96</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Sulfanilamide</td>
<td>4:1</td>
<td>4</td>
<td>96</td>
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<td>100</td>
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<td></td>
<td>8:1</td>
<td>99</td>
<td>1</td>
<td>100</td>
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<td></td>
<td></td>
<td>16:1</td>
<td>99</td>
<td>1</td>
<td>100</td>
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<tr>
<td></td>
<td></td>
<td>32:1</td>
<td>99</td>
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<td>100</td>
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</tbody>
</table>

Glucose was not added to the incubation mixtures. All concentrations are expressed as a per cent of the initial total hemoglobin concentration.

Fig. 4.—Determinations of the “intact” hemoglobin concentration, methemoglobin concentration and hematocrit of the venous blood of a dog before and after a single intravenous injection of buffered phenylhydrazine, in a dose of 50 mg. per kilogram body weight.
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cent within 30 minutes and decreased to 0.1 Gm. per cent 6 hours after the injection. The “intact” hemoglobin concentration decreased from a preinjection level of 14.0 Gm. per cent to 11.5 Gm. per cent 6 hours after the injection.

A single intravenous injection of menadione sodium bisulfite in a dose of 75 mg. per kilogram of body weight was administered to an adult mongrel dog. Cyanosis of the animal was noticed almost immediately after the injection, and the methemoglobin concentration in a sample taken after 5 minutes was 3.5 Gm. per cent. The methemoglobin concentration reached a maximum of 4.2 Gm. per cent after 90 minutes, then gradually decreased to 0.9 Gm. per cent 10 hours after the injection.

A single intravenous injection of 4-amino-2-methyl-1-naphthol in a dose of 25 mg. per kilogram was administered to an adult rabbit. The methemoglobin concentration increased from a preinjection level of less than 0.1 Gm. per cent to 0.4 Gm. per cent after 30 minutes, then decreased to 0.2 Gm. per cent after 6 hours. The “intact” hemoglobin concentration decreased from a preinjection level of 12.5 Gm. per cent to 11.0 Gm. per cent 6 hours after the injection.

DISCUSSION

This study was designed to determine the changes in hemoglobin, especially with regard to the formation of methemoglobin, that occur when erythrocytes are incubated with compounds capable of inducing Heinz body formation. Initially, erythrocytes were incubated for 4 hours with each test substance in a molar ratio to hemoglobin of 4:1. Significant concentrations of methemoglobin were found in erythrocytes incubated with some of the test substances. In addition, with certain of these substances the sum of the concentrations of oxyhemoglobin and methemoglobin was also found to have decreased. The term “intact” hemoglobin was adopted to describe the total hemoglobin present as oxyhemoglobin and methemoglobin, in order to distinguish forms of hemoglobin actually or potentially capable of carrying oxygen from degradation products no longer directly convertible to oxygen-carrying pigments. Because of the large number of late products of hemoglobin breakdown described as resulting from the action of toxic agents, the rate of destruction of “intact” hemoglobin appears to be a more satisfactory index of the effect on hemoglobin of a given test substance than the rate of production of a single late product.

Destruction of “intact” hemoglobin was not demonstrated without methemoglobin production with any test substance. Acetyl phenylhydrazine, phenylhydrazine, 8-naphthol, menadione sodium bisulfite, 4-amino-2-methyl-1-naphthol and primaquine each produced high concentrations of methemoglobin and significant degrees of destruction of “intact” hemoglobin. Methemoglobin production without significant destruction of “intact” hemoglobin was demonstrated with vitamin K1, menadiol sodium diphosphate and nitrofurantoin. Obviously, it is possible that destruction of “intact” hemoglobin might also have been demonstrated if higher concentrations of these substances had been used. Added glucose was shown to protect the erythrocyte from the action of each test substance, both with regard to the degree of methemoglobin production and of “intact” hemoglobin destruction.
The different patterns of effect with different test substances were of considerable interest. Phenylhydrazine not only produced considerably more destruction of "intact" hemoglobin than equivalent concentrations of acetyl phenylhydrazine, but also appeared to produce its maximal effect more rapidly. The degree of change produced by preparations of vitamin K decreased in the order: 4-amino-2-methyl-1-naphthol, menadione sodium bisulfite, vitamin K₁, and menadiol sodium diphosphate.

The effects of varying concentrations of test substances were of particular interest. In low concentrations, both acetyl phenylhydrazine and phenylhydrazine caused methemoglobin production with minimal destruction of "intact" hemoglobin, whereas in higher concentrations marked destruction also occurred. Even in higher concentrations acetyl phenylhydrazine initially caused considerable methemoglobin production with minimal destruction of "intact" hemoglobin, followed later by simultaneous decreases in the concentrations of methemoglobin, oxyhemoglobin and "intact" hemoglobin. Obviously, a measure of the methemoglobin concentration in erythrocytes is no index of the kinetics of methemoglobin formation.

The absence of demonstrable change in intra-erythrocytic hemoglobin after 4 hours incubation with a given test substance, in a molar ratio to hemoglobin of 4:1, does not deny the possibility that such change may be produced by the test substance, either in higher concentration in vitro or administered in vivo. Demonstrable changes were produced in vitro by serotonin, potassium chlorate and sulfisoxazole only in molar ratios to hemoglobin of more than 4:1. Quite possibly other test substances may produce an effect only after conversion in vivo to a more active derivative.

The in vitro production of high concentration of methemoglobin by β-naphthol is in contrast to a report of failure to produce methemoglobin, either in vivo or in vitro, with this substance.¹² The demonstration of methemoglobin production by phenylhydrazine, both in vivo and in vitro, is also at variance with previous observations.³⁴¹³ The demonstration of methemoglobin production by serotonin was stimulated by the observations that serotonin disappears when incubated with washed erythrocytes,¹⁴ and that oxyhemoglobin, when undergoing denaturation, can oxidize serotonin.¹⁵ Methemoglobin production without "intact" hemoglobin destruction in erythrocytes incubated with nitrofurantoin is consistent with the observation that incubation of glucose-6-phosphate dehydrogenase deficient erythrocytes with nitrofurantoin resulted in a marked decrease in reduced glutathione concentration without Heinz body formation.

Our animal experiments demonstrated that significant concentrations of methemoglobin were formed following the administration of single large doses of phenylhydrazine, menadione sodium bisulfite and 4-amino-2-methyl-1-naphthol. The transient nature of the methemoglobin formation may well explain the failure to demonstrate methemoglobinemia following the repeated administration of small amounts of certain hemolytic agents. Of interest in this regard is the observation of Horecker¹⁷ that the mean concentration of methemoglobin in the blood of a group of trinitrotoluene workers, although low, was higher than that of a group of normal controls.
With the demonstration that certain drugs can produce methemoglobin, both in vitro and in vivo, the question arose as to the significance of methemoglobin formation in drug-induced Heinz body production and erythrocyte destruction. On the basis of unpublished observations, and of the observations of Finch, Beutler has quoted the purely hemolytic effect of phenylhydrazine as an example of the essentially independent nature of drug-induced methemoglobinemia and of drug-induced hemolysis. After suggesting that "the over-all picture is best represented as a spectrum with methemoglobin formation at one end and extensive red cell destruction at the other," Finch expressed the opinion that phenylhydrazine acts in vivo only to produce red cell destruction, without suggesting a mechanism whereby phenylhydrazine might damage the erythrocyte. However, Magos and Sziza considered that the methemoglobin formation produced by the administration of nitrobenzol to rats was the first step in the peroxidase oxidation of hemoglobin and the formation of Heinz bodies in erythrocytes.

The results presented here do not deny the possibility that methemoglobin production need not be an essential step in the breakdown of oxyhemoglobin to later oxidation products, but do suggest that methemoglobin formation may be a stage in at least one process whereby oxyhemoglobin is destroyed by various toxic agents. Certain test substances in low concentration have been shown to produce methemoglobin without destruction of "intact" hemoglobin, whereas in higher concentration such destruction also occurred. In appropriate concentrations, certain substances have also been shown to produce, initially, significant concentrations of methemoglobin with minimal destruction of "intact" hemoglobin, followed later by simultaneous decreases in the concentrations of methemoglobin, oxyhemoglobin and "intact" hemoglobin. These findings would tend to support a hypothesis that methemoglobin production is an essential stage in the conversion by drugs of oxyhemoglobin to late degradation products. Further support for such a hypothesis is provided by the observations of Beavan and White, that destromatized oxyhemoglobin is oxidized by phenylhydrazine to methemoglobin alone when the hemoglobin is present in excess, and that regardless of concentration, methemoglobin formation only occurs when oxyhemoglobin and either of the phenylhydrazine derivatives, 2:4-dinitrophenyhydrazine and asym-diphenylhydrazine are incubated together.

Certain agents which are known to cause Heinz body formation and erythrocyte destruction in vivo have thus been shown to produce methemoglobin formation and "intact" hemoglobin destruction in vitro. Some of these agents have also been shown to produce these effects in vivo. The results presented suggest that destruction of "intact" hemoglobin is associated with the destruction of erythrocytes by these agents. Considerable evidence also favors a hypothesis that methemoglobin production is an essential stage preceding the destruction of "intact" hemoglobin. The facts that certain agents produce methemoglobin alone; that others, such as nitrates, may, after prolonged administration, also produce Heinz bodies and erythrocyte destruction, and that agents which typically produce Heinz bodies and erythrocyte destruction have been shown, both in vitro and in vivo, to produce significant concentrations of
methemoglobin, all suggest that methemoglobin formation may represent an essential step in this destructive process.

**Summary**

A study of the changes in hemoglobin of erythrocytes incubated with various test substances has been reported. The concentrations of oxyhemoglobin and methemoglobin in these erythrocytes were measured and the term “intact” hemoglobin was introduced, in order to distinguish these pigments from later degradation products of hemoglobin. Certain agents which are known to cause Heinz body formation and erythrocyte destruction in vivo have been shown to cause methemoglobin formation and “intact” hemoglobin destruction in vitro, and some of these agents were also shown to produce these changes in vivo. The possible significance of these findings in relation to the role of methemoglobin formation in Heinz body production has been discussed.

**Summario in Interlingua**

Es reportate un studio del alterationes in le hemoglobina de erythrocytos incubate con varie substantias experimental. Le concentrationes de oxyhemoglobina e de methemoglobina in iste erythrocytos eseva mesurate, e le termino hemoglobina “intacte” es introducita a fin do distinguere iste pigmentos ab le subsequente productos del degradation de hemoglobina. Eseva monstate que certe agentes—que cognoscitemente causa in vivo le formation de corpcres de Heinz e le destruction de erythrocytos—causa in vitro le formation de methemoglobina e le destruction de hemoglobina “intacte.” Eseva etiam monstate que certes de iste agentes produce le mesme alterationes in vivo. Es discutite le possible signification de iste constatationes in relation al rolo del formation de methemoglobina in le production de corpores de Heinz.

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In a continuation of a previous study the nature of the four carbon chain compound condensing with glycine was investigated, although previous work strongly suggested it was succinyl-coenzyme A. When particle matter from anemic chicken erythrocyte hemolysates are dry-frozen, the capacity to form δ-aminolevulinic acid from glycine and α-oxoglutarate or succinate is lost, but if either synthetic or enzymatically generated succinyl-coenzyme A is used as a C₄ source the formation of δ-aminolevulinic acid is largely restored. Freeze-dried particles form little or no δ-ALA from α-oxoglutarate unless DPN is added, and it is suggested that the synthesis of δ-ALA from α-oxoglutarate involves the formation of succinyl-coenzyme A by the action of α-oxoglutaric dehydrogenase.

That pyridoxal phosphate is a cofactor is suggested by experimental evidence. Additional information suggests that the α-carbon of glycine is activated, before condensing with succinyl-coenzyme A, by the formation of a Schiff's base between the amino group of glycine and the enzyme bound pyridoxal phosphate. This may explain the inhibition of synthesis of δ-ALA by cyanide penicillamines and cysteine.—G. W. J., III.
Studies on the Formation of Heinz Bodies. I. Methemoglobin Production and Oxyhemoglobin Destruction

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