Experience with an Extended Methemoglobin Reduction Test

Factors Influencing Hemoglobin Destruction

By Helen Robin and J. D. Harley

Previously, we reported from 0 to 46 per cent loss of intact hemoglobin (methemoglobin plus oxyhemoglobin) when the methemoglobin reduction test of Brewer et al. was applied to samples of blood obtained from normal placentas, infants, children and adults. The propensity to intact hemoglobin destruction appeared to be increased in subjects with hemoglobin H, acute lead poisoning, and drug-induced hemolysis, but to be decreased in glucose-6-phosphate dehydrogenase (G6PD)-deficient males. Quite marked species differences in the degree of loss of intact hemoglobin, with the surprising observation that susceptibility to such loss increased on induction of a young cell population by exsanguinating adult rabbits have also been recorded briefly.

This communication outlines experiments designed to define the factors influencing the degree of intact hemoglobin destruction in samples of whole blood subjected to the methemoglobin reduction test. The details and implications of differences noted on application of the test to various mammalian species will be discussed in a subsequent publication.

Materials and Methods

Most of the methods used have previously been described in detail.

The methemoglobin reduction test was done as described by Brewer et al. after correction (if necessary) of the packed cell volume to more than 35. Simultaneously, a control mixture in which 0.2 ml. of isotonic buffered saline at pH 7.4 was substituted for the nitrite and methylene blue solutions was prepared and incubated. After 3 hours incubation, the concentrations of methemoglobin, oxyhemoglobin and intact hemoglobin (methemoglobin plus oxyhemoglobin) in the test and control mixtures were determined by a modification of the methods of Evelyn and Malloy and Michel and Harris. The concentrations of methemoglobin, oxyhemoglobin and intact hemoglobin in the test mixture were then expressed as a percentage of the concentration of intact hemoglobin in the control mixture, from which the per cent loss of intact hemoglobin in the test mixture could be readily estimated.

Reincubation mixtures for the measurement of rates of methemoglobin reduction were prepared as described. Washed erythrocytes were incubated with nitrite, to cause more than 90 per cent methemoglobin formation, then washed thoroughly again and reincubated

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in glucose buffer, both in the presence and absence of methylene blue. The initial concentration of heme in these mixtures was always between 0.45 and 0.50 mM/L, and the initial molar ratio of methylene blue:heme was always 1:1000. At the commencement and at appropriate intervals during reincubation, the concentration of methemoglobin was measured and expressed as a percentage of the intact hemoglobin content of the incubation mixture, from which readings the rate of reduction of methemoglobin was then calculated in terms of the intact hemoglobin content of the incubation mixture.

Erythrocytic G6PD activity was measured by Zinkham’s modification of the method of Glaser and Brown. The G6PD-deficient subjects were all of Caucasian extraction, and the males were characterized by very low levels of enzyme activity (1 to 5 units/100 ml. of erythrocytes).

Separation of relatively old and relatively young erythrocytes was achieved by repeated centrifugation in 30 per cent bovine albumin as described by Prankerd.

For animal experiments, adult rabbits were exsanguinated or treated with phenylhydrazine as described in the course of the results. In some of these experiments, the total hemoglobin content was determined by the cyanmethemoglobin method for the determination of hemoglobin concentration from a single reading at wave length 540 mµ, as outlined previously.

RESULTS

Intact Hemoglobin Destruction in Normal and Abnormal Subjects Following Application of an Extended Methemoglobin Reduction Test

Results obtained previously were first confirmed and elaborated. There was no obvious or statistical difference between the degrees of intact hemoglobin destruction after application of the methemoglobin reduction test to samples of blood from 39 normal adult males, 34 normal adult females, 34 normal infants and children, and 13 G6PD-deficient females. However, the range and mean destruction for 11 G6PD-deficient males was obviously lower than for the other groups, with statistically significant differences as between G6PD-deficient males and each of the other categories (p < 0.02). In contrast, markedly increased degrees of intact hemoglobin destruction were observed in three subjects with hemoglobin H, two of three children with acute lead poisoning, and five patients with erythrocytic changes induced by aromatic drugs.

Subjects with a wider variety of hematologic conditions were also tested. No evidence of increased susceptibility to intact hemoglobin destruction was observed in five adults heterozygous for β-thalassemia, two adults heterozygous for hemoglobin S disease, two adults with mildly deficient reduced diphosphopyridine nucleotide (DPNH)-dependent methemoglobin reduction, one adult with pyruvate kinase deficiency, one adult heterozygous, and one child homozygous for galactose-1-phosphate uridyl transferase deficiency, two children with hereditary spherocytosis, two children with cyanotic congenital heart disease, one infant with moderate methemoglobinemia due to defective DPNH-dependent methemoglobin reduction, or ten infants with neonatal jaundice due to isoimmune hemolytic disease. A loss of intact hemoglobin of 44 per cent, near the upper limit of the normal range, occurred in an adult male with a congenital nonspherocytic hemolytic anemia of unknown etiology.
Effect of Exclusion of Methylene Blue or Nitrite from the Incubation Mixture

The effect of varying the conditions in the methemoglobin reduction test was next investigated. Simultaneously with the usual test and control mixtures, samples were exposed to test mixtures in which either methylene blue or nitrite had been excluded and replaced by 0.1 ml. of isotonic buffered saline at pH 7.4 (table 1). Exposure to either nitrite or methylene blue alone practically excluded destruction of intact hemoglobin in those normal subjects and G6PD-deficient females in whom such destruction occurred when both reagents were present.

In patients with hemoglobin H, destruction of intact hemoglobin was considerably reduced in the presence of nitrite alone, and virtually eliminated when only methylene blue was present. In partial contrast, in two patients with drug-induced hemolytic anemia and one patient with congenital non-spherocytic hemolytic anemia of unknown etiology, the degree of intact hemoglobin destruction was markedly decreased on the separate exposure to either nitrite or methylene blue.

“Simple” oxidation by nitrite thus achieved substantial intact hemoglobin destruction only in erythrocytes containing hemoglobin H. Under other conditions, the additional presence of methylene blue was necessary for such loss to occur. Since methylene blue stimulates reduced triphosphopyridine nucleotide (TPNH)-dependent methemoglobin reduction, the relation of the rate of reduction of intact hemoglobin destruction will now be considered.

Relation of Intact Hemoglobin Destruction to Rates of Methemoglobin Reduction

Figure 1A shows that the degree of intact hemoglobin destruction in normal adults tended to vary directly with the rate of methemoglobin reduction in the presence of glucose and methylene blue. This trend was accentuated in samples from G6PD-deficient males, with very low rates of reduction associated with slight or negligible intact hemoglobin destruction.

A direct relation was also observed between the rates of methemoglobin reduction in the absence of methylene blue and the degree of intact hemoglobin destruction during the methemoglobin reduction test (fig. 1B). However, this trend was less striking than in the presence of methylene blue, involving samples from normal and G6PD-deficient subjects to approximately the same degree.

Intact Hemoglobin Destruction in Young and Old Erythrocytes

Samples presumed to contain mainly young and old erythrocytes were prepared by repeated centrifugation from whole blood taken from normal adult human subjects and from normal adult rabbits, resuspended in plasma to a packed cell volume of 35–45, and then subjected to the methemoglobin reduction test. Table 2 shows that the degree of intact hemoglobin destruction in the top fraction, expected to contain mainly young cells, was consistently more than in the bottom fraction, with a presumed preponderance of old
<table>
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<tr>
<th>Hematologic Condition</th>
<th>Subjects</th>
<th>Test Incubation Mixture</th>
<th>Nitrite + Methylene Blue</th>
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<th>Methylene Blue</th>
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<td>Intact Hemoglobin %</td>
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<td>Intact Hemoglobin %</td>
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<td>15 23</td>
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<td>89 96</td>
<td>2 98</td>
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**Fig. 1.**—Comparison of rates of methemoglobin reduction in washed erythrocytes with degrees of intact hemoglobin destruction in whole blood subjected to the methemoglobin reduction test. Ordinate: A. Methemoglobin reduced in 2 hours with glucose and methylene blue; B. Methemoglobin reduced in 5 hours with glucose alone. Blackened circles = normal subjects. Open circles = G6PD-deficient males.

cells. Furthermore, the degree of intact hemoglobin destruction in whole blood samples simultaneously tested was consistently between the results obtained from the top and bottom fractions.

**Intact Hemoglobin Destruction in Exsanguinated Rabbits**

To observe the effect of a young cell population in vivo, healthy adult rabbits were exsanguinated. A typical experiment in figure 2 depicts the changes resulting from removal of 150 ml. of blood over 8 days. The upper segment shows that early reticulocytosis was associated with more gradual increase in G6PD activity and in the rates of methemoglobin reduction in the presence and absence of methylene blue. Pari passu with these gradual changes, there occurred (as seen in the lower segment) a corresponding increase in the degree of intact hemoglobin destruction when samples were subjected to the methemoglobin reduction test. As the G6PD activity and rates of methemoglobin reduction declined toward normal, so did the amount of intact hemoglobin remaining after the methemoglobin reduction test increase to the previous level.

**Intact Hemoglobin Destruction in the Phenylhydrazine-Treated Rabbit**

Lastly, the effect was studied of phenylhydrazine-induced changes on the results of the methemoglobin reduction test. A typical experiment, involving
Table 2.—Concentrations of Intact Hemoglobin after Application of the Methemoglobin Reduction Test to the Top and Bottom Cell Fractions Obtained after Repeated Centrifugation of Samples of Whole Blood

<table>
<thead>
<tr>
<th>Subject</th>
<th>Whole blood</th>
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<th>Bottom fraction</th>
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<td>98</td>
</tr>
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<td>73</td>
<td>82</td>
</tr>
<tr>
<td>Normal adult rabbit</td>
<td>90</td>
<td>49</td>
<td>97</td>
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The residual methemoglobin concentration was in no test greater than 10 per cent of the control intact hemoglobin concentration.

intramuscular injection of a single dose of 2.0 ml. of a 3.3 per cent solution of phenylhydrazine hydrochloride, pH 7.0, is depicted in figure 3. The top segment of this figure shows that transient methemoglobinemia was followed rapidly by Heinz body formation in every cell, by anemia with preponderance of total hemoglobin over intact hemoglobin, and then by more gradual reticulocytosis.

Pronounced parallel variations in the response to the methemoglobin reduction test are shown in the lower segment. Initially, the degree of intact hemoglobin destruction increased without methemoglobin formation. Subsequently, there was a phase of increasing residual methemoglobin concentrations with a rebound decrease in destruction of intact hemoglobin. Finally, intact hemoglobin destruction increased again, then gradually returned to normal, in association with residual methemoglobin concentrations which gradually decreased from mildly abnormal to the normal range.

Similar results were obtained in repeated experiments, indicating that aromatic drugs such as phenylhydrazine may cause a falsely positive methemoglobin reduction test in terms of residual methemoglobin, and also an increase in the degree of intact hemoglobin destruction with normal or slightly increased residual methemoglobin levels.

**DISCUSSION**

The work has provided good evidence that the rate of methemoglobin reduction is a significant direct determinant of the degree of intact hemoglobin destruction during the methemoglobin reduction test. Since negligible intact hemoglobin destruction normally occurs if either nitrite or methylene blue is omitted from the incubation mixture, it seems very likely that the rapid reduction achieved by the pentose phosphate pathway in the presence of methylene blue is the more important factor. The increased destruction observed in young cells would then reflect the increased capacity of the pentose...
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Fig. 2.—Changes in the response to the methemoglobin reduction test compared with other changes following exsanguination of a normal adult rabbit.

phosphate pathway of methemoglobin reduction. However, the possibility must be considered that this propensity for intact hemoglobin destruction in young cells is potentiated by increased rates of methemoglobin reduction via the Embden-Meyerhof pathway, by the presence of such defective or short-lived erythrocytes as may result from a rapid regenerative phase,\textsuperscript{13,14} or perhaps by other factors.

The investigation has also provided further evidence that either congenital or acquired instability of the hemoglobin molecule may be an important determinant of intact hemoglobin destruction in the methemoglobin reduction test. Degradation of hemoglobin H was achieved by simple oxidation with nitrite alone, as might be expected from the work of Bigas and Koler,\textsuperscript{15} but was increased by the additional presence of methylene blue. In patients exposed to aromatic drugs, the degree of intact hemoglobin destruction in the methemoglobin reduction test was markedly decreased by elimination of nitrite or methylene blue from the incubation mixture. Although administration of phenylhydrazine to rabbits caused increased propensity to both methemoglobin formation and intact hemoglobin destruction in the methemoglobin reduction test, these effects tended to occur consecutively rather than concurrently, increased intact hemoglobin destruction preceding either met-
hemoglobin formation or increased rates of methemoglobin reduction. It thus seemed that an increased rate of methemoglobin reduction was an important but by no means consistently significant factor in the increased destruction of intact hemoglobin on testing subjects with an unstable hemoglobin molecule.

An explanation should now be possible of most results obtained in the extended methemoglobin reduction test. On the one hand, an inadequate pentose phosphate pathway and some defects of hemoglobin stability favor the accumulation of methemoglobin, which in turn tends to diminish the destruction of intact hemoglobin. On the other hand, such loss of intact hemoglobin is increased by the rapid reduction of hemoglobin, particularly via the pentose phosphate pathway, by young cell age, and by various congenital or acquired defects favoring instability of the hemoglobin molecule. While largely of theoretic interest, these considerations emphasize the practical and diagnostic value when assessing methemoglobin formation in the methemoglobin
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reduction test, of understanding the simultaneous changes in the intact hemoglobin contents of the incubation mixtures.

Summary

Experiments have been designed to elucidate the varying degrees of destruction of intact hemoglobin (methemoglobin plus oxyhemoglobin) observed on application of the methemoglobin reduction test to normal and abnormal subjects. Surprisingly, the degree of intact hemoglobin destruction was found to vary directly with the rates of methemoglobin reduction, particularly via the pentose phosphate pathway in the presence of methylene blue, and inversely with cell age. In addition, confirmation was obtained of increased susceptibility to destruction of intact hemoglobin in subjects with such congenital or acquired defects of hemoglobin stability as might result from hemoglobin H, acute lead poisoning, or prior exposure to aromatic drugs.

Further evidence was presented that accumulation of methemoglobin militates against intact hemoglobin destruction. It was suggested that the methemoglobin content of samples of blood after application of the methemoglobin reduction test should be assessed in relation to the simultaneous changes in the intact hemoglobin concentrations of the incubation mixtures.

Summary in Interlingua

Esseva concepite experimentos visante a elucidar le variacions de la destruction de hemoglobina intacte (methemoglobina plus oxyhemoglobina) observate post le application del test de reduction methemoglobinica a subjectos normal e anormal. Esseva trovate surprendentemente que le grado del destruction de hemoglobina intacte varia directemente con le reduction de methemoglobina, particularmente con su reduction via le circuito de phosphato de pentosa in le presentia de blau methylenic, e inversemente con le etate del celularas. Esseva obtenite, in plus, confirmation del augmentate susceptibiliteit de hemoglobina intacte de esser destruite in subjectos con congenite o acquiriti defectos del stabilitate hemoglobinica resultante ab hemoglobina H, ab acute invenenamento per plumbo, o ab le previe exposition a pharmacon aromatic.

Es presentate evidentia additional pro le theses que le accumulation de methemoglobina interfere in le destruction de hemoglobina intacte. Es proponite que le contento de methemoglobina de specimens de sanguine al quales le test de reduction de methemoglobina ha essite applicate deberea esser evaluata con respecto a alterationes simultaneae in le concentrationes de hemoglobina intacte in le mixturas de incubation.

REFERENCES

Experience with an Extended Methemoglobin Reduction Test: Factors Influencing Hemoglobin Destruction

HELEN ROBIN and J. D. HARLEY