A novel method of measuring reduction of nitrite-induced methemoglobin applied to fetal and adult blood of humans and sheep

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Power GG, Bragg SL, Oshiro BT, Dejam A, Hunter CJ, Blood AB. A novel method of measuring reduction of nitrite-induced methemoglobin applied to fetal and adult blood of humans and sheep. J Appl Physiol 103: 1359–1365, 2007. First published July 5, 2007; doi:10.1152/japplphysiol.00443.2007.—The reaction of nitrite with deoxymethemoglobin results in the production of nitric oxide and methemoglobin, a reaction recently proposed as an important oxygen-sensitive source of vasoactive nitric oxide during hypoxic and anoxic stress, with several animal studies suggesting that nitrite may have therapeutic potential. Accumulation of toxic levels of methemoglobin is suppressed by reductase enzymes present within the erythrocyte. Using a novel method of measuring methemoglobin reductase activity in intact erythrocytes, we compared fetal and adult sheep and human blood. After nitrite-induced production of 20% methemoglobin, the blood was equilibrated with carbon monoxide, which effectively stopped further production. Methemoglobin disappearance was first order in nature with specific rate constants (k × 1,000) of 12.9 ± 1.3 min⁻¹ for fetal sheep, 5.88 ± 0.26 min⁻¹ for adult sheep, 4.27 ± 0.34 for adult humans, and 3.30 ± 0.15 for newborn cord blood, all statistically different from one another. The effects of oxygen tensions, pH, hemolysis, and methylene blue are reported. Studies of temperature dependence indicated an activation energy of 8,620 ± 1,060 calories/mol (2.06 ± 0.15 kJ/mol), appreciably higher than would be characteristic of processes limited by passive membrane diffusion. In conclusion, the novel methodology permits absolute quantification of the reduction of nitrite-induced methemoglobin in whole blood.

methemoglobin reductase; fetus; whole blood

NITRITE IS PRESENT IN THE plasma at concentrations ranging from the low- to mid-nanomolar range in most mammalian species studied (9, 23). Circulating nitrite is derived primarily from the oxidation of endogenous nitric oxide (NO) produced by NO synthase (23) and is cleared from the plasma by reaction with oxyhemoglobin to form nitrate or with deoxyhemoglobin to form NO (10, 25, 44). In both of these reactions, the iron in hemoglobin normally present in the ferrous form (Fe2⁺) is oxidized to the ferric form and methemoglobin is produced. The reaction of nitrite with deoxyhemoglobin has received much attention over the past 4–5 years because of its potential as a physiologically important, hypoxia-sensitive, and NO synthase-independent source of NO (13). Animal studies of NO production from the reaction of nitrite with deoxyhemoglobin have demonstrated therapeutic potential for infused nitrite in models of myocardial infarction (11), intracerebral hemorrhage (33), and pulmonary hypertension (19).

The oxygen-carrying properties of hemoglobin depend on oxygen binding to ferrous iron at each of the four heme groups. Once iron is in the ferric (Fe3⁺) state, as in methemoglobin, it is unable to combine reversibly with oxygen and transport it in the body. Clinically, methemoglobin concentrations >10–20% result in obvious cyanosis, with headaches, weakness, and breathlessness becoming apparent at levels of 35% or greater (7). Approximately 2–3% of all hemoglobin in the body is converted to methemoglobin each day as a result of endogenous oxidative stresses (15). Given the 120-day lifespan of the erythrocyte and endogenous rates of methemoglobin production, nearly all hemoglobin would be in an oxidized form were it not for the activity of methemoglobin-reducing enzymes within the erythrocyte. By reducing iron back to its ferrous state, these enzymes restore functional hemoglobin. Thus the competing processes of methemoglobin production and reduction are in equilibrium such that methemoglobin levels are typically <1% of total pigment.

Although an NADPH-dependent flavin reductase capable of reducing methemoglobin is known to exist in erythrocytes, a large majority of methemoglobin reduction is carried out by the enzyme cytochrome b₅ reductase (cytb₅r), a soluble 245-amino-acid protein found in the cytosol of the erythrocyte (4, 31). Although membrane-bound versions of the protein have been found, they normally confer only a minor portion of the overall methemoglobin reductase activity. The cytb₅r enzymes facilitate the reduction of cytochrome b₅ by transfer of hydride anions from NADH (39). Cytochrome b₅, in turn, rapidly reduces methemoglobin (18). The predominant source of NADH within the erythrocyte is via the metabolism of glucose in the Embden-Meyerhof pathway (15). Methemoglobin reductase activities vary widely across species (24, 34, 35, 40) and with age. Human newborns, for example, are known to be at greater risk of methemoglobinemia than adults (15, 21). This increased risk has been attributed to less methemoglobin reductase activity in neonatal erythrocytes (14, 21) and a greater tendency of fetal hemoglobin to assume forms that are stable in the ferric state (15, 32).

As noted, the reaction of nitrite with either oxy- or deoxyhemoglobin results in hemoglobin oxidation to methemoglobin (10, 25), but the chemistry in each instance is unusually complex. The stoichiometry of these reactions is complicated by formation of products, such as NO, that are themselves capable of hemoglobin oxidation or regeneration of nitrite.
Such reactions would result in measurements of a >1:1 ratio of methemoglobin production to nitrite metabolism. Conversely, recent evidence suggests that nitrite and NO may act together to reduce methemoglobin independent of methemoglobin reductase enzymes in a reaction known as reductive nitrosylation (12). Experiments that use whole blood with intact erythrocytes are needed to determine the physiological relevance of either of these processes to rates of methemoglobin reduction. However, reported cytb5r activities are most commonly measured indirectly by spectrophotometric measurement of the enzyme’s reduction of ferrocytochrome-c from nitric oxide in hemolysates as alternative substrates for methemoglobin (3, 42). Alternatively, cytb5r activities have been studied in whole blood, but with the use of dilute suspensions of red blood cells washed after pharmacological induction of methemoglobinemia with hemoglobin-oxidizing agents (2, 40). Interestingly, however, the functional activity of overall methemoglobin reduction, i.e., the decline in methemoglobin levels per unit time in whole blood at body temperature, does not appear to have been measured in any species, perhaps because of the complications introduced by the continuous formation of methemoglobin that normally occurs coincident with its removal.

The present study was designed to test the hypothesis that carbon monoxide (CO), by converting hemoglobin to carboxyhemoglobin, would block nitrite-induced methemoglobin production. We further hypothesized that this treatment would enable the measurement of methemoglobin-reducing processes in the absence of competing methemoglobin production. Using this simplified method, we compared rates of methemoglobin reduction in the presence of nitrite in the blood of fetal and adult sheep and humans.

MATERIALS AND METHODS

Collection of human blood samples was performed by following protocols that had been approved by the Loma Linda University Institutional Review Board. Collection of sheep blood samples was performed by following protocols that had been approved by the Loma Linda University Institutional Animal Care and Use Committee. Venous blood samples were collected in heparinized syringes from in-dwelling catheters in fetal sheep at 130–140 days of gestation (term 145 days), from adult sheep and adult humans, and from human umbilical cords immediately after separation from infants at parturition of uncomplicated pregnancies.

In a typical study, 10.0 ml of blood were added to a sealed flask maintained in a water bath at body temperature (39 and 37°C for sheep and humans, respectively). Two baseline samples (0.3 ml) were collected during a 10-min equilibration period for measurement of methemoglobin saturation (MetHb%), blood gases, oxyhemoglobin saturation, total hemoglobin, and pH. One milliliter of sodium nitrite dissolved in 0.5 ml of saline was then added to the flasks, which provided an initial concentration of 1.4 mM nitrite. This level of nitrite established an initial molar ratio of ~1:2 nitrite to hemoglobin, equivalent to 1% nitrite to heme. After addition of nitrite, the increase of MetHb% was followed with periodic sampling until MetHb% reached ~20%, at which time the gas phase in the flask was replaced with CO and the blood swirled for 2 min. The subsequent decline in MetHb% was followed with 0.1-ml sampling at 20-min intervals during the next 3–4 h. Blood gases and pH were measured at hourly intervals. One to eight disappearance curves were recorded from blood of each animal or subject.

Experiments were carried out to assess whether CO was effective in completely preventing the formation of methemoglobin. In these control experiments, blood was treated with CO before introduction of nitrite and levels of MetHb% were measured with time. Results were obtained after standard doses of nitrite and, in addition, after introducing initial concentrations 5- and 10-fold higher than standard to amplify the effect of this potential error. To verify that methemoglobin was not being formed by xanthine oxidase during reduction, the experiments were repeated after addition of 100 μM allopurinol.

To study the pH dependence of methemoglobin disappearance, pH was varied from ~7.2 to 7.6 by addition of 0.1 M HCl or NaOH to 10 ml of swirled blood. To study the oxygen dependence of methemoglobin reduction, the oxygen tension of the blood was varied from 10 to 100 Torr by equilibration of the blood with gas mixtures containing 3% CO2 and varying oxygen concentrations in nitrogen. To study the importance of the erythrocyte membranes and intact nature of the red blood cells, the experiments were repeated after the red blood cells were lysed by one of three alternative methods. These were osmotic rupture with distilled water (2:1 water-blood), sonication, and repeated cycles of freeze-thawing. The method of lysis did not appear to have an effect on results, and the results of the freeze-thaw experiments are presented herein. The temperature dependence of methemoglobin disappearance was studied by repeating experiments at temperatures ranging from 16 to 39°C.

To study the effects of methylene blue, a 1% solution was prepared and 0.025–0.1 mg of the dye was added to the equilibration flasks at the time of equilibration with CO. This provided concentrations ranging from 7 to 30 μM.

Methemoglobin measurements. Methemoglobin, oxyhemoglobin, and carboxyhemoglobin saturations were measured to the nearest 0.1% using a hemoximeter (model OSM3; Radiometer). The coefficient of variation for methemoglobin saturation measurement was 0.5% of measured values with MetHb% in the range studied and 13% of measured values with MetHb% at baseline levels. The accuracy of hemoximetry measurements of methemoglobin in the presence of saturating amounts of CO was assessed by preparing and measuring methemoglobin in mixtures of predetermined ratios of 100% carboxyhemoglobin and 100% methemoglobin (created by exposing 100% oxygenated blood to 10 mM nitrite for ~20 min followed by 3 centrifuge-wash cycles with saline) using whole adult sheep blood samples. Measured methemoglobin concentrations were plotted against actual concentrations, and the resulting data points were fitted to a linear regression analysis. Blood gases and pH were measured with appropriate specific electrodes (model ABL-5; Radiometer).

Calculation of methemoglobin reduction rates. Rates of methemoglobin reduction were determined from the linear regression analyses of semilog plots of methemoglobin concentration vs. time after saturation of the blood with CO. The relationship between the logarithm of methemoglobin concentration and time was highly linear, and the rate constant (k) for methemoglobin reduction was taken as the slope of the linear relationships. For each set of experiments, the k value was calculated as a mean of k values determined for each individual experiment, rather than a linear regression analysis of all experiments combined.

Statistical analysis. ANOVA was used to analyze differences between experimental groups. When significant effects were found, the post hoc test used was Fisher’s protected least significant difference. For all tests, α was set at 0.05. Means are presented with their SEs. The software DATAMSTR (courtesy of R. A. Brace), GraphPad Prism v4.0, and TableCurve were used for the analyses.

RESULTS

The accuracy of hemoximetry measurements of methemoglobin in saturating levels of carboxyhemoglobin in whole adult sheep blood is shown in Fig. 1. Methemoglobin was measured at levels ranging from <1% to 80%. The resulting linear regression demonstrated an r2 value of 0.988, a slope of 0.900, and a y-intercept of 3.455 ± 1.329.
The curves analyzed in this study. The specific was 0.00396 unit time was calculated from the slope, and in this experiment it first-order kinetics and providing the fractional disappearance per

is shown in Fig. 2. After addition of nitrite at 150.99/H11006 changes in COHb% after exposure of the blood sample to CO.

C stopping further oxidation by nitrite; 99% confidence limits are shown.

after conversion of the remaining non-MetHb to carboxyhemoglobin (COHb), nitrite at in a 10.0-ml blood sample at 37°C after introduction of 1.0 mg of sodium

Fig. 2. Time course of changes in cord blood of newborn 15 is shown in Fig. 2. After addition of nitrite at time 0, MetHb% increased from a baseline level of 0.7% to 19.6% during the next 30 min. After equilibration with CO, MetHb% decreased, more rapidly at first and then more slowly, reaching 8.1% during the next 230 min. When MetHb% was plotted semilogarithmically during the decay phase, as shown in Fig. 2B, a linear relationship was observed, as is characteristic of first-order kinetics. A similar linear relationship (average \( r^2 \) of >0.98) was observed for each of the curves analyzed in this study. The specific \( k \) characterizing first-order kinetics and providing the fractional disappearance per unit time was calculated from the slope, and in this experiment it was 0.00396 ± 0.00007 min⁻¹.

A representative curve showing changes in methemoglobin as a percentage of total pigment from cord blood of human newborn 15 is shown in Fig. 2. After addition of nitrite at time 0, MetHb% increased from a baseline level of 0.7% to 19.6% during the next 30 min. After equilibration with CO, MetHb% decreased, more rapidly at first and then more slowly, reaching 8.1% during the next 230 min. When MetHb% was plotted semilogarithmically during the decay phase, as shown in Fig. 2B, a linear relationship was observed, as is characteristic of first-order kinetics. A similar linear relationship (average \( r^2 \) of >0.98) was observed for each of the curves analyzed in this study. The specific \( k \) characterizing first-order kinetics and providing the fractional disappearance per unit time was calculated from the slope, and in this experiment it was 0.00396 ± 0.00007 min⁻¹.

The summary results of fitting linear slopes to the decay curves for the various experimental groups are shown in Fig. 3 and summarized in Table 1. The results given are for 9 fetal sheep at 130–145 days gestation, 20 adult ewes, 8 adult human subjects (4 men and 4 women with ages ranging from 16 to 70 yr), and cord blood from 21 human term newborns. The fractional loss per minute was highest in fetal sheep blood (averaging 0.0129 ± 0.0012 min⁻¹), which is more than twofold higher than that for adult sheep (0.00552 ± 0.00022 min⁻¹; \( P < 0.001 \)). By way of contrast, \( k \) was least in human newborns, averaging 0.00330 ± 0.00015 min⁻¹, and less than that in adult humans (0.00427 ± 0.00034 min⁻¹; \( P < 0.01 \)). The apparent half-lives averaged 54 ± 5 min for fetal sheep blood, 125 ± 5 min for adult sheep blood, 162 ± 13 min for adult human blood, and 210 ± 10 min for cord blood from term newborns (Table 1).

The results of testing the extent to which CO prevents methemoglobin formation are shown in Fig. 4. After hemoglobin had been converted to carboxyhemoglobin at the beginning of the experiment followed by nitrite addition, MetHb% increased 3% in 4 h. After nitrite was introduced at a dose fivefold higher than standard, MetHb% increased 1.2% in 4 h and after 10-fold excess it increased 2.2%. With the use of nitrite concentrations typically employed, \( k \) values were recalculated and found to alter 2.3% from values as reported here. These data indicate the near complete effectiveness of equilibration with and continuous exposure of blood to 100% CO in preventing methemoglobin formation in the presence of 1.4 mM nitrite. After addition of allopurinol, a xanthine oxidase inhibitor, the measured rate of methemoglobin reduction varied 5% or less, indicating that reduction of nitrite to NO by xanthine oxidase does not contribute to continued methemoglobin production after CO saturation in these experiments (data not shown).

The influence of oxygen tension and pH in the blood during the reduction process was studied in adult human blood; \( k \) was not measurably affected by changes in oxygen tension from 7 to 100 Torr (\( r = 0.22, P = 0.15, n = 44 \)). In addition, \( k \) was not affected by changes in pH from 7.2 to 7.6 (\( r = 0.14, P = 0.38, n = 44 \)) (data not shown).
blood in the equilibrium chamber increased lysis. Nor by replacement of the plasma by phosphate buffer before membrane-solubilizing agent Triton X-100 to the hemolysates, pronounced decrease in rate was not restored by addition of the water, sonication, or repeated freeze-thaw cycles. The prolongation observed whether the lysis was carried out by distilled water, sonication, or repeated freeze-thaw cycles. The pronounced decrease in rate was not restored by addition of the membrane-solubilizing agent Triton X-100 to the hemolysates, nor by replacement of the plasma by phosphate buffer before lysis.

Addition of 0.025–0.10 mg of methylene blue to 10 ml of blood in the equilibrium chamber increased k to 0.0487 ± 0.0065 min⁻¹ (P < 0.001), some sixfold higher than in untreated blood. Methylene blue had little effect when added to lysed blood. The combination of methylene blue and 1–5% Triton X-100 also did not alter the rate of reduction appreciably.

Figure 6 shows an Arrhenius plot in which the logarithm of k is plotted against the reciprocal of the absolute temperature. The data shown are for the blood of adult sheep. The slope of the line is −1.883 ± 231 using least squares regression analysis; after we multiplied the slope by the gas constant and 2.303 to allow for base 10 logarithms, we were able to calculate an activation energy of 8,620 ± 1,060 cal/mol or 2,060 J/mol. This value is two- to threefold higher than is characteristic of processes limited by passive diffusion. Finally, methemoglobin is reduced manyfold more slowly after disruption of red cell membranes, and the rate is not restored to normal by addition of methylene blue or detergent to the hemolysates or by replacement of plasma with phosphate buffer before lysis.

DISCUSSION

The present results provide data characterizing the rate that methemoglobin is reduced to functional hemoglobin in whole blood at body temperature and are thus directly relevant to the safety of administering nitrite to humans. Methemoglobin is reduced more slowly in humans than in shear, and the process is measurably slower in cord blood of newborn humans than in adults. The effective half-life of methemoglobin in newborn human blood is 210 min, 30% longer than in adult human blood. In both species and irrespective of age, the reduction of methemoglobin closely follows first-order kinetics in a 3-h period of observation. The reduction of methemoglobin is temperature dependent to an extent that is characteristic of enzyme-catalyzed reactions rather than a process limited by passive diffusion. Finally, methemoglobin is reduced manyfold more slowly after disruption of red cell membranes, and the rate is not restored to normal by addition of methylene blue or detergent to the hemolysates or by replacement of plasma with phosphate buffer before the lysis.

To our knowledge, the present study is the first to provide specific rate constants that characterize the rate of reduction of elevated methemoglobin, without concomitant methemoglobin production, in undiluted whole blood at body temperature. Earlier work has established that this reductive process is brought about largely by methemoglobin reductase, alternat-

Figure 4. Time course of changes in MetHb% after pretreatment with CO. Small increases with time are seen at initial doses of nitrite 5- and 10-fold higher than standard (dashed lines) but not observed with standard doses (triangles), demonstrating near complete effectiveness of CO at inhibiting MetHb production. Also shown are the effects of CO treatment of whole blood (squares) and lysed blood (circles) approaching 20% MetHb, demonstrating the marked reduction of MetHb reduction by hemolysis.

Figure 5. Box and whisker plots of adult sheep blood showing the first-order rate constant k in whole blood, after lysis, after addition of methylene blue (0.025 mg), and after Triton X-100 (final concentration of 5%) in combination with methylene blue.
Soluble cytochrome b₅ and cytochrome b₅r, which are located in the interior of red blood cells. The rate of methemoglobin reduction in red blood cells has also been found to be dependent on glycolytic rate and therefore the rate at which NADH can be generated as a source of reducing equivalents for the methemoglobin reduction. It is worth noting that, although the processes of methemoglobin reduction are enzyme dependent, we have elected to describe the rates using first-order equations rather than Michaelis-Menten kinetics. This is because methemoglobin reduction is a result of at least two distinct enzyme systems (cytb₅r and flavin reductases) with two different cofactors (NADH and NADPH, respectively). In addition, the relative contribution of these two enzyme systems to overall methemoglobin reduction is likely to vary significantly with methemoglobin concentrations and between species (22). As a result, it was decided that the use of first-order equations, which accurately described the experimental data, was more appropriate.

The NADH-methemoglobin reductase system comprises a soluble cytochrome b₅ and cytochrome b₅r in the interior of erythrocytes (20). Six electrophoretically distinct variants of NADH-methemoglobin reductase have been found in studies of human blood (16). The enzyme exists in two forms, one fraction being tightly bound to the inner face of the red blood cell membrane and a second fraction dissolved in the cytosol (8). Earlier work has examined the difference in methemoglobin reductase activity among species and at different ages (1, 26, 27, 36, 43). Typically, these earlier investigators have used alternative substrates such as NADH-ferricyanide and diluted hemolysates in their analyses and have not sought to measure functional activity at body temperature. Nevertheless, where comparisons are possible, results of the present study are in acceptable accord with earlier work. For instance, methemoglobin reductase activity in 1:20 dilutions of erythrocytes in a stabilizing solution were comparable in adult sheep and humans (27), in agreement with measured first-order rate constants also being of like magnitude in the present work (Fig. 3). Similarly, methemoglobin reductase activity has been reported to be 23% less in low-birth-weight human newborns than in adults (29) in accordance with the 30% lower k values reported here. Thus, in two species and at different ages, the rate of reduction reported here appears to correlate reasonably well with indirectly measured indicators of methemoglobin reductase activity.

In vivo rates of methemoglobin reduction have been previously determined in humans after treatment with inhaled NO gas (45, 46). Comparison of rate constants calculated from these previous studies with the present study suggest that methemoglobin reduction may occur more rapidly in vivo (k × 1,000 ranging from 11 to 25 s⁻¹) than observed in the present experiments. The mechanism for this difference is not clear but may be due to the increased presence of reducing equivalents in vivo.

In the present study, the effective half-life of methemoglobin in human newborn blood was 30% longer than in adult human blood (P < 0.01). This difference is in agreement with earlier work that reported lower levels of methemoglobin reductase activity in the newborn human (26, 27). The slower rate in newborns clearly points out the greater care required in the therapeutic use of NO, nitrite, and other methemoglobin formers in newborns than in adults. The difference is judged not to be so large, however, that it would preclude the therapeutic use of nitrite. This conclusion is supported by clinical experience in which methemoglobin levels seldom increased above 5% in humans during experiments testing the safety of inhaled NO (45) or in newborns given NO.

Methylene blue is thought to be reduced both intra- and extracellularly by enzymes present in the erythrocyte membrane (28). Inside the erythrocyte, the reduced dye is capable of reducing methemoglobin. Both NADPH (6, 38) and NADH (37) are required for reduction of methylene blue. After addition of methylene blue in the present study, k increased about sixfold. This result is in agreement with the four- to sixfold increases in the rate of methemoglobin reduction observed when methylene blue is given to patients therapeutically (5) and the sevenfold increase in methemoglobin reduction that has been reported in human erythrocytes studied in vitro (40). Methylene blue was less effective when added to hemolysates (Fig. 5), and this is in agreement with the decline in methylene blue efficacy that has been noted in patients with hemolysis (5) and may be due to disruption of the methylene blue-reducing enzymes present in the red blood cell membrane (28) or cytosol (17).

The mechanism by which lysis causes a marked slowing in the reduction of methemoglobin is not known, but some possibilities can be largely discounted by the present work. It seems unlikely to be inhibition induced by plasma chloride, a known inhibitor of methemoglobin reductase (21) whose extracellular concentration is ~10-fold higher than intracellular chloride concentration, because the rate was not restored to normal by replacement of plasma with phosphate buffer. It is also not likely to be via increased binding of the reductases to red blood cell membranes after lysis because treatment with 1–5% Triton X-100, an agent known to be effective in solubilization of membranes (30), did not restore k to normal values, although this treatment may not necessarily restore the protein-protein interactions of membrane-associated proteins. Rather, it seems more likely that lysis disrupted the assembly of proteins that are closely associated with one another primarily by electrostatic interaction in the cell interior and responsible for the reduction of methemoglobin. The disruption would break the close-linked chain that transfers electrons from NADH to the flavin of cytb₅r and then to cytochrome b₅ and finally on to methemoglobin. Other possibilities for loss of
function after lysis include dependency on the relatively high intraerythrocytic concentrations of components of the methemoglobin reductase system that would be diluted after lysis or decreased activity of glycolytic enzymes that supply NADH, which is known to occur with hemolysis (17).

In conclusion, the present study reports the rate constants and effective half-times that characterize the disappearance of experimentally induced methemoglobinemia in blood samples in vitro at body temperature. Rates of reduction are measurably less in newborn human blood than in adult blood. The 30% longer half-life in newborns is not so extended, however, that it should be taken to preclude the therapeutic use of nitrite in the newborn period, provided caution is exercised with periodic monitoring and the ready availability of methylene blue.

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