The Oxygen and Sulfide Binding Characteristics of Hemoglobins Generated from Methemoglobin by Two Erythrocytic Systems

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SUMMARY

Methemoglobinemia induced in mice and other animals protects against death from inhaled hydrogen sulfide or injected sodium sulfide. Equivalent circulating levels of methemoglobin impart equivalent degrees of protection irrespective of the agent used to induce the methemoglobinemia (nitrite, *p*-aminopropiophenone, or hydroxylamine). In each case 2–4 moles of sulfide are apparently inactivated *in vivo* for each available ferric heme group. The ferric iron of methemoglobin is one site available for hydrosulfide anion binding. No information is available about the nature of the additional sulfide binding sites, but they are inferred to be associated with the globin moiety.

Protection against sulfide poisoning is not demonstrable in mice after methemoglobin levels have been reduced to normal concentrations by spontaneous methemoglobin reductase activity. However, if methemoglobin reductase activity is increased by methylene blue, a ferrous hemoglobin which retains significant sulfide-inactivating properties exists transiently *in vivo*. This protective effect is also lost after a time interval equivalent to that required for spontaneous activity to reduce methemoglobin levels to normal in the absence of methylene blue. In mouse erythrocytes exposed to sodium nitrite the non-methemoglobin heme produced as a result of methylene blue-induced reductase activity has only one-tenth of the oxygen capacity of normal mouse hemoglobin or the product of normal mouse methemoglobin reductase activity. A fully functional mouse hemoglobin in terms of its oxygen capacity is eventually generated in the presence of both methylene blue and nitrite if enough time is allowed for the spontaneous reductase to act. Similarly, the total ferrous hemoglobin products of methylene blue-induced reductase activity in nitrited human cells have less than half the oxygen-binding capacity of normal human hemoglobin. These findings suggest that spontaneous reductase activity restores a fully functional hemoglobin whereas methylene blue-induced reductase activity only reduces the heme iron. The methylene blue therapy of methemoglobinemia bears reexamination in terms of these findings.

INTRODUCTION

Previous work (1) has shown that methemoglobinemia induced in mice and other animals protects against the lethal effects of inhaled hydrogen sulfide or injected sodium sulfide. This protective effect is due to a trapping and/or inactivation of free hydrosulfide anion by methemoglobin (1, 2). Mortality studies indicate that 2–4 moles of sulfide are inactivated for each mole of available ferric heme, whereas both cyanide and azide are detoxified in an amount consistent with one mole of either combining with each ferric heme group (2).

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Since cyanmethemoglobin prepared *in vitro* and injected into the peritoneal cavity of mice still protects the animals against injected sulfide, the molecule must possess binding or inactivation sites for sulfide in addition to the ferric heme site already occupied by cyanide. Although the precise location of these additional sulfide inactivation sites has not been established, they will be referred to in this communication as "globin" sites to distinguish them from the ferric iron-binding site. In order to demonstrate the existence of these globin sites, however, cyanmethemoglobin must have been exposed originally to nitrite; these sites are either absent or masked if the hemoglobin was exposed to ferricyanide instead (2).

The reaction of nitrite with hemoglobin, therefore, appears to produce at least two kinds of changes: (a) oxidation of the heme iron which produces one binding site per heme group available to a variety of ligands, and (b) "globin" alterations which result in the production of 1–3 specific sulfide inactivation sites per heme group. Since these changes are demonstrable in isolated heme pigments, they cannot be related to alterations in red cell ratios of reduced to oxidized glutathione. The latter also protects against sulfide poisoning, but nitrite does not deplete reduced glutathione levels when administered to mice in this dose (3). Furthermore, it does not appear likely that these changes are related to disulfide bond formation on the hemoglobin molecule (2).

In mouse red cells vigorous spontaneous reductase activity rapidly reduces ferric heme groups, as judged by a standard spectrophotometric method for measuring methemoglobin levels (4, 5). Since the operating definition of methemoglobin adopted in this work is a blood pigment which reacts with cyanide in near neutral media to produce a decrease in absorbance at 635 mμ, it is assumed that this definition dictates the state of oxidation of the heme iron. No *a priori* information on the status of the globin sulfide-binding sites is gained by this measurement of circulating methemoglobin levels. The present work was undertaken primarily to ascertain whether or not the globin changes produced by nitrite are reversed by methemoglobin reductase activity.

METHODS

Absorption spectra were traced from 1-cm cells in a Bausch and Lomb Spectronic 505 recording instrument. Methemoglobin solutions were prepared from twice-crystallized human hemoglobin as previously described (2). Commercially available purified bovine globin or the hemoglobin above was exposed to 20-fold molar excesses of either nitrite or ferricyanide in neutral solutions, followed by exhaustive dialysis against distilled water. Methemoglobin solutions were then diluted with phosphate buffer for spectrophotometric examination or used directly for total iron analyses by atomic absorption spectrophotometry (Perkin-Elmer 303) based on spectral lines of 248 or 372 mμ. The various methemoglobin solutions were adjusted to the same heme concentration on the basis of their absorbances at 540 mμ after conversion to cyanmethemoglobin. The globin solutions were lyophylized, and aliquots were redissolved in dilute hydrochloric acid for iron determinations as above. Total protein was measured by a biuret procedure (7).

Methods for determining circulating methemoglobin levels in 25–30 g Swiss-Webster female mice have been previously described (6). All chemicals were injected intraperitoneally in water except *p*-aminopropiophenone (*p*-APP) which was dissolved in propylene glycol. Doses and injection sequences are described in the appropriate tables.

For the experiments of Tables 4 and 5 washed mouse or human erythrocytes were incubated in Krebs-Ringer-phosphate-glucose (4). Initial heme concentrations were adjusted to 5.0 mM. Some incubation mixtures also contained 0.01 mM methylene blue or 6.75 mM sodium nitrite or both. Incubation mixtures were maintained at 38°, and methylene blue was always added 15 min prior to nitrite (4). Methemoglobin levels were determined as above, and total pigment was independently assayed as cyanmethemoglobin. Total oxygen capacity

was measured manometrically with a Bronwill Warburg apparatus. Reaction vessels contained 0.2 ml 20% KOH in the center well, 0.2 ml of ferricyanide-saponin-capryl alcohol solution in the side arm, and 1-ml aliquots of red cell incubation mixtures in the reaction chamber. After temperature (25°) and gas (room air) equilibration, the side-arm solution was tipped in and oxygen evolution was measured after 30 mins. At the end of 2 hr of incubation the hemoglobin in all red cell suspensions containing only sodium nitrite was totally in the form of methemoglobin. Aliquots from these suspensions, therefore, served as reagent blanks in the oxygen evolution measurements. This correction was always very small, and both positive and negative values were observed. Similarly an aliquot from a red cell suspension containing neither nitrite nor methylene blue served as a total oxygen capacity reference. The oxygen released per mole of heme was corrected for that fraction of the total pigment present in the red cell suspension as methemoglobin.

In experiments with mouse red cells all incubation mixtures were washed three times with fresh buffer-substrate after the 2-hr sampling and resuspended to the same volume. This procedure probably accelerates methemoglobin reduction by removing excess nitrite (8). Aliquots removed for methemoglobin analyses were washed three times in saline prior to hemolysis to effect dissociation of the nitrite-methemoglobin complex (8).

RESULTS

Figure 1 shows the visible absorption spectrum of nitrite-generated human methemoglobin following prolonged dialysis to remove inorganic reaction products and nitrite bound to heme sites (8). Also shown are absorption spectra of three aliquots of the same pigment preparation in the presence of an excess of sodium cyanide, sodium sulfide, or sodium azide (2). Although not shown, a similar set of spectra were prepared from methemoglobin originally treated with potassium ferricyanide. Be-

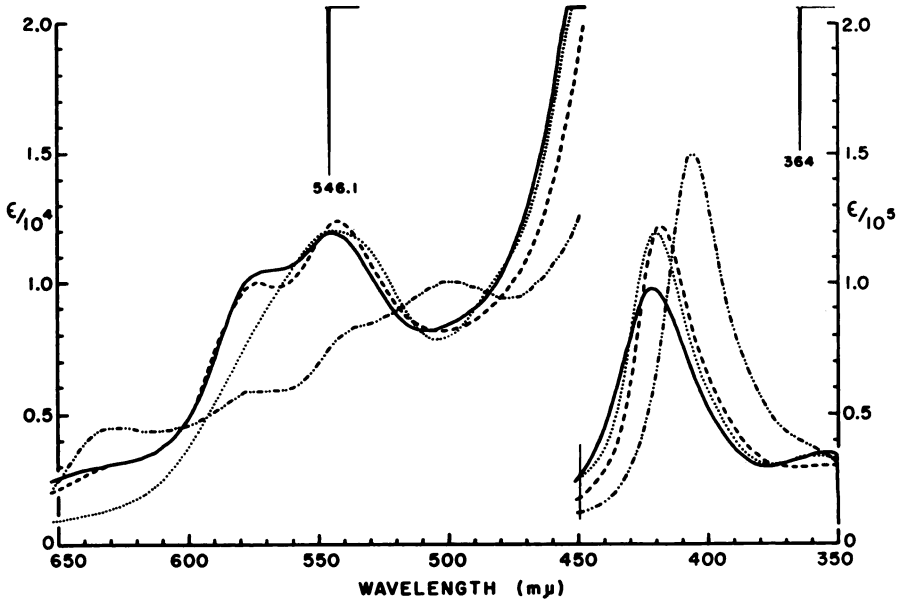

FIG. 1. *Visible absorption spectra of methemoglobin in 0.1 M phosphate buffer at pH 7.4* (-•••-•••-) *after treatment with sodium nitrite and prolonged dialysis*

Other spectra are the same pigment preparation in the presence of excess sodium cyanide (•••••), sodium sulfide (———), and sodium azide (-----). All preparations were diluted 10-fold to record the Soret bands. Calibration lines are mercury emission.

cause of differences in the sulfide-binding capacity previously reported (2), particular attention was paid to a comparison of the hydrosulfide complexes of nitrite- and ferricyanide-treated hemoglobins. These spectra appeared to coincide within experimental error.

TABLE 1

Iron concentrations of various human methemoglobin and bovine globin solutions

Human hemoglobin and bovine globin were incubated with 20-fold excesses of oxidants in neutral media followed by exhaustive dialysis against distilled water (Visking membrane). The methemoglobin dialysants were adjusted to 1.0 mM in heme as cyanmethemoglobin and the globin dialysants were adjusted to 1 mg of biuret protein (7) per ml. These solutions were used directly for atomic absorption spectrophotometry of their total iron concentrations.

Preparation	Methemoglobin (mmoles Fe/l)	Globin (μmoles Fe/g protein)
Control	0.95	<1.8
Nitrite treated	1.0	<1.8
Ferricyanide treated	2.9	35.8

Table 1 confirms the observations of many others (e.g., 9) that ferricyanide-treated hemoglobin even after prolonged dialysis retains bound iron. Although no information is available about the form of the bound iron or the nature of the binding site, it is bound to the extent of two extra iron atoms per heme group. Purified bovine globin treated with ferricyanide also binds iron in a form resistant to dialysis (Table 1). Moreover, the bovine globin preparation binds as much iron per gram of protein as the human hemoglobin sample.

Table 2 shows that the mortalities of female mice following intraperitoneal sodium sulfide are not significantly different at equivalent circulating methemoglobin levels irrespective of whether $NaNO_2$, *p*-APP, or H_2NOH was used to induce the methemoglobinemia. The rationale for inferring an *in vivo* inactivation ratio of 2–4 moles of sulfide for each nitrite-induced ferric heme group has been discussed previously (2). The data in Table 2 indicate that both hydroxylamine (H_2NOH) and *p*-APP share with nitrite the property of inducing a methemoglobinemia that can inactivate sulfide in excess of available ferric heme groups.

In Table 3 mice pretreated with methylene blue were then injected with the same dose of nitrite as in Table 2. The methemoglobinemic response to nitrite after 20 min was reduced by methylene blue pretreatment from a mean of 34.1% of the total blood pigment to only 3.4%. The normal circulating methemoglobin level in mice by this method is $1.3 \pm 0.6\%$ (5, 6). Despite a low circulating methemoglobin level at the time of the sulfide injection, methylene blue-pretreated animals were significantly resistant to sulfide when compared to a control group that received methylene blue but saline instead of nitrite. Table 3 also shows that when the sodium sulfide injection was delayed for 3 hr, a time period

TABLE 2

Sulfide inactivation capacity of methemoglobinemias induced in mice

Agent	Dose (mmoles/kg)	Percent methemoglobin at time of sulfide challenge[a]	Mortality after 2.4 LD_{50}'s sodium sulfide[b]	
			Dead/total	Percent dead
$NaNO_2$	1.1	34.1 ± 8.4	8/14	57
p-APP	0.1	37.3 ± 7.0	5/10	50
H_2NOH	1.1	35.7 ± 7.1	10/15	67

[a] Mean ± SD for 6 animals. The percent methemoglobin shown for $NaNO_2$ represents 0.22 mmole of methemoglobin heme per kilogram body weight of mice (6).

[b] Given intraperitoneally (2.4 LD_{50}'s Na_2S = 1.3 mmoles/kg) 20 min after $NaNO_2$ or 10 min after either *p*-APP or H_2NOH. No two of the indicated mortalities are significantly different from each other.

TABLE 3
Sulfide inactivation capacity of hemoglobins generated from methemoglobin in mice

Treatment	Duration (min)	Percent methemoglobin at time of sulfide challenge[a]	Mortality after 0.71 mmole/kg Na_2S[b] Dead/total		
			Control	Exptl.	
Methylene blue	0				
$NaNO_2$	20				
Na_2S	40	3.4 ± 1.1	12/15	4/15	$p < 0.02$
Methylene blue	0				
$NaNO_2$	20				
Na_2S	180	1.1	10/10	9/10	$p > 0.99$
p-APP	0				
Na_2S	120	2.4 ± 0.8	10/12	8/12	$p > 0.5$

[a] Mean ± SD for six animals or simple average for 3.

[b] Control mice received saline for nitrite or propylene glycol for *p*-APP. In the first two experiments the control group of mice also received methylene blue, 0.134 mmole/kg, intraperitoneally; therefore, methylene blue alone does not protect against sulfide.

required for spontaneous reductase activity to reduce this methemoglobin concentration to normal in the absence of methylene blue (5), protection against sulfide is no longer demonstrable. Similarly, protection against sulfide induced by *p*-aminopropiophenone, as shown in Table 2, also disappeared 2 hr after injection of this amine (Table 3). At this time spontaneous reductase activity had lowered methemoglobin levels to a mean of 2.4% of the total blood pigment.

Table 4 shows that control mouse red cell suspensions at 25° exposed to atmospheric oxygen tensions released about 22.3 μl of oxygen per μmole of heme following lysis and treatment with ferricyanide. A similar red cell suspension after 2 hr in the presence of 6.75 mM sodium nitrite was totally in the form of methemoglobin. The slight gas exchanges observed with such cell suspensions on tipping in ferricyanide-saponin solution (±1 μl O_2 per μmole of heme) were used as reagent blank corrections. A red cell suspension with added methylene blue released about 20 μl O_2 per μmole of heme, but red cell suspensions containing both nitrite and methylene blue released only about 1.5 μl O_2 per μmole of heme. An independent assay of the total hemoglobin concentration as cyanmethemoglobin showed that all incubation mixtures contained identical concentrations. In each case, however, this total amount of blood pigment was corrected for that fraction present as methemoglobin in

TABLE 4
Oxygen released by saponin-ferricyanide mixture after aerobic incubation of mouse erythrocytes with nitrite, methylene blue, or both

Gas evolution determinations were made in duplicate on each incubation mixture. The total heme pigment in all incubation mixtures measured by the cyanmethemoglobin method was 5.0 mM. In each case the oxygen capacity was calculated on the basis of this concentration minus the concentration of methemoglobin heme present at the time of oxygen evolution (see column 2).

Treatment	Percent methemoglobin	μl O_2/μmole heme
		After 2 hr incubation
Control	0	22.4, 22.3
Nitrite	100	0/0, 0/0
Methylene blue	0	19.8, 20.6
Methylene blue + nitrite	23.3	1.8, 1.3
		After 6 hr incubation
Control	1.8	20.6
Nitrite	31.8	25.2, 20.6
Methylene blue	5.4	21.0, 22.6
Methylene blue + nitrite	9.9	13.6, 15.0

any particular cell suspension when the oxygen saturation per mole of heme was computed.

Four hours later spontaneous reductase activity in the cell suspension containing only nitrite (Table 4) had reduced the methemoglobin level to about a third of the total pigment. The oxygen capacity of hemoglobin from these cells, after correcting for the methemoglobin fraction, is seen to be comparable to either the control cell suspensions or cell suspensions exposed to only methylene blue. The oxygen released by mouse erythrocyte suspensions exposed to both methylene blue and nitrite at this time is seen to be improved over the 2-hr samples to about 14 μl O_2 per μmole of heme. Even 6 hr after nitrite addition, Table 4 shows that about a third of the total pigment in mixtures containing only nitrite had not yet been reduced by spontaneous reductase activity. If the oxygen capacity of cell suspensions containing both methylene blue and nitrite is corrected upward to account for the fraction of total pigment not yet acted upon by spontaneous reductase at 6 hr, all conditions of incubation yield blood pigments with comparable oxygen capacities.

The non-methemoglobin heme of human red cells exposed to both nitrite and methylene blue as shown in Table 5 has less than half the total oxygen capacity of normal human hemoglobin or human hemoglobin exposed to only methylene blue.

TABLE 5

Oxygen released by saponin-ferricyanide mixture after aerobic incubation of human erythrocytes with nitrite, methylene blue, or both

Gas evolution determinations in duplicate for two complete experiments, i.e., mean ± SD for four Warburg vessels. The methemoglobin levels were slightly different as shown for the two experiments, but the total pigment was always 5.0 mM in heme. See legend to Table 4.

Treatment	Percent methemoglobin	μl O_2/μmole heme After 2 hr incubation
Control	1, 6	22.3 ± 3.1
Nitrite	100, 100	0/0
Methylene blue	15, 17	20.9 ± 0.8
Methylene blue + nitrite	7, 22	7.9 ± 2.3

DISCUSSION

The absorption spectra shown in Fig. 1 support evidence previously presented that the ferric heme binding site of methemoglobin is available to cyanide, hydrosulfide, and azide (2). Yet many kinds of chemically induced methemoglobinemias inactivate sulfide in amounts greater than available ferric heme groups (Table 2). This circumstance and other evidence (2) suggests that certain kinds of methemoglobin have sites available for sulfide detoxication in addition to ferric iron. Only one kind of methemoglobin has been encountered which appears to be devoid of extra-ferric heme binding sites for sulfide, namely, methemoglobin prepared by treatment of hemoglobin solutions with ferricyanide.

Ferricyanide prepared methemoglobin is unique in other respects: it contains more disulfide bonds, or at least fewer free sulfhydryl groups than nitrite-treated or autoxidized hemoglobin (2), and it contains iron apparently bound to the globin moiety in a form resistant to dialysis (9) (Table 1). Although the estimated number of extra sulfide binding sites on methemoglobins prepared with $NaNO_2$, *p*-APP, or H_2NOH and the number of extra moles of iron bound to ferricyanide-treated hemoglobin are equal, no other evidence is presented here to suggest that ferricyanide iron is bound to methemoglobin to the exclusion of sulfide. The fact that the visible absorption spectra of the nitrite- and the ferricyanide-prepared hydrosulfide complexes are not significantly different, together with the iron-binding studies above and other evidence (2), suggests that the extra binding sites are associated with the globin moiety. An interaction of *N,N*-dimethylaniline *N*-oxide with the globin moiety in the production of methemoglobin is also implicit in the recent findings of Kiese (10). One type of inactivation site which fulfills the criterion of specificity for sulfide is a disulfide bond. At least the rates of reaction of cyanide and azide with some disulfides are

apparently too slow *in vivo* to be of significance in preventing death due to these toxic anions (3). Yet, ferricyanide-treated hemoglobin, which has fewer free sulfhydryl groups than nitrite-treated hemoglobin, is less protective against sulfide poisoning (2).

Except in the case of ferricyanide, the appearance of globin sulfide-binding sites may always occur when the heme iron is oxidized (Table 2). That these changes are reversible as the heme iron is acted upon by the spontaneous red cell methemoglobin reducing system is shown by the data in Table 3. The mouse red cell, like that of man, apparently has two methemoglobin reducing systems, one coupled to NADH and the other to NADPH (4). The activity of the latter can be greatly increased by methylene blue so that in the presence of this dye methemoglobin levels are reduced to normal concentrations apparently before the NADH-coupled enzyme has acted upon any of the pigment (4) (Table 4). By taking advantage of this large difference in rates, one can effectively generate a pool of ferrous blood pigment that has been selectively reduced by the NADPH-coupled enzyme via methylene blue. That this pigment has sulfide-binding properties different from the ferrous product of spontaneous reductase activity is shown by the data in Table 3.

These findings are consistent with the hypothesis that the methylene blue-mediated reaction reduces the heme iron, but does not reverse the inferred globin changes. Not only is the product of the NADH-coupled enzyme reaction apparently devoid of sulfide inactivating properties, but the experiments of Tables 3 and 4 indicate this enzyme can act sequentially on the product of the NADPH-coupled enzyme to restore a normal blood pigment in terms of both its sulfide- and oxygen-binding characteristics. Although the relationship between sulfide-inactivation sites and a decreased capacity for oxygen remains unknown for the present, these two properties demonstrate the transient existence of an abnormal blood pigment when red cells are exposed to both nitrite and methylene blue.

Since a ferrous hemoglobin product that does not combine stoichiometrically with oxygen is contrary to all experience, a reasonable interpretation of these results is that red cell incubation mixtures in the presence of both methylene blue and nitrite probably contain methemoglobin, oxyhemoglobin, and an unknown hemoprotein that does not combine with oxygen. An alternative explanation, which is in some ways equivalent, is that the unknown pigment contains some heme groups which combine with oxygen and some which do not. A competitive ligand (e.g., NO) may be responsible for the absence of oxygen on some heme groups. Experiments are presently underway to elucidate these points.

It would appear highly likely that this phenomenon is related to recent findings of Robin and Harley (11, 12), in which they refer to "intact hemoglobin destruction" seen in red cells incubated in the presence of methylene blue and nitrite. Intact hemoglobin is defined as the sum of the spectrophotometrically determined methemoglobin and oxyhemoglobin fractions. This sum is often significantly less than the total pigment determined as cyanmethemoglobin after several hours of incubation. As shown here their incubation mixtures probably contained appreciable quantities of the ferrous hemoglobin product of the NADPH-coupled reductase. That Robin and Harley have demonstrated variations in this phenomenon with certain abnormal and pathological conditions may imply that the NADPH-coupled enzyme is activated in some states.

Although definite recommendations are not yet justified, it is apparent that the methylene blue therapy for methemoglobinemia bears reexamination in terms of the findings of Tables 4 and 5. Methylene blue therapy is not without hazard (e.g., 13) and it is less effective in reversing the methemoglobinemias of other oxidants than nitrite-induced methemoglobinemia both in mice (5) and in human red cells (R. P. Smith, unpublished observation).

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