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EFFECTS OF CHLORIDE AND BICARBONATE ON METHEMOGLOBIN REDUCTION IN MOUSE ERYTHROCYTES*

GREGOR KLURFELD and ROGER P. SMITH†

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire, U.S.A.

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Abstract—A methemoglobinemia generated by phenylhydroxylamine persists longer in mouse red cells suspended in Krebs–Ringer-phosphate-glucose (K-R-PO_4-G) than *in vivo* or in shed blood. Heparin or bovine serum albumin added to K-R-PO_4-G did not shorten the duration of the methemoglobinemic response to phenylhydroxylamine, and phosphate added to shed blood did not prolong it. When a Krebs–Ringer-bicarbonate-glucose (K-R-HCO_3-G) medium was used instead of K-R-PO_4-G, the pattern of the methemoglobinemic response to phenylhydroxylamine was altered to resemble that of shed blood. When methemoglobinemic cells originally exposed to nitrite are washed, methemoglobin reductase activity, as assessed by net decreases in methemoglobin concentration, is inversely related to the extracellular chloride concentration. This effect of chloride in prolonging a methemoglobinemia is shared by nitrate. Rates of methemoglobin reduction were not influenced by changes in red cell volume as modified by alterations in extracellular osmolality. The addition of bicarbonate to a chloride-free medium did not further stimulate reductase activity, but in the presence of a critical extracellular chloride concentration, bicarbonate stimulates methemoglobin reduction. Since these effects are still seen when lactate is substituted for glucose, they are apparently mediated through the spontaneous NADH-linked reductive system (diaphorase). These findings suggest that the chloride shift may regulate the rate of methemoglobin reduction in mouse red cells.

PREVIOUS reports[1, 2] have indicated some advantages of mice as an experimental species for the study of chemically induced methemoglobinemias. Mouse and rabbit erythrocytes possess unusually brisk spontaneous methemoglobin reductase activity.[1] The substrate requirements for this activity suggest that the enzyme, like that of human red cells (diaphorase), is NADH-dependent.[1, 3] Increases in reductase activity of mouse erythrocytes occur when they are incubated in the presence of methylene blue and glucose. By analogy to similar findings in human red cells, this increase has been attributed to activation of a separate NADPH–methemoglobin reductase.[1, 3] Although the products of methylene blue-stimulated reductase may have oxygen and sulfide-binding characteristics which differ from normal hemoglobin or the product of spontaneous methemoglobin reductase activity, this enzyme appears to reduce the ferric heme iron, as does diaphorase.[4]

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The time course of a nitrite-generated methemoglobinemia is about the same whether it is followed *in vivo* or in suspensions of mouse red cells in Krebs–Ringer-phosphate-glucose (K-R-PO_4-G). Equivalent concentrations of methemoglobin generated by phenylhydroxylamine, however, persist longer in such a washed red cell suspension than in the intact animal.[2] This finding suggested that factors external to the mouse red cell may be important in terminating some kinds of chemically induced methemoglobinemias.

METHODS

For reasons which will be apparent in the Results section, it was originally assumed that the phenomenon under study was peculiar to a phenylhydroxylamine-generated methemoglobinemia.[2] In the initial experiments of Figs. 3 and 4 and Table 2, this chemical was employed to generate the methemoglobin. As this investigation progressed, it was discovered that the effects observed with phenylhydroxylamine could be duplicated in nitrited red cells if they were washed after exposure to nitrite. A major disadvantage of phenylhydroxylamine for these studies is that its methemoglobin-forming ability is mediated at least in part through interference with erythrocytic metabolism.[5] Therefore, an observed net effect could not be ascribed with confidence to an effect on methemoglobin reduction as opposed to an effect on methemoglobin formation. In subsequent experiments (Figs. 5–7 and Table 3) mouse red cells were preincubated with nitrite for 2 hr in K-R-PO_4-G; then aliquots of this reaction mixture were washed three times with 8 vol. of fresh media of the indicated compositions.

Mice were of the same sex, weight and genetic strain as in previous studies.[1, 2] Methods for following methemoglobin levels *in vivo* were as previously described.[6] Blood for studies *in vitro* was sometimes taken by open-chest cardiac puncture from pentobarbital- or ether-anesthetized animals. In other experiments it was collected after decapitation of either anesthetized or conscious animals. The method of collection of the blood or the presence or absence of the anesthetics had no influence on the experimental results. Heparin was employed as the anticoagulant. Methods of incubation (with the exception of the modification above), sampling and methemoglobin analyses were as previously described.[1, 2]

Chloride analyses were performed with a Buchler–Cotlove chloridometer; osmolality determinations were made in an Advanced Instruments Osmometer; microhematocrits were determined with the aid of an Adams Autocrit centrifuge; total protein was assessed by a Biuret procedure;[7] and total hemoglobin determinations were based on absorbance measurements as cyanmethemoglobin.[1, 2] Relative erythrocyte volumes in different media are expressed in arbitrary units as the ratio of the decimal-hematocrit (e.g. 50 per cent = 0·50) to the o.d. of a standard 1–250 dilution of cell suspension in Drabkin's reagent at 540 mμ (1 cm light path).* The compositions of the two basic incubation media used in these experiments are shown in Table 1. Modifications in these formulae are indicated in legends to the appropriate figures and tables. All incubation mixtures were adjusted to a heme concentration of 5·0 $\pm$ 0·2

* This expression is, therefore, proportional to the *reciprocal* of the "mean corpuscular hemoglobin concentration" (MCHC) as defined in M. M. Wintrobe, *Clinical Hematology*, 5th edn, p. 404, Lea and Febiger, Philadelphia (1961). For a hematocrit to hemoglobin ratio of 1·0, the corresponding MCHC has been calculated as 34 per cent.

TABLE 1. COMPOSITION BY CALCULATION IN M-MOLE/L. OF pH 7·4 INCUBATION MEDIA USED IN THESE EXPERIMENTS

Species	Krebs–Ringer-phosphate-glucose (K-R-PO_4-G)	Krebs–Ringer-bicarbonate-glucose* (K-R-HCO_3-G)
Na^+	140	155
K^+	5	6
Mg^{2+}	1	1
Ca^{2+}	1	1
Cl^-	140†	140†
HCO_3^-		24
Phosphate buffer, pH 7·4	6	
SO_4^-	1	1
Glucose	10	10
Sucrose	30–40‡	
Osmolality	290	320

* In equilibrium with 5% CO_2–95% O_2.

† In some experiments chloride was partially or wholly replaced by gluconate or nitrate.

‡ Sometimes added to raise osmolality of K-R-PO_4-G up to that of K-R-HCO_3-G.

mM. In experiments with whole blood, dilution to this pigment concentration was made with physiological saline. In some experiments cells were preincubated for 15 min with 10^{-5} M methylene blue prior to the addition of oxidants.

RESULTS

The patterns of the methemoglobinemic responses to sodium nitrite and to phenylhydroxylamine both in mice and in various types of mouse red cell suspensions are shown in Figs. 1–3. The numerals on the graphs of the results *in vivo* (Fig. 1) refer to

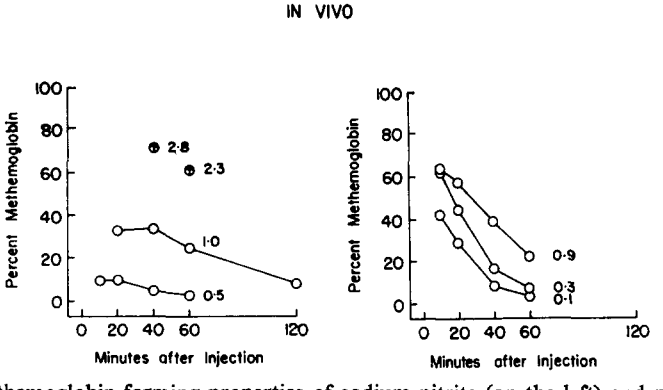

FIG. 1. The methemoglobin-forming properties of sodium nitrite (on the left) and phenylhydroxylamine (on the right) in mice. The mean methemoglobin levels (3–6 mice) are shown at various times after the i.p. doses (m-mole/kg) indicated by the numerals. The single point at 2·3 m-mole/kg (the LD_{50} of sodium nitrite) shows the mean methemoglobin level at the mean time of death for animals that succumbed. The single point at 2·8 m-mole/kg (essentially an LD_{100}) shows the mean methemoglobin levels at the mean time of death.

the intraperitoneal doses of the oxidants in m-mole/kg. The single result at 2·3 m-mole/kg of nitrite, which is its LD_{50}, shows methemoglobin levels at the mean time of death in those animals that succumbed. Similarly, the result at 2·8 m-mole/kg

(essentially an LD_{100} for nitrite) shows mean methemoglobin levels at the mean time of death. None of the indicated doses of phenylhydroxylamine produced death or observable signs other than cyanosis. All results *in vivo* represent average values obtained with 3–6 mice. Some of these data have been reported previously.[2, 6]

In Figs. 2 and 3, the top two panels of the results *in vitro* are derived from washed red cells incubated in K-R-PO_4-G, whereas the bottom two panels show results from red cells in homologous heparinized plasma diluted to the same heme concentration with physiological saline. These suspensions were not gassed with carbon dioxide, but the initial and final pH's did not differ from that of suspensions in K-R-PO_4-G. The top and bottom panels on the extreme right show the influence of prior incubation of the suspensions with 10^{-5} M methylene blue. Numerals on these graphs refer to final nitrite or phenylhydroxylamine concentrations (mM) added to separate reaction mixtures.

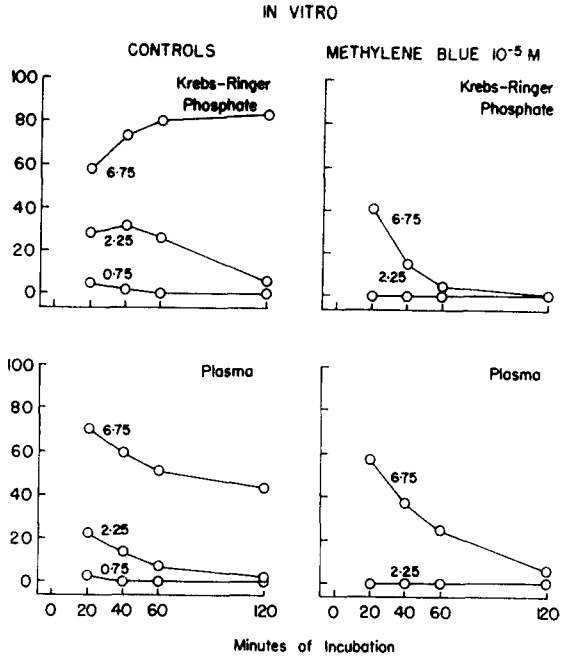

FIG. 2. The methemoglobin-forming properties of sodium nitrite *in vitro*. The four panels show the responses of various kinds of red cell suspensions to nitrite added at zero time to the final concentrations (mM) indicated by the numerals. The top two panels are red cell suspensions in K-R-PO_4-G, whereas the bottom two panels are cells in homologous "plasma" (see Methods). The top and bottom panels on the extreme right show the influence of preincubation for 15 min with 1×10^{-5}M methylene blue before the addition of nitrite.

Fig. 1 shows that the pattern of the methemoglobinemic response to sodium nitrite *in vivo* is closely mimicked by red cell suspensions in K-R-PO_4-G (Fig. 2). The response may be somewhat attenuated in diluted whole blood (control "Plasma" panel of Fig. 2). Fig. 3 shows the results of an identical series of experiments where phenylhydroxylamine was used to generate methemoglobin. Since phenylhydroxylamine is more potent than nitrite, its absolute concentrations are 10-fold lower in order to produce comparable methemoglobin concentrations. As previously reported[2]

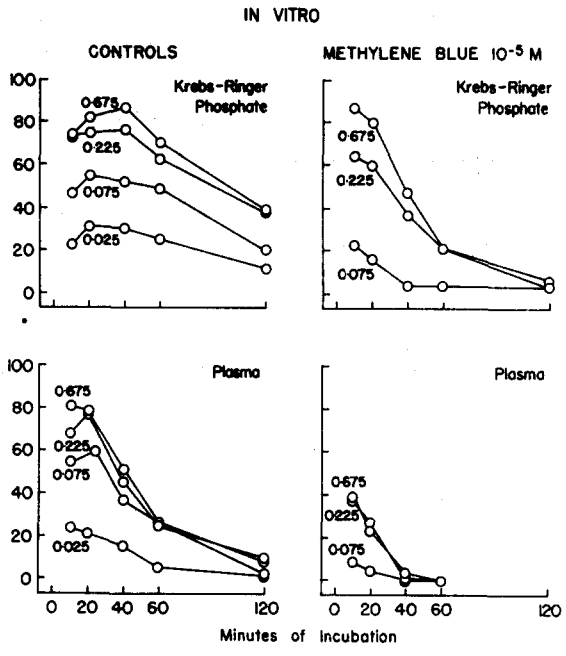

FIG. 3. The methemoglobin-forming properties of phenylhydroxylamine *in vitro*. See legend to Fig. 2.

equivalent levels of methemoglobin generated by phenylhydroxylamine persist longer in K-R-PO_4-G red cell suspensions than *in vivo*. The diluted whole blood response to phenylhydroxylamine (control "Plasma" panel of Fig. 3) seems to be intermediate between these two responses (also compare Fig. 1). This finding suggests that either a plasma factor in mouse blood accelerates methemoglobin reductase activity or that some constituent of K-R-PO_4-G inhibits it.

Irrespective of whether the methemoglobin was generated by nitrite or by phenylhydroxylamine or whether cells were incubated in K-R-PO_4-G or plasma, the presence of methylene blue attenuates the methemoglobinemic response (top and bottom panels on the right in Figs. 2 and 3). The effect of methylene blue on phenylhydroxylamine-generated methemoglobin is perhaps less dramatic than on nitrite-generated methemoglobin.

In separate experiments, the addition of 4 mg/ml crystalline sodium heparin or of 27 mg/ml bovine serum albumin to mouse red cell suspensions in K-R-PO_4-G did not change the response to phenylhydroxylamine from that of control incubations in which these substances were omitted. The concentration of bovine serum albumin added was the total Biuret protein equivalent of blood as diluted to produce a heme concentration of 5·0 mM. When shed blood was diluted with iso-osmotic buffer, pH 7·4, to a final added phosphate concentration of 6mM (equivalent to K-R-PO_4-G), the response to phenylhydroxylamine appeared essentially the same as in blood diluted with saline. Although not shown singly, the results of these and similar experiments are pooled in Table 2 by arbitrarily assigning them to two categories: (1) a sustained response like that in K-R-PO_4-G, or (2) an attenuated response like that in plasma. In a sustained response (K-R-PO_4-G or its equivalent) the net methemo-

TABLE 2. PHENYLHYDROXYLAMINE-GENERATED METHEMOGLOBINEMIA IN MOUSE ERYTHROCYTES SUSPENDED IN K-R-PO_4-G OR HOMOLOGOUS "PLASMA"*

Media	Phenyl-hydroxylamine (mM)	No. of Experiments	Per cent methoglobin ± S.D.		Net Methoglobin reduction†
			10 min	120 min	
K-R-PO_4-G	0·675	6	74 ± 5	39 ± 17	38
or its equivalent	0·225	6	73 ± 10	38 ± 4	38
"Plasma"	0·675	7	81 ± 8	10 ± 6	77
or its equivalent	0·225	7	67 ± 14	8 ± 6	64

* Experiments conducted as in Fig. 2. For definition of "plasma" see Methods section.

† Expressed as the per cent methemoglobin reduced per 2 hr of incubation as extrapolated from the mean difference between 10-min and 120-min values.

globin reduction in a 2-hr incubation is about half of the net reduction in plasma or its equivalent.

Fig. 4 shows an alteration in the response of mouse red cells to phenylhydroxylamine when suspensions in Krebs–Ringer-bicarbonate-glucose (K-R-HCO_3-G) are compared with suspensions in K-R-PO_4-G. The major effect of K-R-HCO_3-G is seen during the

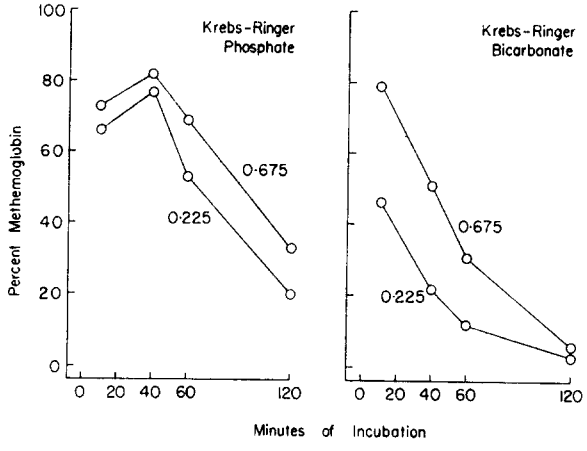

FIG. 4. The responses *in vitro* of mouse red cells to phenylhydroxylamine. On the left cells were suspended in K-R-PO_4-G; on the right, in K-R-HCO_3-G (see Table 1). Numerals show the final concentrations of phenylhydroxylamine (m-mole/l.) added at zero min.

initial time period of the incubation. After 40 min in K-R-PO_4-G, rates of methemoglobin reduction are roughly equivalent to those in K-R-HCO_3-G. These results suggest that a component of K-R-PO_3-G inhibits or delays the achievement of maximal rates of reductase activity and that bicarbonate can antagonize or overcome this effect. Since bicarbonate is well known to exchange for intra-erythrocytic chloride, these observed alterations in methemoglobin reductase activity suggested a link with the chloride shift.

Table 1 shows that both Krebs–Ringer incubation media used in these experiments contain 140 m-Equiv chloride/l. Heparinized mouse plasma samples from 5 animals gave a mean chloride concentration (± S.D.) of 99 ± 4·2 m-Equiv/l. The extracellular

chloride concentrations of the incubation media are therefore higher than that *in vivo* or in shed blood incubations even when the blood was diluted with physiological saline. Mouse blood is about 9·0 mM in heme. For these experiments it was diluted to 5·0 mM with 150 mM sodium chloride. The resulting chloride concentration in such a dilution is between 120 and 125 mM. The sustained methemoglobinemic response to phenylhydroxylamine in K-R-PO_4-G (Fig. 3), therefore, may represent the net result of the absence of bicarbonate and the presence of an abnormally high extracellular chloride. concentration.

Figs. 1–3 show that the difference in rates of reductase activity in K-R-PO_4-G as opposed to rates in mice or in mouse whole blood is not as evident when nitrite is used to generate methemoglobin. The more sustained methemoglobinemia produced by nitrite relative to other oxidants has been noted previously[2] and may be due, at least in part, to the transient formation of a nitrite–methemoglobin complex.[8] Thus, the mere presence of nitrite may be a more important determinant of the red cell response to nitrite than the influences of bicarbonate and chloride demonstrable with phenylhydroxylamine. Even nitrite complexed with methemoglobin is easily dissociated by washing nitrited red cells.[8] In all subsequent experiments mouse red cells in K-R-PO_4-G were incubated with nitrite for 2 hr; the methemoglobin concentration of this suspension was measured and then aliquots of this mixture were washed at least three times with fresh buffer solutions before reincubation.

Fig. 5 shows the results of such an experiment in which mouse erythrocytes previously exposed to 6·75 mM nitrite in K-R-PO_4-G were washed with fresh buffers

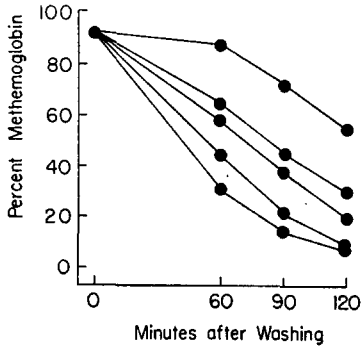

FIG. 5. Methemoglobin reductase activity in media of various extracellular chloride concentrations Mouse red cells were preincubated in K-R-PO_4-G with 6·75 mM nitrite for 2 hr (methemoglobin level shown at zero min). Aliquots of this reaction mixture were then washed three times with fresh media in which various concentrations of gluconate were substituted for chloride. The chloride concentrations of these media reading from top to bottom were 140, 70, 35, 18 and 0 m-equiv. liter·

in which various amounts of chloride were replaced by gluconate. Although again the major effect is seen during the first hour after washing, it is clear that methemoglobin reductase activity in the absence of bicarbonate is exquisitely sensitive to the extracellular chloride concentration. Maximal rates of reductase activity were achieved in a medium in which the chloride of K-R-PO_4-G was totally replaced by gluconate.

When the extracellular osmolality of both media was 290 mOsm, the hematocrit to hemoglobin ratio in K-R-PO_4-G was 1·3, but in K-R-PO_4-G in which chloride was totally replaced by gluconate the ratio was 1·0. These results indicate that gluconate

penetrates into mouse red cells rather poorly; the 22 per cent decrease in cellular volume presumably reflects a loss of water. Similar experiments performed with human cells resulted in a 13 per cent decrease in red cell volume. If nitrate is substituted for chloride in K-R-PO_4-G, the hematocrit to hemoglobin ratio changes from 1·18 to 1·16, representing a change in mean cell volume of less than 2 per cent. As shown in Fig. 6, however, substitution of nitrate for chloride does not increase reductase activity as does substitution of gluconate for chloride.

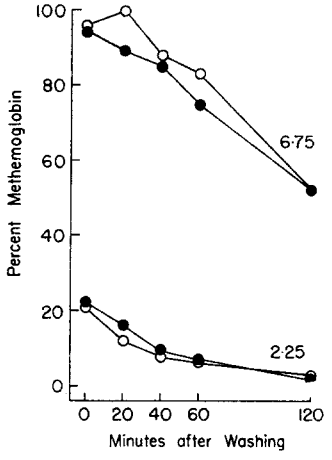

FIG. 6. Methemoglobin reductase activity in K-R-PO_4-G (●), or in a modified K-R-PO_4-G in which nitrate was totally substituted for chloride (○). The numerals refer to nitrite concentrations (mM) originally used to generate the methemoglobin in cells in K-R-PO_4-G. At zero min, however, aliquots were washed three times with fresh media of the indicated composition.

To demonstrate that reductase activity is not dependent on red cell volume *per se*, a series of K-R-PO_4-G media were prepared to which were added various concentrations of raffinose. A control red cell suspension in K-R-PO_4-G (285 mOsm) had a hematocrit to hemoglobin ratio of 1·1. A similar suspension with added raffinose (508 mOsm) had a hematocrit to hemoglobin ratio of 0·8, representing a decrease in mean cell volume of 28 per cent. Methemoglobin reductase activity in these two preparations was equal, indicating that it was unrelated to red cell volume *per se*. In the absence of other evidence, these results are consistent with an inhibitory action of chloride on methemoglobin reductase activity. Presumably this effect of prolonging a methemoglobinemia is shared by nitrate.

Fig. 7 shows that in a chloride-free K-R-PO_4-G medium 10 mM lactate is able to support methemoglobin reduction in mouse red cells as well as, or even better than, 10 mM glucose.

The results of Fig. 4 indicate that bicarbonate is able to overcome partially or at least to modify the "reductase inhibitory" effect of chloride. The results of a more detailed examination of this question are summarized in Table 3. Here, estimates of reductase activity, expressed as the net per cent methemoglobin reduced in 2-hr incubations, are given for media containing various concentrations of chloride and bicarbonate. It can be seen that when the extracellular concentration of chloride is zero the addition of bicarbonate (equilibrated with an appropriate carbon dioxide

tension) does not result in increases in reductase activity. Presumably, rates of methemoglobin reduction are already maximal in the absence of chloride; the mean methemoglobin reductase activity for these six experiments was 80 per cent in 2 hr (S.D. = ± 5·1 per cent). Increasing concentrations of chloride in the absence of

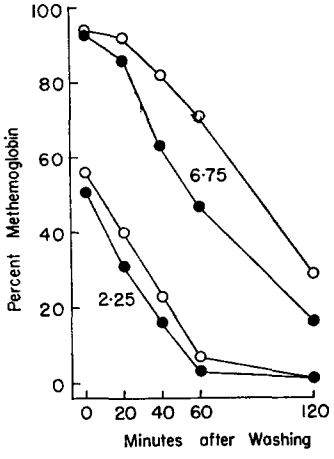

FIG. 7. Methemoglobin reductase activity in a modified K-R-PO_4-G in which gluconate was totally substituted for chloride. The substrate was either glucose (○) or the same molar concentration of lactate (●). See also legend to Fig. 5.

TABLE 3. NET METHEMOGLOBIN REDUCTIONS IN 2-HR INCUBATIONS IN KREBS–RINGER SOLUTIONS OF VARIOUS CHLORIDE AND BICARBONATE CONCENTRATIONS*

(Cl^-) (m-Equiv/l.)	(HCO_3^-) (m-Equiv/l.) 0	10	25	50
0	71 85	78	82 84	80
18	83			
35	73			
50			68	
70	63			
95	47		62	
120		58		75
140	48 39		47 48	

* Methemoglobin originally generated by exposing mouse red cells in K-R-PO_4-G to 6·75 mM sodium nitrite for 2 hr. After a methemoglobin determination, aliquots of this reaction mixture were washed three times with fresh buffers of the indicated composition. Phosphate was substituted for bicarbonate, and gluconate was substituted for chloride to produce equivalent osmolaties.

bicarbonate, as already demonstrated in Fig. 5, result in decreased rates of methemoglobin reduction. A maximal effect appears to be reached at chloride concentrations above 95 m-Equiv/l. i.e. the normal extracellular chloride concentration of mouse plasma appears to be critical. At critical chloride concentrations (95–120 m-Equiv/l.) bicarbonate seems to have an ability to reverse chloride inhibition. The bicarbonate effect also reaches a maximum at its normal physiological concentrations in plasma (25 m-Equiv/l.). Bicarbonate does not appear to be able to reverse the inhibitory effects of chloride when the chloride extracellular concentration is as high as 140 m-Equiv/l.

DISCUSSION

A previously reported experimental observation,[2] namely that the methemoglobinemic response to phenylhydroxylamine is more sustained in a Krebs–Ringer-phosphate-glucose medium than *in vivo* or in shed blood, has been traced to differences in extracellular ionic concentrations. Spontaneous methemoglobin reductase activity in mouse erythrocytes has been demonstrated to be sensitive to the extracellular chloride concentration. The K-R-PO_4-G medium employed in the original observation contained 140 m-Equiv. chloride/l., a concentration that is 40 per cent higher than normal mouse plasma (reported here as $99 \pm 4{\cdot}2$ m-Equiv/l.). This concentration difference in chloride alone might be sufficient to result in a detectable difference in rates of methemoglobin reduction (Fig. 5). But, this difference is further magnified *in vivo* and in shed blood by the presence of bicarbonate, which at critical chloride concentrations can partially reverse the inhibitory effects of chloride (Table 3). Originally it was assumed that these effects were unique to phenylhydroxylamine generated methemoglobin (compare Figs. 1–3). If nitrited red cells are washed after methemoglobin formation, these ionic influences can be demonstrated. Perhaps the presence of nitrite is a major determinant of the overall response of red cells in a nitrite-generated methemoglobinemia.[8]

Although not demonstrated here, it is presumed that decreases in the extracellular chloride concentration, accomplished by substituting gluconate for chloride, result ultimately in decreases in the intracellular chloride concentration. It is well known that added extracellular bicarbonate rapidly exchanges with intracellular chloride in the so-called chloride shift.[9] Whereas an inhibitory action of chloride on methemoglobin reduction fits all observations presented here, it seems unlikely that bicarbonate directly stimulates methemoglobin reduction. Added bicarbonate does not further stimulate reductase activity in chloride-free media, and it is unable to reverse inhibition when the extracellular chloride concentration is as high as 140 m-Equiv/l. (Table 3). That the bicarbonate effect on reductase activity is manifested only at critical physiological concentrations of both chloride and bicarbonate further suggests that bicarbonate increases methemoglobin reduction by substituting for intracellular chloride.

This inhibitory effect of chloride, whatever its mechanism, is ultimately manifested through the NADH-linked methemoglobin reductase system (diaphorase). Mouse erythrocytes contain two methemoglobin reductase systems. The spontaneous or NADH-linked enzyme can utilize either glucose or lactate as a substrate.[1] The NADPH-linked enzyme is thought to have an absolute requirement for exogenously supplied methylene blue.[3] It also needs a substrate that can enter the pentose phosphate shunt.[1,3] Although the NADPH-reductase is not thought to be active in the

absence of methylene blue, it seemed possible that in past experiments its activity had been masked by the presence of chloride. A hypothesis which fits all the above observations is that chloride inhibits the NADPH-reductase instead of the spontaneously active enzyme. In the absence of chloride, both enzyme systems may be functional to accelerate reductase activity. A corollary to this hypothesis is a novel explanation for the mechanism of action of methylene blue in reversing methemoglobinemia, namely that it antagonizes the reductase-inhibitory action of chloride. If chloride inhibits the NADPH-reductase, this inhibitory influence would be unmasked in a chloride-free medium. If the resulting increase in reductase activity were due to activation of the NADPH-enzyme, glucose, which is available to the shunt, should produce faster rates of methemoglobin reduction than lactate, which can only generate NADH. The results of Fig. 7 do not support this hypothesis. If there is any real difference between these two substrates in a chloride-free medium, lactate supports methemoglobin reduction better than glucose. Apparently chloride inhibition is manifested through the NADH-linked enzyme.

Chloride may inhibit some step in the glycolytic sequence to produce ultimately a critical depletion of NADH, or it could directly inhibit methemoglobin reductase. Chloride inhibition of enzymatic activity in the concentrations used here is not unknown.[10] Preliminary experiments indicate that chloride also inhibits methemoglobin reduction in rabbit erythrocytes. This species also has unusually brisk spontaneous reductase activity.[1] It has not yet been possible, however, to demonstrate conclusive acceleration of methemoglobin reductase activity in human erythrocytes in chloride-free media.

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