Cobalt Lysis and Methemoglobin Accumulation in Human Erythrocyte Suspensions[1] (35345)

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Because methemoglobin accumulates in human red cell suspensions exposed to about 8 m*M* cobaltous chloride, it has been suggested that this metal inhibits the spontaneously active, intraerythrocytic enzyme, methemoglobin reductase (1). A similar explanation has been offered for the unusually persistent protection against acute cyanide poisoning in mice pretreated with sodium cobaltinitrite (2). If the cobalt moiety of this complex inhibited methemoglobin reductase in the erythrocytes of treated animals, the methemglobinemia due to its nitrite content would be more persistent than that induced by nitrite alone, as with sodium nitrite. It has been shown, however, that a cobaltinitrite–methemoglobinemia is not more prolonged than a nitrite–methemoglobinemia where equivalent peak blood levels were generated in mice (3). Many others have demonstrated protective effects of certain salts and chelates of cobalt against cyanide. At least cobaltous chloride and cobaltinitrite appear to have direct effects which cannot be entirely ascribed to methemoglobin formation (3). Thus, the prolonged protective effects of cobaltinitrite can be ascribed to the persistence of cobalt in treated animals instead of the persistence of methemoglobin (3).

Experiments with rabbit and mouse erythrocyte suspensions indicated that significant inhibition of methemoglobin reductase activity occurred only at concentrations of cobaltous chloride which also produced gross hemolysis (3). Since hemolysis alone virtually abolishes methemoglobin reductase activity [*e.g.*, (4)], it appeared likely that methemoglobin accumulation was secondary to hemolysis rather than to a specific inhibition of methemoglobin reductase by cobalt. A hemolytic effect of cobalt could equally well explain all previously reported observations (1) except one, namely, that methemoglobin accumulation in the presence of cobalt was intensified by low oxygen tensions. The opposite was said to be true of methemoglobin accumulation in control red cell suspensions and in suspensions exposed to either *p*-aminophenol or methylene blue. If hemolysis were the only determinant of methemoglobin accumulation in erythrocyte suspensions exposed to cobalt, one would predict that the lytic activity of the metal would be intensified at low oxygen tensions.

Materials and Methods. Pooled human blood samples originally drawn into K_3HEDTA were centrifuged and the plasma and buffy coat were removed by aspiration. The cells were washed 3 times in 5 vol of isotonic saline and the final supernatants were discarded. Two different incubation media were employed in these experiments, but both contained 0.5 mg/ml of sodium penicillin G and of streptomycin sulfate. One buffer was 0.1 *M* sodium phosphate, pH 7.4, with 200 mg/100 ml of glucose. Sucrose was used to adjust the osmolality to 280 mOsm. The other buffer was a slightly modified Krebs–Ringer–bicarbonate–glucose (KRBG) also at 280 mOsm and pH 7.4, when equilibrated with the appropriate carbon dioxide gas phase. Three ml of washed, packed red cells were mixed with 3 ml of buffer and either 0.15 ml of cobaltous chloride solution

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TABLE I. Final pH of Incubation Mixtures.

Gas phase	Buffer	pH		*p* value
		Control	Cobalt	
95% O_2–5% CO_2	Bicarbonate	6.4 ± 0.06	6.3 ± 0.02	<0.05
95% N_2–5% CO_2	Bicarbonate	6.4 ± 0.08	6.4 ± 0.07	—
	p value	—	—	
100% O_2	Phosphate	6.9 ± 0.20	6.8 ± 0.09	—
100% N_2	Phosphate	7.0 ± 0.05	6.9 ± 0.02	<0.02
	p value	—	—	

(4 g/100 ml of water to give a final concentration in the suspension of 8.0 m*M*) or 0.15 ml of physiological saline. These mixtures in 50-ml Erlenmeyer flasks were incubated 24 hr at 37° in a metabolic shaker. The gas phase for the phosphate buffer was either 100% O_2 or 100% N_2; whereas for the KRBG it was either 95% O_2–5% CO_2 or 95% N_2–5% CO_2. After incubation the pH values of the mixtures were determined with a glass electrode. The methemoglobin levels (5) were measured in whole suspensions, supernatants after centrifugation, and in 100-μl aliquots of packed intact cells after 3 saline washes. The total extracellular pigment was also determined as cyan-methemoglobin (5). Four separate cell suspensions were each evaluated in the two different buffers under all conditions in completely paired experimental designs. The results were compared statistically by paired samples *t* tests.

Results. Table I summarizes the mean (± SD) pH values after incubation for 24 hr under the conditions shown. The bicarbonate buffer was obviously less effective in maintaining the starting pH over the 24-hr incubation than was the phosphate buffer. Although the pH dropped an entire unit in bicarbonate buffer, the same magnitude of change occurred under all four experimental conditions. Only one of the four cited comparisons in bicarbonate buffer was significant, namely, the difference between control and cobalt-treated mixtures under 95% O_2–5% CO_2. With phosphate buffer the pH fell only 0.4 to 0.6 unit. Of the four comparisons in phosphate buffer only the difference between control and cobalt-treated mixtures under 100% N_2 was significant. Shen *et al.* (1) incubated defibrinated blood for 24 hr under 95% O_2–5% CO_2, but final pH values were not reported.

Table II indicates the mean (± SD) methemoglobin levels as a percentage of the total hemoglobin in the incubation mixture after 24 hr. The addition of cobalt to bicarbonate buffer resulted in significant increases in the methemoglobin levels whether the suspensions were incubated under O_2 or under N_2. However, as also shown in Table II, incubation under N_2 even without cobalt resulted in increased levels of methemoglobin. Although this difference due to the gas phase was not significant in bicarbonate buffer, it

TABLE II. Percentage of Methemoglobin in Incubation Mixtures.

Gas phase	Buffer	Methemoglobin (%)		*p* value
		Control	Cobalt	
95% O_2–5% CO_2	Bicarbonate	3.9 ± 1.0	6.0 ± 0.6	<0.02
95% N_2–5% CO_2	Bicarbonate	5.7 ± 1.4	10.1 ± 0.7	<0.01
	p value	—	<0.01	
100% O_2	Phosphate	2.8 ± 0.2	3.6 ± 0.3	<0.02
100% N_2	Phosphate	6.2 ± 1.0	4.8 ± 1.0	—
	p value	<0.01	—	

TABLE III. Percentage Methemoglobin in Washed Red Cells from Incubation Mixtures.

Gas phase	Buffer	Methemoglobin (%) Control	Cobalt	p value
95% O_2–5% CO_2	Bicarbonate	2.6 ± 0.5	3.2 ± 0.5	—
95% N_2–5% CO_2	Bicarbonate	3.5 ± 2.0	5.8 ± 1.3	—
	p value	—	—	
100% O_2	Phosphate	2.0 ± 0.4	0.9 ± 0.8	—
100% N_2	Phosphate	5.6 ± 0.6	4.9 ± 1.3	—
	p value	<0.01	<0.01	

was highly significant in phosphate buffer. In bicarbonate buffer, cobalt and nitrogen appear to potentiate each other, but in phosphate buffer the potentiation did not occur.

Table III compares the intracellular levels of methemoglobin as a percentage of the total intracellular blood pigment in washed red cells from these same incubation mixtures. Most comparisons in Table III were not significant, but one exception was the effect of N_2 in phosphate buffer which led to significantly more intracellular methemoglobin than paired mixtures under O_2. This effect appeared to be independent of the presence of cobalt.

Table IV lists methemoglobin levels as a percentage of the total extracellular pigment. All incubation mixtures had visible evidence of hemolysis. The results of the methemoglobin determinations in Table IV show considerably more variation than the results in Tables II and III. In part, this variation may be due to the low concentrations of blood pigment involved and the sensitivity limits of the assay. Most comparisons in Table IV were not significant, but in bicarbonate buffer a smaller percentage of the extracellular pigment appeared to be in the form of methemoglobin in the presence of cobalt than in its absence. In phosphate buffer there appeared to be a trend toward higher methemoglobin levels under N_2 than under O_2, but these differences were not significant.

Table V indicates the percentage hemolysis in various incubation mixtures as computed from the ratios of the final total hemoglobin (heme pigment) concentration of the extracellular fluid to the final total hemoglobin concentration of the incubation mixture. In bicarbonate buffer the data in Table V clearly indicate that O_2 produces more hemolysis than N_2 whether or not cobalt is present. However, cobalt and O_2 appear to potentiate each other's lytic effects, whereas under N_2, cobalt did not provoke a significant degree of hemolysis. In phosphate buffer the hemolytic effects of cobalt were significant irrespective of the gas phase, and the hemolytic effects of O_2 were significant only in the absence of cobalt. The major difference between bicarbonate and phosphate buffers in terms of hemolysis was an apparent absence of the cobalt–oxygen potentiation in the latter.

The actual methemoglobin concentrations

TABLE IV. Percentage Methemoglobin in Supernatants from Incubation Mixtures.

Gas phase	Buffer	Methemoglobin (%) Control	Cobalt	p value
95% O_2–5% CO_2	Bicarbonate	40 ± 8	18 ± 3	<0.05
95% N_2–5% CO_2	Bicarbonate	38 ± 8	14 ± 6	<0.05
	p value	—	—	
100% O_2	Phosphate	14 ± 7	19 ± 5	—
100% N_2	Phosphate	49 ± 35	37 ± 7	—
	p value	—	—	

TABLE V. Percentage Hemolysis in Incubation Mixtures.

Gas phase	Buffer	Hemolysis (%)		p value
		Control	Cobalt	
95% O_2–5% CO_2	Bicarbonate	1.6 ± 0.7	18.0 ± 1.0	<0.001
95% N_2–5% CO_2	Bicarbonate	0.1 ± 0.03	0.2 ± 0.04	—
	p value	<0.05	<0.001	
100% O_2	Phosphate	0.7 ± 0.1	3.1 ± 0.2	<0.01
100% N_2	Phosphate	0.4 ± 0.2	2.4 ± 1.1	<0.05
	p value	<0.01	—	

in mmoles of methemoglobin heme/liter of extracellular fluid are listed in Table VI. From these results, it is clear that the presence of cobalt always results in a greater extracellular methemoglobin concentration. These results occurred in the face of an apparently lower percentage of methemoglobin in extracellular bicarbonate buffer (Table IV) because of the intensity of the cobalt-induced hemolysis (Table V). In bicarbonate buffer (Table VI), cobalt produced higher methemoglobin concentrations under O_2 than under N_2, a result which also parallels the intensity of hemolysis shown in Table V.

Discussion. The marked differences in results between bicarbonate and phosphate buffers was puzzling until it was noted that the addition of cobaltous chloride to phosphate buffer resulted in the slow formation of a precipitate, presumably cobaltous phosphate (6). Cobaltous phosphate is described in standard reference works as "insoluble" in water, but no data were found in regard to its solubility product under conditions employed here. No precipitation occurred in bicarbonate buffer. The results obtained in the phosphate buffer must, therefore, represent the exposure of red cells to a lower concentration of cobalt than in the case of bicarbonate buffer.[3] That cobalt was not totally inactivated by precipitation is strongly suggested by the significant effects in phosphate buffer shown in Tables II, V, and VI. It has been previously observed that human red cells can tenaciously bind cobalt and may even compete with phosphate for this metal (7).

The premise behind these experiments was that methemoglobin accumulation in human red cell suspensions exposed to cobalt was a nonspecific response secondary to hemolysis instead of a specific inhibition of methemoglobin reductase activity as previously suggested (1). Although the mechanism remains obscure, it has been known for many years that methemoglobin reductase activity is virtually abolished by hemolysis [*e.g.*, (4, 8, 9)]. Because hemoglobin has a certain tendency toward spontaneous oxidation to methemoglobin, the latter always accumulates more rapidly in lysates than in comparable intact cell suspensions. It was previously demonstrated (1) that cobaltous chloride did not influence the rate of oxidation of crystalline oxyhemoglobin in solution.

In accord with the results of Shen *et al.* (1), Table II shows that in all cases except one (N_2, phosphate) the addition of cobalt led to the accumulation of significant levels of methemoglobin in human red cell suspensions. Also in accord with Shen *et al.* (1), methemoglobin accumulation in the presence of cobalt was greater under N_2 than under O_2 although this difference was significant only in the case of the bicarbonate buffer. In contrast to the results of Shen *et al.* (1), however, in the absence of cobalt, methemoglobin accumulation was greater under N_2 than under O_2 (although this difference was signifi-

[3] $^{60}CoCl_2$ in the same chemical concentration as that employed here was added to water or to 0.1 *M* phosphate buffer. The latter was centrifuged and filtered 1 hr after mixing; and the radioactivity of the filtrate was compared with that in plain water. The filtrate from phosphate buffer had lost 80% of its radioactivity. Thus the free cobalt concentration in phosphate buffer at 1 hr is estimated to be 1.6 m*M* instead of the 8.0 m*M* in bicarbonate buffer.

TABLE VI. Methemoglobin Concentrations in Supernatants from Incubation Mixtures.

Gas phase	Buffer	Methemoglobin heme (mmoles/liter)		p value
		Control	Cobalt	
95% O_2–5% CO_2	Bicarbonate	0.04 ± 0.01	0.22 ± 0.05	<0.01
95% N_2–5% CO_2	Bicarbonate	0.04 ± 0.06	0.11 ± 0.03	<0.05
	p value	—	<0.05	
100% O_2	Phosphate	0.01 ± 0.008	0.04 ± 0.03	<0.05
100% N_2	Phosphate	0.01 ± 0.003	0.06 ± 0.02	<0.05
	p value	—	—	

cant only in the case of the phosphate buffer). Incubation under N_2 favors the accumulation of deoxyhemoglobin. Although still unexplained, it has been known for many years that deoxyhemoglobin has a greater tendency for auto-oxidation to methemoglobin than does oxyhemoglobin (10, 11). Thus, high concentrations of O_2 prevent methemoglobin accumulation by preventing formation of deoxyhemoglobin.

With respect to methemoglobin accumulation, therefore, O_2 and cobalt have effects in opposite directions. Cobalt induces hemolysis (Table V) which favors methemoglobin accumulation, but O_2 prevents methemoglobin accumulation by keeping the pigment in the form of oxyhemoglobin (Tables II and III). However, oxygen itself also has an additional effect, which favors the accumulation of methemoglobin, namely, O_2 also promotes hemolysis (Table V). Thus, in bicarbonate buffer (Table V) cobalt potentiates the hemolytic effect of oxygen more than 10-fold and oxygen potentiates the cobalt effect 90-fold. Yet, in terms of methemoglobin accumulation in the supernatant (Table VI), cobalt potentiates oxygen only 5-fold and oxygen potentiates cobalt only 2-fold. Presumably, the absence of a dramatic cobalt–oxygen interaction in phosphate buffer is due to partial inactivation of the added cobalt by precipitation with phosphate.

The data of Table IV are more troublesome to interpret. It is clear that in all cases a much greater proportion of the extracellular pigment is in the form of methemoglobin than is the intracellular pigment (Table III). In phosphate buffer N_2 appears to favor methemoglobin accumulation although the differences were not significant. However, in bicarbonate buffer, significant results suggest that cobalt prevents the accumulation of methemoglobin in extracellular fluid (Table IV). It is possible that these results are artifactual. It was previously observed that high concentrations of cobalt interact with methemoglobin in solution to form a blood pigment which has a decreased reactivity toward cyanide (7). Since the fraction of the extracellular pigment present as methemoglobin is determined on the basis of the decrease in absorbance at 635 mμ after the addition of cyanide, it is possible that the methemoglobin estimates in bicarbonate buffer of Table IV are erroneously low. Although cobalt may interfere in methemoglobin estimation, previous observations suggest that the metal has little influence on the determination of the total extracellular pigment as cyanmethemoglobin (7). Therefore, the estimates of hemolysis in Table V are probably more reliable than estimates of the extracellular methemoglobin concentration (Table IV and VI).

Because the extracellular concentration of methemoglobin (Table VI) in human red cell suspensions exposed to cobalt was always significantly higher than control suspensions, it seems likely that methemoglobin accumulation in the total incubation mixture (Table III) is largely an extracellular phenomenon secondary to hemolysis as already suggested (3). This view is further supported by the data in Table III, where no significant differences were found in the intraerythrocytic levels of methemoglobin between control cells and those exposed to cobalt. Since no evidence exists to show that methemoglobin

formation *per se* predisposes erythrocytes to hemolysis, one would expect a specific inhibitor of methemoglobin reductase to increase intracellular levels of methemoglobin. The only significant differences found in the intact cell (Table III) were in phosphate buffer and are ascribed to the effect of the gas phase instead of cobalt.

Summary. Cobaltous chloride in concentrations up to 8.0 m*M* in human red cell suspensions in a Krebs–Ringer–bicarbonate–glucose media produced significant hemolysis under O_2 but not under N_2. Both O_2 and cobalt have significant hemolytic effects and appear to greatly potentiate each other. Hemolysis favors methemoglobin accumulation in the extracellular fluid, but O_2 has an additional effect in the opposite direction. By keeping the hemoglobin saturated, oxygen tends to prevent auto-oxidation of the pigment to methemoglobin. Thus, methemoglobin accumulation in erythrocyte suspensions exposed to cobalt is a net result of several opposing effects, but no evidence was obtained to suggest that cobalt is a specific inhibitor of methemoglobin reductase.

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