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INHIBITION OF METHEMOGLOBIN AND METMYOGLOBIN REDUCTION BY COBALT

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Abstract—Because cobalt compounds tend to form stable complexes, there has been continued interest in the use of the salts and chelates of cobalt in cyanide poisoning, and continued uncertainty about the precise nature of their protective effects. We have found that cobalt ions inhibit the enzymatic reduction of both methemoglobin and metmyoglobin. Virtually total inhibition of methemoglobin and metmyoglobin reductase activity occurred with the addition of 2.5 mM cobalt acetate to the assay system. Both enzymes were inhibited by lower levels of cobalt in a dose-dependent manner. The similarity in susceptibility of cobalt inhibition is further evidence that the enzymes which reduce methemoglobin and metmyoglobin are functionally comparable. The inhibition of methemoglobin reductase activity may be, in part, responsible for the therapeutic effectiveness of cobalt salts and chelates in cyanide poisoning.

There has been continued interest in the therapeutic efficacy of cobalt salts in cyanide poisoning since 1894. The history of the use of various cobaltous antidotes has been reviewed briefly [1]. Despite a long history of use, and widespread investigation, the precise antidotal mechanism of cobalt remains uncertain.

In 1954, Shen *et al.* [2] demonstrated the formation of methemoglobin in normal human blood incubated in the presence of cobaltous chloride, and they concluded that cobalt inhibits the intracellular system which maintains hemoglobin iron in the ferrous state. These studies were performed before any of the putative methemoglobin reductases had been described. In more recent studies of suspensions of intact red blood cells, there was no evidence to suggest that cobalt is a specific inhibitor of methemoglobin reductase; however, specific assays of methemoglobin reductase activity were not performed [3]. Until such assays are performed, any action or lack of action on methemoglobin reductase activity which is ascribed to cobalt must remain speculative, and must be clearly distinguished from the evaluation of methemoglobin formation and reduction which occur in intact red blood cells.

In 1967, Hegesh and Avron [4, 5] described an active methemoglobin reductase in the erythrocyte which requires ferrocyanide for *in vitro* study. We have isolated from muscle a metmyoglobin reductase which actively reduces metmyoglobin *in vitro*, and have reported some of its properties and characteristics [6, 7]. The metmyoglobin reductase is similar to the methemoglobin enzyme described by Hegesh and Avron [4, 5] in that it requires ferrocyanide for *in vitro* activation, but otherwise the enzymes appear to be distinct entities [6]. The isolation of these two met-heme protein-reducing enzymes allows an evaluation of the effect of cobalt on both methemoglobin and metmyoglobin reductase activity. Therefore, we examined the effect of cobalt on the ferrocyanide activated methemoglobin and metmyoglobin reductases and found that cobalt inhibited the activity of these two enzymes *in vitro*. This paper summarizes our findings.

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MATERIALS AND METHODS

Materials and methods were the same as those previously reported [6, 7]. Hemoglobin and myoglobin substrates were prepared as previously described [6]. Bovine blood and cardiac muscle were used as the sources of crude enzyme and as the starting materials for the partially purified enzyme solutions. Other purification and analytical procedures were identical to those previously reported [6, 7] with the following exceptions.

The amounts of buffer in both the methemoglobin and metmyoglobin reductase assays were adjusted to keep the final pH values in the optimum range. A 0.1 M solution of cobalt acetate (pH 7.34) was used as the source of the cobalt ion.

Partially purified bovine metmyoglobin reductase, carried through the DEAE-cellulose step of purification, was utilized in these studies. This material had a specific activity of about 1000 units/mg protein which represents an 11-fold purification over the starting material [6].

Partially purified methemoglobin reductase was prepared as follows. At the slaughterhouse, whole beef blood was collected with the equivalent of 1.2 g of ammonium oxalate and 0.8 g of potassium oxalate added per liter to prevent clotting. The blood was placed on ice immediately. All subsequent procedures were carried out at 4°. The blood was centrifuged at 2000 *g* in a Sorvall RC-5 centrifuge (Dupont Co., Newton, CT) for 10 min; the plasma was separated and discarded. the remaining packed

red blood cells were washed with normal saline and recentrifuged twice at 2000 *g* for 10 min, following which the packed cells were frozen. After thawing, 300 ml of the resulting hemolysate was mixed with 700 ml of 0.001 M sodium phosphate buffer, pH 7.0, plus 80 g of DE 52 cellulose (Whatman, Inc., Clifton, NJ) which had been equilibrated previously with the same buffer. Following 15 min of gentle mixing, the mixture was centrifuged at 2000 *g* for 15 min. The supernatant fraction was discarded, and the precipitated DE 52 was resuspended in about 100 ml of 0.001 M sodium phosphate buffer, pH 7.0.

This suspension was poured into a 2 × 56 cm column and washed with the pH 7.0, 0.001 M sodium phosphate buffer until effluent protein was no longer detectable by measuring absorbance at 280 nm. At this point the column was washed with 0.01 M sodium phosphate buffer, pH 7.0, until effluent absorbance at 280 nm, which had risen sharply, returned to baseline levels. The column was then washed with 0.06 M KCl in 0.05 M citrate buffer, pH 5.2, with 0.00005 M EDTA, and 15 ml fractions were collected. Fractions containing measurable methemoglobin reductase activity were pooled, concentrated, and dialyzed against 0.03 M sodium phosphate buffer, pH 6.0.

A 2 × 19 cm column of Affi-gel Blue (Bio-Rad Laboratories, Richmond, CA) was equilibrated with 0.03 M sodium phosphate buffer, pH 6.0. A 6.3 ml sample of the enzyme solution was applied to the column and washed with the same buffer until the effluent absorbance returned to baseline levels. Elution was accomplished with a linear gradient formed by 350 ml of starting buffer plus 350 ml of the same buffer which contained 1.0 M NaCl and 0.001 M NADH. Fractions of about 13 ml were collected. Those containing enzyme activity were pooled, concentrated, and dialyzed to remove NADH. This partially purified methemoglobin reductase had a specific activity of 62 units/mg protein which represents an 11-fold purification over the starting material.

All spectrophotometric measurements were carried out in duplicate on a Beckman Acta M VII dual beam spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA) at room temperature (22–23°).

Table 1. Influence of cobalt on the enzymatic reduction of methemoglobin*

	Percent of change			
	Added cobalt (μmoles/ml)			
Enzyme†	0.1	1.0	1.5	2.5
1	−3	−36	−97	−100
2	−3	−6	−66	−99
3	+2	−14	−74	−98
4	+2	−6	−75	−100

* Values represent the percent change in activity from control values obtained without added cobalt.

† Sources of enzyme (control values, as nmoles of substrate reduces per min per mg protein): (1) red blood cells (5.5); (2) partially purified methemoglobin reductase (62.1); (3) heart muscle supernatant fraction (13.0); and (4) partially purified metmyoglobin reductase (87.8).

Table 2. Influence of cobalt on the enzymatic reduction of metmyoglobin*

	Percent of change			
	Added cobalt (μmoles/ml)			
Enzyme†	0.1	1.0	1.5	2.5
1	−13	−53	−92	−100
2	−3	−11	−72	−100
3	0	−10	−77	−97
4	+7	+11	−50	−99

* Values represent the percent change in activity from control values obtained without added cobalt.

† Sources of enzyme (control values, as nmoles of substrate reduced per min per mg protein): (1) red blood cells (19.2); (2) partially purified methemoglobin reductase (223); heart muscle supernatant fraction (42.6); and (4) partially purified metmyoglobin reductase (268).

RESULTS

The activities of the crude and partially purified enzymes against hemoglobin substrate are shown in Table 1. A dose response is clearly evident. The activities of the same enzyme sources against myoglobin substrate are shown in Table 2. Once again the dose response is obvious. Irrespective of the substrate, inhibition was apparent over a narrow range of cobalt concentrations, and was virtually complete when the concentration of cobalt in the assay system reached 2.5 mM. Because of the small number of experimental observations, the statistical significance of differences in the degree of inhibition of each of the various enzymes against each heme protein substrate cannot be ascertained. Despite these differences, the responses of both the crude and partially purified enzymes to the inhibitory effects of cobalt were similar, irrespective of whether methemoglobin or metmyoglobin was used as substrate.

DISCUSSION

Cobalt salts have been known for many years to be effective antidotes for cyanide poisoning [1, 8–10] and, because high concentrations of cobalt salts have their own toxicity, it has been suggested recently that as an antidote a cobalt chelate, such as dicobalt edetate, is as effective and is less toxic [11–14]. It is known that cobalt forms stable complexes with cyanide in the form of cobaltocyanides $[—CO(CN)_6]^{4-}$ and cobalticyanides $[—CO(CN)_6]^{3-}$ so that each mole of cobalt could bind up to six moles of cyanide [8]. If this relationship were maintained *in vivo*, then only relatively small amounts of cobalt would be required to counteract the usual levels of cyanide in cases of poisoning. The continued interest in the salts and chelates of cobalt is based, in part, on their ability to form these stable complexes with cyanide.

We chose cobalt acetate for these studies because its pH (7.34) required only a minimal adjustment in the amount of buffer necessary to keep the final pH of the enzyme assay in the optimum range, and because other cobalt salts may present solubility

problems [3]. Evans [8] used cobalt acetate for his studies in rabbits. Other investigators have used cobaltinitrite in mice [9, 10] and in dogs [1] and have found consistent protective effects against cyanide. Cobaltous chloride was ineffective in dogs [1], but it did protect mice against potentially lethal doses of sodium cyanide. These studies indicate that the effects in cyanide poisoning may be related to the specific cobalt salt and the species in which it is utilized.

Apart from their ability to form stable complexes, cobalt salts may have other effects which influence the outcome of cyanide poisoning. One such effect, which concerns the relationship between cobalt and methemoglobin levels, has received considerable attention and remains an issue of continuing controversy. Shen *et al.* [2] demonstrated the formation of methemoglobin in normal human blood incubated in the presence of cobaltous chloride, and they concluded that cobalt inhibits the normal intracellular reduction system which maintains hemoglobin iron in the reduced state. Goldenberg and Mann [9] found that mice, pretreated with cobaltinitrite, were protected against acute cyanide poisoning. They argued that the effectiveness of cobaltinitrite was due to prolongation of the nitrite-induced methemoglobinemia by cobalt. Smith [10] demonstrated that mice which had been pretreated with cobaltous chloride or sodium cobaltinitrite were protected against potentially lethal doses of sodium cyanide. Only in the case of cobaltinitrite was the protective mechanism ascribed to the formation of methemoglobin. He concluded that cobaltous chloride did not inhibit methemoglobin reductase activity in the intact erythrocyte, and did not increase methemoglobin levels in mice. He attributed the protective effect of cobaltous chloride to hemolysis. His data clearly indicate that cobaltous chloride prolongs the methemoglobin induced by *p*-aminopropiophenone. Irrespective of whatever direct chemical relationship exists between cobalt and cyanide, it can be argued that Smith's results are due to inhibition of methemoglobin reductase by cobaltous chloride.

Other inferential evidence supports the argument against any relationship between cobalt and methemoglobin reduction. Shen *et al.* [2] cite the absence of increased methemoglobin levels in the blood of patients and animals treated with cobalt. In one such study [15], rats were treated with approximately 1 mg of cobalt daily for 20 weeks. The authors concluded that the methemoglobin levels in the cobalt-treated animals were not different from the controls. Careful inspection of the data reveals an apparent elevation of methemoglobin levels in the cobalt-treated animals and scatter (due to methodology) which hampers interpretation. Furthermore, the possibility of differences in the effects of acute and chronic cobalt administration on methemoglobin reduction was not addressed. There are species-related differences in the rates of methemoglobin reduction [16] and in ferrocyanide-activated methemoglobin reductase (L. Hagler, unpublished observations). The high rates of methemoglobin reduction in rodents may not have been considered in the interpretation of many of the foregoing studies. Finally, it can be argued that the effects of specific activators or inhibitors on enzyme activity can best be evaluated in well-defined biochemical systems in which numerous, possibly confounding, reactions are not present to obscure or preclude correct interpretation.

It therefore appeared worthwhile to evaluate the relationship between cobalt and methemoglobin reduction by performing the appropriate studies with a known, specific, methemoglobin reductase. It is clear from the results that, despite whatever other effects cobalt might exert, it does specifically inhibit the enzymatic reduction of methemoglobin *in vitro*. Cobalt salts and chelates do ionize to variable degrees, and cobalt binds to red cell membranes and penetrates the red cell to bind to hemoglobin [17]. Cobalt levels in red blood cells are from two to twenty times greater than in plasma [18, 19]. Smith [3, 10] has argued that methemoglobin accumulation in red cell suspensions exposed to cobalt was a non-specific response secondary to hemolysis instead of a specific inhibition of methemoglobin reductase as previously suggested [2]. It is possible, although unproven, that a level of cobalt sufficient to inhibit methemoglobin reductase activity in intact red cells might result from conventional therapeutic doses. Smith's argument cannot be applied to our findings, since there can be no effect due to hemolysis in an *in vitro* system in which hemolysis precedes measurement of enzyme activity. Our *in vitro* data confirm the results of Shen *et al.* [2] demonstrating cobalt inhibition of methemoglobin reduction.

It is clear from the data reported herein that methemoglobin reductase activity is inhibited by cobalt *in vitro*. It is by no means clear that such as inhibitory effect occurs or plays any significant role in the antidotal effect of cobalt on cyanide poisoning *in vivo*. The simultaneous occurrence of numerous, possibly confounding reactions (which are eliminated by study of an isolated enzyme system *in vitro*), represents the real state of affairs *in vivo*. From what is known about the chemistry of cyanide, hemoglobin, and cobalt, the inhibition of methemoglobin reduction could be an effective antidotal mechanism and would be in keeping with our experimental results. Whether or not cobalt acts as an antidote for cyanide poisoning by inhibition of methemoglobin reductase *in vivo* remains uncertain.

The inhibition of metmyoglobin-reducing activity by cobalt is not surprising in view of the similarities of methemoglobin and metmyoglobin reductase. Studies of these two enzymatic activities confirm their many similarities as well as their distinct differences ([6, 7] and unpublished observations). Cobalt excess is not associated with any well-defined entity related to muscle dysfunction with the possible exception of cardiomyopathy in heavy beer drinkers [20]. In cyanide poisoning, any effect of therapeutic doses of cobalt on muscle would undoubtedly be masked by the more prominent symptoms related to the underlying toxic state.

The physiological and biochemical effects of cobalt have been reviewed recently [21]. The current understanding of the actions of cobalt includes effects on a number of enzyme systems as measured *in vitro*. The significance of these *in vitro* effects in intact organisms is not known; the effects are currently under investigation [21]. While the significance of cobalt inhibition of methemoglobin and metmyoglobin reductase activity remains unknown, particularly in regard to cyanide poisoning, the inhibition by cobalt *in vitro* can be added to the known effects.

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