METHEMOGLOBIN FORMATION IN HUMAN BLOOD BY COBALT *IN VITRO* [1]

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Although the erythropoietic effect of cobalt has been amply demonstrated in experimental animals (1–3), in normal human beings (4, 5), and in certain types of anemia (4, 6–8), the mechanism of this increased blood formation has not been elucidated. No evidence has been adduced that administration of cobalt causes a decrease in oxygen capacity (9) or alters the oxygen dissociation curve (10, 11) in the blood either of experimental animals or of human beings. It has been suggested, therefore, that cobalt interferes with the cellular oxidative processes of the marrow, or of some other hypothetical regulatory center for erythropoiesis, thus producing a histotoxic anoxia (4), leading in turn to increased erythropoietic activity.

Some support for this hypothesis is offered by observations which show that cobalt induces the formation of reversible complexes of oxygen with histidine or cysteine (12, 13). Moreover, Barron and Barron (14) have shown that the addition of cobalt *in vitro* diminishes the oxygen consumption of suspensions of red cells derived from rabbits made polycythemic by the previous administration of cobalt. These authors and Davis (15) have also demonstrated that the development of cobalt-induced polycythemia is prevented by the simultaneous administration of ascorbic acid, a reducing agent known to be effective in directly reducing methemoglobin to hemoglobin (16). It seems possible, then, that cobalt may exert its histotoxic effect by blocking the enzymatic reducing systems of cells, thus interfering with the utilization of oxygen by the cells.

In normal intact erythrocytes *in vitro* methemoglobin is reconverted to hemoglobin spontaneously at the rate of 1.2 gm. per 100 cc. of blood per hour (17) by an enzymatic reducing system within the cells involving glucose and glycolysis (18–21). Consequently, it was considered appropriate to examine the action of cobalt in relation to the formation of methemoglobin in intact erythrocytes and to determine whether any methemoglobin thus formed was a result of direct oxidation of hemoglobin by cobalt or of an inactivation of the normal methemoglobin reducing mechanism.

METHODS

Quantitative estimation of the methemoglobin in blood was performed by the method of Evelyn and Malloy (22). Examination of the pigments in blood was made with the Beckman Spectrophotometer, Model B. The pH of the blood was measured with a glass electrode on the Beckman pH Meter. Blood sugar was determined by the method of Somogyi (23).

REAGENTS

Cobaltous chloride. "Baker's Analyzed" $CoCl_2 \cdot 6H_2O$; 4 gm. of the crystals were dissolved in 100 ml. of distilled water. The solution was autoclaved before use.

p-Aminophenol. Eastman Kodak Co.; 25 mg. were dissolved in 50 ml. of 0.85 gm. per cent NaCl. This 50 mg. per cent solution was freshly prepared immediately before use.

Ascorbic acid. Hoffmann-LaRoche, Inc. Vitamin C, Injectable; 1 ml. containing 250 mg. was diluted with 9 ml. of 0.85 gm. per cent NaCl to give a 2.5 gm. per cent solution. This dilution was freshly prepared immediately before use.

Methylene blue. National Aniline Pharmaceutical Laboratories; 100 mg. of this powder were dissolved in 100 ml. of 0.85 gm. per cent NaCl. This 0.1 gm. per cent solution was then diluted 1:9 with 0.85 gm. per cent NaCl to give an 0.01 gm. per cent solution.

Glucose. "Baker's Analyzed" Dextrose; 5 gm. were dissolved in 100 ml. of distilled water.

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Hemoglobin crystals (24). The red cells from 25 ml. of defibrinated blood from normal individuals were washed three times with 0.85 gm. per cent NaCl after the removal of the serum. The packed cells were then laked with twice their volume of distilled water, and 0.4 volume of toluene was added. After being kept in the refrigerator overnight, the mixture was centrifuged at 3,000 r.p.m. for 30 minutes. Both stroma and toluene were removed, and the supernatant hemoglobin solution was then placed in a cellophane sac and dialyzed against 1 liter of saturated ammonium sulfate solution for 24 hours at 4° C. The resultant precipitate had the microscopic appearance of hemoglobin crystals. The crystals thus prepared were made ready for use by dissolving in approximately 20 ml. of 0.85 gm. per cent NaCl.

EXPERIMENTAL

Formation of Methemoglobin in Erythrocytes Incubated with Cobalt

Blood was drawn from a normal subject and defibrinated under sterile conditions. The hematocrit was then adjusted to 50 per cent with the subject's serum, as it was in all subsequent experiments. Sixteen ml. of this blood were mixed with 0.8 ml. of 4 gm. per cent $CoCl_2$ (yielding a final concentration of cobaltous chloride of 0.2 per cent). Another 16 ml. were mixed with 0.8 ml. of 0.85 gm. per cent NaCl and one-half of each sample was incubated for 24 hours at 37° C. On the remainder of each sample the pH was determined and adjusted with CO_2 to approximately 7.0. The methemoglobin content of the unincubated samples and of the incubated samples after appropriate periods of incubation was measured and expressed as per cent of total hemoglobin.

The results of six experiments are shown in Table I and demonstrate that an appreciable amount of methemoglobin was formed in the cells incubated with cobalt. The identity of the pigment was confirmed by its light absorption at 635 millimicrons and the amount determined by the change in extinction upon the addition of neutralized sodium cyanide.

TABLE I

Formation of methemoglobin upon incubation of cobaltous chloride with normal human defibrinated blood

Blood sample incubated with	Number of expts.	Hours at 37° C.	Methemoglobin % of total hemoglobin
Sodium chloride	6	0	0
	6	24	0 to 0.85 Av. 0.53
Cobaltous chloride	6	0	0
	6	24	9.6 to 15.0 Av. 11.85

Action of Cobalt Contrasted with p-Aminophenol in the Formation of Methemoglobin in Erythrocytes

The following experiments were designed to determine whether the formation of methemoglobin induced by cobaltous chloride in normal blood results from increased oxidation of hemoglobin or from inhibition of the normal reduction mechanism. The action of cobaltous chloride was, therefore, compared to that of p-aminophenol, an agent known to produce methemoglobin by accelerated oxidation of hemoglobin (25).

The rate of formation of methemoglobin

Ten ml. of normal defibrinated blood were mixed with 0.5 ml. of 4 gm. per cent $CoCl_2$ solution. A 5 ml. sample of the same blood was mixed with 5 ml. of freshly prepared 50 mg. per cent solution of p-aminophenol. An additional 5 ml. sample was mixed with 5 ml. of 0.85 gm. per cent NaCl. From each sample 13 aliquots of about 0.5 ml. were placed in separate 10 × 100 mm. test tubes, which were then placed in a water bath at 37° C. A methemoglobin determination was made from one of the 0.5 ml. aliquots of the three types of mixture every two hours for 24 hours.

The results of an experiment typical of the two performed are shown in Figure 1 and indicate striking differences in the rates of formation of methemoglobin. The formation of methemoglobin induced by p-aminophenol was rapid for the first few hours and then proceeded relatively slowly. No significant amount of methemoglobin was formed in the blood incubated with cobaltous chloride for the first 10 hours. Thereafter, methemoglobin formation gradually increased so that at the end of 24 hours 8 per cent of the hemoglobin had been oxidized to methemoglobin. In the sample incubated with NaCl, no methemoglobin was formed at the end of 24 hours.

The effect of anoxia on methemoglobin formation

It has been shown that in the presence of oxygen, p-aminophenol will directly oxidize the hemo-

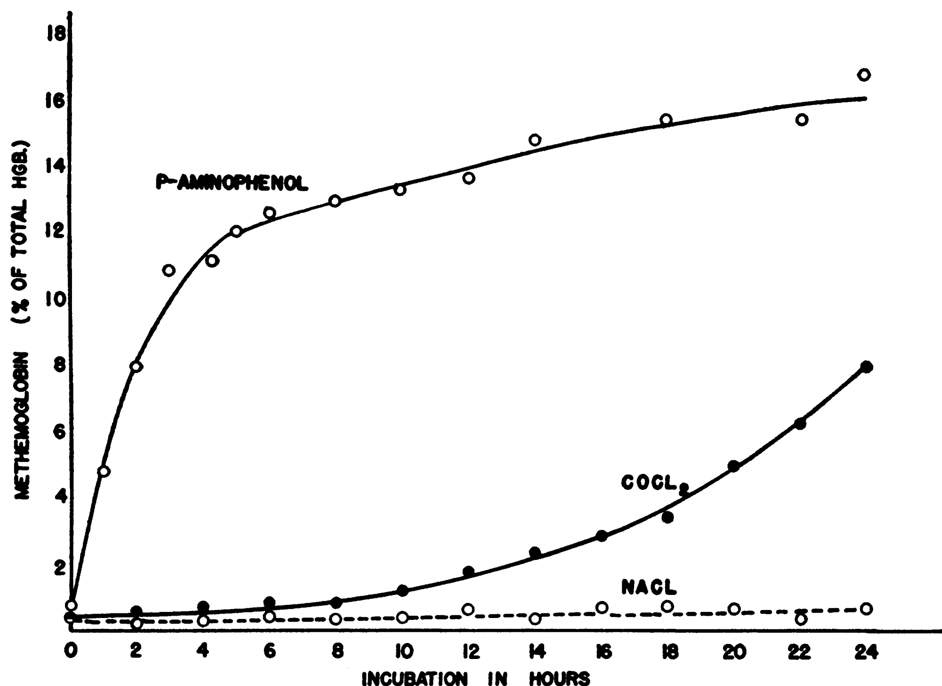

FIG. 1. RELATION BETWEEN METHEMOGLOBIN FORMATION AND DURATION OF INCUBATION OF HUMAN DEFIBRINATED BLOOD WITH P-AMINOPHENOL AND COBALTOUS CHLORIDE

globin of normal blood to methemoglobin, whereas in the absence of oxygen, little or no methemoglobin is formed (25). Methylene blue may either catalyze the oxidation of hemoglobin to methemoglobin or reduce the latter to hemoglobin (26). It was of interest, then, to study the effect of anoxia on the formation of methemoglobin by cobalt and especially in comparison with p-aminophenol and methylene blue.

Two 8 ml. samples of human defibrinated blood were mixed with 4 ml. of 0.85 gm. per cent NaCl. Two 8 ml. samples were mixed with 0.4 ml. of 0.01 gm. per cent methylene blue. Two 8 ml. samples were mixed with 4 ml. of 50 mg. per cent p-aminophenol. Finally, two 8 ml. samples were mixed with 0.4 ml. of 4 gm. per cent $CoCl_2$. The eight samples were then placed in tonometers; and one of each pair was equilibrated with a mixture of 90 per cent O_2 and 10 per cent CO_2, the other with 90 per cent N_2 and 10 per cent CO_2. All samples were then incubated for 24 hours at 37° C. At the end of the incubation period, methemoglobin determinations were done on each sample.

The results presented in Table II indicate that in the presence of oxygen, p-aminophenol and methylene blue readily formed methemoglobin; whereas, in the absence of oxygen, they formed relatively little methemoglobin. In contrast, cobalt permitted the formation of substantially more methemoglobin in the absence of oxygen than in the oxygenated sample. The control samples incubated with sodium chloride solution showed no significant amount of methemoglobin in either gas mixture.

TABLE II

Effects of p-aminophenol and of cobaltous chloride upon methemoglobin formation in blood incubated in presence and in absence of oxygen

Blood sample incubated with	Number of expts.	Hours at 37° C.	Methemoglobin % of total hemoglobin — Gas mixture: 90% O_2 10% CO_2	90% N_2 10% CO_2
Sodium chloride	6	24	0 to 0.85 Av. 0.53	0
Methylene blue	2	24	8.2 and 13.6 Av. 10.9	2.8 and 6.7 Av. 4.7
p-Aminophenol	2	24	59.3 and 63.1 Av. 61.2	4.7 and 7.6 Av. 6.1
Cobaltous chloride	6	24	2.0 to 5.3 Av. 3.7	6.3 to 34.3 Av. 15.2

The effect of methylene blue on the formation of methemoglobin by cobalt and by p-aminophenol

It has been demonstrated that methylene blue reduces preformed methemoglobin in intact erythrocytes by accelerating the normal reconversion mechanism (27, 28). Since the preceding experiments suggested that cobalt induces methemoglobin formation by inhibiting the normal reconversion mechanism, whereas p-aminophenol directly oxidizes hemoglobin to methemoglobin, the effects of cobalt and of p-aminophenol in the presence of appropriate amounts of methylene blue were contrasted.

To 10 ml. of normal human defibrinated blood were added 5 ml. of 50 mg. per cent p-aminophenol in 0.85 gm. per cent NaCl. To another 10 ml. sample, 0.5 ml. of 4 gm. per cent $CoCl_2$ was added. Each sample was then divided into two equal parts. To one part of each sample, 0.5 ml. of 0.85 gm. per cent NaCl containing 0.01 gm. per cent methylene blue was added; to the other, 0.5 ml. of 0.85 gm. per cent NaCl. The samples were then incubated at 37° C., and methemoglobin determinations were done on the samples containing p-aminophenol after 8 and after 24 hours and on those samples containing cobalt after 24 hours.

The results obtained at 24 hours presented in Table III indicate that the addition of methylene blue substantially prevented methemoglobin formation in the blood containing p-aminophenol. In contrast, methylene blue did not suppress methemoglobin formation in the blood containing cobalt.

TABLE III

Effects of methylene blue on the formation of methemoglobin during incubation of normal blood with p-aminophenol and with cobaltous chloride

Blood sample incubated with	Number of expts.	Hours at 37° C.	Methemoglobin *% of total hemoglobin*
p-Aminophenol and Sodium chloride	4	24	48.0 to 68.4 Av. 58.1
p-Aminophenol and Methylene blue	4	24	0.9 to 7.7 Av. 4.2
Cobaltous chloride and Sodium chloride	4	24	6.0 to 19.5 Av. 10.6
Cobaltous chloride and Methylene blue	4	24	8.1 to 18.2 Av. 13.8

TABLE IV

Inhibitory action of ascorbic acid on the formation of methemoglobin during incubation of normal blood with cobaltous chloride or p-aminophenol

Blood sample incubated with	Number of expts.	Hours at 37° C.	Methemoglobin *% of total hemoglobin*
Sodium chloride	4	24	0.4 to 0.7 Av. 0.6
Cobaltous chloride and Sodium chloride	6	24	7.1 to 15.0 Av. 11.5
Cobaltous chloride and Ascorbic acid	6	24	0 to 3.4 Av. 1.2
p-Aminophenol and Sodium chloride	5	24	29.7 to 71.3 Av. 44.6
p-Aminophenol and Ascorbic acid	5	24	1.1 to 8.1 Av. 2.8

The effect of ascorbic acid on the formation of methemoglobin by cobalt and by p-aminophenol

In contrast to methylene blue, ascorbic acid has been shown to reduce methemoglobin directly rather than by way of the normal cellular enzymatic process (29). The following experiment was designed to determine whether ascorbic acid would prevent or inhibit methemoglobin formation induced by either cobalt or p-aminophenol.

To 10 ml. of normal human defibrinated blood 5 ml. of 50 mg. per cent p-aminophenol in 0.85 gm. per cent NaCl were added. To another 10 ml. of blood 0.5 ml. of 4 gm. per cent cobaltous chloride was added. Each sample was then divided into two parts. To one part was added 0.8 ml. of 2.5 gm. per cent solution of ascorbic acid; to the other was added 0.8 ml. of 0.85 gm. per cent NaCl. Another 5 ml. aliquot of blood was mixed with 0.8 ml. of 0.85 gm. per cent. NaCl to serve as a control. All samples were incubated at 37° C. Methemoglobin determinations were done on all samples at the end of 8 hours and of 24 hours.

The results presented in Table IV indicate that the addition of ascorbic acid markedly inhibits the formation of methemoglobin by either cobalt or p-aminophenol.

Formation of Methemoglobin in a Solution of Hemoglobin Crystals Incubated with Cobalt

If the transformation of hemoglobin to methemoglobin by the action of cobalt is due entirely to

the inhibition of the cellular reconversion mechanisms, the exposure of crystalline hemoglobin to cobalt, in the absence of the requisite enzymes and their substrates, should produce no more methemoglobin than exposure to any inert substance. The following experiment was done to test this assumption.

Hemoglobin crystals prepared from 20 ml. of normal defibrinated blood as described above were dissolved in 20 ml. of sterile distilled water and the solution divided into two parts. To one part was added 0.5 ml. of 0.85 gm. per cent NaCl and to the other 0.5 ml. of 4 gm. per cent $CoCl_2$. Each part was then further divided into 0.5 ml. aliquots, which were placed in 10 × 100 mm. test tubes and incubated at 37° C. Methemoglobin determinations were done at appropriate intervals during 24 hours.

The results of an experiment typical of four are plotted in Figure 2 and show that the addition of cobaltous chloride to the solution of hemoglobin gave no significantly greater yield of methemoglobin than did the addition of sodium chloride.

DISCUSSION

In normal erythrocytes, both in the circulation and *in vitro*, hemoglobin is constantly being oxidized to methemoglobin presumably by the process of auto-oxidation (26). The methemoglobin thus formed is constantly being reconverted to hemoglobin, chiefly by an enzymatic process which functions so efficiently that both *in vivo* and *in vitro* (26) only small amounts of methemoglobin can be found as long as the necessary glycolytic enzymes and their substrates are present. In addition to this process involving glycolysis, methemoglobin may also be reduced directly by the action of several agents, such as ascorbic acid, cysteine, glutathione, glyceraldehyde, and BAL (dimercaptopropanol) (26, 30).

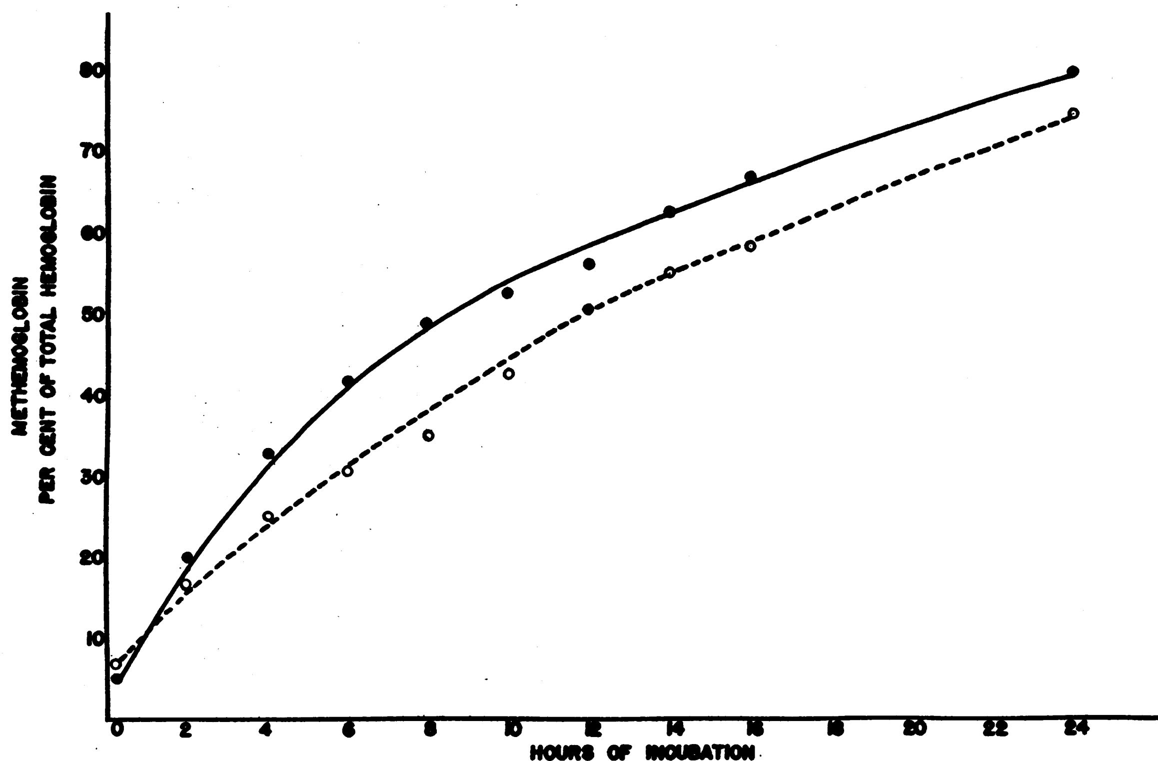

FIG. 2. FAILURE OF COBALT TO AUGMENT THE FORMATION OF METHEMOGLOBIN IN A SOLUTION OF HEMOGLOBIN CRYSTALS INCUBATED IN ROOM AIR AT 37° C.

O—O Hemoglobin crystals in water and NaCl.
●—● Hemoglobin crystals in water and $CoCl_2$.

Under abnormal circumstances methemoglobin may accumulate in erythrocytes due either to an acceleration of the oxidative process beyond the capacity of the normal reduction mechanisms or to a failure of the reduction mechanisms in the presence of the normal oxidative process. It would appear from the experiments cited above that the formation of methemoglobin in blood exposed to cobaltous chloride may best be explained by the latter mechanism. Comparison of the methemoglobin formation by p-aminophenol with that by cobalt, with respect to both rate of formation and effect of anoxia (Table I and II), strongly implies that cobalt does not produce methemoglobin by the same mechanism as does p-aminophenol, a substance known to oxidize hemoglobin directly. Methylene blue both *in vitro* and *in vivo* may, in appropriate concentrations, either accelerate the formation of methemoglobin or catalyze its reduction (26). The former action presumably explains the moderately enhanced formation of methemoglobin by methylene blue alone in oxygen illustrated in Table II. In the first portion of Table III the opposite effect of methylene blue in inhibiting the oxidizing action of p-aminophenol is clearly shown.

Since the reduction of methemoglobin by methylene blue depends on an intact enzymatic reduction system (27, 28), the failure of methylene blue to reduce the methemoglobin induced by cobalt illustrated in the second portion of Table III implies an interference by cobalt with this reduction system. That the methemoglobin so formed may be reduced in the presence of cobalt without participation of the enzymatic system is demonstrated by the action of ascorbic acid (Table IV), an agent known to reduce methemoglobin directly. Finally, when hemoglobin is purified and the enzymatic reduction mechanism is no longer present to be inhibited by cobalt, no appreciably greater amounts of methemoglobin are induced by incubation with cobalt than with an inert substance, such as NaCl (Figure 2).

The implication that cobalt interferes with this normal enzymatic reduction system *in vitro* may be of importance in relation to the action of cobalt in stimulating erythropoiesis *in vivo*. If erythropoietic activity depends on the relative oxygen saturation of the marrow cells, or of some other hypothetical regulatory center, erythropoietic activity will be stimulated by any agent which interferes either with the delivery of oxygen to or with the uptake or utilization of oxygen by the regulatory cells (31). An agent which blocks intracellular enzymatic reduction systems and so inhibits the utilization of oxygen might thus cause histotoxic anoxia of the regulatory cells and so lead to increased erythropoiesis, even though the supply of oxygen brought to these cells was within normal limits. It would appear from the observations reported above that cobalt does in fact inhibit the enzymatic reduction system concerned with the reconversion of methemoglobin to hemoglobin. The failure to find increased levels of methemoglobin in the blood of patients and experimental animals treated with cobalt makes doubtful a relationship between the inhibition of this particular enzyme system of the adult red cell and the development of cobalt polycythemia. Nevertheless, a similar inhibitory effect by cobalt in other and perhaps more sensitive intracellular enzyme systems of other cells might well eventuate there in histotoxic anoxia and hence in erythropoietic stimulation.

SUMMARY

Normal human blood was incubated at 37° C. for 24 hours in the presence of 0.2 per cent cobaltous chloride. Formation of methemoglobin was observed.

Study of the mechanism of methemoglobin formation under these circumstances suggests that cobalt inhibits the normal intracellular reduction system which maintains hemoglobin in the ferrous state.

Others have shown that administration of cobalt to normal animals or man results in polycythemia without evidence of methemoglobin formation or decrease in oxygen capacity of the peripheral blood. Consequently, the physiological relevance of the present observations to such enhanced erythropoiesis is not direct. However, they are at least consistent with the hypothesis that cobalt, by its interference with enzymatic reduction systems, induces relative anoxia of tissues, including those controlling erythropoiesis, and thus may stimulate production of erythrocytes.

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