THE EFFECT OF COBALT ON THE SYNTHESIS OF GLOBIN AND HAEM IN RETICULOCYTES

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1. Introduction

There is some disagreement in the literature as to the effect of cobalt ions on the synthesis of haemoglobin in reticulocytes. The early work suggested that cobalt stimulated the production of globin, whilst at the same time depressing the synthesis of haem [1]. Schulman and Jobe [2], however, claimed that if the globin was purified before assaying, then globin synthesis was depressed concomitantly with the synthesis of haem. They proposed that there was a highly radioactive contaminant of globin which was only found if ^{14}C-glycine was used as a precursor to measure globin and haem synthesis simultaneously. These findings are not in agreement with those of Waxman and Rabinowitz [3] who showed that in the absence of iron, cobalt was able to stimulate globin synthesis and stabilise polysomes. It has also been reported that cobalt protoporphyrin is effective in stimulating globin synthesis when added to intact cells or the cell free lysate [4]. We wish to report that the cause of the discrepancy appears to be the ability of cobalt protoporphyrin to bind irreversibly to proteins in intact cells.

2. Methods

Reticulocytes were washed and incubated as previously described [5]. ^{14}C-Phenylalanine (50 mCi/mmole) was present at a final concentration of 1 μCi/ml where indicated. ^{14}C-δ-Aminolaevulinic acid (53 mCi/mmole) was added to the incubation at 0.5 μCi/ml. No unlabelled δ-aminolaevulinic acid was added. Cobalt was added as cobaltous chloride, iron as ferrous ammonium sulphate and 2,2'-dipyridyl in 25% alcoholic solution.

Incubations were terminated by the addition of acid acetone (conc. HCl-acetone, 1:100). The precipitated protein was collected by centrifugation and the resultant pellet was dissolved in 0.3 N NaOH (containing 1 mg/ml phenylalanine where necessary) and incubated for 30 min at 37°. The protein was reprecipitated by addition of 8% trichloroacetic acid (containing 1 mg/ml phenylalanine where necessary). The precipitate was collected on Whatman GF/C glass fibre filters and washed with 2 × 5 ml washes of 8% trichloroacetic acid. The filters were glued to cardboard discs and dried, before being counted in an end window Nuclear Chicago gas flow counter (^{14}C-counting efficiency about 20%). Washing the protein precipitate with acetone did not alter the amount of radioactivity incorporated in the presence of ^{14}C-δ-aminolaevulinic acid.

For measurement of incorporation of radioactivity into haem, the acid acetone extract was freed of protein by centrifugation and taken to dryness *in vacuo*. The residue was dissolved in a small volume of acid acetone and haem and protoporphyrin were precipitated by addition of water and carrier haemin. After allowing precipitation to occur for 30 min at room temperature, the precipitates were filtered onto glass fibre filters, which were glued to cardboard discs. The radioactivity was estimated as described above. By addition of 2 N HCl instead of water to the acid acetone resuspension, it was possible to precipitate haemin selectively [6]. The results obtained by isolation of haemin were qualitatively much the same as those ob-

tained by the precipitation of protoporphyrin and haemin together. Between 20 and 30% of the radioactive material obtained from ^{14}C-δ-aminolaevulinic acid appeared to be in the form of protoporphyrin or precursors of protoporphyrin as judged by this method. This value did not rise in the presence of cobalt.

3. Results

Fig. 1 shows that in the absence of added iron incorporation of ^{14}C-phenylalanine by our preparation of reticulocytes proceeded linearly for about 30 min and then tailed off. Presumably this reflects the utilisation of an endogenous pool of iron. In order to study the effects of total iron deprivation we have used the ferrous ion chelator, 2,2'-dipyridyl. It can be seen that the incorporation of radioactivity was markedly inhibited in the presence of 10^{-3} M dipyridyl (fig. 1). Addition of either cobaltous or ferrous ions

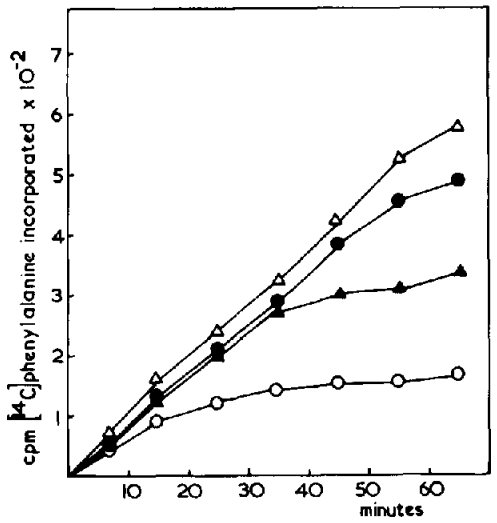

Fig. 1. Incorporation of ^{14}C-phenylalanine into protein in the presence of cobalt. Reticulocytes were incubated as described in Methods at 37° in the presence of ^{14}C-phenylalanine. 50 μl incubations were terminated by the addition of acid acetone. The precipitated proteins were removed by centrifugation and were assayed for radioactivity as described in Methods. Cobalt (1 mM) (△—△), iron (0.1 mM) (●—●) and dipyridyl (1 mM) (○—○) were present at the concentrations indicated. One series of incubations were performed in the absence of added iron (▲—▲).

Table 1
Incorporation of ^{14}C-δ-aminolaevulinic acid into haem and protein fractions.

	+ Iron (0.1 mM)	+ Cobalt (1 mM)	+ Dipyridyl (1 mM)
Control			
Haem fraction	247	201	85
Haem + protoporphyrin fraction	311	253	219
Protein fraction	105	440	80
+ Cycloheximide (0.1 mM)			
Haem fraction	221	174	–
Haem + protoporphyrin fraction	290	250	–
Protein fraction	56	367	–

The results are expressed as cpm per incubation. Reticulocytes were incubated as described in Methods at 37° for 60 minutes in the presence of ^{14}C-δ-aminolaevulinic acid. 50 μl incubations were terminated by the addition of acid acetone. The precipitated proteins were collected by centrifugation and assayed for radioactivity as described in Methods (protein fraction). The acid acetone supernatant was treated as described in Methods to provide either a partially purified haemin preparation (haem fraction) or else a fraction containing both haem and protoporphyrin (haem + protoporphyrin fraction). All the incubations were performed in sixes so that three samples were used for determination of radioactivity in haem and three for haem + protoporphyrin. The concentration of cycloheximide used inhibited incorporation of ^{14}C-phenylalanine by more than 97% in a control experiment.

to the reticulocytes resulted in a prolongation of incorporation in a linear fashion (fig. 1). The optimal added concentration for both ions was in the region of 10^{-4} M, but concentrations as high as 10^{-3} M were not inhibitory. Cobaltous ions appeared to stimulate incorporation slightly more effectively than do ferrous ions. When the synthesis of haem was examined using ^{14}C-δ-aminolaevulinic acid as a labelled precursor, 10^{-3} M cobalt was found to inhibit incorporation of radioactivity into a fraction containing haem and protoporphyrin in a manner similar to dipyridyl (table

1). If, however, the amount of radioactivity incorporated into acid-precipitable alkali-stable material was measured, the figure was very much greater in the presence of cobalt (table 1). By hydrolysis and electrophoresis of this protein fraction, it was possible to show that the radioactivity was not present in the form of δ-aminolaevulinic acid or amino acids. This suggests that cobalt protoporphyrin or a precursor of it, is much more stably bound to globin or some other protein than is iron protoporphyrin. The binding of cobalt protoporphyrin is stable to acid acetone dissociation, and to treatment with acid and alkali. Furthermore the amount of radioactive protein produced in the presence of ^{14}C-δ-aminolaevulinic acid and cobalt was not diminished by the inclusion of 10^{-4} M cycloheximide in the incubation medium (table 1). Under these conditions no protein synthesis occurs but haem synthesis continues to a large extent [7]. The binding of cobalt protoporphyrin to protein is therefore apparently not dependent on the synthesis of new globin. Since, however, there is a large pool of globin in reticulocytes [7], cobalt protoporphyrin might bind to this.

4. Discussion

Our experiments confirm the findings of Waxman and Rabinowitz [3] and of Adamson, Herbert and Kemp [4] on the action of cobalt on haemoglobin synthesis in reticulocytes. Cobalt behaves in a fashion analogous to iron in promoting globin synthesis. The synthesis of cobalt protoporphyrin occurs at the same rate or faster than the synthesis of iron protoporphyrin, when the amount of protoporphyrin bound to protein is taken into account. Cobalt protoporphyrin is as effective as iron protoporphyrin in promoting the initiation of new globin chains and therefore in stabilising the polysomes.

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