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Inhibition of Haem Synthesis Caused by Cobalt in Rat Liver

EVIDENCE FOR TWO DIFFERENT SITES OF ACTION

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Cobalt inhibits liver haem synthesis *in vivo* by acting at least two different sites in the biosynthetic pathway: (1) synthesis of 5-aminolaevulinate and (2) conversion of 5-aminolaevulinate into haem. The first effect is largely, if not entirely, due to inhibition of the activity of 5-aminolaevulinate synthase, rather than to inhibition of the formation of the enzyme. The second effect results from diversion of 5-aminolaevulinate into an unidentified liver pool with solubility properties similar to those of cobalt protoporphyrin.

Rats given cobalt show a marked loss of haemoproteins, particularly cytochrome *P*-450, from their livers (Tephly & Hibbeln, 1971; Yasukochi *et al.*, 1974). Studies conducted *in vivo* with labelled precursors of the haem moieties of microsomal cytochromes and catalase have indicated that cobalt interferes with haem metabolism (Tephly *et al.*, 1973; Yasukochi *et al.*, 1974), both by accelerating haem breakdown (De Matteis & Unseld, 1976; Maines & Kappas, 1976) and also by inhibiting haem synthesis (Nakamura *et al.*, 1975; De Matteis & Gibbs, 1976).

The present results, which are an extension of a recent study (De Matteis & Gibbs, 1976), indicate that cobalt inhibits liver haem synthesis at least two different stages in the biosynthetic sequence: (1) formation of 5-aminolaevulinate and (2) conversion of 5-aminolaevulinate into haem. The first effect is probably due to decreased activity of 5-aminolaevulinate synthase (EC 2.3.1.37), rather than to inhibition of the formation of the enzyme, or to limited generation of its substrate, succinyl-CoA (3-carboxypropionyl-CoA), and the second to a diversion of 5-aminolaevulinate into an unidentified liver component(s) which is different from haem and has solubility properties similar to those of cobalt protoporphyrin.

Materials and Methods

Male rats (160–180g) of the Porton strain were first starved overnight, then injected subcutaneously with $CoCl_2,6H_2O$ (60mg in 10ml of 0.85% NaCl/kg body wt.) and kept starved for the duration of the experiments. Some rats were given 3,5-diethoxycarbonyl-1,4-dihydrocollidine dissolved in arachis oil either by intraperitoneal injection (100mg/kg) or orally (200mg/kg) before being injected with $CoCl_2$. Cycloheximide was given intraperitoneally (40mg/kg), dissolved in 0.85% NaCl. The incorporation of precursors into liver proteins or liver haem was studied by injecting rats with L-[1-^{14}C]leucine (4μCi, 67nmol) subcutaneously or with 5-amino[4-^{14}C]laevulinate (2μCi, 37nmol) intraperitoneally and killing them 15min and 10min to 24h later respectively. Haemin was isolated from the homogenates of livers, after addition of horse erythrocyte lysate, and crystallized (Labbe & Nishida, 1957); the liver proteins were isolated as described by Villa-Treviño *et al.* (1963). Their radioactivity and that of the total liver homogenate was determined by liquid-scintillation counting (after combustion in an Intertechnique Oxymat in the case of haemin).

5-Aminolaevulinate synthase was assayed in whole liver homogenates (Abbritti & De Matteis, 1971) or in isolated mitochondria as described by De Matteis (1971), except that mitochondria were sonicated for 10s (with a Dawe Soniprobe type 7530A at 3A) before incubation and pyridoxal phosphate was present in the assay medium at a final concentration of 0.8mM. Succinyl-CoA was prepared as described by Simon & Shemin (1953) and determined by the method of Lipmann & Tuttle (1945). On account of the instability of succinyl-CoA, when measuring formation of 5-aminolaevulinate, succinyl-CoA was added in successive amounts calculated to maintain a constant concentration throughout the 30min incubation: an initial 1μmol was followed by 0.4μmol every 5min. The disappearance of succinyl-CoA was measured by adding 10μmol to the complete incubation mixture at the beginning of the incubation and measuring the amount remaining after 15min.

Results and Discussion

Inhibition of 5-aminolaevulinate synthase activity

When starved rats were given a single subcutaneous injection of $CoCl_2$ (256μmol/kg), there was a rapid and marked loss of 5-aminolaevulinate synthase activity

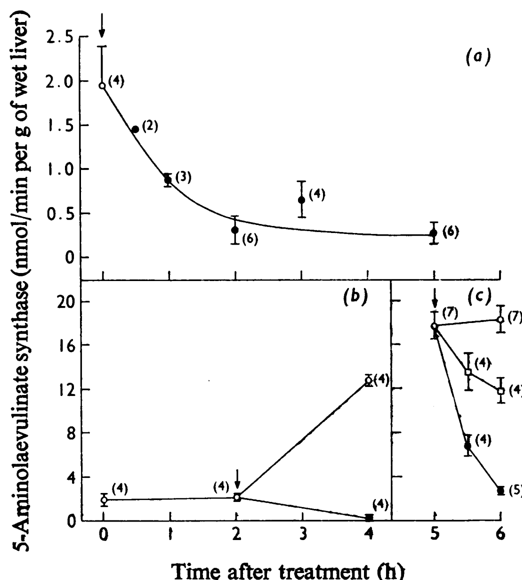

Fig. 1. *Effect of a single dose of $CoCl_2$ on the 5-aminolaevulinate synthase activity of rat liver homogenate*
Rats were starved overnight, then injected (where indicated by an arrow) with $CoCl_2$ (●) or NaCl (○) subcutaneously, or cycloheximide (□) intraperitoneally. In Expts. (*b*) and (*c*) rats were also given at zero time a single dose of 3,5-diethoxycarbonyl-1,4-dihydrocollidine intraperitoneally or orally respectively. Note that the scale of the ordinates has been expanded in (*a*). Results given are means ±S.E.M. of the numbers of observations in parentheses.

in their liver homogenates, and low enzyme activity persisted for at least 5h (Fig. 1*a*). Cobalt was also found (Fig. 1*b*) to prevent the stimulation of 5-aminolaevulinate synthase activity caused by 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Granick & Urata, 1963).

The method of assay of 5-aminolaevulinate synthase in liver homogenates depends on the endogenous generation of succinyl-CoA from citrate; the 5-aminolaevulinate formed accumulates, as the main pathway of its utilization [that involving 5-aminolaevulinate dehydratase (EC 4.2.1.24)] is inhibited by the high concentration of EDTA present. The following possibilities for the effect of cobalt were therefore considered. (1) Cobalt might activate an alternative pathway of 5-aminolaevulinate utilization (other than the one involving the dehydratase). This possibility seems unlikely, since the recovery of added 5-aminolaevulinate (4–23 μM) from incubated liver homogenates was not significantly different in control (79–86% recovery) or $CoCl_2$-treated rats (82–93% recovery). (2) Cobalt might decrease the concentration of succinyl-CoA, either by inhibiting production (Dingle *et al.*, 1962) or by increasing deacylation. These possibilities were tested by incubating mitochondria with assay medium containing either succinyl-CoA or citrate. The mitochondria were obtained from rats pretreated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine 4h before killing. Some rats also received $CoCl_2$ 2h before killing. Without cobalt treatment succinyl-CoA could stimulate 5-aminolaevulinate formation as effectively as did citrate. After cobalt treatment the activity of 5-aminolaevulinate synthase as measured with citrate was decreased to 4% of the control value, and the activity could not be restored by replacing citrate with succinyl-CoA. The amount of succinyl-CoA remaining after incubation was the same with either mitochondrial preparation. Therefore the loss of 5-aminolaevulinate synthase activity caused by cobalt cannot be due to limited availability of succinyl-CoA. (3) A more likely possibility is that the observed loss of 5-aminolaevulinate synthase is due to decrease of either the amount or the activity of the enzyme itself.

Maines *et al.* (1976) have reported that cobalt inhibits the stimulation of 5-aminolaevulinate synthase caused by 2-allyl-2-isopropylacetamide, an effect similar to that shown in Fig. 1(*b*) for 3,5-diethoxycarbonyl-1,4-dihydrocollidine. They suggest that cobalt inhibits the induced synthesis of the enzyme. An alternative explanation is that cobalt inhibits the enzyme activity, rather than its formation. In order to distinguish between these two mechanisms cobalt was administered to rats 5h after 3,5-diethoxycarbonyl-1,4-dihydrocollidine, when the activity of 5-aminolaevulinate synthase was already stimulated. The rate of loss of activity was greater after cobalt administration than after cycloheximide, suggesting that cobalt could not act merely by blocking the formation of the enzyme (Fig. 1*c*). Under these conditions cycloheximide inhibited by 99% the incorporation of [^{14}C]leucine into liver proteins when the label was given either 30min or 2h after the inhibitor. The most likely interpretation for these findings is that cobalt causes *in vivo* an inhibition of the activity of 5-aminolaevulinate synthase and that this effect can be demonstrated *in vitro*, even when washed mitochondria are used as the source of the enzyme. If Co^{2+} itself inhibits 5-aminolaevulinate synthase *in vivo*, as shown by Scholnick *et al.* (1972) for the partially purified enzyme, it must do so by binding to a site from which it is not readily displaceable, not even by the high concentration of EDTA (8mM) present in the assay mixture. Alternatively a cobalt derivative may be formed *in vivo* (for example cobalt protoporphyrin) and this may then inhibit 5-aminolaevulinate synthase (see below).

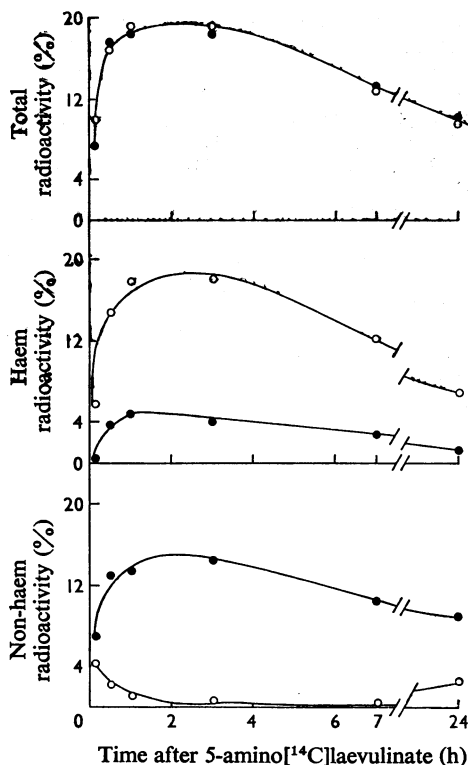

Fig.2. *Recovery of radioactivity from 5-amino[^{14}C]-laevulinate in the liver of animals pretreated with $CoCl_2$ and of their controls*

Rats were starved overnight, then treated with $CoCl_2$ (●) or NaCl (○) subcutaneously, injected with 5-amino[4-^{14}C]laevulinate intraperitoneally 1h later and killed at different times after injection of the label. Radioactivity was determined: (A) in the total liver homogenates and (B) in the haem isolated from them. 'Non-haem' radioactivity was calculated from the difference (A−B). Results given are averages of at least two observations. The radioactivity values are percentages of the injected radioactivity recovered in total liver.

Effect of cobalt on the metabolism of 5-aminolaevulinate in vivo

When cobalt-treated rats are injected with radioactive 5-aminolaevulinate the radioactivities recovered either as haemin from the liver (Tephly *et al.*, 1973; Nakamura *et al.*, 1975; De Matteis & Gibbs, 1976) or as bilirubin from bile (De Matteis & Gibbs, 1976) are both markedly decreased. This suggests that cobalt inhibits liver haem synthesis also by acting at some stage in the pathway after the synthesis of 5-aminolaevulinate. Guzelian & Bissell (1976) have concluded, on the other hand, that cobalt does not inhibit liver haem synthesis.

To elucidate the nature of the inhibition by cobalt of the uptake of radioactive 5-aminolaevulinate into crystallizable liver haem, intact rats were injected with cobalt, and after 1h with labelled 5-aminolaevulinate, and they were killed at different times after the label. At all times the total radioactivity recovered in the liver was not significantly different in cobalt-treated rats from that in controls. In contrast, the radioactivity isolated as crystalline haemin from the liver homogenate was significantly decreased in cobalt-treated rats (Fig. 2).

These results indicate that cobalt pretreatment caused accumulation of radioactivity from 5-aminolaevulinate in a form that could not be extracted and crystallized as haemin and also that this non-crystallizable radioactivity was not readily eliminated from the liver. This effect of cobalt cannot arise from an interference with either the extraction or the crystallization of haem, since, when rats were first given radioactive 5-aminolaevulinate and were then treated with cobalt 2h later, after the incorporation of the label into haem was essentially complete, no increase in non-haem radioactivity was found (De Matteis & Unseld, 1976). Therefore the accumulation of non-haem radioactivity must represent a change in the liver metabolism of 5-aminolaevulinate with diversion of radioactivity into one or more relatively stable liver pools, different from haem.

The nature of the non-haem compound(s) that are labelled in the liver under the conditions of these experiments is not known. The following preliminary experiment indicates that most of this non-haem radioactivity is not readily extractable by acidified acetone, a behaviour similar to that of authentic cobalt protoporphyrin. When liver homogenates from rats killed 1h after injection of 5-amino[^{14}C]-laevulinate (Fig. 2) were repeatedly extracted with acetone/conc. HCl (99:1, v/v), the percentage of the total radioactivity remaining in the extracted pellet was 68% for cobalt-treated livers, but only 26% for control livers (with the balance of radioactivity being present in the extracts in both cases). When authentic cobalt protoporphyrin or authentic haem was added to liver homogenates, the percentage of the total amount added remaining in the pellet after extraction was 79 and 30% respectively. If, in fact, cobalt protoporphyrin is formed and accumulates under these conditions, then the possibility should be considered (De Matteis, 1975) that this haem-like compound may be responsible for the inhibition of 5-aminolaevulinate synthase by a mechanism similar to that involved in feedback inhibition of the enzyme by haem.

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