The Effect of Cobalt on the Uptake of Plasma Iron by the Liver

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1. After an intraperitoneal injection of ^{59}Fe the recovery of radioactivity in the liver, but not in other tissues, was increased in cobalt-pretreated rats. There was no proportional increase in the radioactivity recovered from liver haem. 2. Rats were injected intravenously with serum containing protein-bound ^{59}Fe and ^{125}I -labelled albumin as a marker. At various times after injection the specific radioactivities of iron in plasma and of non-haem iron in liver were determined; corrections were applied for the content of plasma in samples of liver. In cobalt-pretreated rats there was a more rapid loss of ^{59}Fe radioactivity from the plasma and a corresponding increase in the uptake of ^{59}Fe into liver non-haem iron. 3. The results are discussed in relation to the possible sites of action of cobalt, and the possibility is considered that only a fraction of the liver non-haem iron may be involved.

There is evidence that administration of cobalt to rats produces inhibition of the synthesis of haem in the liver (Tephly & Hibbeln, 1971; De Matteis & Gibbs, 1976). A possible site of action is ferrochelatase, where cobalt is known to act as an alternative substrate to iron (Labbe & Hubbard, 1961; Jones & Jones, 1969). The present study was undertaken to obtain evidence for an inhibition of the incorporation of iron into liver haem *in vivo*. The results obtained were unexpected, since cobalt was found to stimulate markedly the uptake of ^{59}Fe from blood into the liver. The present paper describes this phenomenon.

Experimental

Materials

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (A.R. grade) and Amberlite resin IRA-400 (Cl^- form; A.R. grade) were from BDH, Poole, Dorset, U.K. [^{59}Fe]Ferric citrate, $^{59}\text{FeCl}_3$, [^{59}Fe]ferrous ascorbate and ^{125}I -labelled human serum albumin ($2.5\ \mu\text{Ci}/\text{mg}$ of albumin) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Methods

Treatment of animals. Male rats of the Porton (Wistar-derived) strain weighing 180–220 g were used. These rats had been maintained on MRC diet 41B (Bruce & Parkes, 1956), the iron content of which varied between 200 and 250 mg of iron/kg of diet. On the day preceding an experiment, food was removed at 09.30 h and rats received a subcutaneous injection of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (60 mg in 10 ml of 0.85% NaCl/kg

body wt.) or of NaCl alone between 16:00 h and 17:00 h.

Experiments with labelled iron. Rats received ^{59}Fe (as either FeCl_3 or ferrous ascorbate) intraperitoneally [$1\text{--}4\ \mu\text{g}$ of Fe (approx. $10\text{--}30\ \mu\text{Ci}$)/kg body wt. in a volume of 1 ml/kg]. The label was diluted with a solution containing 8.5 mg of NaCl and 0.6 mg of ascorbic acid/ml in the case of [^{59}Fe]ferrous ascorbate, or with 8.5 mg of NaCl and $10\ \mu\text{mol}$ of HCl/ml in the case of $^{59}\text{FeCl}_3$. At various times after injection of the radioisotope rats were killed by decapitation and bled out. Heart, liver, kidneys and spleen were removed and homogenized (10%, w/v) in ice-cold 0.25 M-sucrose. Samples of the homogenates were taken for measurement of total radioactivity. Haemin was extracted from liver homogenates after addition of horse blood haemolysate as carrier and crystallized by the $\text{SrCl}_2/\text{acetone}$ method (Labbe & Nishida, 1957). Preliminary experiments established that the recovery of authentic ^{14}C -labelled haemin added to liver preparations, after extraction and crystallization, was 70–75%. The dry crystals of haemin were dissolved in pyridine; a small sample was taken for measurement of haem as the pyridine haemochrome (Falk, 1964) and the remainder counted for radioactivity.

Double-labelling experiments. In order to correct the ^{59}Fe radioactivity of the liver for the contribution by the plasma, rats were injected with serum containing ^{59}Fe complexed to proteins and also ^{125}I -labelled human serum albumin; the ^{125}I and ^{59}Fe contents of samples of liver were then compared with those of plasma obtained at the same time from each rat. The double-labelled serum was prepared as follows. Donor rats were injected via a tail vein with $5\ \mu\text{Ci}$ of ^{125}I -labelled human serum albumin and left overnight

as a test for any component of the label that might be removed rapidly from the circulation. Blood was then collected under diethyl ether anaesthesia from the aorta into 0.05 vol. of 0.3M-sodium citrate; the plasma was separated by centrifugation at 5000g for 10 min. CaCl_2 (0.025 vol. of 1M) was then added and after incubation at 37°C for 30 min the clot was removed on a swab stick. A small volume of [^{59}Fe]ferric citrate solution was added to the [^{125}I]-labelled serum and the whole incubated at 37°C for 30 min. The ^{59}Fe that had not become complexed with serum proteins was removed by passage of the serum through Amberlite resin (Cavill, 1971) and the double-labelled serum kept for up to 24 h at 0°C before use. The serum was warmed to room temperature (18–20°C) and a weighed amount (approx. 0.2 ml/rat) injected via a tail vein into cobalt-treated rats and their controls. Rats were decapitated at intervals after injection, blood was collected into tubes containing heparin and plasma separated by centrifugation. The livers were excised and dropped into liquid N_2 ; homogenates (10%, w/v) in ice-cold deionized water were prepared. Samples of the injected serum, of the heparinized plasmas and of the liver homogenates were taken for assay of radioactivity and for measurement of iron.

Measurement of iron. All glassware had been soaked overnight in HCl (2M) and rinsed repeatedly in deionized water. For determination of total iron content of liver, samples (2 ml) of homogenate were digested with 3 ml of $\text{HNO}_3/\text{HClO}_4$ (4:1, v/v) and the dry residue was dissolved in 5 ml of 0.6M-HCl. Plasma iron and liver non-haem iron were measured in the supernatant obtained after treatment of tissue with hot 20% (w/v) trichloroacetic acid (Olson & Hamlin, 1969). This method produces precipitation of protein and release of inorganic iron with small interference from haem iron. Iron was measured by atomic-absorption spectroscopy with a Perkin Elmer model 306 instrument, calibrated with appropriate standards. Total iron-binding capacity of plasma was determined by the method of Ramsay (1958), by using a test kit [Boehringer Corp. (London), Lewes, Sussex, U.K.].

Measurements of radioactivity. ^{59}Fe and ^{125}I were measured by using a Packard Auto Gamma Spectrometer model 5330, with a suitable correction for overlap of ^{59}Fe counts into the ^{125}I channel, when required.

Results

Subcutaneous injection of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (60 mg/kg body wt.) in rats kept at 18–20°C was followed by peripheral vasodilation and a marked decrease in body temperature to 32.5°C. After 3 h the body temperature began to recover. The present experiments were carried out 17 h after the injection of CoCl_2 , when the body temperature was only 0.5–1°C below that of controls. At this time there was a significant increase in liver weight (Table 1), with no change in the amount of iron when expressed on a total-liver basis (Table 1). The concentration of iron in the plasma of treated rats was slightly lower, on average, than in controls, but the difference was not statistically significant. No significant differences were found in the haematocrit values or in total iron-binding capacity of the plasma (Table 1).

Effect of cobalt on the distribution of intraperitoneally administered ^{59}Fe in the rat

The effect of CoCl_2 on the recovery of ^{59}Fe from various tissues after intraperitoneal injection of $^{59}\text{FeCl}_3$ was studied in five treated rats and five control rats over a period of 3 h, and results analysed in terms of a two-way analysis of variance (time $\times$ treatment) (Snedecor & Cochran, 1967). In treated rats the accumulation of radioactivity in total liver was significantly increased ($P < 0.05$) and the effect persisted for at least 3 h. No significant differences were observed for spleen, heart and kidneys, although in the latter case, recovery from treated rats tended to be less than that for controls.

An increased uptake of ^{59}Fe by livers of cobalt-treated rats was also obtained when the label was administered intraperitoneally as ferrous ascorbate. In this experiment the radioactivity recovered in the blood was also determined and haem was isolated in

Table 1. *Effect of a single dose of CoCl_2 on liver size, liver and plasma iron concentration, total iron-binding capacity and haematocrit values in rats 17–20 h after treatment*

Results are expressed as means $\pm$ S.E.M., with numbers of observations in parentheses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, when compared with corresponding control value by using Student's *t* test.

	Control	+ CoCl_2
Liver wt. (g/100g body wt.)	3.26 $\pm$ 0.03 (26)	3.94 $\pm$ 0.03 (23)***
Liver total iron (μg)	115 $\pm$ 5 (26)	104 $\pm$ 2 (23)*
Liver non-haem iron (μg)	70.6 $\pm$ 3.7 (26)	58.8 $\pm$ 1.8 (23)**
Plasma iron ($\mu\text{g}/\text{ml}$)	1.18 $\pm$ 0.08 (25)	0.99 $\pm$ 0.09 (23)
Haematocrit (%)	44.1 $\pm$ 0.4 (26)	44.8 $\pm$ 0.4 (23)
Total iron-binding capacity ($\mu\text{g}/\text{ml}$)	4.99 $\pm$ 0.31 (5)	4.86 $\pm$ 0.24 (5)

Fig. 1. Effect of cobalt on the rate of disappearance of ^{59}Fe from the plasma

Changes of specific radioactivity of iron in plasma after an intravenous injection of serum-bound ^{59}Fe are shown for cobalt-treated ($\square$) and control ($\circ$) rats. Data are normalized to an injected dose of 10^5 c.p.m. of $^{59}\text{Fe}/100$ g body wt., and are presented as means $\pm$ S.E.M. with the numbers of rats in parentheses. The zero-time specific radioactivities were calculated as described in the text.

crystalline form from the liver. When the haem radioactivity was expressed as a percentage of the total liver radioactivity, it was found to be lower in the cobalt-treated animals. This effect is consistent with the finding (F. De Matteis & A. H. Gibbs, unpublished work) that the initial rate of incorporation of 5-amino-[4- ^{14}C]aevulinate into liver haem is decreased 17 h after a single dose of CoCl_2 . In these experiments the blood radioactivity was greater in cobalt-treated rats, especially at the early time-points, suggesting that cobalt may stimulate the uptake of ^{59}Fe from the peritoneal site of injection into the blood as well as from the blood into the liver. In order to overcome the problem of absorption of iron from the peritoneal cavity and to administer the labelled iron in a more physiological form, in the work described below ^{59}Fe was complexed to serum proteins and injected by an intravenous route.

Distribution of ^{59}Fe between plasma and liver after intravenous injection of serum-bound ^{59}Fe

The change in the specific radioactivity of iron in plasma with time after intravenous injection of ^{59}Fe

bound to serum protein is shown in Fig. 1. The specific radioactivity at zero time was calculated from the injected dose and from the plasma iron concentrations given in Table 1; since there was no significant difference in the mean haematocrit value of the two groups of rats (Table 1), the assumption was made that the plasma volume was the same in both. This was taken to be $4.2\text{ ml}/100\text{ g}$ body wt. (Cunningham, 1975). The specific radioactivity of iron in the plasma of cobalt-treated rats was significantly lower than that of controls by 1 h and was about 50% of that of controls by 3 h (Fig. 1), indicating a more rapid rate of exchange of plasma iron with non-plasma iron in treated rats. This effect was accompanied by a more rapid appearance of radioactivity in the liver of treated rats (see below).

The ^{59}Fe contents of liver samples were corrected for ^{59}Fe present in plasma entrapped in the samples by using an ^{125}I -labelled serum albumin marker for plasma as described under 'Methods'. There was no significant difference between treated and control rats in the apparent volume of trapped plasma. The mean apparent volume ($\pm$ S.D.) in 36 animals was 0.077 (± 0.016) ml/g of liver. It was assumed that the apparent trapped volume was the true plasma volume retained by the liver, and this correction was made in all calculations of liver ^{59}Fe content. For example, at 1 h, when the difference between treated and control rats was most marked (see below), about 2.5% of the total ^{59}Fe in liver samples was attributable to plasma in treated rats and about 12% in controls.

Throughout the time-course of these experiments, about 90% of the total ^{59}Fe in liver in both groups of rats was extracted by hot trichloroacetic acid and was taken as non-haem iron (see 'Methods'). The variation of non-haem iron specific radioactivity in liver with time is shown in Fig. 2. In control rats the specific radioactivity increased slowly over the 3 h period, whereas in treated rats the specific radioactivity increased rapidly up to 1 h (reaching a value about three times that of controls), after which the specific radioactivity was apparently constant up to 3 h. The specific radioactivity of liver non-haem iron observed over this period was much lower than the corresponding specific radioactivity of iron in plasma (Fig. 1) and at 3 h was about 20% of that of plasma.

Discussion

This work has shown that treatment of rats with CoCl_2 stimulates the transfer of ^{59}Fe from plasma to liver (but not other organs studied) and that this effect occurs irrespective of (a) the chemical form or (b) the route by which the ^{59}Fe is administered. Over the period 0–1 h after intravenous administration of ^{59}Fe , when most of the exchange of label takes place, the increased loss of label from the plasma of treated rats can be accounted for by the increased

Fig. 2. Effect of cobalt on the rate of uptake of ^{59}Fe from the plasma into the liver

Changes of specific radioactivity of liver non-haem iron are shown after intravenous injection of ^{59}Fe into cobalt-treated ($\square$) and control ($\circ$) rats. Data are expressed as described in the legend to Fig. 1.

accumulation of radioactivity in their livers, consistent with a specific, or at least preferential, effect on the liver.

The increased uptake of ^{59}Fe was shown to involve predominantly the non-haem pools of iron in the liver and an apparent equilibrium was reached when the specific radioactivity of liver non-haem iron of cobalt-treated rats was only 20% of the plasma iron specific radioactivity. One possible explanation for this finding is that only a small proportion of the total non-haem iron of the liver is involved in this phenomenon.

There are two possible, and not mutually exclusive, mechanisms to account for the increased uptake of ^{59}Fe by the liver of treated rats. Cobalt treatment may only have caused an accelerated exchange between plasma iron and a pool of non-haem iron in the liver. Alternatively there may have been a net uptake by the liver of a small amount of plasma iron of relatively high specific radioactivity. It is not possible to distinguish between these two possibilities on the basis of the present results.

Three possible sites of action of cobalt can be considered. (1) Cobalt may promote ^{59}Fe uptake by liver from plasma, by altering the concentration or binding characteristics of plasma transferrin. Although multiple doses of cobalt induce erythropoiesis and decrease plasma iron concentration (Morgan, 1966),

our studies show that 17h after a single injection of cobalt, blood haematocrit values and plasma iron concentrations are unchanged (Table 1). If total iron-binding capacity is used as an indirect measure of plasma transferrin then the circulating concentration of transferrin appears unchanged after cobalt treatment (Table 1). Therefore, if both the total iron-binding capacity and plasma iron concentration are unaltered, this would discount the possibility that after cobalt treatment there was an increased saturation of transferrin with iron, a situation in which iron uptake by the liver increases (Fletcher, 1971). There is no evidence so far to link cobalt with an effect on plasma iron metabolism at the level of transferrin.

More likely sites for the action of cobalt on the uptake of iron by the liver are (2) the membranes involved in the transfer of iron between plasma and liver, or (3) an intracellular iron-acceptor molecule. Intermediates involved in the intrahepatic transport of iron between transferrin and ferritin have not been described, but there is circumstantial evidence for a pool of iron that may be the precursor of iron destined for ferritin and into which iron from haem degradation is drawn (Lipschitz *et al.*, 1971).

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