EFFECTS OF COBALT CHLORIDE ON HAEM SYNTHESIS IN ISOLATED HEPATOCYTES

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1. Introduction

Administration of cobalt to rats leads to the inhibition of amino-laevulinate synthetase (EC 2.3.1.37) and the induction of haem oxygenase (EC 1.14.99.3) synthesis [1,2]. These effects have been ascribed to a direct action by the metal ion [1] or the formation of a cobalt porphyrin [3]. No direct evidence for the latter theory could be found however, in spite of the well-known ability of ferrochelatase (EC 4.99.1.1), the metal ion inserting enzyme of haem biosynthesis, to insert a variety of metal ions into a number of porphyrin co-substrates [4]. Recently cobalt protophorphyrin has been isolated from rats injected with large doses of cobalt chloride [5], rat liver homogenates have also been shown to produce this metalloporphyrin [6]. Isolated hepatocytes in culture, which are increasingly used to study hepatic function, have so far been reported unable to produce cobalt protoporphyrin when supplied with the metal ion [7,8]. In view of the findings in [5,6] the effects of this metal ion on hepatocyte cultures was re-examined.

In the presence of cobalt chloride it was found that there was a decreased rate of haem synthesis from exogenously supplied amino-laevulinate (ALA), a specific haem precursor [9]. Under these conditions the metalloporphyrin concentration associated with the cell fraction increased relative to the control. A metalloporphyrin was extracted from cells cultured in the presence of ALA and $CoCl_2$ which had spectral properties consistent with its being cobalt protoporphyrin.

2. Methods and materials

2.1. *Isolation of hepatocytes*

Hepatocytes were isolated as in [10] modified to exclude the post-perfusion incubation step. The isolated cells were washed (3 X 40 ml) in Waymouth MB 752/1 tissue culture medium supplemented with 10% foetal calf serum, penicillin (100 units/ml), streptomycin (100 mcg/ml), mycostatin (50 units/ml) and Hepes buffer (final conc. 2.5 mM) at pH 7.3. After the final wash the cells were resuspended in 40–50 ml of this medium.

2.2. *Hepatocyte culture*

The cell suspension was diluted to 3.5×10^6 cells/ml, with tissue culture medium, and 1.5 ml of this suspension seeded into one 9.5 cm^2 well of a Costar 6 well cluster dish and incubated at 37°C.

After 4 h fresh medium was added. Experimental additions were made either at this point (for 24 h incubations) or after 18 h at the next media change (0–10 h time courses).

2.3. *Haem determinations*

Reaction was stopped by harvesting the cells in: (a) the reaction medium; or (b) after removal of the medium into 0.9% (w/v) NaCl. The total haem concentration was determined as in [11].

2.4. *Metalloporphyrin extraction*

After 24 h incubation the cells and media from 10 cluster dishes were harvested, combined and the metalloporphyrin extracted as in [5].

2.5. *Spectroscopy*

Spectra were obtained using a chopped split-beam spectrophotometer (Applied Photophysics Ltd., London) interfaced to an Apple ITT 2020 Microprossesor used for baseline corrections and spectral manipulations.

2.6. *Protein assays*

Protein concentrations were determined as in [13].

2.7. *Cobalt protoporphyrin*

This was synthesized as described in [14].

3. Results and discussions

The ability of primary monolayer cultures of hepatocytes to incorporate exogenously supplied ALA in cell haem is well documented (e.g., [7,9]) however since optimal conditions may be expected to vary from system to system, as a preliminary to this study the optimal ALA concentration for conversion to haem was determined. The data obtained is shown in fig.1. In the complete incubation mixture (cells + media, fig.1A) there was a maximal rate of haem production at concentrations of ALA in the range 400–500 μM. Differences in haem concentrations became more significant when the cell fraction was examined (fig.1B). There was a sharp increase between ALA concentrations of 250 and 500 μM. This increase may be a reflection of haem transport limitations within the cell [15] which would in turn effect the rate of tetrapyrole transport into the surrounding medium [12]. At the higher concentrations the rate of haem production may be such that it is able to saturate the transport proteins and/or haem binding sites in the cell. Alternatively at the lower ALA concentration the activity of haem oxygenase may be sufficient to degrade the newly synthesized haem. In [7] maximum haem production is reported at an ALA range of 40–60 μM. These authors however only measured cell associated haem concentration increases and used maximum ALA concentration of only 200 μM.

In view of this result 500 μM was chosen at the incubating concentration of ALA. A time course for ALA conversion to haem in the presence and absence of 500 μM $CoCl_2$ is shown in fig.2 measurements refer to the complete incubation mixture. In the absence of $CoCl_2$ the haem concentration increases by 180%, $CoCl_2$ acts to reduce both the rate of ALA to haem conversion and the overal increase in haem concentration. Haem determinations relied on the conversion of the non-fluorescent metalloporphyrin to the fluorescent porphyrin, so could not be due to spectral differences between metalloporphyrins or direct inhibition of haem synthesis with subsequent porphyrin over-production. The difference in the overal rate is consistent with the known activity of ferrochelatase when assayed using protoporphyrin IX and Fe^{2+} or Co^{2+} as reported in [4].

The metalloporphyrin distribution between cells and media were also measured under these conditions (table 1) in a separate experiment. The single point (24 h) determination was consistent with the time course data. In the complete incubation sample metalloporphyrin production from ALA was again significantly greater in the absence of $CoCl_2$. In the cell fraction the reverse was true, there was a greater increase in metalloporphyrin in the presence of $CoCl_2$. These distributions are consistent with data obtained from rats injected with $CoCl_2$ [2].

The properties of the metalloporphyrin produced

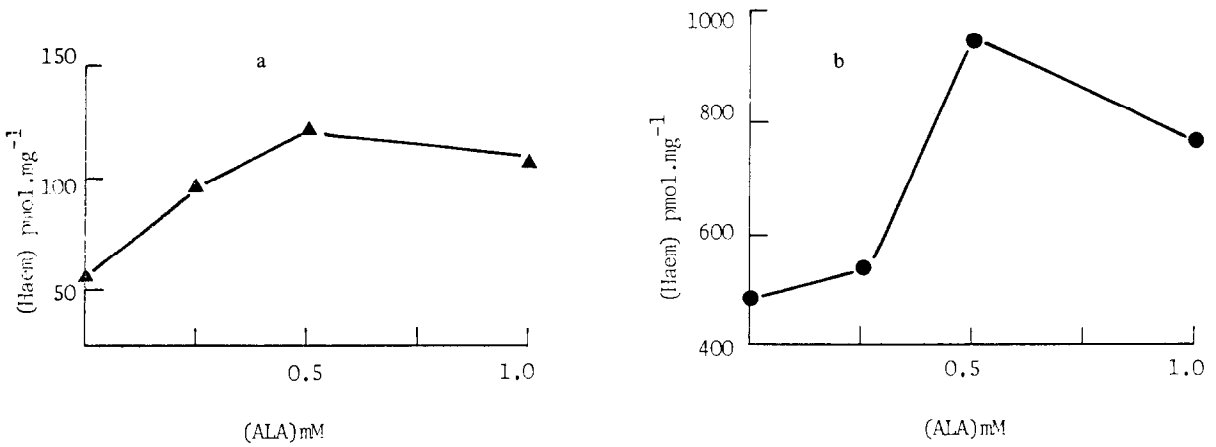

Fig.1. Production of haem by hepatocyte cultures, when supplied with an exogenous source of ALA: (a) increase associated with the complete incubation (cells + media); (b) medium was removed and the culture washed with 1 ml 0.9% (w/v) NaCl prior to harvesting the cells into this solution. Haem concentrations were determined as in [11].

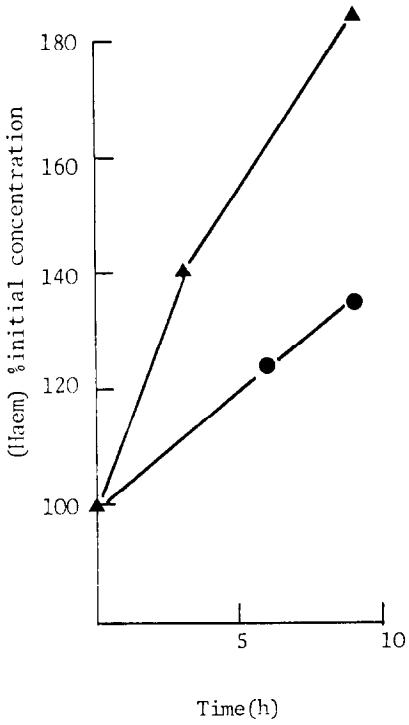

Fig.2. Time course of haem production from exogenously supplied ALA (500 μM) in the presence (●) and absence (▲) of $CoCl_2$ (500 μM). Haem was assayed in the complete incubation mixture as in fig.1.

Table 1
Metalloporphyrin production by hepatocyte cultures over a 24 h period when supplied with ALA (500 μM) in the presence or absence of $CoCl_2$ (500 μM)

Conditions	Cells + medium pmol haem/mg	Cells
No additions	59 (100%)	465 (100%)
+ ALA	216 (366%)	538 (117%)
+ ALA + $CoCl_2$	92 (156%)	605 (130%)
+ $CoCl_2$	66 (111%)	514 (110%)

The control incubations had no additions or just $CoCl_2$ (500 μM)

in the presence of $CoCl_2$ were entirely consistent with its being cobalt protoporphyrin. To confirm this $CoCl_2$-treated cultures were extracted as in [5]. The spectra data are shown in fig.3. The $CoCl_2$-treated cell extract spectrum is directly comparable with that in [5], and with the genuine cobalt porphyrin, except that the contaminant reported in the range 450–500 nm is absent. The difference in absorption maximum between the cell extract and authentic cobalt porphyrin probably reflects minute traces of pyridine and/or acetic acid in the final extract. Under the same conditions heam had a soret peak at 406 nm, an α peak at 518 nm and a β peak at 545 nm.

In conclusion it has been shown that, contrary to

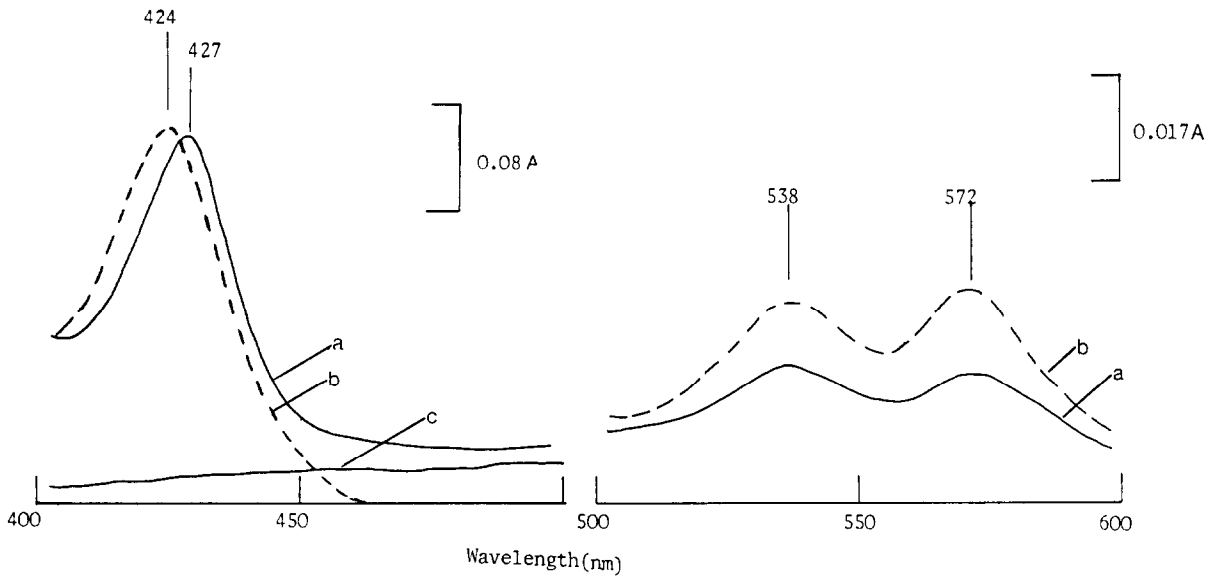

Fig.3. Absolute spectrum of the chloroform extract of hepatocyte cultures incubated with ALA (500 μM) and $CoCl_2$ (500 μM) for 24 h (a). Also shown is the spectrum of geniune cobalt protoporphyrin synthesised as in [14] (b) and the extract from a control sample (c). The metalloporphyrin extraction procedure is described in [5].

previous reports and in agreement with in vivo data isolated hepatocyte cultures in the presence of $CoCl_2$ are able to produce cobalt protoporphyrin. This again demonstrates the usefulness of hepatic culture systems in the study of liver metabolism. The possibility also exists that this system may now be used to monitor the properties of the iron insertion enzyme (ferrochelatase) in situ.

Acknowledgement

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