EVIDENCE FOR A RAPIDLY TURNED OVER POOL OF HAEM IN ISOLATED HEPATOCYTES

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1. Introduction

Mammalian tissues contain a variety of haemoproteins with protohaem as a prosthetic group and these haemoproteins serve widely different metabolic functions. There is evidence from the labelling of bile pigments, the breakdown products of protohaem, that a fraction of the haem in mammalian tissues undergoes rapid synthesis and degradation. An early labelled peak of bilirubin is found only 1–2 h following labelled glycine injection into a whole rat. A prolonged 'late phase' of labelled bilirubin production persists for 2–3 days. This rapidly formed and degraded protohaem is not derived from haemoglobin breakdown in the spleen but arises in the main from the liver [1–3] although some is contributed by immature erythroid cells [4]. Protohaem may serve as a regulator substance within the liver cell, controlling the formation of 5-aminolaevulinate synthetase [5,6] and consequently the induction of cytochrome *P*-450. Within te reticulocyte protohaem controls protein synthesis by controlling the formation of an inhibitor of translation [7–9]. It is interesting to speculate that the regulatory pool of protohaem may coincide with the rapidly turned over pool of protohaem. In order to determine the characteristics of the protohaem synthetic system in vivo under conditions which may be closely controlled and monitored we have studied this pathway in isolated adult rat hepatocytes. We have found that these cells have a capacity to take up iron from the medium and incorporate it into protohaem. A rapidly labelled protohaem fraction was detected in these hepatocytes; the specific activity of the haem increased to a maximum at about 60–120 min after the addition of labelled iron and then declined.

The limiting intermediate in protohaem synthesis is 5-aminolaevulinate; in studies on haem metabolism this has been commonly injected into animals as a specific labelled precursor of protohaem. It is a little surprising that such a key intermediate, whose concentration is closely regulated, should apparently freely pass through the cytoplasmic membrane. We have measured the rate of uptake of 5-aminolaevulinate and found, using hepatocytes, that there is a biphasic uptake of 5-aminolaevulinate, an instantaneous process accounting for 50% of the uptake followed by a slower uptake process which equilibrated approximately 30 min after addition of the label.

2. Materials and methods

2.1. *Isolation of hepatocytes*

Hepatocytes were isolated from female Wistar rats (200–300 g) as in [10]. The isolated hepatocytes were resuspended in Earls tissue culture media, supplemented with 10% foetal calf serum, at a concentration of approx. 5 mg cell protein/ml and usually had a viability of 80–95% as determined by trypan blue exclusion.

2.2. *Iron solutions*

Solutions of ferric iron were prepared by mixing a solution of ferric chloride with a 20-fold excess of sodium citrate solution, and solutions of ferrous iron by mixing with a 20-fold excess of sodium ascorbate.

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2.3. *Hepatocyte cultures*

Where hepatocytes were to be used in culture, the perfusion buffer used to prepare the cells was supplemented with 100 units/ml penicillin and the culture medium with 1 unit/ml penicillin and 1 mg/ml streptomycin. The cells were seeded at a density of 16×10^6 cells/ml, in 27 cm^2 Nunclon culture flasks containing 3 ml medium. After 4 h monolayers had formed and the culture medium was replaced with 3 ml fresh medium. Experimental additions were made after a further 18 h, at the next medium change.

2.4. *Incorporation of exogenous iron into the cell*

2.4.1. Using cell suspensions

Suspended cells, 16 ml, were transferred to a 100 ml stoppered plastic bottle in an atmosphere of air supplemented with 5% CO_2, on a shaking water-bath at 37°C. The experiment was started by the addition of 1 ml carrier-free iron solution (0.6 μg ^{55}Fe, 2 μCi). At intervals 1 ml samples were withdrawn and centrifuged (4000 × *g* for 15 s); the supernatant was removed and 2 ml acetone/HCl solution (2%, v/v) added to the packed cell pellet. The samples were stored at room temperature until the end of the experiment.

2.4.2. Using cell cultures

To 100 ml tissue culture medium was added 2 ml carrier-free iron solution (1.2 μg ^{55}Fe, 4 μCi). Incorporation studies were initiated by adding 3 ml of this medium to the cell monolayers at the second medium change. At intervals the labelled culture medium was removed, and after addition of 1 ml unlabelled culture medium, the cells were scraped off the culture dish, transferred to a centrifuge tube, and centrifuged (4000 × *g* for 15 s). The supernatant was removed and 2 ml acetone/HCl solution (2% v/v) was added to the cell pellet. The samples were stored at room temperature until the end of the experiment. At the end of the experiment, using either cell suspensions or culture, the protein was sedimented from the acetone/HCl suspension by centrifugation (2000 × *g* for 2 min) and the pellet re-extracted with a further 2 ml acetone/HCl. The bulked supernatants from each sample were evaporated to dryness on a rotary evaporator and the residue resuspended in 1 ml alkaline pyridine solution [11]. The alkaline pyridine suspension was centrifuged (18 000 × *g* 2 min) to remove any precipitated iron hydroxide and the supernatant, which contained the extracted and solubilised haem was retained for scintillation counting.

2.5. *Incorporation of 5-aminolaevulinate into haem*

The incubation procedure was identical to that used for the iron studies above. Reaction was started by the addition of 3.6 nmol 5-[G-^{3}H]aminolaevulinate (12 μCi) and the extraction of haem at the end of the incubation was as described above. To the acetone/HCl residue was added 3 ml glacial acetic acid and after the addition of carrier haem, the haem was crystallised as in [12].

2.6. *Total iron uptake studies*

To 15 ml liver cell suspension, inoculated as described above, was added 1 ml iron solution (1.2 μg ^{55}Fe, 10 μCi). At intervals 1 ml samples were withdrawn, the cells sedimented by centrifugation (4000 × *g* for 5 s), the supernatant removed and the inside of the centrifuge tube carefully wiped with a tissue. The pellet was dissolved in 1 ml formic acid.

2.7. *Total 5-aminolaevulinate uptake by liver cells*

To 2 ml suspension of liver cells was added various concentrations of 5-[G-^{3}H]aminolaevulinate and the cells incubated with shaking, at 37°C for 20 min after which time a 0.5 ml aliquot was removed and centrifuged (4000 × *g* for 5 s) after the supernatant was removed the centrifuge tube was wiped with a tissue and the cells dissolved in 0.5 ml formic acid. The degree of supernatant contamination of the dissolved pellet was determined by inclusion of [^{14}C]-inulin, which does not penetrate the liver cells, in the inoculation media.

2.8. *Protein assays*

Protein concentration was determined by the method in [13].

2.9. *Liquid scintillation counting*

Samples to be counted were spotted on Whatman 2.5 cm GF/C discs and dried for 30 min under an infrared lamp. The radioactivity on the discs was determined after suspension in scintillant, in a Nuclear Chicago Isocap/300. Where the samples to be counted were highly coloured, after spotting and drying the

discs were bleached with a minimum volume of hypochlorite solution and counted as above.

3. Results and discussion

Liver cells in suspension rapidly take up exogenously supplied iron (provided in the ferrous or ferric form), equilibration of the uptake process is rapid and occurs within 10–20 min of iron addition (fig.1a). At the same time there is rapid incorporation of the iron into cell haem, whether the iron is supplied in the ferrous or ferric form to cells in suspension or in the ferric form to cells in primary monolayer culture; in fig.1b are results obtained using ferric iron and cell suspensions. During experiments described in fig.1a, b labelled iron was always present and it was surprising to obtain a peak in haem labelling after about 60 min. Such a time course of labelling could indicate the rapid synthesis and breakdown of a cellular component and is the type of result normally associated with a pulse experiment, where exposure to the label is relatively brief. With our system a linear uptake would be expected. Four possible explanations were considered for the results obtained:

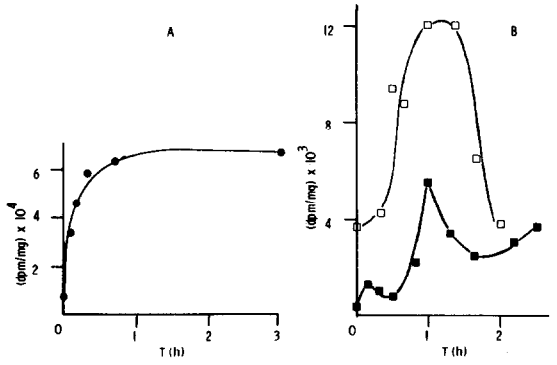

Fig.1. Uptake of iron by liver cells and subsequent incorporation into haem. To 15 ml liver cell suspension was added 1 ml iron solution (1.2 μg Fe^{3+}, 10 μCi) at intervals samples were withdrawn and the cells sedimented. To determine total iron uptake (A) the cells were dissolved in 1 ml formic acid prior to scintillation counting, while for haem incorporation measurements (B) the cells were extracted with acetone/HCl and after evaporation either resuspended in alkaline pyridine for scintillation counting (□) or the haem crystallised (■) as described in the text. Each point is an average of 2 determinations.

1. The labelling peak is an artefact associated with the haem isolation technique.

2. Cell 'death' was occurring after the 1 h incubation which caused a rapid breakdown of cell haem.

3. There is an artefact associated with iron metabolism.

4. A limiting supply of iron for haem synthesis, that is a 'self-imposed' pulse of iron.

We have investigated these possibilities. The use of acetone/HCl solution to extract haem is widely used (e.g. [11]). The acetone/HCl extracts were evaporated to dryness and resuspended in alkaline pyridine (pH ~ 10); had any free iron been carried over it should have been converted to ferric hydroxide and removed by centrifugation. Since at t=0 there should be no labelled haem, the number of counts recovered at this time are a measure of the degree of contamination by free (labelled) iron. An average value of 2.1×10^3 dpm/mg cell protein was obtained from 6 separate determinations. Also recrystallisation of the extracted haem did not affect the pattern of labelling obtained after the incubation with labelled iron for varying times.

The stability of the liver cells, during the time course of the incubations, was measured by the trypan blue vital staining technique and by monitoring the cell ATP levels, as a test of cell viability. As shown in table 1, during the time course of the experiment the ATP levels did not fall and the percentage of trypan blue staining cells remained constant. To test the reliability of these assays of cell viability, a sample of cells was allowed to become anaerobic and

Table 1
Viability of cell suspensions during incubation as determined by ATP levels and trypan blue exclusion

Time (min)	ATP (nmol/mg)	% Cells trypan blue staining
0	14.0 (4)	< 20
30	16.6 (4)	< 20
60	14.9 (4)	< 20
90	16.6 (4)	< 20
120	17.9 (4)	< 20
'dead' control	0.1 (3)	100

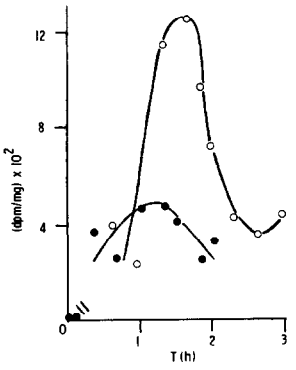

Fig.2. Pulse labelling of isolated liver cells. Liver cell suspensions were pulsed with either 5-aminolaevulinate (•) or iron citrate (○) for 7 min prior to incubation at 37°C. At intervals samples were removed and the haem extracted and either crystallised (•) or resuspended in alkaline pyridine (○) prior to scintillation counting, as described in the text.

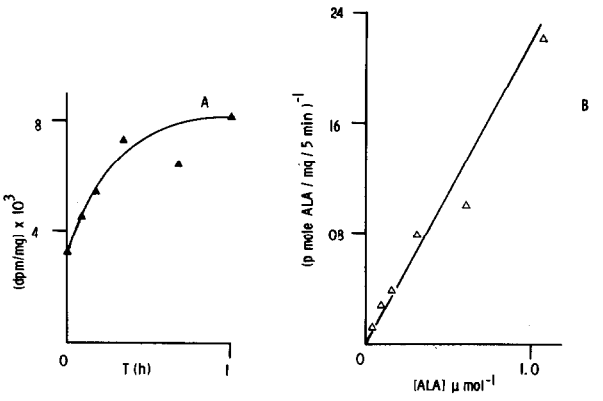

Fig.3. Uptake of 5-aminolaevulinate by liver cells. Liver cell suspensions were incubated in 5-[G-^{3}H]aminolaevulinate (A) 0.36 nmol (1.2 μCi)/ml), at intervals aliquots were removed and after sedimentation dissolved in NaOH prior to scintillation counting. The degree of supernatant contamination was determined using [^{14}C]inulin which does not penetrate the cells. The effect of varying the concentration of 5-aminolaevulinate (B) on slow uptake phase was also examined and the data plotted as the concentration of 5-aminolaevulinate taken up by the cells against the concentration of 5-aminolaevulinate in the incubation.

kept in this condition for 1 h, the ATP levels and the trypan blue staining were then tested. It was found that the ATP level dropped dramatically and all the cells were permeable to trypan blue.

Using a pulse of 5-aminolaevulinic acid, a specific biosynthetic precursor of protohaem (supplied at 0.3 μM, near tissue levels), or iron (Fe^{3+}), a peak of haem labelling was again observed (fig.2). It was found however that 5-aminolaevulinic acid, an amino acid specific for haem synthesis and commonly used exogenously in studies of its effects on the regulation of haem metabolism (e.g. [14]) was poorly incorporated into haem in our experiments. To see if this was due to limiting uptake, the uptake process of aminolaevulinic acid by isolated cells was studied. As shown in fig.3, there are two phases of aminolaevulinate uptake, an instantaneous process which accounts for 40–50% of the total uptake, followed by a slow uptake to equilibrium. From a study of the concentration dependence of the slow uptake, it seems that during this phase 5-aminolaevulinate enters the cell by simple diffusion, there was no indication of carrier system that could become saturated. It was thus possible to determine from the uptake studies that during the pulse experiment the low rate of incorporation of 5-aminolaevulinate into haem was a result of a limited supply of the precursor. However, these low concentrations of aminolaevulinate are closer to those found in the liver cell in vivo and it is thought that the use of unnaturally high levels of aminolaevulinate might result in non-physiological labelling patterns.

It thus appears that both a continuous supply of iron and a pulse of iron or 5-aminolaevulinate result in a peak of haem labelling. It is likely that the iron result obtained is due to a 'self-imposed' pulse of iron by the liver cells. Under the conditions of the experiment approx. 5 times as much iron entered the cells as was incorporated into cell haem (fig.1). Such a pulse effect could arise if some iron transport carrier or process within the cell became limiting during the period of iron feeding. It is possible that iron has to be transported through the cytosol and across the mitochondrial membrane before becoming available to ferrochelatase for haem synthesis [15]. Subsequently haem is probably transported from the mitochondrial matrix to the microsomal membranes for breakdown by haem oxygenase or incorporation into haem proteins. No matter what the explanation for the pulse effect, our experiments clearly show that in isolated liver cells haem is rapidly broken down. This

process may be part of some fine control of the level of haem proteins within the cell such as has been suggested by numerous authors (e.g. [8]).

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