The role of cytosolic proteins in the intracellular transport of haem in rat liver

A dual-label approach

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After consecutive injections of δ -amino[^3H]- and [^{14}C]-laevulinic acid, the incorporation of the two labels into haem associated with different subfractions of the liver was determined. Marked differences in the $^{14}\text{C}/^3\text{H}$ ratios were observed between haem associated loosely and tightly with microsomes and mitochondria and haem associated with three subfractions of the cytosol obtained by gel filtration. The effect of changing the amounts of δ -aminolaevulinic acid injected and of changing the interval between injections and killing of the animal on the ratios of labels in the haem of each subfraction was studied. The results are discussed in terms of the flow of haem from the mitochondria to other parts of the cell via putative cytosolic carrier proteins.

Shortly after administration of radiolabelled ALA, labelled haem is found to be associated with the mitochondrial, microsomal and soluble supernatant fractions of rat liver (Lazarow & DeDuve, 1973; Ketterer *et al.*, 1976; Yannoni & Robinson, 1978). Previous investigations *in vitro* (Yoda & Israels, 1972; Taketani & Tokunga, 1980; Husby *et al.*, 1980) have indicated that haem transport is a protein-mediated event. These proteins may also function in the export of haem from the liver cell (Bissell *et al.*, 1979). Although newly synthesized haem was shown previously to be associated with cytosolic protein components, the binding of haem alone does not necessarily imply that these proteins function in the transport of haem. In the present paper, we report on studies utilizing successive administration of [3,5- ^3H]ALA and [4- ^{14}C]ALA to demonstrate the direction and relative magnitude of the flow of haem through the liver. This approach is a modification of the dual labelling by Arias *et al.* (1969), which has been used previously with ALA to

demonstrate the greater susceptibility of the more rapidly turning-over haem pool(s) to degradation by the agent 2-allyl-2-isopropylacetamide (De Matteis, 1971). By using this technique, we attempted to delineate the function of cytosolic proteins in the transport of haem from mitochondria to other cellular components. The results of the present work are consistent with the function of cytosolic proteins in haem transport, although no specific protein has as yet been identified.

Materials and methods

Chemicals

[3,5- ^3H]ALA (sp. radioactivity 1.19 Ci/mmol) and [4- ^{14}C]ALA (sp. radioactivity 51.5 Ci/mol) were purchased from New England Nuclear. ALA, purchased from Sigma, was purified by chromatography on Whatman no. 1 paper with n-butanol/acetic acid/water (25:4:10, by vol.). ALA was quantified with Ehrlich's reagent after cyclization with acetyl acetone (Mauzerall & Granick, 1956). All other chemicals were of analytical grade.

Experimental protocol

Male Wistar rats weighing 280–310 g were supplied with food and water *ad libitum*. For the time-course experiments, 170 nmol of [^3H]ALA in 0.15 M-NaCl buffered with 0.01 M-sodium phosphate, pH 7.4, was injected via a lateral tail vein. After 1 h, 770 nmol of [^{14}C]ALA was injected into the same vein. The animals were killed at selected

Abbreviations used: haem, iron-protoporphyrin IX; ALA, δ -aminolaevulinic acid; Hepes, 4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid.

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intervals (0.25–3.0 h) after the second injection. In a second series of experiments the amount of ALA injected was varied. In each instance the second label was administered 1 h after the first and the animals were killed 15 min thereafter. Immediately after killing, the livers were perfused with 100 ml of the ice-cold buffered-saline solution and minced. Homogenates were prepared in ice-cold 0.25 M-sucrose buffered with 2 mM-Hepes, pH 7.4 (homogenization buffer), with three strokes of a Potter–Elvehjem homogenizer.

Subcellular fractionation

The cytosolic fraction was obtained after centrifugation of a portion of an homogenate, containing 2 ml of the homogenization buffer/g of liver, at 100 000 *g* for 1 h and by carefully separating the supernatant from the pellet and floating lipid layer with a syringe. A portion of an homogenate containing 10 ml of buffer/g of liver was initially centrifuged at 1000 *g* to remove the nuclear fraction and cell debris. To obtain the mitochondrial fraction this supernatant was centrifuged at 7800 *g* for 10 min and the pellet was washed with homogenization buffer and resuspended to a concentration of approx. 15 mg of protein/ml. The microsomal fraction was prepared by centrifugation of the second portion of the 10 ml/g of liver homogenate at 10 000 *g* for 20 min to remove the nuclear fraction, cell debris and mitochondria. The supernatant was centrifuged at 105 000 *g* for 1 h. The pellet was resuspended, centrifuged a second time and resuspended to a final concentration of 15 mg of protein/ml. Cross contamination of microsomes by mitochondrial outer-membrane fragments was less than 15% as assayed by monoamine oxidase activity with benzylamine as a substrate (Schnaitman *et al.*, 1967). Contamination of the mitochondria with microsomes was negligible as determined by cytochrome *P*-450 content measured from the decreased carbon monoxide absorbance at 450 nm of cytochrome *P*-450 (Omura & Sato, 1964). The presence of other organelles in the three fractions was not investigated.

Loosely bound haem was operationally differentiated from the more firmly bound haem by the method of White *et al.* (1978). In this procedure the mitochondrial and microsomal fractions were treated for 6 min each with a solution containing 1.5 mM-human serum albumin and 1.5 mM-dithioerythritol at 37°C. The mixtures were centrifuged at 105 000 *g* for 1 h at 4°C to separate the supernatant containing haem associated with albumin from haem remaining bound to the membranes.

The liver cytosol was fractionated by gel filtration on a column (1.5 cm × 60 cm) containing LKB Ultrogel AcA-44. Cytosol (2 ml) was applied to the column equilibrated with 10 mM-Hepes, pH 7.4,

containing 0.1 M-NaCl. The column was eluted at a flow rate of 10 ml/h, and 1.5 ml fractions were collected. Representative results are shown in Fig. 1. Haem analysis was carried out on the four peak fractions containing radioactivity.

The preparation of subcellular fractions and the gel-filtration experiments were carried out at 0–4°C. The fractions were stored at –70°C after freezing in liquid N₂. No differences were observed in results obtained for freshly processed samples and those stored frozen.

Determination of labelled haem

Labelled haem was recovered from the samples after addition of 100 µl of a 10% (w/v) haemoglobin solution to provide carrier haem. The protein in the sample was precipitated by trichloroacetic acid (10% final concentration) and the haem was extracted from the precipitate with ethyl acetate/acetic acid (3:1, v/v). The extract was washed twice with an equal volume of saturated sodium acetate and once each with water and 15% (w/v) HCl. The sample was dried under a stream of N₂ and purified on 0.1 mm MN polyamide-6-chromatography plates developed with 2.5% acetic acid in methanol (Lamson *et al.*, 1973). The band corresponding to haem was eluted from the plate with pyridine and dried and dissolved in 0.5 ml of *NN*-dimethylformamide. Yields of haem recovered by this method were determined by adding purified labelled haem to the fractions immediately before the addition of the carrier haemoglobin. Recoveries were 20 ± 3%. In the process of fractionation and isolation of the haem, approx. 50% of the protohaem was converted into haematohaem (observed on polyamide t.l.c.). Since haematohaem is more water soluble than protohaem it is more likely to be redistributed among the fractions. The separation procedure employed in the present study resolved protohaem from haematohaem, thereby avoiding this potential artefact.

To determine the radioactivity of the haem, 10 ml of Econofluor was added to the haem solution and the ³H and ¹⁴C radioactivities were determined in low- and high-energy channels by liquid-scintillation spectrophotometry. The radioactivity counts were at least three times background. The efficiency of counting was determined from external standard calibration curves, with haem to correct for quenching. In the low-energy channel the counting efficiency for ³H was greater than 35% and was less than 18% for ¹⁴C. In the high-energy channel, the counting efficiency for ¹⁴C was greater than 43%, whereas that for ³H was zero.

The measured ratio of second to first label in the haem (after correction for loss of ³H during haem synthesis) was divided by the ratio of the two isotopes in the administered ALA. A corrected ratio

of 1.0 in a particular fraction thus indicates that equal proportions of each of the injected doses of ALA are incorporated into the haem of that fraction. The correction factor for the loss of ^3H during haem synthesis was determined from the ratio of labels in haem after administration of a known mixture of [^3H]ALA and [^{14}C]ALA. Three experiments gave an average value of 29.7% incorporation with a standard deviation of $\pm 0.2\%$. The following equation was used when [^{14}C]ALA was the second label:

$$\begin{aligned} \text{Corrected ratio} &= 29.7 \times \frac{^{14}\text{C (d.p.m.)}}{^3\text{H (d.p.m.)}} \\ &\quad \text{ratio in haem extracted} \\ &\times \frac{[\text{total } ^3\text{H}]\text{ALA injected (d.p.m.)}}{[\text{total } ^{14}\text{C}]\text{ALA injected (d.p.m.)}} \end{aligned}$$

When [^3H]ALA was the second label the equation was:

$$\begin{aligned} \text{Corrected ratio} &= \frac{100}{29.7} \times \frac{^3\text{H (d.p.m.)}}{^{14}\text{C (d.p.m.)}} \\ &\quad \text{ratio in haem extracted} \\ &\times \frac{[\text{total } ^{14}\text{C}]\text{ALA injected (d.p.m.)}}{[\text{total } ^3\text{H}]\text{ALA injected (d.p.m.)}} \end{aligned}$$

Results

The incorporation of haem synthesized from ^3H - and ^{14}C -labelled precursors into microsomes, mitochondria and cytosol was examined. The percentage of counts administered that appears as haem in each of these fractions was similar to that observed in experiments by Yoda & Israels (1972) and Garner & McLean (1969). In addition, the cytosol fraction was analysed by gel filtration for the presence of a subfraction that displays an exceptionally high ratio of $^{14}\text{C}/^3\text{H}$ (second label/first label) and that might therefore be implicated as containing a component instrumental in intracellular haem transport. Fig. 1 shows the distribution of ^3H and ^{14}C labels in the subfractions resolved by gel filtration for two rats, one killed at 0.25 h and the other at 1.33 h after administration of the second label. Four prominent peaks of radioactivity were resolved in both cases as well as in experiments with other animals.

The highest-molecular-weight peak (A) contained catalase activity as assessed by the method of Greenfield & Price (1956). The radioactivity in this subfraction increases greatly over the 3-h experimental period. By contrast, the amount of ^{14}C and ^3H in peaks B and C showed much less change. Glutathione *S*-transferase activity was detected in these fractions by using 1-chloro-2,4-dinitrobenzene as substrate (Habig *et al.*, 1974), suggesting these two peaks might contain the cytosolic protein ligandin, which is known to bind haem (Ketterer *et*

al., 1976). Fractions B and C contained approx. 60% and 40% respectively of the glutathione *S*-transferase activity. However, the presence of ligandin was detected by radial immunodiffusion only in fraction B.

The fourth and lowest-molecular-weight peak, constituting the salt fraction, fractions 55–70, although exhibiting a relatively high absorbance at 280 nm, contained no measurable protein when assayed by the method of Bensadoun & Weinstein (1976). The ^{14}C content of these fractions was very low even at 15 min after administration of [^{14}C]ALA. The total content of ALA, determined by the method of Mauzerall & Granick (1956) was also very low. The ^3H content in this subfraction may be attributed to protons that are a by-product of haem formation derived from the [3,5- ^3H]ALA.

Haem was isolated from the peak fractions of the three cytosolic subfractions A, B and C and the ratio

Fig. 1. Fractionation of cytosol by gel filtration. Total radioactivity was measured in fractions (1.5 ml) eluted from an Ultrogel AcA-44 gel column (1.5 cm $\times$ 60 cm). Recorded are A_{280} (—), ^{14}C (d.p.m.) (●) and ^3H (d.p.m.) (○). Animals were treated as described in the text and were killed 0.25 h (a) and 1.33 h (b) after the second injection ([^{14}C]ALA), which was given 1 h after the first ([^3H]ALA). Peak A contained catalase activity. Peaks B and C showed glutathione *S*-transferase activity, and ligandin was detected immunologically in peak B. The lowest-molecular-weight peak of radioactivity exhibited a high absorbance at 280 nm but contained no protein as assayed by the method of Bensadoun & Weinstein (1976).

of $^{14}\text{C}/^3\text{H}$ incorporated into the haem of these fractions was determined for experiments in which the $[^3\text{H}]\text{ALA}$ was administered 1 h before the $[^{14}\text{C}]\text{ALA}$. The animals were subsequently killed at different times (Fig. 2) after the administration of the second label. In all three cases, the ratios were observed to vary with time. However, the temporal behaviour of the ratio for fraction A differed from that observed for fractions B and C. The $^{14}\text{C}/^3\text{H}$ ratio in the latter fractions displayed an initial rise to a value of approx. 2.0, followed by a gradual decline to a value that remained greater than 1.0 at the 3-h time point. On the other hand, the ratio of the two labels incorporated into the haem of peak A was less than 1.0 at the earliest time points, but was essentially unity for time points exceeding 1 h. All of the radioactivity in this fraction could be attributed to haem. This was not the case for peaks B and C, which showed several labelled species other than haem. One of these was identified as protoporphyrin after its isolation by the method of Doss (1974) in the presence of carrier porphyrins. A non-homogeneous distribution of porphyrins may have contri-

buted to the differences in the temporal change in total radioactivity observed in peaks B and C when Figs. 1(a) and 1(b) are compared. In contrast, similar ratios are observed for haem derived from these fractions (Fig. 2).

The ratio of $^{14}\text{C}/^3\text{H}$ incorporated into haem was also measured for haem loosely and firmly bound to the mitochondrial and microsomal subfractions. The loosely bound haem was removed from the mitochondrial and microsomal fractions by the human serum albumin/dithioerythritol treatment described by White *et al.* (1978). The temporal behaviour of the $^{14}\text{C}/^3\text{H}$ ratio of the haem firmly bound to mitochondria and microsomes was similar to that of cytosol subfraction A (Fig. 2). For both the microsomal and the mitochondrial fractions this ratio was initially less than 1.0 and subsequently approached unity within 1 h. By contrast, the temporal changes of the ratios for haem loosely attached to the two subcellular fractions differed. The ratio of $^{14}\text{C}/^3\text{H}$ haem loosely associated with the microsomal fraction fluctuated near unity throughout the experimental period. In contrast, the ratio of haem loosely bound to mitochondria was greater than the one at the earliest time point, but declined to unity at approx. 1 h.

The effect of the ALA dosage on the ratio of $^{14}\text{C}/^3\text{H}$ incorporated into haem was examined in a separate set of experiments. The results are listed in Table 1. We can conclude that the ratios (see lines 2 and 3 of Table 1) are independent of the order in which the two labels were administered. For the largest second dose of ALA (see line 4 of Table 1) the ratios in all subcellular fractions were approximately one-third lower than those observed for the smaller doses. This finding indicates that for large amounts of ALA given as a second dose, there may be a delay in the time of completion of haem formation or a less complete utilization of ALA for haem synthesis after the second label is administered. All other doses used showed similar ratios in each of the subcellular fractions.

Fig. 2. Ratio of $[^{14}\text{C}]\text{haem}$ to $[^3\text{H}]\text{haem}$ extracted from subfractions of mitochondria, cytosol and microsomes. Data are the averages of four experiments for the first time point (data of line 1 in the table) or two experiments for the remaining points (the values differed less than 10%). For mitochondria and microsomes, O represents haem removed by human serum albumin/dithioerythritol treatment (White *et al.*, 1978) and ● represent residual haem. The data for the cytosol subfractions B (□), C (○) and A (●) are taken from peak fractions of the gel-filtration eluates as shown for a representative experiment in Fig. 1. The ratio of radioactivity (d.p.m.) of $[^{14}\text{C}]\text{haem}$ to $[^3\text{H}]\text{haem}$ was corrected by a factor accounting for the different amounts of each label administered and for the stoichiometry of the incorporation of ^3H from $[3,5\text{-}^3\text{H}]\text{ALA}$ into haem, determined as described in the Materials and methods section. Each time point shown refers to the time elapsed after the administration of the second label, $[^{14}\text{C}]\text{ALA}$, which was injected 1 h after the first label, $[^3\text{H}]\text{ALA}$.

Discussion

The association of haem with various cellular fractions was traced by using a double-isotope labelling technique. The incorporation of $[^{14}\text{C}]\text{ALA}$ and $[^3\text{H}]\text{ALA}$ into haem was used to distinguish, in time-course experiments, newly synthesized haem from haem synthesized 1 h before. In this manner, we hoped to identify proteins that bind and subsequently transfer haem rapidly, since turnover of haem is necessary if these proteins function to transport haem from its site of synthesis in mitochondria to its site of incorporation into haem proteins.

It has been suggested that administration of ALA

Table 1. *Dependence of the ratio of second label to first label in haem on the amount of ALA given†*
 Values in parentheses are standard deviations ($n = 4$). The range of duplicate experiments was less than 10% of the mean.

Experimental protocol line no.	Amounts of ALA injected (nmol)		Corrected ratio of second label/first label in haem bound to liver subfractions*						
			Mitochondria		Microsomes		Cytosol		
	First Label	Second Label	Tightly bound	Loosely bound	Tightly bound	Loosely bound	A	B	C
1	770 (^{14}C)	170 (^3H)	0.46	2.5	0.44	0.78	0.50 (± 0.12)	2.11 (± 0.41)	1.89 (± 0.18)
2	770 (^{14}C)	770 (^3H)	0.51	1.71	0.47	1.21	0.60	2.00	1.89
3	770 (^3H)	770 (^{14}C)	0.45	2.25	0.47	1.05	0.49	2.08	2.49
4	770 (^{14}C)	8170 (^3H)	0.16	0.94	0.15	0.27	0.21	0.75	0.83
5	170 (^3H)	770 (^{14}C)	0.57	1.52	0.51	1.01	0.47 (± 0.15)	1.70 (± 0.24)	1.59 (± 0.33)

* Corrections for the different amounts of each label administered and the percentage incorporation of ^3H from [3,5- ^3H]ALA were made as described in the Materials and methods section.

† Animals were given the second injection of ALA at 1 h after the first and killed 15 min thereafter. Line 1 is the average of two (mitochondria, microsomes) or four (cytosolic fractions) experiments. These data were used in Fig. 2. Lines 2 and 3 represent single experiments. Line 4 is the average of two experiments and line 5 the average of two (mitochondria, microsomes) or four (cytosolic fractions) experiments. The amounts of [^3H]ALA in lines 2, 3 and 4 were made by adding unlabelled ALA to the usual amount of labelled ALA.

may result in the formation of a 'free' haem pool in the liver (e.g. Druryan & Kelly, 1972). This pool may exist because of the excess production of haem from the large doses of ALA utilized in the present study. Since this haem may be associated non-specifically with the mitochondrial and microsomal membranes, the fractions were washed with human serum albumin. White *et al.* (1978) have shown that human serum albumin in the presence of dithioerythritol removes free haem associated with the microsomal fraction.

The haem loosely bound to the mitochondrial fraction initially exhibited a high ratio of newly synthesized [^{14}C]haem (second label) to [^3H]haem (first label). This is consistent with the known function of this organelle as the site of the terminal step in haem synthesis (Jones & Jones, 1969). The decrease in ratio of the more recently synthesized [^{14}C]haem to the older [^3H]haem with time after the successive administration of [^3H]ALA and [^{14}C]ALA indicates that the turnover of the newly synthesized haem does not proceed in a simple first-order manner, which would yield a constant ratio of the two labels. The decrease in the ratio of the more recently synthesized haem might arise if the loosely bound haem comprised two pools with different rates of turnover, or if the two labels equilibrated via exchange with a pool of haem exhibiting a lower ratio, such as the haem loosely bound to microsomes. The time dependence of the $^{14}\text{C}/^3\text{H}$ ratios of the haem loosely associated with the microsomal fraction cannot be easily explained. Fluctuations in the ratio at the earliest time points (Table 1) could have masked the true behaviour of this pool of haem.

The ratio of [^{14}C]haem/[^3H]haem associated with the cytosol subfractions B and C increases to a value above unity at about 30 min after administration of the second label, whereafter it decreases gradually. This behaviour is consistent with components of the subfractions B and C being instrumental in the flow of haem from the mitochondria to other parts of the cell, as suggested by Ketterer *et al.* (1976). The subsequent gradual decrease in ratios may indicate a protracted exchange of haem between components of the subfractions B and C with fractions containing haem with lower ratios of $^{14}\text{C}/^3\text{H}$ or it may indicate a heterogeneous turnover of haem within subfractions B and C. In the latter case, the behaviour of a rapidly depleted haem-binding component could be masked at longer times by a gradual depletion of other haem-binding components of subfractions B and C. Had equilibrium occurred, the ratio of [^{14}C]haem/[^3H]haem in subfractions B and C would have reached unity. Since unity was not reached and the ratios at the 1.33- and 3.0-h time points are similar, a heterogeneous turnover due to several haem-binding components is the more likely possibility. Further resolution of subfractions B and C may reveal components solely responsible for haem transport. However, the limited amount of material obtained from the gel-filtration experiment did not allow us to further resolve the components contained in the subfractions.

The observation of a gradual increase to unity of the ratio associated with haem in cytosol subfraction A, which contains catalase, and that in haem tightly bound to mitochondria and microsomes, both of which contain large concentrations of cytochromes, suggests that components of these

subcellular fractions are the ultimate destination of haem. This is also apparent in Fig. 1, where the total radioactivity in peak A can be seen to increase at 1.33 h relative to 0.25 h when Fig. 1(b) is compared with Fig. 1(a).

These results suggest that haem, formed from injected ALA in the mitochondrion, flows by reversible pathways to other parts of the cell. The data are consistent with components of cytosol subfractions B and C, perhaps ligandin (Ketterer *et al.*, 1976), being instrumental in this flow. Under conditions of increased concentrations of haem in tissues, e.g., with increased haem uptake by the liver in states of haemolysis (Eyster *et al.*, 1972) or treatment for porphyria (Watson *et al.*, 1977), the flow of haem to the mitochondria may predominate and cause the well-recognized feedback inhibition of mitochondrial ALA synthase (Granick *et al.*, 1975) or prevent the uptake of this enzyme by the mitochondria (Yamauchi *et al.*, 1980). In keeping with this concept, we found haem associated with cytosol subfractions B and C after intravenous administration of [^{59}Fe]haem-haemopexin (Davies *et al.*, 1979).

We conclude that the flow of haem from the mitochondria to other cellular fractions is potentially a reversible process and that cytosol subfractions B and C contain protein carriers that may be implicated in intracellular haem transport.

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