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The Structural Organization of Haem Synthesis in Rat Liver Mitochondria

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1. Anaerobic conditions are normally necessary for incorporation of iron into haems and only ferrous iron is used. After addition of succinate to an incubation mixture containing intact or ultrasonically treated mitochondria, Fe^{3+} is used, but only if no inhibitors prevent the transfer of electrons from the mitochondrial respiratory chain to oxygen. 2. A dual-wavelength spectrophotometric assay for ferrochelatase is described that has been used for the continuous assay of incorporation of metal ions into porphyrins. Constants are given for the determination of rates of formation of protohaem and cobalt protoporphyrin, mesohaem, cobalt mesoporphyrin and zinc mesoporphyrin. For cobalt mesoporphyrin formation the K_m for Co^{2+} is 11×10^{-6}M and that for mesoporphyrin is 5×10^{-6}M. 3. An improved method for the separation of inner and outer membranes of mitochondria is described. Mitochondria swollen in hypo-osmotic media were contracted in hyperosmotic potassium chloride solution containing ATP and the outer membranes detached by mild ultrasonic treatment. Sucrose inhibited the ATP-induced contraction and decreased the yield of outer membranes. 4. Ferrochelatase is associated with cytochrome oxidase, which is used as a marker for inner mitochondrial membranes. 5. By using as substrate porphyrin dissolved in phospholipid micelles, ferrochelatase activity of intact mitochondria was shown to be latent, and to be liberated by ultrasonic treatment. 6. No ferrochelatase was detectable in microsomes or soluble cell components.

The final stage in haem biosynthesis, the incorporation of iron into protoporphyrin, is catalysed by ferrochelatase, a mitochondrial enzyme (Nishida & Labbe, 1959). In rat liver mitochondria the enzyme may be presumed to function in the formation of the prosthetic groups of cytochromes and possibly of catalase and peroxidase. In our experiments (M. S. Jones & O. T. G. Jones, unpublished work) we have failed to detect any ferrochelatase activity in microsomes when protoporphyrin or mesoporphyrin was substrate, nor was such activity found in other cell fractions. This suggests that haem synthesis may occur at a site different from that of the protein of the cytochrome, since at least with cytochrome *c* it appears that synthesis of the cytochrome apoprotein takes place on the microsomes (Roodyn, Suttie & Work, 1962; Gonzalez-Cadavid & Campbell, 1967). Combination of the haem and the protein may then take place in a later reaction, perhaps analogous to the combination of haem and globin. Since there may be a requirement for haem production to be closely integrated with apoprotein synthesis it appeared possible that ferrochelatase was located on the outer membrane of the mitochondrion, in proximity to the microsomes, and in the present paper we describe experiments to determine the submitochondrial distribution of ferrochelatase by using an improved method for the separation of mitochondria into inner-membrane, outer-membrane and soluble matrix fractions. We have used a new assay for ferrochelatase based on the continuous measurement of disappearance of porphyrin by dual-wavelength spectroscopy and have investigated the accessibility of the enzyme in intact mitochondria to externally added substrates. Our results suggest that ferrochelatase is located on the inside of the inner mitochondrial membrane.

MATERIALS AND METHODS

Preparation of mitochondria. Rat livers were homogenized in 8vol. of medium containing (final concentrations) 0·25M-sucrose, 4mM-tris–HCl buffer, pH7·2, and 1mM-ethylenedioxybis(ethyleneamino)tetra-acetic acid. After sedimentation of the nuclear fraction by centrifugation at 800*g* for 10min. the supernatant was centrifuged at 17000*g* for 4min. and the mitochondrial pellet was washed two or

three times in the same buffer. During the washing procedure any freely flowing reddish material was discarded. Ultrasonically treated mitochondria were prepared by using an MSE 60w Sonicator (3min.).

Preparation of microsomal fraction. Microsomes were prepared by differential centrifugation of the 17000*g* supernatant obtained during preparation of the mitochondrial fraction; the remaining mitochondria were removed by centrifugation at 15000*g* for 15min., and the microsomes were sedimented by centrifugation at 60000*g* for 90min. and resuspended in 0·25M-sucrose.

Soluble ferrochelatase. This was prepared from mitochondria, in Tween 80 at pH10·0, as described by Porra, Vitols, Labbe & Newton (1967).

Preparation of membrane fractions. Freshly prepared mitochondria (about 200mg. of protein) were allowed to swell for 7min. in 30ml. of phosphate (10mM)–tris buffer, pH7·5. Then 10ml. of a solution containing (final concentrations) 0·48M-KCl, 2mM-ATP and 2mM-$MgSO_4$, adjusted to pH7·4 with tris, was added and after 5min. 5ml. samples of the suspension were ultrasonically treated by using an MSE 60w Sonicator (10sec., 1·5A) to free outer membranes from the contracted inner membranes. The two membrane fractions were separated by application of swollen, shrunk and ultrasonically treated mitochondria (20ml.) to a sucrose gradient consisting of 1·32M-sucrose (22ml.) and 0·76M-sucrose (11ml.) in 60ml. centrifuge tubes. The tubes were centrifuged at 24000rev./min. for 3½hr. in the SW 25.2 rotor of a Beckman–Spinco preparative centrifuge.

Ferrochelatase assays. The development of the continuous dual-wavelength assay is described in the Results section. Formation of [^{59}Fe]haem was measured as described by Jones (1968). Haem formation was also measured by the pyridine haemochrome assay (Porra & Jones, 1963).

Assay of marker enzymes. Malate dehydrogenase was assayed by the method of Ochoa (1955); monoamine oxidase was assayed as described by Schnaitman, Erwin & Greenwalt (1967); cytochrome b_5 and cytochromes a and a_3 were assayed as described by Jones & Jones (1968).

Porphyrins. Mesoporphyrin IX was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks. Protoporphyrin IX was prepared as described by Falk (1964). Approx. 1mM solutions of porphyrins were prepared in 2% (v/v) Tween 80 in 40mM-tris–HCl buffer, pH8·2. The porphyrin concentrations of the solutions were calculated from the intensity of the Soret maximum in acid solution (Falk, 1964).

Porphyrin micelles. Lecithin (phosphatidylcholine) prepared from egg yolk was a gift from Dr J. D. McGivan. A solution of lecithin in chloroform was evaporated to dryness under a jet of N_2 and the residue taken up in an ether solution of the porphyrin. The ether was evaporated under N_2 and the residue suspended in 200mM-tris–HCl buffer, pH8·2, to give a final concentration of porphyrin of approx. 0·5–1mM and of lecithin of 5–10mM. The suspension was ultrasonically treated for 3min. in an MSE 60w Sonicator and the concentration of the porphyrin was determined spectroscopically in acid solution (Falk, 1964).

The dual-wavelength spectrophotometer (Chance & Legallais, 1951) used in the present work was constructed in the workshops of Bristol University Medical School by using two Hilger D330 monochromators.

RESULTS

Development of the ferrochelatase assay. In studies of the submitochondrial distribution of ferrochelatase it was necessary to develop an assay for the enzyme that was as sensitive as those used for assaying the enzymes used as markers for membrane and matrix enzymes. Although the incorporation of ^{59}Fe into a cyclohexanone-soluble complex had been used to determine protohaem formation by chloroplasts (Jones, 1968) this method gave activities that did not vary linearly with the concentration of mitochondria above 1mg. of mitochondrial protein/ml.; concentrations in excess of this were sometimes necessary for examination of the less active samples that were produced in the fractionation. By measuring the disappearance of porphyrin substrate when metal ions were added to the incubation mixture it was, however, possible to measure ferrochelatase with continuous rate readings and with great sensitivity. Two metal ions, Co^{2+} and Fe^{2+}, were commonly used in our study; Co^{2+} is not readily autoxidized and is a good substrate for ferrochelatase; Fe^{2+} is much less stable and Fe^{3+}, the product of its autoxidation, is not a substrate. Special conditions were used when incorporation of Fe^{2+} was measured; anaerobicity was produced in the incubation mixture by the addition of freshly harvested actively respiring yeast cells (*Torulopsis utilis*). If intact or ultrasonically treated mitochondria were the source of ferrochelatase then addition of succinate maintained a reducing environment and Fe^{3+} was available for incorporation unless electron transport to oxygen was inhibited by cyanide or antimycin A.

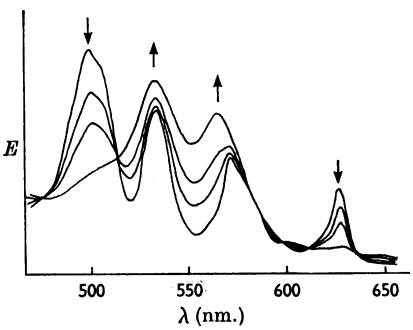

Fig. 1. Spectroscopic changes during the enzymic incorporation of Co^{2+} into mesoporphyrin. Mesoporphyrin (80μM) was incubated in a 1cm.-light-path cuvette in a conventional spectrophotometer, at 37°, with 1·0ml. of solubilized ferrochelatase and 1·0ml. of 100mM-tris–HCl buffer, pH8·2, containing 1% of Tween 80. Spectra were recorded before addition of 200μM-Co^{2+} and at 30min. intervals after its addition. Arrows indicate direction of change of extinction.

The isosbestic points for the reactions where metal complexes were formed from porphyrins were determined in a conventional spectrophotometer, with repetitive scanning over 2–3hr. An example of such a reaction is given in Fig. 1. By correlating the fall in extinction at the absorption maximum of band IV of the porphyrin during these plots with a simultaneous determination of porphyrin remaining in the cuvette (assayed by removing samples into 0·1M-hydrochloric acid and measuring the absorption of the Soret band) it was possible to obtain the constants used in determining rates of metal ion incorporation by the dual-wavelength assay (Table 1). The results obtained by these assays agreed with results obtained by prolonged incubation and determination of haem and cobalt porphyrin formation by the alkaline pyridine assay of Porra & Jones (1963) or of Johnson & Jones (1964), as shown in Table 2.

When Co^{2+} was added to an incubation mixture that also contained Fe^{2+}, the rates of disappearance of porphyrin were not additive but were between those obtained when Fe^{2+} and Co^{2+} were added separately; a similar effect was obtained with mixtures of Zn^{2+} and Co^{2+} or Fe^{2+} (Table 3). We have also found that the ratios of rates of metalloporphyrin formation from Zn^{2+}, Co^{2+} or Fe^{2+} were not changed during procedures for the fractionation and solubilization of mitochondria (see Fig. 6). In our work we have assumed that the same enzyme

Table 1. *Data used in determining rates of formation of metal porphyrins by ferrochelatase preparations*

Isosbestic points were taken from curves of the type shown in Fig. 1. Porphyrin solutions of known concentration were converted into metal porphyrins by incubation in cuvettes either with solubilized ferrochelatase or with ultrasonically treated mitochondria (see the Materials and Methods section). The wavelength of maximum decrease of the absorption spectrum of porphyrin was noted and the change in extinction at this wavelength was correlated with the concentration of porphyrin remaining unchanged, as determined in samples removed from the cuvette and added to 0·1M-HCl for assay, by using ϵ_{mM} from Falk (1964). With zinc mesoporphyrin and cobalt mesoporphyrin formation, the reaction went rapidly to completion and the extinction coefficients were obtained directly from a curve such as that shown in Fig. 1. Incorporation of Fe^{2+} was measured with ultrasonically treated mitochondria where anaerobic conditions were maintained by adding succinate (3mM) or yeast (5μl.).

Porphyrin substrate	Metal ion substrate	Wavelength (nm.) of maximum E decrease (λ_m)	Wavelength (nm.) of isosbestic point (λ_i)	$\Delta\epsilon_{mM}$ ($\lambda_m-\lambda_i$)
Protoporphyrin	Fe^{2+}	505	521	7·6
	Co^{2+}	505	521	7·32
Mesoporphyrin	Fe^{2+}	498	512·5	8·9
	Co^{2+}	498	511	7·75
	Zn^{2+}	498	524	9·2

Table 2. *Comparison of the determination of metalloporphyrin synthesis by continuous dual-wavelength spectrophotometric assay and from the difference spectra in alkaline pyridine*

Porphyrin (100nmoles) and rat liver mitochondria (40mg. of protein/ml.) in 3·0ml. of 200mM-tris–HCl buffer, pH8·2, were incubated at 37° in the dual-wavelength spectrophotometer; 10μl. of concentrated yeast suspension (*Torulopsis utilis*) or 5mM-succinate was added to promote anaerobic conditions. The reaction was started by addition of 100nmoles of $FeSO_4$, $FeCl_3$ or $CoSO_4$, and metalloporphyrin formation was measured continuously by using the constants given in Table 1. After a 20min. incubation the contents of the cuvette were tipped into a tube containing 1ml. of pyridine, 0·5ml. of 1M-NaOH and 1ml. of water. Metalloporphyrins were determined by difference spectroscopy (Porra & Jones, 1963; Johnson & Jones, 1964).

Substrates	Method of producing anaerobiosis	Specific activity (nmole of metalloporphyrin/min./mg. of protein): Dual-wavelength assay	Specific activity (nmole of metalloporphyrin/min./mg. of protein): Alkaline pyridine difference-spectra assay
Protoporphyrin + Fe^{2+}	Yeast (10μl.)	0·164	0·174
Protoporphyrin + Co^{2+}	—	0·119	0·127
Mesoporphyrin + Fe^{2+}	Yeast (10μl.)	0·259	0·245
Mesoporphyrin + Co^{2+}	—	0·215	0·223
Mesoporphyrin + Fe^{3+}	Succinate (5mM)	0·132	0·137

Table 3. *Effect of mixtures of metal ions on the rate of mesoporphyrin utilisation by ferrochelatase of rat liver mitochondria*

Metalloporphyrin formation (i.e. porphyrin utilization) was measured by the dual-wavelength spectrophotometric method at the wavelengths used in cobalt mesoporphyrin formation (see Fig. 2). Reaction was started by addition of the metal ion, alone or in mixtures, as indicated, at concentrations known to give the maximum rate of mesoporphyrin utilization for each metal ion separately. Experiments involving Fe^{2+} were carried out under N_2 in Thunberg cuvettes, or in the presence of yeast (see Table 3).

Enzyme used	Metal ion	Porphyrin utilization (nmole/min./mg. of protein)
Solubilized ferrochelatase	Co^{2+} (40 μM)	0·213
	Zn^{2+} (40 μM)	0·108
	Co^{2+} (40 μM) + Zn^{2+} (40 μM)	0·141
Solubilized ferrochelatase	Zn^{2+} (40 μM)	0·061
	Fe^{2+} (60 μM)	0·047
	Zn^{2+} (40 μM) + Fe^{2+} (60 μM)	0·052
Ultrasonically treated mitochondria	Co^{2+} (40 μM)	0·485
	Fe^{2+} (60 μM)	0·459
	Co^{2+} (40 μM) + Fe^{2+} (60 μM)	0·468

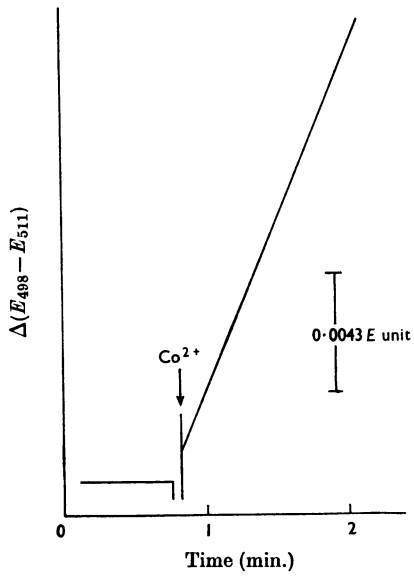

Fig. 2. Linearity of ferrochelatase activity with time. The cuvette of the dual-wavelength spectrophotometer contained 100nmoles of mesoporphyrin and 8·0mg. of mitochondrial protein in 2·5ml. of 200mM-tris–HCl buffer, pH8·2, at 37°. At the point indicated 100nmoles of Co^{2+} were added in 20 μl. of water, and the change in extinction, $\Delta(E_{498}-E_{511})$, was recorded.

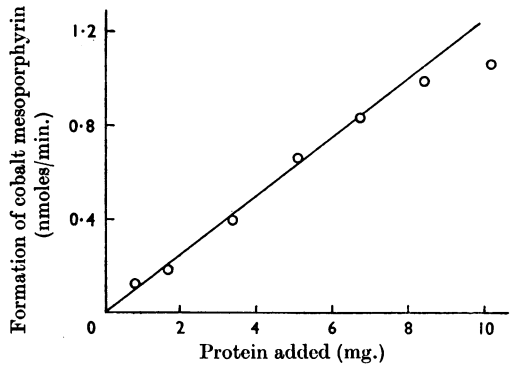

Fig. 3. Effect of mitochondrial protein concentration on ferrochelatase activity. Assay conditions were as described in Fig. 2, except that the concentration of mitochondria was varied as shown. Formation of cobalt mesoporphyrin was determined by using the constants given in Table 2.

catalyses the incorporation of either metal ion and have generally assayed ferrochelatase by incorporation of Co^{2+}. This avoided the use of special measures to remove oxygen when Fe^{2+} was the substrate.

The rate of formation of cobalt mesoporphyrin by mitochondria was linear with time and was proportional to the amount of mitochondria present (Figs. 2 and 3). The K_m for incorporation of Co^{2+} into mesoporphyrin was 11×10^{-6}M (Fig. 4*a*). For mesoporphyrin the K_m was 5×10^{-6}M (Fig. 4*b*). High concentrations of Co^{2+} were inhibitory, and the addition of Zn^{2+} increased the inhibition obtained at lower Co^{2+} concentrations.

Preparation of inner-membrane, outer-membrane and matrix fractions from rat liver mitochondria: effect of contraction medium. In our early experiments we prepared membrane fractions from mitochondria by the method of Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967). The mitochondria were allowed to swell in hypo-osmotic buffer, which caused the rupture of the outer membrane. The

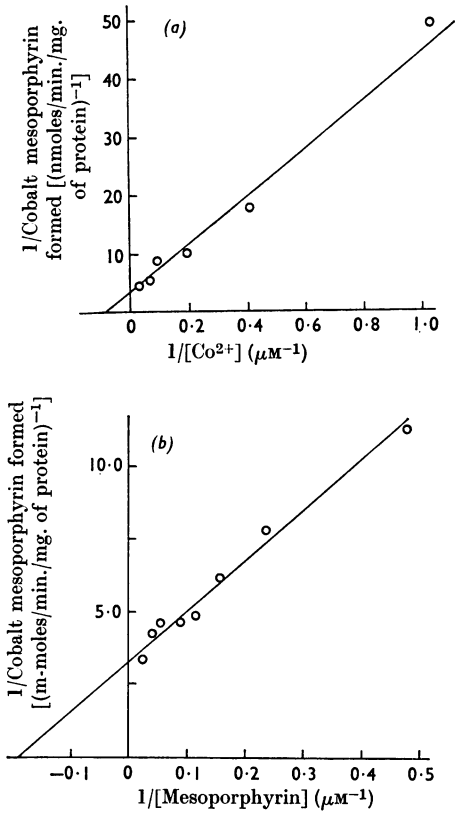

Fig. 4. Lineweaver–Burk plots of the effect of substrate concentration on synthesis of cobalt mesoporphyrin by rat liver mitochondria. (*a*) Porphyrin (100 nmoles) in 2·5 ml. of 200 mM-tris–HCl buffer, pH 8·2, containing 8 mg. of mitochondrial protein, was incubated at 37° in the dual-wavelength spectrophotometer and the $\Delta(E_{498}-E_{511})$ was recorded after the addition of increasing concentrations of Co^{2+}. Rates of synthesis of cobalt mesoporphyrin were calculated by using the constants given in Table 2. (*b*) Conditions were as in (*a*), except that the concentration of porphyrin was varied, with a constant amount (100 nmoles) of Co^{2+}.

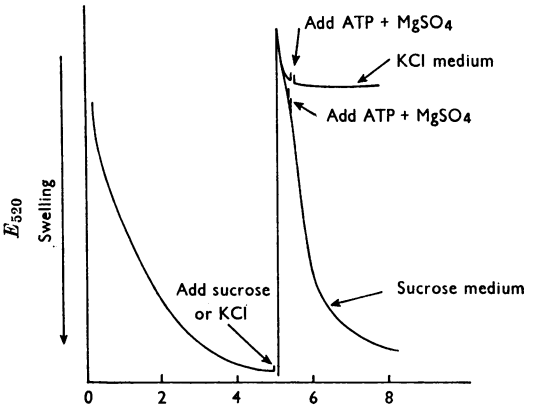

Fig. 5. Effect of ATP on the contraction of osmotically swollen rat liver mitochondria in hyperosmotic media. Freshly prepared mitochondria (2 mg. of protein) were added to 3·0 ml. of tris–phosphate (10 mM) buffer, pH 7·5, in a cuvette and the swelling was measured by following the decrease in E_{520}. At the point marked by an arrow the medium was made hyperosmotic by the addition of 200 μl. of a strong solution of KCl (final concn. 0·12 M) or sucrose (final concn. 0·45 M). After a few seconds ATP (0·5 mM) and $MgSO_4$ (0·5 mM) were added.

inner membrane was then made to contract away from the outer membrane by transfer to hyperosmotic sucrose solution containing ATP. After the usual separation by density-gradient centrifugation we found that the yields of outer-membrane fraction were small and that the inner membrane was contaminated with outer membrane. Since it was possible that the sucrose inhibited the ATP-induced contraction of the inner-mitochondrial-membrane fraction (Chappell & Greville, 1958) we measured the effects of different media on the contraction of mitochondria (Fig. 5). Mitochondria were transferred to hypo-osmotic buffer and the rate of swelling was measured spectrophotometrically at 520 nm. After swelling for 5 min. at room temperature the mitochondria were made hyperosmotic by the addition of either potassium chloride solution or sucrose solution followed by the addition of ATP. The increase in extinction (representing contraction) in the sucrose medium was transient, whereas in the potassium chloride medium contraction was permanent. In the absence of ATP the contraction in hyperosmotic potassium chloride was not permanent, probably because the mitochondria were permeable to K^+ ions, but contraction could still be caused by addition of ATP. This ATP-induced contraction was not obtained in mitochondria in the hyperosmotic sucrose medium.

Mitochondria swollen and shrunk in potassium chloride were then treated ultrasonically briefly to free outer membranes from the contracted inner membranes and then fractions were separated by centrifugation on a sucrose density gradient (Sottocasa *et al.* 1967). At the top of the gradient was the fraction S_1 containing soluble proteins, at an interface of 1·32 M-sucrose and 0·76 M-sucrose was the M_1 fraction (probably outer membrane), and the pellet (M_2) appeared to contain the inner-membrane and matrix enzymes.

Distribution of ferrochelatase and of marker enzymes. Proteins characteristic of the outer membrane are believed to be cytochrome b_5

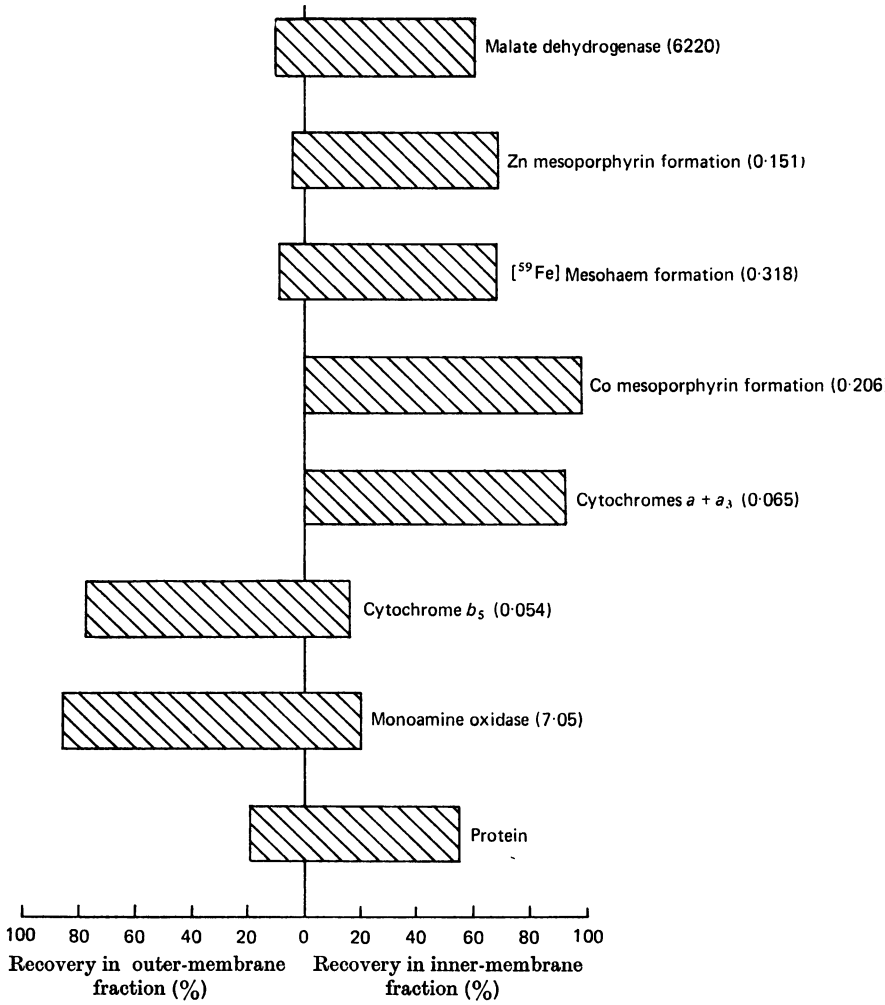

Fig. 6. Distribution of protein and enzyme activities in inner-membrane and outer-membrane fractions of rat liver mitochondria (see the Materials and Methods section). Results are expressed as the proportions of the initial activity of the mitochondria recovered in the appropriate fractions. Only the particulate fractions are shown. The soluble supernatant contained 22·1% of the total protein, but none of the enzymes assayed except malate dehydrogenase, of which 35·5% was recovered in this soluble fraction. The specific activities of the marker proteins in the intact mitochondria are given in parentheses (as nmoles/min./mg. of protein); cytochromes are given as nmoles/mg. of protein.

(Sottocasa *et al.* 1967) and monoamine oxidase (Schnaitman *et al.* 1967; Beattie, 1968); characteristic of the inner membrane is cytochrome oxidase and of the soluble matrix enzymes is malate dehydrogenase. The distribution of these enzymes is shown in Fig. 6, which also includes the distribution of ferrochelatase as determined by utilization of porphyrin in the presence of Zn^{2+} or Co^{2+} or by the formation of [^{59}Fe]haem. A good separation of the markers was obtained and the distribution of ferrochelatase closely followed that of cytochrome oxidase. Confirmation of the membrane fractionation was also obtained by electron microscopy of fractions fixed in glutaraldehyde and stained as described by Schnaitman *et al.* (1967) (M. S. Jones & O. T. G. Jones, unpublished work). No ferrochelatase or monoamine oxidase activity was detected in the microsome fraction.

Evidence for the location of ferrochelatase on the inside of the inner membrane. It has not previously

Table 4. *Latency of ferrochelatase in intact rat liver mitochondria*

The dual-wavelength spectrophotometric assay was used (see Fig. 2), but the incubation medium was 80mM-KCl–20mM-tris–HCl buffer, pH 8·2. Porphyrin–lecithin micelles (see the Materials and Methods section) and Co^{2+} were used as substrates.

Mitochondrial preparation	Enzyme activity (nmoles/min./mg. of protein)	
	Malate dehydrogenase	Ferrochelatase
Intact (*A*)	0·9	0·123
Ultrasonically treated (*B*)	7·35	0·80
B/*A* ratio	8·15	6·52

been possible to determine whether ferrochelatase exhibits latency, since the poorly soluble porphyrin substrates are normally present in a detergent solution that disrupts the mitochondrial membrane. We have found that porphyrins can be made up into phospholipid micelles that are optically clear and have very sharp absorption bands. These porphyrin–lecithin micelles do not cause the breakdown of mitochondrial membranes, since malate dehydrogenase remains latent, and the dissolved porphyrin is available as a substrate for ferrochelatase. It was found that the rates of incorporation of metal ions into these micellar porphyrins by intact mitochondria were very much less than those for ultrasonically treated mitochondria and that the degree of latency of ferrochelatase was very similar to that for the matrix enzyme malate dehydrogenase (Table 4). This suggests that ferrochelatase also is on the inside of the inner mitochondrial membrane.

DISCUSSION

With Co^{2+} as metal ion substrate a quick assay for ferrochelatase with continuous rate readings could be based on the measurement of use of porphyrin substrate; this rate of disappearance measured in the dual-wavelength spectrophotometer was directly proportional to the amount of mitochondria taken and to the time of incubation (Figs. 2 and 3); this assay has proved useful in further studies on the properties of the enzyme. By using slightly modified incubation conditions it was possible to study the incorporation of iron into porphyrins without the use of anaerobic cuvettes. Fe^{3+} was used only when reducing substrates were provided and when the electron-transport pathway was functional, either in whole mitochondria or in ultrasonically treated mitochondria. Since the utilization of Fe^{3+} iron for haem synthesis in incubation mixtures with succinate as an electron donor was inhibited by either cyanide or antimycin it is likely that the oxidation of succinate lowers the oxygen concentration within the incubation system, a process blocked by the inhibitors of electron flow, and so prevents the autoxidation of any Fe^{2+} formed. The direct reduction of Fe^{3+} must still occur, possibly at the site for non-haem iron of succinate dehydrogenase. The association of ferrochelatase with the mitochondrial electron-transport chain thus provides a mechanism for the production of the required Fe^{2+} from iron that is present in the cells almost entirely in the Fe^{3+} form.

The total haem content of the mitochondria used in our work was approx. 1nmole/mg. of protein and so the protohaem-synthesizing activity of ferrochelatase (approx. 0·4nmole/min./mg. of protein) is far in excess of the activity required for mitochondrial biogenesis; the half-life of mitochondrial components appears to be about 10 days (Fletcher & Sanadi, 1961; Bailey, Taylor & Bartley, 1967). Ferrochelatase would synthesize the haem required in mitochondria in less than 3min. and little longer would be needed for the formation of extramitochondrial haemoproteins (cytochromes b_5 and *P*-450 of microsomes, and catalase and peroxidase).

It is possible that haem synthesis is limited by earlier steps in porphyrin metabolism. Thus Granick & Urata (1963) found that there was no detectable δ-aminolaevulate synthetase in guinea-pig liver mitochondria and other workers found low activities of this enzyme in normal mitochondria (see Matsuoka, Yoda & Kikuchi, 1968). Possibly haem synthesis is controlled at this level.

Another factor controlling haem synthesis might be the oxygen concentration around the mitochondria. Since it is unlikely that the liver cells are anaerobic, Fe^{2+} would be rapidly oxidized when formed and the concentration of Fe^{2+} would not reach the K_m value. A high ferrochelatase activity would still permit haem to be synthesized under these conditions.

By using a potassium chloride medium when inducing the contraction of swollen mitochondria a consistent improvement in the subsequent separation of inner and outer membranes by the method of Sottocasa *et al.* (1967) was obtained (Jones & Jones, 1968). As shown in Fig. 5, the contraction induced by hyperosmotic potassium chloride solution and ATP was permanent, unlike that induced by hyperosmotic sucrose solution and ATP. Almost certainly this lack of permanent contraction in sucrose solution was due to its effect in inhibiting ATP-induced shrinkage of mitochondria (Chappell & Greville, 1958). The gentle ultrasonic treatment of potassium chloride-shrunk mitochondria completed the separation of outer from inner membranes without completely destroying the integrity of the

inner membrane (malate dehydrogenase in these preparations was still latent). Density-gradient centrifugation then resolved the membranes, as shown by the use of enzyme markers. In agreement with Schnaitman *et al.* (1967) and Beattie (1968) we found that monoamine oxidase is a satisfactory marker for outer membranes; its specific activity rose in this fraction (Fig. 6) to a value higher than that in microsomes or in the fraction containing cytochrome oxidase. In our work we have from time to time used other markers, such as rotenone-insensitive NADH-cytochrome *c* reductase, glucose 6-phosphatase, cytochrome b_5 and glutamate dehydrogenase, and have found a distribution of enzyme activities in agreement with that found by Sottocasa *et al.* (1967). Our finding that the distribution of ferrochelatase followed that of cytochrome oxidase (Fig. 6) was supported by finding a latency of ferrochelatase (Table 4) that further suggested that this enzyme is on the inside of the inner membrane.

This distribution of activity means that ferrochelatase is separated from the microsomes by two membranes and that cytochrome synthesis may require either transport of haem to the microsomes for attachment to the apoprotein, followed by the return of the completed cytochrome, or possibly the movement of apocytochrome through the membranes and assembly of the completed cytochrome on the inner membrane of the mitochondrion. Alternatively the protein of the insoluble cytochromes *a* and *b* may be synthesized within the mitochondrion, by using mitochondrial DNA and RNA (for a review of mitochondrial protein synthesis see Roodyn & Wilkie, 1968), and then the structural organization of haem synthesis would appear more logical. It is possible that the synthesis of cytochrome *c* takes place by a different route, through porphyrin cytochrome *c* (a porphyrin covalently bound to protein) into which insertion of iron takes place catalysed by a special ferrochelatase. Such a proposal is not unreasonable since the combination of protoporphyrinogen with cysteine or thiol-containing peptides proceeds smoothly at neutral pH and room temperature (Popper & Tuppy, 1963) and model porphyrins *c* can be prepared in this way (Sano, Nanzyo & Rimington, 1964).

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