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Intramitochondrial Localization of δ-Aminolaevulate Synthetase and Ferrochelatase in Rat Liver

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Intramitochondrial loci for δ-aminolaevulate synthetase and ferrochelatase, the initial and final enzymes in haem synthesis, have been found in rat liver. Two different methods of fractionation were applied to mitochondria: (*a*) sonication and density-gradient centrifugation; (*b*) treatment with digitonin and differential centrifugation. Similar results were obtained with each technique. δ-Aminolaevulate synthetase is distributed similarly to two known matrix enzymes, malate dehydrogenase and glutamate dehydrogenase. Ferrochelatase is firmly bound to the the inner mitochondrial membrane. These results are considered in terms of the regulation of haem synthesis and in relation to mitochondrial biogenesis.

The metabolic pathway for haem synthesis in mammalian cells involves integration of mitochondrial and cytoplasmic enzyme activities (Sano & Granick, 1961). The initial reaction, a condensation of glycine and succinyl-CoA to form ALA,* is catalysed by the mitochondrial enzyme ALA synthetase (succinyl-CoA–glycine succinyltransferase) (Shemin & Kikuchi, 1958). Subsequent reactions that convert ALA into coproporphyrinogen occur in soluble cell fractions, and the final two enzymic reactions of this pathway, catalysed by coproporphyrinogen oxidase and ferrochelatase (protohaem ferrolyase, EC 4.99.1.1), take place within the mitochondrion (Granick & Levere, 1964). Although this compartmentalization has been suggested as a possible control mechanism in haem synthesis (Sano & Granick, 1961), evidence that the mitochondrial enzymes are segregated from the cytoplasm is lacking. Permeability barriers to substrates imposed by the outer and inner mitochondrial membranes may be quite different. An enzyme bound to the external mitochondrial membrane may be freely accessible to substrates generated outside the mitochondrion, whereas a matrix or inner-membrane locus may limit access to the substrate. It was therefore decided to determine the intramitochondrial localization of ALA synthetase and ferrochelatase.

The intramitochondrial locus of ALA synthetase is of relevance to the biogenesis of this organelle. From turnover rates of cytochrome *c*, soluble and insoluble protein, DNA and phospholipids (Fletcher & Sanadi, 1961; Beattie, Basford & Koritz, 1967; Gross, Getz & Rabinowitz, 1968) it has been suggested that mitochondria, or, more likely, the inner mitochondrial membrane, turn over as an entity with a half-life of 8–10 days. ALA synthetase, however, is known to be readily induced (Granick & Urata, 1963) and to turn over with a half-life of 90 min. (Tschudy, Marver & Collins, 1965). Inducibility and rapid turnover have also been reported for two other mitochondrial enzymes, alanine aminotransferase and ornithine aminotransferase (Swick, Rexroth & Stange, 1968). Exact localization of enzymes such as ALA synthetase would help to evaluate the hypothesis of synchronous turnover of the inner mitochondrial membrane. If ALA synthetase were an enzyme of either the matrix or the outer membrane, then such a hypothesis would still be tenable. If, however, ALA synthetase were firmly bound to the inner membrane, the hypothesis would require radical modification, or would have to be discarded.

Development of techniques for isolation of mitochondrial components now permits examination of these questions. Inner and outer mitochondrial membranes have been characterized morphologically and biochemically (Parsons, Williams & Chance, 1966; Parsons, Williams, Thompson, Wilson & Chance, 1967; Schnaitman, Erwin & Greenwalt, 1967; Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967; Beattie, 1968; Schnaitman & Greenwalt, 1968). Marker enzymes for the inner membrane, matrix and outer membrane have been identified. In the present study we

* Abbreviation: ALA, δ-aminolaevulate.

have used monoamine oxidase activity as a marker for the outer membrane (Sottocasa *et al.* 1967; Beattie, 1968; Schnaitman & Greenwalt, 1968), malate dehydrogenase and glutamate dehydrogenase activities for the matrix (Sottocasa *et al.* 1967), and cytochrome *c* reductase and cytochrome *c* oxidase activities for the inner membrane (Parsons *et al.* 1966, 1967).

Although resolution of mitochondria into membrane components potentially constitutes a probe into the relation of structure and function, disruption of mitochondrial structure requires the assumption that structure-dependent changes in enzymic activity are equal for all fractions. To justify this assumption, we have used two different methods of mitochondrial fractionation: chemical disruption with digitonin (Schnaitman *et al.* 1967) and mechanical disruption by swelling, shrinking and sonication (Sottocasa *et al.* 1967).

A preliminary account of this work has appeared (McKay, Druyan & Rabinowitz, 1968).

MATERIALS AND METHODS

To study ALA synthetase activity, we induced chemical porphyria in adult male Sprague–Dawley rats by tube-feeding with diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (400mg./day/rat) for 2 days (Figge, 1959). In untreated animals ALA synthetase activity was negligible. The rats were kept without food for 18–24hr. before being killed by stunning and exsanguination. Three to six rat livers were combined in 0·3M-sucrose for homogenization. Mitochondria were separated by differential centrifugation (Gross, Getz & Rabinowitz, 1969). Enzyme assays were completed on the same day as membrane isolation.

Mitochondria were fractionated after incubation with digitonin as described by Schnaitman *et al.* (1967), but in most experiments 1mg. of digitonin was added/5mg. of mitochondrial protein. All pellets were suspended in 0·25M-sucrose for enzyme assays.

For disruption by swelling, contraction and sonication (referred to as sonicated mitochondria in the text), mitochondria were initially suspended in 0·01M-tris–phosphate buffer, pH 7·5 (Sottocasa *et al.* 1967). After the mitochondria had swollen for 5min. at 0°, the suspension, which contained 10–12mg. of protein/ml., was diluted with $\frac{1}{3}$ vol. of 1·8M-sucrose containing 2mM-ATP and 2mM-Mg^{2+}. This suspension was kept at 0° for 5min. before sonication (Branson model S110, 3A for 10sec.). Then 6–9ml. of sonicated mitochondria was layered on top of a discontinuous sucrose gradient; the bottom layer was 10ml. of 1·32M-sucrose and the middle layer was 5ml. of 0·76M-sucrose. Centrifugation for 3hr. at 90000*g* (in a Spinco SW25.1 rotor) resolved three fractions: a brown pellet (heavy fraction), a band at the lower interface (light fraction) and a clear yellow supernatant fluid (soluble fraction). Less concentrated material on either side of the interface band was collected as the intermediate fraction. The pellet (heavy fraction) was rinsed with 0·25M-sucrose and resuspended in 0·25M-sucrose for enzyme assays. The light fraction and soluble fraction were removed from the gradient with J-shaped Pasteur pipettes and were assayed as collected.

Ferrochelatase activity was measured spectrophotometrically (Porra & Jones, 1963). Each assay mixture was incubated for 90min. at 35° with constant agitation. Before use, protoporphyrin IX was purified by precipitation from acidic and from basic solutions (Labbe & Hubbard, 1960). Triplicate samples were incubated under a stream of N_2. Blank samples were treated identically, but were incubated aerobically. After incubation, particulate material was removed by centrifugation at 12000*g* for 30min. at 4°. Difference spectra between reduced and oxidized pyridine haemochromogens were obtained with a recording spectrophotometer (Cary model 14).

To measure ALA synthetase activity, mitochondrial fractions were incubated for 60min. at 35° with constant agitation. The assay medium contained (per ml.): 100μmoles of glycine, 10μmoles of succinate, 5μmoles of α-oxoglutarate, 1μmole of pyridoxal phosphate, 0·5μmole of ATP, 0·5μmole of NAD^+, 10μmoles of EDTA, 20μmoles of Mg^{2+} and 100μmoles of tris–chloride adjusted to final pH 7·4. Trichloroacetic acid (300μmoles) was added to terminate the reaction. After centrifugation, the concentration of ALA was measured in the protein-free supernatant solution (Granick, 1966). For blank samples, trichloroacetic acid was added before incubation. Preliminary experiments showed that addition of succinyl-CoA increased ALA synthetase activity by up to 20% in internal-membrane, external-membrane and soluble fractions. Hence a total of 4·4μmoles of freshly prepared succinyl-CoA (Shemin & Russell, 1953) was added to all assay tubes in equal portions at 15min. intervals. The concentration of succinyl-CoA was measured by the hydroxamate method (Stadtman, 1957).

Succinate–cytochrome *c* reductase activity was measured at 37° (Rabinowitz & DeBernard, 1957). Albumin was omitted from the assay medium. Cytochrome *c* oxidase was assayed spectrophotometrically after 4–5min. preincubation at room temperature (Cooperstein & Lazarow, 1951; de Duve, Pressman, Gianetto, Wattiaux & Applemans, 1955). Malate dehydrogenase activity was measured by the method of Ochoa (1955); 90μmoles of sodium Amytal were added to each cuvette. Glutamate dehydrogenase was assayed by the method of Sottocasa *et al.* (1967). After preincubation for 3min. at 37°, monoamine oxidase activity was determined with benzylamine as substrate (Tabor, Tabor & Rosenthal, 1954).

Digitonin, pyridoxal phosphate and protoporphyrin IX were from Calbiochem, Los Angeles, Calif., U.S.A. ATP, NADH and CoA were from P-L Biochemicals, Milwaukee, Wis., U.S.A. Benzylamine, cytochrome *c*, NAD^+, α-oxoglutarate and oxaloacetic acid were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Protein concentrations were measured by the method of Lowry, Rosebrough, Farr & Randall (1951), or by the biuret reaction (Gornall, Bardawill & David, 1949). Crystalline bovine serum albumin was used as a protein standard.

RESULTS

When observed by electron microscopy, isolated mitochondria appeared intact, free from nuclear material and almost free from microsomal contamination. Treatment with digitonin followed by differential centrifugation released four submitochondrial fractions, as shown in Table 1. The pellet

Table 1. *Comparison of mitochondrial fractionation procedures*

Fractions after digitonin treatment: 9·5-P, pellet obtained after 10 min. centrifugation at 9500*g*; 40-P, 15 min. at 40000*g*; 144-P, 60 min. at 144000*g*; 144-S, supernatant after final centrifugation. The procedure for sonication and the resulting fractions are described in the text. Numbers in parentheses indicate protein distribution calculated as 100 × (mg./fraction)/(total mg. recovered).

Mitochondrial fraction	Digitonin	Sonication	Marker enzyme
Internal membrane	40-P (36–39)	Heavy (60–71)	Cytochrome *c* oxidase; succinate–cytochrome *c* reductase
	9·5-P (32–35)		
Matrix	144-S (25–32)	Soluble (18–20)	Glutamate dehydrogenase; malate dehydrogenase
External membrane	144-P (3–5)	Light (2–8)	Monoamine oxidase

Table 2. *Effect of digitonin/protein ratio on the distribution of monoamine oxidase and protein*

The fractions are as defined in Table 1.

	Digitonin/protein ratio 0·13:1		Digitonin/protein ratio 0·20:1	
Fraction	Monoamine oxidase (%)	Protein (%)	Monoamine oxidase (%)	Protein (%)
9·5-P	33	21	1	32
40-P	7	48	13	39
144-P	50	10	35	3
144-S	10	10	51	25

that sedimented at 9500*g* (9·5-P) corresponded to the inner membranes plus some matrix, that at 40000*g* (40-P) to internal membranes without matrix and the pellet recovered at 144000*g* (144-P) to external membranes. The 144000*g* supernatant (144-S) fraction contained solubilized and matrix enzymes. Density-gradient centrifugation of sonicated mitochondria yielded three fractions, corresponding to internal membranes, external membranes and matrix (Table 1).

Preliminary experiments with digitonin/protein ratio 0·13:1 reproduced the distribution of monoamine oxidase activity and protein reported by Schnaitman *et al.* (1967). Subsequently more complete separation of the external membrane from the 9·5-P and the 40-P fractions was sought. At an increased digitonin/protein ratio (Table 2) the monoamine oxidase activity associated with inner-membrane fractions was decreased from 40 to 14%. Concomitantly more monoamine oxidase activity and protein were released into the 144-S fraction. The appearance of a large proportion of the monoamine oxidase activity in the 144-S fraction is ascribed to the higher digitonin/protein ratio employed.

The biochemical characterization of the products of typical fractionations by digitonin or sonication performed on mitochondria from normal rats is summarized in Tables 3 and 4 respectively. Cytochrome *c* oxidase activity, used as a marker for the internal membrane, was found with the 9·5-P, 40-P and heavy fractions, whereas monoamine oxidase activity, used to trace the behaviour of outer membranes, was limited to the 144-P, 144-S and light fractions. After fractionation with digitonin (Table 3), the increase in specific activity for cytochrome *c* oxidase in the 40-P fraction was 33% and for ferrochelatase it was 27%. The overall distribution was also similar for these enzymes: 45% of cytochrome *c* oxidase activity and 55% of ferrochelatase activity were associated with the 40-P fraction. After sonication (Table 4), 94% of both cytochrome *c* oxidase and ferrochelatase activities were found with the heavy fraction. The specific enzyme activity for ferrochelatase increased by 220% and that of cytochrome *c* oxidase rose by 90% in the heavy fraction. Total recovery of ferrochelatase was 215% in this experiment, but similar distributions were obtained in experiments where recovery was 60%. From the parallel behaviour of ferrochelatase and cytochrome *c* oxidase, in two different fractionation schemes, with respect to recovery in each fraction, as well as increase of specific enzyme activity, we conclude that ferrochelatase is an inner-membrane enzyme.

With the digitonin fractionation method, the theoretical distribution for a matrix enzyme would be with the 9·5-P fraction or the 144-S fraction.

Table 3. *Enzyme distributions in mitochondria from normal rats: fractionation with digitonin*

Fractions are as defined in Table 1. Specific enzyme activity: ferrochelatase, nmoles of haem formed/hr./mg. of protein; cytochrome *c* oxidase, μmoles of cytochrome *c* oxidized/min./mg. of protein; malate dehydrogenase, μmoles of $NADH^+$ reduced/min./mg. of protein; monoamine oxidase, nmoles of benzylamine oxidized/min./mg. of protein.

Yield = 100 × (activity/fraction)/(activity in all fractions).

Recovered activity of intact mitochondria = 100 × (activity in all fractions)/(activity in intact mitochondria).

	Ferrochelatase		Cytochrome *c* oxidase		Malate dehydrogenase		Monoamine oxidase	
Fraction	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield
Intact mitochondria	60	—	303	—	1·3	—	11	—
9·5-P	54	37	442	46	1·1	29	7	15
40-P	76	55	402	45	1·3	35	4	9
144-P	62	8	341	5	1·0	3	56	16
144-S	0	0	38	4	1·3	32	29	60
Recovered activity of intact mitochondria	83		107		105		144	

Table 4. *Enzyme distributions in mitochondria from normal rats: fractionation by swelling, sonication and density-gradient centrifugation*

The units of specific enzyme activity, yield and recovered activity of intact mitochondria are given in Table 3.

	Ferrochelatase		Cytochrome *c* oxidase		Malate dehydrogenase		Monoamine oxidase	
Fraction	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield
Intact mitochondria	94	—	106	—	2·7	—	16	—
Heavy fraction	306	94	197	94	2·9	69	5	26
Light fraction	78	3	72	4	0·3	1	83	53
Soluble fraction	32	3	10	2	4·9	30	12	21
Recovered activity of intact mitochondria	215		123		118		75	

Experimentally malate dehydrogenase was partitioned almost equally among the 9·5-P, 40-P and 144-S fractions, and in no single fraction was there a marked enhancement of the specific enzyme activity. In the sonication procedure malate dehydrogenase should have been found primarily in the soluble fraction, corresponding to its matrix locus. This activity, however, was found to be distributed between the heavy and the soluble fractions.

Representative findings for ALA synthetase and marker enzyme distribution for fractionations of mitochondria from porphyric rats are shown in Tables 5 and 6. The distribution of marker enzymes was similar to that found in mitochondria from normal animals. ALA synthetase activity was recovered in the 9·5-P, 40-P and 144-S fractions after fractionation with digitonin (Table 5). The heavy and soluble fractions from the sonication fractionation experiments contained almost all of the ALA synthetase activity (Table 6). In both types of fractionation procedure the distribution of ALA synthetase activity was similar to that found for malate dehydrogenase.

Further release of ALA synthetase activity from inner membranes was explored by resonication of heavy fractions. After resonication for various times, inner-membrane suspensions were centrifuged at 144000*g* for 1hr., and the pellets and supernatant fractions were then assayed for ALA synthetase, glutamate dehydrogenase and cytochrome *c* oxidase activities. As shown in Table 7, with increasing time of sonication ALA synthetase activity was released from the inner-membrane fraction. The pattern of response is similar to that found with glutamate dehydrogenase, which has been designated a matrix enzyme (Sottocasa *et al.* 1967). In contrast, only trace amounts of cytochrome *c* oxidase activity were released from the inner membrane even by prolonged sonication. Variable inactivation of malate dehydrogenase activity precluded its use in similar experiments.

Table 5. *Enzyme distributions in mitochondria from porphyric rats: fractionation with digitonin*

Fractions are as defined in Table 1. The units of specific activity, yield and recovered activity of intact mitochondria are given in Table 3.

	ALA synthetase		Cytochrome c oxidase		Malate dehydrogenase		Monoamine oxidase	
Fraction	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield
Control mitochondria	0·9	—	252	—	1·6	—	6	—
9·5-P	0·9	29	565	44	1·9	37	0·2	1
40-P	1·0	40	562	53	1·3	33	2	13
144-P	0·6	2	331	3	1·2	3	66	35
144-S	1·1	29	8	0	1·6	27	12	51
Recovered activity of intact mitochondria	98		157		94		101	

Table 6. *Enzyme distributions in mitochondria from porphyric rats: fractionation by swelling, sonication and density-gradient centrifugation*

Specific activity: succinate–cytochrome c reductase, μmoles of cytochrome c reduced/min./mg. of protein; other units, yield and recovered activity of intact mitochondria are defined in Table 3.

	ALA synthetase		Succinate–cytochrome c reductase		Malate dehydrogenase		Monoamine oxidase	
Fraction	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield	Sp. activity	Yield
Intact mitochondria	2·2	—	131	—	3·3	—	21	—
Heavy fraction	2·5	75	100	94	3·1	68	9	24
Light fraction	1·8	1	89	3	0·6	0	173	17
Soluble fraction	2·8	24	0	0	4·6	29	14	13
Intermediate fractions	—	—	26	3	1·2	3	115	46
Recovered activity of intact mitochondria	118		63		103		113	

Table 7. *Resonication of inner membranes: effect on solubilization of enzymes*

Supernatant (%) = 100 × (activity in supernatant)/(activity in total sonicated fraction). Specific activity: glutamate dehydrogenase, μmoles of $NADH^+$ oxidized/min./mg. of protein; other units as in Table 3. Sonication was performed on inner membranes prepared as described by Sottocasa *et al.* (1967). For resonication, 15sec. bursts of energy were employed until the total required period of sonication was achieved. To ensure against heating, intervals of cooling of at least 2min. were employed between bursts. The results reported with zero resonication refer to the distribution of the original heavy fraction prepared as described in the Materials and Methods section and recentrifuged at 144000*g* for 1hr.

	ALA synthetase		Glutamate dehydrogenase		Cytochrome c oxidase		Protein
Length of resonication (sec.)	Supernatant (%)	Sp. activity	Supernatant (%)	Sp. activity	Supernatant (%)	Sp. activity	Supernatant (%)
0	33	3·54	40	0·42	0·1	1	40·0
15	58	5·10	52	0·51	2·2	18	58·5
30	85	5·53	53	0·55	3·3	28	58·9
60	73	4·53	62	0·52	6·2	47	66·1
120	76	5·34	65	0·66	4·2	41	64·2

The distribution of enzyme activities was calculated as the percentage of the recovered enzymic activity, with a sample of unfractionated mitochondria that had been either incubated with digitonin, or swollen, contracted and sonicated, as a reference. Recoveries, shown in the bottom row

of each of Tables 3, 4, 5 and 6, indicated that it was not always possible to recover the expected activity. As has been suggested (Schnaitman *et al.* 1967; Sottocasa *et al.* 1967) this change probably reflects structural changes resulting from the fractionation techniques. Recovery of total protein in these experiments ranged from 95 to 107%.

Membrane fractions isolated either by digitonin or by sonication were examined by electron microscopy. In both the 40-P and heavy fractions inner membranes were found, minimally contaminated by external membranes. The 144-P and light fractions, however, showed significant contamination by small fragments of the inner membrane.

DISCUSSION

Our results indicate that ferrochelatase activity is associated with the internal membrane of rat liver mitochondria. Both fractionation methods give high recoveries and enhanced specific enzyme activities with internal-membrane fractions. Similar results were reported by Jones & Jones (1968). In some experiments ferrochelatase recovery was 200% of the initial activity. The remote possibility therefore exists that differential activation of ferrochelatase in different fractions could alter our conclusions with regard to an inner-membrane locus, We believe this to be unlikely, however, as the distribution of cytochrome *c* oxidase and ferrochelatase was parallel in all experiments performed, with either fractionation method. Total recovery of ferrochelatase ranged from 60–215%, and highest specific activities were always associated with the 40-P or heavy fractions. A small amount of ferrochelatase, albeit of high specific activity, is recovered with external membranes. This finding parallels our finding for cytochrome *c* oxidase, or succinate–cytochrome *c* reductase, and correlates with morphological evidence of contamination of external membranes by fragments of the inner membrane. However, neither ALA synthetase nor ferrochelatase appears to be an external-membrane enzyme, and therefore this contamination does not affect our conclusions.

Segregation of ALA synthetase activity in a single mitochondrial fraction is less obvious. With both fractionation methods the distributions of malate dehydrogenase and ALA synthetase are parallel. However, neither ALA synthetase nor malate dehydrogenase is partitioned uniquely to one component of the mitochondrion, as is, for example, cytochrome *c* oxidase. In contrast with a previous report (Schnaitman *et al.* 1967), we find only 20–30% of malate dehydrogenase activity in the soluble (144-S) fraction, whereas more activity (30–60%) remains associated with inner-membrane fractions. Significant enhancement of specific activity of malate dehydrogenase is not observed among these fractions.

The release of matrix marker enzymes and ALA synthetase from inner-membrane fractions was studied further, as a function of progressive disruption of the heavy fraction. With increasing duration of sonication of this latter fraction, the amount of ALA synthetase and glutamate dehydrogenase released into the supernatant increases in a similar manner; specific activities of the released enzymes also rise (Table 7). Negligible amounts of cytochrome *c* oxidase activity are released under the same conditions. Malate dehydrogenase is partitioned similarly, but with increasing sonication recovery of the activity decreases, presumably owing to enzyme inactivation. Both the partition of ALA synthetase among isolated submitochondrial fractions and the release of this activity from heavy fractions are entirely parallel to the behaviour of two enzymes generally characterized as biochemical markers of the mitochondrial matrix.

A matrix enzyme is operationally defined as an enzyme that is readily solubilized by osmotic lysis (Schnaitman *et al.* 1967), treatment with detergent (Schnaitman *et al.* 1967), or sonication (Sottocasa *et al.* 1967). On this basis malate dehydrogenase and glutamate dehydrogenase have been designated as matrix markers. In none of these solubilization procedures, however, have matrix enzymes been quantitatively released from sedimentable membrane fractions. From these criteria, the behaviour of ALA synthetase is entirely consistent with its being a matrix enzyme. Hackenbrock (1968) has described the mitochondrial matrix as a reticular network, physically linked to the inner membrane. These observations provide an ultrastructural explanation for the inability of ourselves and others to achieve quantitative solubilization of matrix enzymes by the procedures described above. Hackenbrock's (1968) observations also offer a basis for considering the localization of one isoenzyme of malate dehydrogenase within the mitochondria. Such a localization could be explained on the basis of the binding of this isoenzyme to the reticular matrix network. Munkres & Woodward (1966) have reported that a mutation involving structural protein of *Neurospora* alters the kinetic properties of malate dehydrogenase.

These findings may be pertinent to problems of mitochondrial biogenesis and turnover. The known rapid turnover of ALA synthetase (Tschudy *et al.* 1965) together with its locus in the matrix implies that synchronous turnover of a structural complex may be limited to the inner-membrane unit. In yeast ALA synthetase appears to be made outside the mitochondrion (R. Druyan, A. Rubenstein, G. S. Getz & M. Rabinowitz, unpublished work). It is possible that matrix enzymes are synthesized

in the endoplasmic reticulum, and are then independently inserted into and deleted from the intact mitochondrion.

Haem synthesis in mammalian tissue is regulated via de-repression of ALA synthetase synthesis (Granick, 1966). The very short half-life of ALA synthetase may make it possible to achieve fine regulation via this mechanism alone (Tschudy *et al.* 1965). Product inhibition effected by haem is a second possible regulatory mechanism, and has been demonstrated in *Rhodopseudomonas spheroides*, where ALA synthetase occurs in soluble form (Burnham & Lascelles, 1963). Kinetic experiments with tissue or mitochondria have failed to demonstrate feedback inhibition of ALA synthetase (Granick, 1966; Marver, Tschudy, Perlroth & Collins, 1966; Vavra & Poff, 1966). In these studies, however, haem was added to intact cells or to intact mitochondria, and its actual concentration in the matrix is unknown. The proximity of the initial (ALA synthetase) and terminal (ferrochelatase) steps of this synthetic pathway makes it attractive to speculate that feedback inhibition may yet be demonstrated.

The intracellular compartmentalization of haem synthesis has itself been cited as a possible regulatory mechanism (Sano & Granick, 1961). Of the seven enzymes required for haem synthesis four are in the cytosol and three are in the mitochondrion. Our results localize the first and final steps to the matrix and inner membrane respectively, and provide structural evidence that may implicate the transport of intermediates across mitochondrial membranes as a factor in regulating haem synthesis.

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