Inner- and Outer-Membrane Enzymes of Mitochondria during Liver Regeneration

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1. Marker enzymes for the mitochondrial matrix, inner membrane, inter-membrane space and outer membrane were measured in mitochondria isolated from control and regenerating rat liver. The specific activity of these enzymes was then followed for up to 30 days after operation. 2. The specific activity of marker enzymes for the matrix, inner membrane and inter-membrane space remained constant during liver regeneration. 3. However, the specific activities of monoamine oxidase and kynurenine hydroxylase, both outer-membrane markers, fell by 67% and 49% respectively from their control values at 4 days after operation, and returned to normal by about 3 weeks. 4. The repression of kynurenine hydroxylase activity was shown to be unrelated to any independent variation in tryptophan catabolism, based on tryptophan pyrrolase assays. 5. These results are considered to indicate that enzymes of the inner and outer mitochondrial membranes are synthesized asynchronously during morphogenesis. 6. The enzyme complement of purified outer membrane at 4 days after operation was about 50% of that of the appropriate control. Thus the composition of the outer membrane itself may vary dramatically, and supports the concept that constitutive enzymes may turn over independently of a membrane's existence. 7. The behaviour of the rotenone-insensitive, NADH cytochrome *c* reductase did not parallel the other outer-membrane enzymes for intact mitochondria, but did so when assayed in highly purified fractions of outer membrane. This suggests a labile binding to the outer membrane during the early stages of morphogenesis. 8. Electrophoresis of inner- and outer-membrane proteins revealed little difference between control and experimental mitochondria at 4 days, except for an increase in several, high-molecular-weight components of the outer membrane. These bands closely correspond to similar bands derived from smooth endoplasmic reticulum. 9. The results are discussed in relation to the biogenesis and turnover of mitochondria, and are considered to provide evidence for turnover as a unit, at least for the matrix, inner membrane, inter-membrane space and possibly some form of primary outer membrane.

The outer and inner membranes of mitochondria differ in their complement of enzymes (Parsons, Williams & Chance, 1966; Beattie, 1968; Schnaitman & Greenawalt, 1968), as well as in chemical composition (Parsons & Yano, 1967; Parsons, Williams, Thompson, Wilson & Chance, 1967). In addition, isotope-incorporation studies, both *in vivo* and *in vitro*, have revealed distinctive characteristics for the two membranes (Kadenbach, 1968a; Bygrave & Bücher, 1968; Druyan, DeBernard & Rabinowitz, 1969; Beattie, 1969). Evidence has been obtained by Brunner & Neupert (1968) that the half-life of proteins associated with the outer mitochondrial membrane is only 4.2 days, in contrast with a half-life of 12.6 days for inner-membrane proteins.

In view of these differences, it is of considerable interest to determine whether a synchronous development of the two membranes occurs during mitochondrial morphogenesis, or alternatively is there a differentiation of the inner and outer membranes during a period of rapid mitochondrial morphogenesis? To answer these questions, the following approach was taken: specific enzyme markers were chosen for the outer membrane, the space between the inner and outer membrane, the inner membrane and the matrix. In accordance with generally accepted data (Lardy & Ferguson, 1969), kynurenine hydroxylase, monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase were taken as markers for the outer mitochondrial membrane, adenylate kinase for the

inter-membrane space, succinate cytochrome *c* reductase and rotenone-sensitive NADH cytochrome *c* reductase for the inner mitochondrial membrane, and finally NAD^+ malate dehydrogenase and NAD^+ glutamate dehydrogenase as markers for the mitochondrial matrix.

A series of experiments measuring the activity of these marker enzymes was then carried out on mitochondria isolated from regenerating rat liver. Partial hepatectomy of the rat induces a massive biogenesis of mitochondria, concomitant with the complete regeneration of liver mass (Gear, 1965a,b; Bucher, 1963). This system thus provides a good opportunity for studying inner- and outer-membrane synthesis, since in normal liver the daily turnover of mitochondria is only about 5%.

The results of the present study show that enzymes of the mitochondrial inner membrane and matrix, with the exception of cytochrome *c* oxidase (Gear, 1965b), appeared synchronously during a period of massive mitochondrial biogenesis. In sharp contrast, however, were two enzymes of the outer mitochondrial membrane, monoamine oxidase and kynurenine hydroxylase. The restoration of their activity lagged behind that of the inner membrane and matrix enzymes, and suggests that the enzymes of the outer mitochondrial membrane are renewed by a different mechanism than the inner-membrane and matrix enzymes. These results thus support an asynchronous assembly of the two membrane systems.

EXPERIMENTAL

Chemicals. Cytochrome *c* (type III), DL-kynurenine sulphate, benzylamine, oxaloacetic acid, 2-oxoglutaric acid, α -D(+)-glucose, rotenone and tris were all obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; NADH, NADPH and ADP were purchased from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A.; glycylglycine, hexokinase (yeast) and glucose 6-phosphate dehydrogenase (yeast) were obtained from Calbiochem, Los Angeles, Calif., U.S.A.; Lubrol-WX, a non-ionic detergent, was purchased from ICI Organics Inc., Provincetown, R.I., U.S.A. All other reagents were of A.R. grade, or of the highest purity commercially available.

Animals. Male Sprague-Dawley albino rats, obtained from Hormone Assay Laboratories, Chicago, Ill., U.S.A., were used throughout. The animals weighed from 200 to 350 g.

Partial hepatectomy. This was carried out as described by Higgins & Anderson (1931). Rats were anaesthetized with ether and given 10% (w/v) glucose for a day after operation. They were then maintained on a normal diet until killed. Appropriate sham operations were carried out.

Induction of tryptophan pyrrolase. Rats were injected intraperitoneally with a solution of L-tryptophan [1 g/kg body wt. dissolved in 0.9% NaCl solution as described by Feigelson & Greengard (1962)]. The animals were then killed at 4 h, 1 day and 4 days after the initial injections.

Isolation of mitochondria. The technique described by Gear (1965a) was followed. The heavy and light mitochondrial fractions were combined for the washing steps of the differential centrifugation procedure, and the fluffy-layer fraction was discarded for the present experiments. The mitochondrial pellets were finally suspended in 0.25 M-sucrose at a protein concentration of 50 mg/ml, and were kept at 0°C until assay of enzyme activity.

Purification of inner and outer mitochondrial membranes. The fractionation procedure involving digitonin (Schnaitman & Greenawalt, 1968) was modified as follows. A purified inner-membrane fraction was obtained by treating the inner membrane plus matrix fraction with 0.1 mg of Lubrol-WX/mg of original mitochondrial protein, followed by gentle stirring for 30 min at 0°C; the inner- and outer-membrane fractions were diluted to 25 ml with 0.25 M-sucrose and layered on top of a discontinuous sucrose gradient for further purification. The gradient was formed in tubes for a Spinco SW 27 rotor and consisted of 4 ml of 1.4 M-sucrose cushion, followed by 3 ml of 1.2 M-sucrose, 3 ml of 1.0 M-sucrose, and 3 ml of 0.7 M-sucrose. The gradients were then centrifuged for 1 h at 131000g at 3°C, after which the top 26 ml was discarded. The next 7 ml containing the membranes was collected, diluted threefold with water, and centrifuged for 1 h at 100000g in the titanium Spinco 60 rotor. The resultant pellets were then resuspended in small volumes of 0.25 M-sucrose and frozen overnight for protein determination, enzyme assay and commencement of the electrophoresis procedure the following day. At least 100 mg of mitochondrial protein was subjected to the complete procedure to obtain sufficient membrane protein for the subsequent solubilization procedure.

For comparative purposes two other procedures were used for obtaining purified fractions of outer membranes. Both involve swelling and mechanical disruption of mitochondria: Parsons & Williams (1967) and Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967a).

Solubilization and gel-electrophoresis of the inner- and outer-membrane proteins. This was carried out as described by Schnaitman (1969), on the purified inner- and outer-membrane fractions obtained by the digitonin procedure outlined above.

Assay of enzymic activities. (a) Kynurenine hydroxylase. This enzyme was assayed by the procedure described by Hayaishi (1962) by using a Cary 14 spectrophotometer. The mitochondrial fractions were solubilized 15 min before assay by adding Lubrol-WX at a concentration of 0.3 mg of Lubrol/mg of mitochondrial protein, so as to minimize swelling effects. Lubrol-WX does not significantly inhibit enzyme activity (Schnaitman & Greenawalt, 1968).

(b) Monoamine oxidase. The method of Tabor, Tabor & Rosenthal (1954) was used in which benzaldehyde formation is monitored spectrophotometrically at 250 nm. Lubrol-WX was employed as described above to minimize light-scattering effects.

(c) Rotenone-insensitive and rotenone-sensitive NADH cytochrome *c* reductases. These enzymes were assayed exactly as described by Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967b). The rotenone concentration was 0.5 μM . Lubrol-WX was not used, since it inhibited the activities by about 20%.

(d) Adenylate kinase. The coupled assay procedure

outlined by Schnaitman & Greenawalt (1968) was followed, Lubrol-WX being used to activate the enzyme.

(e) Succinate cytochrome *c* reductase. The procedure of Sottocasa *et al.* (1967b) was followed. Lubrol-WX was not used for activation.

(f) NAD⁺ malate dehydrogenase. The method described by Ochoa (1955) was followed with Lubrol-WX being employed to release the enzyme completely from the mitochondrial matrix.

(g) NAD⁺ glutamate dehydrogenase. The procedure outlined by Beaufay, Bendall, Baudhuin & de Duve (1959) was employed except for the omission of Triton X and nicotinamide. The mitochondria were disrupted with Lubrol-WX before assay.

(h) Glucose 6-phosphatase. Assays on both rat liver homogenates and purified mitochondrial fractions were made according to the procedure of Harper (1963).

(i) Tryptophan pyrrolase. Samples (0.5 ml) of the supernatant from the third centrifugation of the mitochondrial isolation procedure described by Gear (1965a) were added to 2.5 ml of the medium described by Knox, Piras & Tokuyama (1966). The enzyme was initially activated by preincubation as suggested by Knox *et al.* (1966) and then assayed spectrophotometrically at 360 nm. A molecular extinction coefficient of $4.53 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Knox *et al.* 1966) was used to calculate the enzyme activities, which are expressed as μmol of *N*-formylkynurenine formed/min per g of liver at 25°C.

All enzymic activities were measured at 25°C with a Gilford automatic-sampling spectrophotometer coupled to a Sargent 10 in SLR recorder, with the exception of kynurenine hydroxylase, which was monitored with a Cary 14 spectrophotometer. Assay of enzyme activity was

always complete within 6 h of the death of the animal, except for the purified outer-membrane fractions, which were frozen before assay.

Respiratory control. This was measured by a polarographic technique using the medium described by Gear & Lehninger (1968).

Protein determination. Protein concentrations were determined by the u.v. absorption method of Murphy & Kies (1960) with bovine serum albumin as reference standard.

RESULTS

Changes in liver weight and mitochondrial protein during regeneration. The effect of partial hepatectomy on liver weight and mitochondrial content is shown in Table 1. The very rapid gain in liver weight as compared with the slower increase of mitochondrial protein agrees with results previously obtained (Gear, 1965a). However, the present experiments are characterized by a larger decrease in the specific content of mitochondria for a given weight of liver, and a more rapid increase in liver weight, than the earlier studies. Such quantitative differences may result from the use of a different strain of rat, Sprague-Dawley instead of Wistar, as suggested by Bucher (1963).

It is possible that the very rapid gain in liver weight might reflect largely intracellular water and not protein. To check this, the protein content of liver was compared with its wet weight for a series

Table 1. *Changes in liver weight and mitochondrial protein during liver regeneration*

Four series of experiments were carried out. A minimum of two rats, their livers being pooled, were killed at every time-interval for each experimental series. The values are expressed as percentages of the control $\pm$ s.d., with absolute control values given in parentheses. The specific content refers to the amount of mitochondria in a given weight of liver.

Days after partial hepatectomy	Liver (wet wt.)	Total mitochondrial protein	Specific content
Control	100% (11.5 $\pm$ 1.9 g)	100% (285 $\pm$ 63 mg)	100% (24.8 $\pm$ 5.5 mg/g)
2	42 $\pm$ 16	28 $\pm$ 8	66 $\pm$ 14
3	66 $\pm$ 14	31 $\pm$ 3	47 $\pm$ 12
4	77 $\pm$ 4	45 $\pm$ 5	59 $\pm$ 8
6	86 $\pm$ 17	49 $\pm$ 15	57 $\pm$ 15
10	88 $\pm$ 15	72 $\pm$ 18	81 $\pm$ 20
15	82 $\pm$ 26	62 $\pm$ 27	75 $\pm$ 15
20	98 $\pm$ 10	76 $\pm$ 20	77 $\pm$ 17
30	103 $\pm$ 12	84 $\pm$ 28	81 $\pm$ 24

Table 2. *Protein content of regenerating rat liver*

Values are from one experiment, two rats being killed at each time-interval after operation, and are expressed as mg of protein/g of liver.

Time after operation (days)	...	2	3	6	10	20	30
Sham-operated		196	237	223	202	202	241
Regenerating liver		210	227	179	229	187	218

of both sham-operated and partially hepatectomized rats. The results (Table 2) show that the protein content of liver expressed on a wet-weight basis did not vary significantly throughout liver regeneration. This means that results (Table 1) expressed on a wet-weight basis do not suffer from

apparent changes caused by variation of the water content of liver.

Changes in mitochondrial marker enzymes after partial hepatectomy. Four separate series of experiments were carried out, with each series involving the use of about 40 rats, and with a minimum of two

Fig. 1. Changes in the specific activity of mitochondrial marker enzymes during liver regeneration: ●, sham-operated; ○, partially hepatectomized animals. Results are all expressed in terms of mitochondrial protein. (a) NAD⁺ malate dehydrogenase; (b) NAD⁺ glutamate dehydrogenase; (c) succinate cytochrome c reductase; (d) rotenone-sensitive NADH cytochrome c reductase; (e) adenylate kinase; (f) rotenone-insensitive NADH cytochrome c reductase; (g) monoamine oxidase; (h) kynurenine hydroxylase.

rats being killed each time. At 2, 3 and 4 days after operation, three or four rats had to be killed for the regenerating series, to obtain sufficient liver for the subsequent manipulations. Sham-operated animals were killed at the same time as partially hepatectomized ones.

The results of the enzyme determinations are illustrated in Fig. 1(a)–1(h). The specific activities of all the enzymes tested, with the exception of the two outer-membrane enzymes, monoamine oxidase and kynurenine hydroxylase, did not change significantly during the course of liver regeneration; i.e. P values were consistently greater than 0.10 in the Students t test comparing the partially hepatectomized animals with the appropriate shams at the same time-interval after operation. The one exception to this was the rotenone-sensitive, or inner-membrane (Lardy & Ferguson, 1969), NADH cytochrome c reductase (Fig. 1d) at 2 days after partial hepatectomy. The specific activity of this enzyme more than doubled from the control value of $0.093 \mu\text{mol/min per mg}$ of mitochondrial protein ($P < 0.001$). The significance of this large early increase is not yet understood.

A noteworthy observation is that the specific activity of both monoamine oxidase and kynurenine hydroxylase (Figs. 1g and 1h) decreased significantly, reaching a minimum at about 3–4 days after partial hepatectomy and then returned to the control values by 3 weeks. At 4 days the specific activity of monoamine oxidase was $4.60 \text{ nmol/min per mg}$ of mitochondrial protein ($P < 0.001$) compared with a sham value of 7.54; that of kynurenine hydroxylase, $1.05 \text{ nmol/min per mg}$ of mitochondrial protein ($P < 0.001$) compared with a sham value of 2.21; the values at 2, 3 and 6 days were also significantly lower ($P < 0.001$) than the appropriate shams. In contrast with this, was the behaviour of rotenone-insensitive NADH cytochrome c reductase shown in Fig. 1(f). The specific activity of this enzyme did not decrease significantly ($P < 0.1$ at 2, 3 and 6 days, $P < 0.05$ at 4 days) during the early course of liver regeneration. The significance of this apparent difference in behaviour of the outer-membrane enzymes will be considered in the Discussion section.

Inducibility of kynurenine hydroxylase. The observation that the specific activity of both kynurenine hydroxylase and monoamine oxidase, but not rotenone-insensitive NADH cytochrome c reductase, decreased sharply after partial hepatectomy (Figs. 1g and 1h), raises the possibility that these changes might not represent a true change in the amount or in the composition of the outer mitochondrial membrane. Rather these two enzymes might be selectively repressed during active liver regeneration. It is known, for example, that monoamine oxidase activity can be either increased or decreased by specific inhibitors, or by the hormonal state of the animal (Zeller, 1968). There is also evidence that kynurenine hydroxylase may be an inducible enzyme. Kizer & Howell (1963) found that the enzyme was missing in several different rat hepatomas. Together with earlier reports by Reid (1962) and Pitot & Morris (1961) that the related tryptophan pyrrolase was likewise depressed in hepatomas, this evidence suggests that rapidly growing tissues might possess lower amounts of enzymes involved in tryptophan catabolism.

To test directly that kynurenine hydroxylase might be inducible under the same conditions as tryptophan pyrrolase, rats were injected intraperitoneally with tryptophan solutions (Feigelson & Greengard, 1962). These conditions are known to induce a rapid synthesis *de novo* of tryptophan pyrrolase. Injections were given daily and the two enzymes assayed at 4h, 28h and 4 days after the initial induction. The experiments were continued up to 4 days since, if kynurenine hydroxylase is induced, it would take 4 days for half the outer mitochondrial membrane to be replaced (Brunner & Neupert, 1968). Alternatively, amounts of outer-membrane components could vary without net replacement of the whole membrane. The results (Table 3) reveal no evidence for any induction of kynurenine hydroxylase.

Tryptophan pyrrolase activity during liver regeneration. The above experiment (Table 3) proves that kynurenine hydroxylase is not synthesized under conditions known to induce the related enzyme, tryptophan pyrrolase. However, there

Table 3. *Inducibility of kynurenine hydroxylase*

Kynurenine hydroxylase and tryptophan pyrrolase were measured at various intervals after intraperitoneal injection of solutions of L-tryptophan. (For details see the Experimental section.) The results are given as ratios of the specific activities of the induced animals as compared with the sham-injected animals. Two rats, their livers being pooled, were killed for each experimental value.

Time of induction ...	4 h	28 h	96 h
Kynurenine hydroxylase	0.97	0.95	0.97
Tryptophan pyrrolase	1.88	1.50	1.84

remains the possibility that when the activity of kynurenine hydroxylase was markedly depressed (Fig. 1*h*) during the early stages of liver regeneration, tryptophan pyrrolase might also be depressed. This possibility was tested and the results in Table 4 show that no decrease in tryptophan pyrrolase activity occurred between 2 and 6 days after partial hepatectomy.

Enzyme activity of purified outer-membrane fractions. To determine directly whether there is a change in either the amount or composition of outer membrane during liver regeneration, purified outer-membrane fractions were prepared from mitochondria 4 days after partial hepatectomy. These fractions were then assayed for monoamine oxidase, kynurenine hydroxylase and rotenone-insensitive NADH cytochrome *c* reductase, as were the corresponding preparations from sham-operated animals. If the outer membrane were to change in composition, but not in amount, then the specific activity of these outer-membrane enzymes should be altered similarly in both the mitochondrial and purified outer-membrane fractions, when each fraction is compared with its appropriate sham. On the other hand, for a change in the amount of outer membrane, the specific activities would be the same in the purified, outer-membrane fractions derived from both sham and experimental animals, but still different for the original mitochondrial fractions.

Purified outer-membrane fractions were obtained by several procedures to help eliminate the possibility that one technique might not work with equal efficiency on experimental and control mitochondria. The procedures used involved prolonged swelling and centrifugation (Parsons & Williams, 1967); swelling followed by contraction and ultrasonic treatment (Sottocasa *et al.* 1967*a*); and the action of digitonin as a detergent (Schnaitman & Greenawalt, 1968). The results (Table 5) are expressed as the ratio of the enzyme specific activities of partially hepatectomized to sham-operated animals 4 days after operation. Purifications of between 10- and 20-fold were obtained for the digitonin procedure and that of Parsons & Williams (1967), but below 10-fold for that of Sottocasa *et al.* (1967*a*). This may explain the greater correspondence between results from the two former procedures than from the latter.

The results in Table 5 clearly show that the enzymic composition of purified, outer-membrane fractions from partially hepatectomized rats is quite different from that of sham-operated animals. What is particularly intriguing is that whereas rotenone-insensitive NADH cytochrome *c* reductase was the same for partial and sham in the whole mitochondria (Fig. 1*f*), a sharp distinction arose during purification of the outer membrane. This is seen as a shift in the ratio of specific activities from 0.99 to about 0.60 for the most highly purified

Table 4. *Tryptophan pyrrolase activity during liver regeneration*

The enzyme was assayed 2, 3, 4 and 6 days after partial hepatectomy. The results are expressed as nmol of *N*-formylkynurenine formed/min per g of liver at 25°C. The numbers of animals used, their livers being pooled, are given in parentheses.

Time after operation (days) ...	2	3	4	6
Sham-operated	29.4 (1)	22.3 (1)	26.7 (1)	25.4 (1)
Partially hepatectomized	23.2 (3)	28.0 (3)	24.9 (2)	25.4 (2)

Table 5. *Enzyme activity of purified outer-membrane fractions*

Purified outer-membrane fractions were prepared by three separate procedures (see the Experimental section) from liver mitochondria isolated 4 days after the partial hepatectomy of six rats, and the sham-operation of three rats. The intact mitochondrial and outer-membrane fractions were both assayed for the activity of outer-membrane enzymes. Results are expressed as the ratio of the specific activity in the fraction derived from the partially hepatectomized animals to that derived from the sham-operated animals.

Enzyme	Intact mitochondria	Purified outer-membrane fractions		
		Detergent (Schnaitman & Greenawalt, 1968)	Mechanical (Parsons & Williams, 1967)	Mechanical (Sottocasa <i>et al.</i> 1967 <i>a</i>)
Monoamine oxidase	0.70	0.44	0.42	0.70
Kynurenine hydroxylase	0.59	0.45	0.47	0.53
Rotenone-insensitive NADH cytochrome <i>c</i> reductase	0.99	0.59	0.62	0.76

fractions. The significance of this will be considered in the Discussion section.

Microsomal contamination. Mitochondrial preparations are always contaminated to some extent by microsomes. It could be argued that small variations in the amount of contamination might have a marked influence on the activity of rotenone-insensitive NADH cytochrome *c* reductase, since the same activity exists in microsomes. However, even though the microsomal dehydrogenase possesses slightly different properties (see Sottocasa *et al.* 1967a), this would not affect the measured enzyme activity. There is also a group (Green, Allmann, Harris & Tan, 1968) that maintains that the dehydrogenase measured in mitochondrial preparations merely reflects microsomal contamination. Consequently it is necessary to determine whether the microsomal content of liver changes during the course of liver regeneration and also whether the amount of mitochondrial contamination by microsomes is low and constant. Glucose 6-phosphatase activity was measured in whole-liver homogenates to reflect microsomal content (Fig. 2), as well as in the purified mitochondrial preparations to monitor microsomal contamination (Fig. 3).

Two important conclusions may be drawn from the results. First, the specific activity of glucose 6-phosphatase in liver homogenates was constant during regeneration (Fig. 2). This result agrees with a study by Weber & Cantero (1955), who demonstrated that restoration of glucose 6-phosphatase activity paralleled increase in liver weight after partial hepatectomy. Secondly (Fig. 3), microsomal contamination of mitochondria was very low, being close to 2% and constant during liver regeneration. Thus the results concerning rotenone-insensitive NADH cytochrome *c* reductase (Fig. 1f

and Table 5) truly reflect the mitochondrial outer-membrane enzyme and not the microsomal one.

Electrophoresis of inner- and outer-membrane proteins. Mitochondrial fractions from 4-day sham-operated and partially hepatectomized rats were separated into inner- and outer-membrane fractions by the use of digitonin as described in the Experimental section. The membrane proteins of these fractions were then solubilized and subjected to electrophoresis as described by Schnaitman (1969). The results are shown as gel-scanner tracings in Fig. 4.

The scans for inner-membrane proteins were essentially identical for both control and experimental animals; however, whereas much of the outer-membrane scans was similar, several noteworthy features emerge concerning some of the high-molecular-weight components. The amount of band 2 (using the designation of Schnaitman, 1969) for the experimental animals was considerably higher than that for the control animals. It should be noted that the mobility of this protein band is identical with that for band 3 of smooth endoplasmic reticulum (see Schnaitman, 1969). In addition, a new band, called 2' here, may be seen. The mobility of this band exactly corresponds to band 2 of smooth endoplasmic reticulum, and to no other band, be it inner membrane or rough endoplasmic reticulum.

An additional observation may be made concerning the outer-membrane proteins. A minor band in sham-operated animals, designated '0', was greatly increased after partial hepatectomy. The mobility of this band appears to correspond to a high-molecular-weight component (not designated by Schnaitman, 1969) of smooth endoplasmic reticulum, remaining close to the origin of the gel. The significance of the increase in these proteins with the same mobility as those of smooth endoplasmic reticulum is discussed below

Fig. 2. Glucose 6-phosphatase activity of liver homogenates during liver regeneration: ●, sham-operated; ○, partially hepatectomized animals.

Fig. 3. Glucose 6-phosphatase activity of isolated mitochondrial fractions: ●, sham-operated; ○, partially hepatectomized animals.

Fig. 4. Gel-scanner traces of inner and outer, mitochondrial membrane proteins, after electrophoresis for 5 h in 7.5% (w/v) acrylamide gels, and staining with Amido Schwartz. The top traces are derived from sham-operated animals, the bottom traces from partially hepatectomized animals, both 4 days after operation.

DISCUSSION

The results presented in this paper support the conclusion that development of the inner and outer mitochondrial membranes is asynchronous. This was shown by following the behaviour of specific marker enzymes for the various mitochondrial compartments during the massive biogenesis induced by partial hepatectomy. Restoration of outer-membrane enzymes, notably kynurenine hydroxylase and monoamine oxidase, lagged behind that for matrix and inner-membrane enzymes. This was seen as a decrease of up to 50% in the specific activity 4 days after operation. On the other hand, the specific activity of the inner membrane, matrix and inner-membrane enzymes remained constant

throughout liver regeneration. Such behaviour emphasizes the differences between the two mitochondrial membranes with regard to biogenesis *in vivo*, and supplements previous data on differences in composition and isotope incorporation (Lardy & Ferguson, 1969). Also some related studies on the development of different functions in yeast mitochondria during biogenesis caused by de-repression from a high to a low glucose medium, have been made (Lenaz, Littarru & Castelli, 1969). These authors base some of their conclusions on what is now considered to be an erroneous localization (Bachmann, Allmann & Green, 1966) of tricarboxylic acid-cycle enzymes in the outer membrane (Lardy & Ferguson, 1969). However, Lenaz *et al.* (1969) do report an asynchronous appearance of

adenosine triphosphatase and succinoxidase activity, and suggest independent synthesis of these components, followed by assembly on the inner membrane.

An important concept arising from the present studies is that marker enzymes for the outer mitochondrial membrane appear to turn over independently of a pre-existing outer membrane. This is supported by the demonstration, in highly purified fractions of outer membrane, that the specific activities of monoamine oxidase, rotenone-insensitive NADH cytochrome *c* reductase and kynurenine hydroxylase were significantly lowered during the early stages of liver regeneration. Consequently, the enzyme complement of the outer membrane may be lowered, rather than the total amount of membrane. It is probable, then, that the membrane exists in a dynamic state where a primary, structural form may gain, or lose, various constitutive enzymes. A similar conclusion was reached by Dallner, Siekevitz & Palade (1966a,b) whose studies on the biogenesis of endoplasmic reticulum led them to propose that enzymes may attach on to an existing membrane in successive steps. The present results do not support the possibility that the outer mitochondrial membrane is assembled from pre-existing components into a static final form.

An unexpected finding resulting from the purification of outer membrane was that the behaviour of rotenone-insensitive NADH cytochrome *c* reductase changed from resembling an inner-membrane enzyme to resembling the other two outer-membrane enzymes, monoamine oxidase and kynurenine hydroxylase. Rotenone-insensitive NADH cytochrome *c* reductase has proved to be localized in the outer membrane (Sottocasa *et al.* 1967b; Schnaitman & Greenawalt, 1968) despite conflicting data (Green *et al.* 1968). What then could be the explanation that the pattern of the three outer-membrane enzymes differed when assayed in whole mitochondria, but not in purified outer-membrane fractions? It could be argued that, since similar enzymic activity exists in microsomes (Sottocasa *et al.* 1967a), excessive microsomal contamination of mitochondria derived from regenerating liver could falsify the true activity of the outer mitochondrial membrane. However, measurement of glucose 6-phosphatase activity in both whole-liver homogenates and purified mitochondrial fractions, revealed that the microsomal enzyme could not influence the observed results (Figs. 2 and 3). Alternatively, it could be that rotenone is a less efficient inhibitor for intact mitochondria isolated from regenerating liver than from control liver. If this were true, then the activity of the rotenone-sensitive enzyme might be expected to be elevated during the first week of liver regeneration.

This was not found to be so (Fig. 1d), except for one time-interval at 2 days after partial hepatectomy.

Consequently, the explanation of this finding remains obscure. One possibility may be mentioned. Outer-membrane enzymes could in fact be bound to the membrane during the early stages of mitochondrial biogenesis, but only loosely so. Then the procedures used for purifying the outer membrane might cause loosely bound enzymes to be lost, the loss being greatest for the mitochondria derived from regenerating liver. In this context it may be noted that the loss, seen as a decrease in the ratio during purification (Table 5), was almost the same for monoamine oxidase and rotenone-insensitive NADH cytochrome *c* reductase (1.62-fold), but much less for kynurenine hydroxylase (1.28-fold).

A noteworthy correlation may be made between the activity of outer-membrane enzymes and the rate of tissue growth and mitosis. The most rapid increase in liver mass occurred 2–4 days after partial hepatectomy after the mitotic peak at about 28 h (Bucher, 1963). It was precisely during this period that the rapid fall in the activity of outer-membrane enzymes occurred, which is noteworthy in that this observation parallels studies made on various hepatomas. For example, Kizer & Howell (1963) showed that kynurenine hydroxylase was absent from the mitochondria of four transplanted rat hepatomas, two transplanted mouse hepatomas and in primary hepatomas induced in the rat. Pedersen, Greenawalt, Chan & Morris (1970) have reported that the specific activity of monoamine oxidase, an outer-membrane enzyme, was directly related to the rate of growth, being lowest in those hepatomas with the highest growth rate. Together with the present observations, these studies indicate that the machinery for synthesis of outer-membrane enzymes may become repressed during rapid cell growth. Alternatively, the enzymes may still be synthesized, but are unable to integrate with a pre-existing basic membrane. This might depend on the existence of a suitably receptive membrane, or some carrier molecule as suggested by Dawson (1966) and Kadenbach (1968b) to transport proteins synthesized on the endoplasmic reticulum to the mitochondria.

Several investigators have pointed out the similarity between the endoplasmic reticulum and the outer mitochondrial membrane (Sottocasa *et al.* 1967a; Parsons & Yano, 1967; Schnaitman, 1969). The results presented in the present paper have a bearing on this relationship. For example, it is noted that outer-membrane proteins derived from partially hepatectomized rats 4 days after operation contained elevated amounts of several high-molecular-weight components, identical in electrophoretic mobility with similar bands derived from smooth endoplasmic reticulum (Schnaitman, 1969).

There is thus a possibility that some of the outer-membrane proteins originate in the smooth endoplasmic reticulum. However, as the enzymic results on the outer membrane revealed a clear lag behind the microsomal marker, glucose 6-phosphatase, the similarity between the two membrane systems does not extend to parallel regeneration during a period of rapid cellular proliferation. It may be that the cell has to attend to first things first; i.e. regenerate endoplasmic reticulum before synthesizing specific enzymes for the outer mitochondrial membrane.

Finally, the results obtained in the present studies have a bearing on the mode of mitochondrial morphogenesis. Fletcher & Sanadi (1961) originally suggested that mitochondria turn over as a unit, basing their conclusions on radioisotope incorporation into lipid and soluble- and insoluble-protein fractions of rat liver mitochondria. They reported a half-life of about 10 days for all these components, a value which has been amply confirmed by others: Gross, Getz & Rabinowitz (1969) provide a summary of available data. Brunner & Neupert (1968) have challenged the concept of turn over as a unit, since they demonstrated a clear difference in the half-life for outer-membrane protein of 4.2 days, compared with a value of 12.6 days for inner-membrane protein. However, other investigators using non-reutilizable isotopes (Swick, Rexroth & Stange, 1968; Druyan *et al.* 1969), or measuring the turnover of individual enzymes (Rechcigl, 1968), have revealed a half-life in the range of 4–6 days. Half-lives for the inner and outer mitochondrial membranes are in fact quite similar; e.g. Druyan *et al.* (1969) were unable to demonstrate a statistically significant difference between the turnover of cytochrome *b* and cytochrome *b_s*, by using ³H-labelled δ -aminolaevulinic acid. Consequently the original hypothesis of Fletcher & Sanadi (1961) has not yet been disproved, even though, as mentioned above, several reports have indicated a more rapid incorporation of isotopes into outer membrane than inner membrane (DeBernard, Getz, Rabinowitz, 1969; Kadenbach, 1968a; Bygrave & Bücher, 1968; Beattie, 1969). Such experiments can be subject to experimental artifacts and problems of interpretation (Waite, 1969; McMurray & Dawson, 1969).

In relation to the concept of mitochondrial turnover as a unit, it now becomes necessary to define what that unit is, since the present investigation shows that constitutive enzymes of the outer mitochondrial membrane appear to turn over independently of the membrane itself. Thus the unit of mitochondrial turnover may not include constitutive enzymes of the outer membrane. What is more probable is that the inner membrane and matrix, surrounded by a primary outer membrane,

do turn over as a unit. The continued existence of an intact outer membrane is supported by the observation that the specific activity of adenylate kinase, an inter-membrane enzyme (Schnaitman & Greenawalt, 1968) that is readily lost from slightly damaged mitochondria, paralleled that for inner-membrane and matrix enzymes during the whole course of liver regeneration (Fig. 1e), and also by the fact that the respiratory control of mitochondria, an index of structural integrity, did not differ significantly from the control value of 3.5 during regeneration. Then, as mitochondria enlarge, specific enzymes could attach to a pre-existing primary outer membrane. The present studies do not attempt to differentiate between morphogenesis by synthesis *de novo* (Criddle & Schatz, 1969) and by division (Luck, 1963).

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