Biochemical Heterogeneity of Rat Liver Mitochondria Separated by Rate Zonal Centrifugation

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1. Rat liver mitochondria were separated on the basis of their sedimentation coefficients in an iso-osmotic gradient of Ficoll–sucrose by rate zonal centrifugation. The fractions (33, each of 40 ml) were collected in order of decreasing density. Fractions were analysed by spectral analysis to determine any differences in the concentrations of the cytochromes and by enzyme analyses to ascertain any differences in the activities of NADH dehydrogenase, succinate dehydrogenase and α -glycerophosphate dehydrogenase. 2. When plotted as % of the highest specific concentration, the contents of cytochrome $a+a_3$ and cytochrome $c+c_1$ were constant in all fractions but cytochrome b was only 65% of its maximal concentration in fraction 7 and increased with subsequent fractions. As a result, the cytochrome b /cytochrome $a+a_3$ ratio almost doubled between fractions 7 and 25 whereas the cytochrome $c+c_1$ /cytochrome $a+a_3$ ratio was unchanged. 3. Expression of the dehydrogenase activities as % of highest specific activity showed the following for fractions 6–26: NADH dehydrogenase activity remained fairly constant in all fractions; succinate dehydrogenase activity was 62% in fraction 6 and increased steadily to its maximum in fraction 18 and then decreased; the activity of α -glycerophosphate dehydrogenase was only 53% in fraction 6 and increased slowly to its peak in fractions 22 and 24. 4. These differences did not result from damaged or fragmented mitochondria or from microsomal contamination. 5. These results demonstrate that isolated liver mitochondria are biochemically heterogeneous. The importance of using a system for separating biochemically different mitochondria in studies of mitochondrial biogenesis is discussed.

Even though histochemical studies have shown liver mitochondria to be heterogeneous, the isolated organelles are usually treated as a homogeneous population. An attempt was therefore made in this investigation to demonstrate biochemical differences in liver mitochondria separated by rate zonal centrifugation on the basis of their sedimentation coefficients.

By a variety of histochemical techniques, a number of investigators have demonstrated that several mitochondrial enzymes have different patterns of distribution within the liver lobule (Seligman & Rutenberg, 1951; Schumacher, 1957; Burstone, 1959; Novikoff, 1959; Pette & Brandau, 1966). Succinate dehydrogenase and cytochrome oxidase have a higher activity in the cells in the periphery of the lobule whereas NAD^+ - and NADP^+ -diaphorases and glutamate and β -hydroxybutyrate dehydrogenases appear to be more active in the centrolobular cells. In contrast with either of these two patterns, α -glycerophosphate dehydrogenase is uniformly distributed in the liver lobule. These dissimilar distribu-

tions indicate that liver mitochondria are heterogeneous in their enzyme complements.

Liver mitochondria have also been shown to exist in a range of sizes. From electron micrographs of liver sections, Novikoff & Shin (1964) observed that the mitochondria in the central cells were 0.2–0.3 μm in width and up to 2 μm long and those in the peripheral cells were 0.5–1.0 μm in width and up to 4 μm in length. Gregson & Williams (1969) estimated the size of isolated mitochondria from negatively stained preparations by averaging two diameters at right angles. These mean diameters had a normal distribution and ranged from 0.3 to 1.1 μm . This size distribution means that isolated mitochondria can be separated centrifugally on the basis of their sedimentation coefficients.

Numerous attempts, using different centrifugation techniques, have been made to demonstrate heterogeneity in isolated liver mitochondria. Mitochondria have been separated by differential centrifugation (Laird *et al.*, 1952; Novikoff *et al.*, 1953; Gear, 1965*a,b*; Lusena & Depocas, 1966), isopycnic

density-gradient centrifugation (Kuff & Schneider, 1954; Pette, 1966; Pollak & Munn, 1970) and rate zonal centrifugation (Swick *et al.*, 1967; Schuel *et al.*, 1969). Critics have suggested that reported heterogeneities are artifacts of the centrifugation procedures. Separation by isopycnic density-gradient centrifugation has been especially criticized. Beaufay & Berthet (1963) and Beaufay *et al.* (1964) believed the bimodal distribution of liver mitochondria on sucrose density gradients was the consequence of density changes caused by high sucrose concentrations. Similarly, the results of rate zonal centrifugation may be questioned since hyperosmotic sucrose concentrations were used for the gradients.

Since previous studies reporting differences in isolated liver mitochondria are held in doubt, experiments were undertaken to try to demonstrate heterogeneity in mitochondria separated by rate zonal centrifugation. It was considered essential to maintain the mitochondria in their physiologic state; therefore, an iso-osmotic gradient of Ficoll-sucrose was used. In analysing the mitochondrial fractions obtained, the first objective was to determine if there were differences in the concentrations of the cytochromes in mitochondria of different fractions. The second objective was to determine if there were differences in the activities of the enzymes NADH dehydrogenase (EC 1.6.99.3), succinate dehydrogenase (EC 1.3.99.1) and α -glycerophosphate dehydrogenase (EC 1.1.99.5) in different fractions. These enzymes were selected for two reasons: they exhibit different patterns of distribution in the liver lobule and their activities are influenced by various treatments e.g. thyroid hormone. Finally, the development of a system for separating biochemically different mitochondria makes possible studies of mitochondrial biogenesis which take into account the heterogeneity of mitochondria occurring in tissues.

Materials and Methods

Chemicals

Ficoll, DL- α -glycerophosphate (disodium salt), D-glucose 6-phosphate (disodium salt) and cytochrome c (type III) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Preparation of mitochondria

Male Sprague-Dawley rats, 225–260 g, were decapitated and the median and left lateral lobes of the liver rapidly removed and placed in ice-cold 0.15 M-NaCl. All subsequent operations were performed at 4°C. The liver (30 g) was sliced, washed and blotted, and a 10% (w/v) homogenate prepared in 0.25 M-sucrose–0.1 mM-EDTA–0.01 M-tris–HCl buffer, pH 7.4, by using a Potter-Elvehjem glass homogenizer and a motor-driven Teflon pestle.

Isolation of mitochondria was done in a Sorval RC-2 centrifuge with an SS-34 rotor. The homogenate was centrifuged for 10 min at 400 g_{av} and the supernatant was recentrifuged for 5 min at 400 g_{av} . The second supernatant was then centrifuged at 7800 g_{av} for 10 min. The mitochondrial pellet was washed twice by resuspending it in 0.25 M-sucrose–0.1 mM-EDTA–0.01 M-tris–HCl buffer, pH 7.4, and recentrifuging it at 7800 g_{av} for 10 min. The supernatant fraction from the original mitochondrial pellet and each washing was removed completely by aspiration. The final pellet was suspended in 0.25 M-sucrose–0.1 mM-EDTA–0.01 M-tris–HCl buffer, pH 7.4, in a total volume of 30 ml (1 ml/g of liver). This mitochondrial suspension was used for rate zonal centrifugation.

Rate zonal centrifugation

Fractionation of the mitochondria was done with the SZ-14 reorienting gradient zonal rotor (Sheeler *et al.*, 1971) in a Sorvall RC-2 centrifuge. The rotor was loaded at 3000 rev./min and unloaded at rest after deceleration. The gradient contained 8.5% (w/w) sucrose throughout but varied from 0 to 11.5% (w/w) Ficoll; the cushion was 45% (w/w) sucrose. All solutions were prepared in 0.01 M-tris–HCl buffer, pH 7.4, and checked for proper density before use with an Abbé refractometer.

The cushion (150 ml) followed by gradient (1150 ml) was introduced into the rotor by means of a variable-speed Masterflex peristaltic pump. By using a hypodermic syringe, the mitochondrial suspension (30 ml) was rapidly sprayed through the opening in the rotor top on to the gradient and then centrifuged for 13 min (including time allowed for acceleration to maximum speed) at 7600 g_{av} . The rotor was decelerated and after it had stopped completely, the contents were collected in 40 ml fractions.

A sample of each fraction was kept for determination of refractive index. Mitochondria were pelleted by centrifuging at 25000 g_{av} for 30 min in an SS-34 rotor. The pellets were used for enzyme analyses, spectral analyses and protein determinations. Spectral analyses were conducted immediately; pellets used for enzyme analyses were quick-frozen in a solid CO₂-acetone bath and stored overnight in solid CO₂.

Analyses

Refractive indices were measured with an Abbé refractometer and converted into their corresponding density values at 4°C.

Cytochrome concentrations were determined from the difference spectra as described by Estabrook & Holowinsky (1961). The recordings were made with a Cary 15 split-beam spectrophotometer. The mito-

chondrial pellets were resuspended in an iso-osmotic buffer, containing 26mM-NaCl, 70mM-KCl, 6mM-MgCl₂, 13mM-K₂HPO₄ and 3mM-KH₂PO₄, pH 7.0, to give a protein concentration of approx. 5–8mg/ml. To 2ml of the mitochondrial suspension, 25μl of 0.1M-ADP was added and the mixture was allowed to warm to room temperature (23 ± 1°C). Mitochondrial ADP suspension (500μl) was added to each cuvette and the baseline of equal light absorption recorded. The cytochromes were reduced by addition of 5μl of 600mM-succinate to the sample cuvette and the reduced-minus-oxidized spectrum was recorded between 650 and 500nm.

The activities of NADH dehydrogenase (Minakami *et al.*, 1962), succinate dehydrogenase (Veeger *et al.*, 1969) and α-glycerophosphate dehydrogenase (Ringler & Singer, 1959) were determined by following the rate of reduction of ferricyanide at 420nm. The mitochondrial pellets were resuspended in 0.044M-potassium phosphate buffer, pH 7.4, and remained in this hypo-osmotic medium for approx. 30min before assaying. For all three enzyme assays, the medium contained 0.04M-potassium phosphate buffer, pH 7.4, and 1mM-KCN. In addition, the reaction mixtures contained the following: for NADH dehydrogenase, 1.68mM-K₃Fe(CN)₆, 0.3mM-NADH and 15–30μg of mitochondrial protein; for succinate dehydrogenase, 1.68mM-K₃Fe(CN)₆, 5.4mM-succinate and 100–200μg of mitochondrial protein; for α-glycerophosphate dehydrogenase, 0.84mM-K₃Fe(CN)₆, 10mM-MgCl₂, 100mM-α-glycerophosphate and 240–800μg of mitochondrial protein. Addition of the substrate initiated the reaction and brought the final volume to 550μl. Two blanks were always determined; one included all reactants except the mitochondria and the second all reactants except the substrate.

Protein was determined by the method of Lowry *et al.* (1951). Because of the interference of Ficoll with the assay, protein determinations were done on the pellets of fractions.

Microsomal contamination was determined by analysing the pellets of various fractions for glucose 6-phosphatase (EC 3.1.3.9), NADPH-cytochrome *c* reductase (EC 1.6.99.1) and RNA. The activities of glucose 6-phosphatase and NADPH-cytochrome *c* reductase were determined immediately; pellets for RNA analysis were quick-frozen in a solid CO₂-acetone bath and stored at -20°C. Glucose 6-phosphatase activity was measured by determining the appearance of orthophosphate in the incubation medium (Nordlie & Arion, 1966). The incubation mixture (final vol. 1.5ml) contained 40mM-sodium cacodylate buffer, pH 6.5, 10mM-glucose 6-phosphate and 0.3–1.2mg (mitochondrial) or 0.1–0.4mg (microsomal) of protein. The reaction was initiated by addition of the enzyme preparation and incubation was done at 30°C for 20min. The reaction was

terminated by addition of 1ml of 12.5% (w/v) trichloroacetic acid and the sample centrifuged. P_i was determined on the supernatant by the method of Fiske & SubbaRow (1925) with a modification of the reducing reagent (Bartlett, 1959). The activity of NADPH-cytochrome *c* reductase was determined by following the rate of reduction of cytochrome *c* at 550nm as described by Masters *et al.* (1967). The assay mixture consisted of 0.05M-potassium phosphate buffer, pH 7.6, containing 0.1mM-EDTA, 1mM-KCN, 32.7μM-cytochrome *c*, 0.1mM-NADPH, and 50–150μg (mitochondrial) or 5–25μg (microsomal) of protein. Addition of NADPH initiated the reaction and brought the final volume to 550μl. RNA was determined by the method of Schmidt & Thannhauser (1945) as modified by Munro & Fleck (1966). When these assays were done on the microsomal fraction, microsomal material was obtained from the mitochondrial supernatant by centrifugation at 78000g_{av} for 90min.

Results

To achieve a good separation of mitochondria based on differences in sedimentation coefficient, the centrifugation procedure was designed so that the mitochondria would span a large number of fractions in the linear region of the gradient. The distribution of mitochondria in mg of protein/ml on the Ficoll-sucrose gradient for three separate experiments is shown in Fig. 1. The protein distribution was similar in the three experiments, spanning 20 fractions and reaching a maximum in fraction 16. Only one gradient

Fig. 1. Mitochondrial protein distribution on the Ficoll-sucrose gradient

The rate zonal centrifugation procedure is described in the Materials and Methods section. The results presented are for three separate experiments. Gradient density (----); protein (Δ, □, ○).

Fig. 2. Distribution of specific concentrations of cytochromes in mitochondrial fractions obtained by rate zonal centrifugation

Cytochrome concentrations were determined from difference spectra reduced by succinate as described in the Materials and Methods section. The results of three separate experiments are presented. The fractions are numbered in order of decreasing density. Cytochrome $a+a_3$ ($\square$); cytochrome b ($\circ$); cytochrome $c+c_1$ (Δ).

curve is shown as the gradient was almost identical from experiment to experiment.

To determine if there were differences in the concentrations of the cytochromes, spectral analyses were conducted on selected fractions. The specific concentrations of the cytochromes were similar in all three experiments (Fig. 2). In general, cytochrome b appeared to increase in concentration with decreasing density, whereas cytochrome $a+a_3$ and cytochrome $c+c_1$ remained constant. When the values were averaged and plotted as % of highest specific concentration, this trend was clearly seen (Fig. 3). The concentration of cytochrome $a+a_3$ and cytochrome $c+c_1$ were constant up to fraction 25 whereas cytochrome b was only 65% of its maximal concentration in fraction 7 and increased with subsequent fractions. Cytochrome b concentration decreased in fraction 25 but not nearly to the extent of cytochrome $a+a_3$ and cytochrome $c+c_1$. As a result, the cytochrome b /cytochrome $a+a_3$ ratio almost doubled between fractions 7 and 25 whereas the cytochrome $c+c_1$ /cytochrome $a+a_3$ ratio did not change (Table

Fig. 3. Specific concentrations of cytochromes expressed as % of highest specific concentration

Cytochrome concentrations from the three experiments were averaged for each fraction and the % of the highest specific concentration determined. The fractions are numbered in order of decreasing density. Cytochrome $a+a_3$ ($\square$); cytochrome b ($\circ$); cytochrome $c+c_1$ (Δ).

1). These results can be seen by directly comparing the spectra of fractions 7 and 25 (Fig. 4). The difference between cytochrome b and cytochrome $a+a_3$ was greater in fraction 7 than in fraction 25 whereas the difference between cytochrome $c+c_1$ and cytochrome $a+a_3$ was similar for the two fractions.

The fractions were also analysed to see if there were differences in the activities of NADH dehydrogenase, succinate dehydrogenase, and α -glycerophosphate dehydrogenase. Plotting specific activity against fraction number revealed a different pattern for each enzyme (Figs. 5, 6 and 7). As with the cytochrome analyses, the results of three separate experiments were similar. When the activities were expressed as % of highest specific activity the results showed the following: NADH dehydrogenase activity remained fairly constant in all fractions; succinate dehydrogenase activity was 62% in fraction 6 and increased steadily to its maximum in fraction 18 and then decreased; the activity of α -glycerophosphate dehydrogenase was only 53% in fraction 6 and increased slowly to its peak in fractions 22 and 24 (Fig. 8).

It was considered important to demonstrate that the differences obtained were not the result of damaged or fragmented mitochondria. Carefully prepared liver mitochondria have been shown to be

Table 1. Ratios of cytochromes in mitochondrial fractions obtained by rate zonal centrifugation

Results are given for Experiments I, II and III. The density of the gradient decreased with increasing fraction number.

Fraction no.	$b/(a+a_3)$			$(c+c_1)/(a+a_3)$		
	I	II	III	I	II	III
7	0.31	0.43	0.38	0.99	1.14	1.02
11	0.43	0.46	0.47	1.25	1.13	1.19
15	0.52	0.52	0.54	1.21	1.17	1.26
19	0.55	0.57	0.60	1.12	1.12	1.32
21	0.50	0.57	0.60	1.12	1.10	1.26
25	0.63	0.65	0.62	1.11	0.99	1.06

Fig. 4. Comparison of cytochrome difference spectra of fraction 7 (high density) and fraction 25 (low density)

The results presented are from Experiment I. ---, Fraction 7; —, fraction 25. Cytochromes were reduced by addition of succinate to the sample cuvette and the reduced-minus-oxidized spectrum was recorded on a Cary 15 split-beam spectrophotometer as described in the Materials and Methods section. The wavelength pairs used to measure the height of the cytochrome peaks were (Estabrook & Holowinsky, 1961): cytochrome $a+a_3$, 605–630nm; cytochrome b , 562–575nm; cytochrome $c+c_1$, 551–540nm.

impermeable to exogenous NADH (Lehninger, 1965). The degree of intactness of the mitochondria was therefore determined by following the rate of oxidation of added NADH at 340nm. This rate was compared with the rate in mitochondria of the same fraction which had been made permeable to NADH by freezing and thawing three times. For fractions 9, 11, 17, 23 and 25, the ratio of the rate of NADH

oxidation in frozen and thawed mitochondria to the rate in untreated mitochondria was 3.8, 3.9, 4.8, 5.1 and 5.0 respectively. These similar ratios indicated that the mitochondria were not separated on the gradient according to varying degrees of mitochondrial damage and that the observed enzyme and cytochrome differences were not artifacts. However, when the intactness of the mitochondria was checked before zonal centrifugation, the ratio of the rate of NADH oxidation in frozen and thawed mitochondria to the rate in untreated mitochondria was 28. An attempt was made to explain this discrepancy. As part of the zonal centrifugation procedure, mitochondria underwent a manyfold dilution when placed on the gradient and remained at this dilution until pelleted approx. 2–3 h later. To investigate the effect of time and dilution mitochondria were resuspended at a protein concentration similar to that encountered on the gradient. One half of the mitochondrial suspension was pelleted after remaining at this dilution for 13 min (length of zonal run). The ratio of the rate of NADH oxidation in mitochondria frozen and thawed to the rate in untreated mitochondria was 15.4. The other half of the mitochondrial suspension was pelleted after remaining diluted for 2 h; the ratio of the rate of NADH oxidation in frozen and thawed mitochondria to the rate in untreated mitochondria decreased to 9. These results suggested that the observed decrease in the ratio of the rate of NADH oxidation in frozen and thawed mitochondria to the rate in untreated mitochondria from 28 to 5 after zonal centrifugation was due to dilution in the gradient and to the time spent at that dilution after centrifugation.

It was also necessary to show that the differences obtained did not result from microsomal contamination. The presence of microsomal material was estimated by determining RNA and the activities of the microsomal enzymes NADPH-cytochrome c reductase and glucose 6-phosphatase. The assays were conducted on different zonal fractions and, for the purpose of comparison, on mitochondrial and

microsomal pellets obtained by differential centrifugation. Analysis of the mitochondrial and microsomal pellets showed the following: for RNA, 15 μ g of RNA/mg of protein in the mitochondrial pellet and 165 μ g of RNA/mg of protein in the microsomal pellet; for NADPH-cytochrome *c* reductase, 4.2 m μ mol of NADPH oxidized/min per mg of mitochondrial protein and 57.9 m μ mol of NADPH oxidized/min per mg of microsomal protein; and for glucose 6-phosphatase, 0.54 μ mol of P_i released/

Fig. 5. Specific activity of NADH dehydrogenase in mitochondrial fractions obtained by rate zonal centrifugation

The assay procedure is described in the Materials and Methods section. The results of three separate experiments are presented (○, Δ, □), and fractions are numbered in order of decreasing density.

Fig. 6. Specific activity of succinate dehydrogenase in mitochondrial fractions obtained by rate zonal centrifugation

For details see Fig. 5.

20 min per mg of mitochondrial protein and 3.2 μ mol of P_i released/20 min per mg of microsomal protein. The results of analysis of the different zonal fractions

Fig. 7. Specific activity of α -glycerophosphate dehydrogenase in mitochondrial fractions obtained by rate zonal centrifugation

For details see Fig. 5.

Fig. 8. Specific activities of NADH dehydrogenase, succinate dehydrogenase and α -glycerophosphate dehydrogenase expressed as % of highest specific activity

The specific activities from the three experiments were averaged for each fraction and the % of highest specific activity determined. The fractions are numbered in order of decreasing density. NADH dehydrogenase (□); succinate dehydrogenase (Δ); α -glycerophosphate dehydrogenase (○).

Fig. 9. Contamination of mitochondrial fractions from rate zonal centrifugation by microsomal material as estimated by distribution of specific activities of NADPH–cytochrome *c* reductase and glucose 6-phosphatase and RNA/mg of protein

The assay procedures are described in the Materials and Methods section. The fractions are numbered in order of decreasing density. NADPH–cytochrome *c* reductase (□); glucose 6-phosphatase (Δ); RNA (○).

Table 2. Reduction of cytochromes of mitochondria and microsomal material with succinate and dithionite

The mitochondria and microsomal material were obtained by differential centrifugation and the cytochrome concentrations were determined as described in the Materials and Methods section. Succinate was first added to reduce the cytochromes and the spectrum was recorded. Dithionite was then added to the same cuvette and the spectrum was re-recorded. The final concentration of dithionite was 15mM.

	Protein (mg/ml)	Cytochrome	Succinate		Dithionite	
			(nmol/ml)	<i>b/a</i>	(nmol/ml)	<i>b/a</i>
I Microsomal material	3.92	<i>b</i> ₅	—	—	1.48	—
II Mitochondria	5.1	<i>a</i> + <i>a</i> ₃	0.94	0.52	0.94	0.96
		<i>b</i>	0.49		0.90	
		<i>c</i> + <i>c</i> ₁	1.02		1.44	
III Mitochondria + micro- somal material	5.1 0.98	<i>a</i> + <i>a</i> ₃	1.00	0.51	1.00	1.31
		<i>b</i>	0.51		1.31	
		<i>c</i> + <i>c</i> ₁	1.03		1.77	

are shown in Fig. 9. Compared with the values given above for mitochondrial and microsomal pellets, the specific activities of NADPH–cytochrome *c* reductase and glucose 6-phosphatase and RNA/mg of protein were very low in fractions 6–24. Between fraction 24 and fractions 30–32, the activities increased five- to nine-fold. These results indicated that microsomal material contaminating the mitochondrial pellet was concentrated by zonal centrifugation in the region of the gradient of lowest density. Since cytochrome analyses were conducted on fractions 7–25 only, and enzyme analyses only on fractions 6–26, microsomal

contamination cannot explain the mitochondrial differences found.

In addition, an experiment was undertaken to determine if microsomal cytochrome *b*₅ could alter the mitochondrial cytochrome *b* concentrations obtained by reduction with succinate. This was considered important since fraction 25 was included in the cytochrome determinations and this fraction was in the region of the gradient of increasing microsomal contamination (Fig. 9). Mitochondria and microsomal material were obtained by differential centrifugation. Reduction of the cytochromes by

succinate and dithionite was compared on the microsomal preparation, on mitochondria and on mitochondria plus microsomal material (Table 2). Addition of succinate to microsomal material alone did not reduce cytochrome b_5 . Similarly, addition of succinate to a cuvette containing mitochondria plus microsomal material resulted in the same cytochrome concentrations and cytochrome b /cytochrome $a+a_3$ ratio as obtained with mitochondria alone. Dithionite did reduce cytochrome b_5 and demonstrated that cytochrome b_5 was present in detectable amounts in all three preparations. Reduction with dithionite also resulted in increased cytochrome $c+c_1$ concentrations, most likely caused by interference from cytochrome b_5 . These results demonstrated that microsomal material did not influence the determinations of the cytochromes obtained by reduction with succinate and that the change in cytochrome b /cytochrome $a+a_3$ ratios between fractions 7 and 25 did not result from interference from microsomal cytochrome b_5 .

Discussion

Several attempts have been made to separate isolated liver mitochondria into populations that differ chemically, enzymically or morphologically. These attempts have followed two basic approaches: separation of mitochondria on the basis of differences in density and separation of mitochondria on the basis of differences in sedimentation coefficient.

Kuff & Schneider (1954), employing a discontinuous sucrose gradient, separated mitochondria on the basis of density into six fractions which differed in activity of succinate dehydrogenase/mg of nitrogen. Isopycnic density centrifugation on a sucrose gradient by Pollak & Munn (1970) resulted in the separation of two distinct bands of intact mitochondria. No chemical or enzymic differences were found between the two bands and it was concluded that their dissimilar densities resulted from a difference in the permeability of their inner membrane to sucrose.

Both differential and rate zonal centrifugation have been used to separate mitochondria on the basis of differences in sedimentation coefficient. Laird *et al.* (1952) separated mitochondria by differential centrifugation into two fractions that had different ratios of succinoxidase activity relative to protein nitrogen. Electron microscopy showed the mitochondria of the light fraction to be about one half the length and width of those of the heavy fraction. Gear (1965b) also isolated rat liver heavy and light mitochondrial fractions differentially and showed differences in their iron and cytochrome a contents and cytochrome oxidase activity. In another use of differential centrifugation, Lusena & Depocas (1966) showed that mitochondria isolated from rat liver could be

separated into five fractions which were distinguishable on the basis of their fragility. Mitochondria separated by rate zonal centrifugation have also been reported to be heterogeneous. Swick *et al.* (1967) showed that there was a significant separation of the midpoints of the distributions of many mitochondrial enzymes on a sucrose gradient. Schuel *et al.* (1969) observed two apparent populations of liver mitochondria on a sucrose gradient, as indicated by the distribution of cytochrome oxidase.

These reports may be criticized, for it was not clearly demonstrated that the observed heterogeneities were not artifacts of the centrifugation procedures. Separation of mitochondria into different fractions by differential centrifugation may simply reflect varying degrees of damage to the mitochondria. In isopycnic density centrifugation sucrose is commonly used to form the gradients. High sucrose concentrations place an osmotic stress on the mitochondria and separation into different populations on a sucrose gradient has been attributed to the hyperosmolarity of the gradient rather than to heterogeneous mitochondria (Beaufay *et al.*, 1964). The sucrose concentrations used in the gradients of rate zonal centrifugation were lower than those of isopycnic density centrifugation but were still hyperosmotic. Therefore, this leaves the results of rate zonal centrifugation also in question.

In light of the criticism of previous reports, special care was taken in this investigation to ensure that the mitochondria were not altered or damaged. To maintain the mitochondria in their physiological state, the gradient consisted of iso-osmotic sucrose with Ficoll added to increase the density. Ficoll is a synthetic polymer of sucrose with negligible osmotic effects since it has a molecular weight of approx. 400000. An experiment was also conducted to see if the observed heterogeneity could be the result of damaged mitochondria. The rate of oxidation of exogenous NADH was used as a measure of the intactness of mitochondria in fractions throughout the gradient. Similar results for all fractions tested indicated that the mitochondria had not been separated on the gradient according to varying degrees of mitochondrial damage. Therefore, the cytochrome and enzymic differences obtained could not be attributed to damaged mitochondria.

The mitochondrial differences may reflect the fact that the liver is not a completely homogeneous tissue; mitochondria in the non-parenchymal cells could be different from those in the parenchymal cells. However, this is considered unlikely, for although only 60–70% of the cells of the liver are parenchymal cells, they constitute approx. 95% of the total cell mass (Harkness, 1957; Bucher, 1963).

Experiments were also done to demonstrate that the heterogeneity obtained did not result from microsomal contamination. Distribution in the gradi-

ent of RNA, glucose 6-phosphatase and NADPH-cytochrome *c* reductase showed that any microsomal contaminants were concentrated in the least-dense region of the gradient, between fractions 25 and 32. Microsomal contamination could therefore not account for the results of the enzyme and cytochrome analyses, which were done on fractions 6–26 and fractions 7–25 respectively. However, the presence of microsomal material in fractions 25–32 probably explains the decreases in the specific activities of the mitochondrial enzymes in fraction 26 and the specific concentrations of the cytochromes in fraction 25. An additional experiment showed that in the analysis of cytochromes reduced by succinate, microsomal cytochrome *b*_s did not interfere with the determination of mitochondrial cytochrome *b*. Even slight microsomal contamination, as in fraction 25, could therefore not explain the differences obtained in cytochrome *b* concentration.

Separation of mitochondria results from differences in sedimentation coefficient. Since the densities of liver mitochondria are centred in a narrow region around 1.19 g/cm³ (Wattiaux & Wattiaux-De Coninck, 1970), separation should be largely determined by size. The shape of the protein curve obtained in this investigation (Fig. 1) should therefore represent the distribution of mitochondrial sizes. This would be consistent with the results of Gregson & Williams (1969) who determined mitochondrial size from electron micrographs and found that the diameters of liver mitochondria from adult rats had a normal distribution.

NADH dehydrogenase, succinate dehydrogenase and α -glycerophosphate dehydrogenase were selected for this investigation because histochemical studies have shown them to have different patterns of distribution in the liver lobule. The results presented here show that these enzymes have different distributions in mitochondria which have been separated mainly on the basis of size by rate zonal centrifugation. There is no obvious correlation between the heterogeneity reported here and the mitochondrial differences observed histochemically. This would require at the very least a thorough analysis of size in this preparation. In addition a more extensive analysis than has been done to date would be necessary to determine distributions of mitochondrial size in various regions of the liver lobule.

The concentrations of the cytochromes of different fractions were determined even though there is a general assumption that all mitochondria have the same cytochrome complement. The histochemical demonstration of enzymatic heterogeneity led us to question whether liver mitochondria might also differ in their cytochrome concentrations. To measure only the cytochromes involved in the electron transfer chain, cytochromes were reduced by succinate for spectral analysis. The results showed mitochondria

to be homogeneous in the content of cytochrome *a*+*a*₃ and cytochrome *c*+*c*₁ but to have different concentrations of cytochrome *b*.

A primary reason for this investigation was to develop a system for approaching the problem of mitochondrial biogenesis from the point of view of biochemically heterogeneous mitochondria. Previous studies on the biogenesis of mitochondria have not taken into account the heterogeneity of liver mitochondria. For example, treatment with thyroid hormone has been reported to cause synthesis of new mitochondria and mitochondrial proteins. Gross (1971) found that mitochondrial DNA (labelled before the administration of thyroid hormone) turned over after the start of thyroid hormone administration more rapidly than newly synthesized DNA. The different turnover rates suggested that the pre-existing population of mitochondria was being replaced by a new population synthesized under different physiological conditions. From electron micrographs of hyperthyroid animals, Kadenbach (1966*a*) observed an apparent increase in the volume of liver mitochondria and the number of cristae per mitochondrion. The mitochondria resulting from thyroid hormone treatment have also been shown to be qualitatively different. The concentrations of cytochromes *a*, *b* and *c*/mg of protein were found to increase 1.5 to 2 times (Roodyn *et al.*, 1965; Kadenbach, 1966*b*) and the specific activities of α -glycerophosphate dehydrogenase and succinate dehydrogenase were reported to increase 20-fold and 2-fold respectively (Lee & Lardy, 1965). All these effects of thyroid hormone were obtained by analysing liver mitochondria as a single population.

The results presented in this investigation demonstrate that liver mitochondria are heterogeneous with regard to cytochrome *b*, succinate dehydrogenase and α -glycerophosphate dehydrogenase. Because of this, it cannot be assumed that all mitochondria will respond to thyroid hormone in an identical manner. Therefore, it seems that the study of mitochondrial biogenesis in tissues of multicellular organisms requires analysis in terms of the biochemical heterogeneity of mitochondria.

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