The Isolation by Isopycnic Density-Gradient Centrifugation of Two Mitochondrial Populations from Livers of Embryonic and Fed and Starved Adult Rats

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(Received 7 January 1970)

1. Mitochondria isolated from livers of fed adult, starved adult, and embryonic rats can be separated into three distinct bands by isopycnic density centrifugation on a sucrose density gradient. The least dense band (B1) has a mean buoyant density of 1.162 and consists mainly of disrupted mitochondria. The middle band (B2) has a mean buoyant density of 1.184. The most dense band (B3) has a mean buoyant density of 1.216. B2 and B3 consist of intact mitochondria. 2. The mitochondria in B2 and B3 have very similar protein/phospholipid ratios, virtually identical phospholipid and fatty acid compositions and similar specific activities for cytochrome oxidase, malate dehydrogenase and monoamine oxidase. Both fractions have very low glucose 6-phosphatase and acid phosphatase activities. 3. As isolated, adult rat liver mitochondria have electron-dense matrices (condensed forms); some embryonic liver mitochondria are condensed, but a significant proportion have dilated matrices. All B2 mitochondria are in the condensed form. B3 mitochondria from adult rats are condensed if fixed in their equilibrium-density sucrose, but when this is diluted rapidly to 0.25M they become swollen. Some B3 mitochondria from embryonic rats are condensed, the others have dilated matrices. They all swell if rapidly diluted to 0.25M-sucrose. B2 mitochondria retain their condensed form on dilution of the sucrose. 4. It is concluded that the matrix space of B2 mitochondria is almost totally inaccessible to sucrose, but that of B3 mitochondria is readily accessible to sucrose. 5. In liver from normally fed adult rats the B2 mitochondria predominate, whereas in starved rats B2 and B3 are present in approximately equal proportions. Mitochondrial preparations from embryonic liver consist predominantly of B3 mitochondria, but the proportion of these decreases progressively as development proceeds. 6. The B2 mitochondria from livers of fed adult rats can be converted into B3 mitochondria by incubation with 10mM-succinate and 10mM-phosphate. 7. Some B2 mitochondria are converted into B3 mitochondria by exposure to high concentrations of sucrose.

It is well known that mitochondria from different tissues differ in characteristic ways both with respect to details of structure (shape, size, numbers and organization of cristae) and functional properties. Mitochondria in any one tissue may also show changes in their structure and enzymic properties in response to alterations in physiological conditions. Thus Wilson & Leduc (1963) showed that the ultrastructure of mitochondria in the liver of normally fed mice was altered by essential fatty acid deficiency or starvation. Similarly, enzymic differences have been observed between mito-

chondria of embryonic and adult rodent liver and progressive morphological changes have been demonstrated in chicken liver mitochondria at different morphogenetic stages (Kafer & Pollak, 1961; Shorey & Pollak, 1967; Mintz *et al.* 1967). Jasper & Bronk (1968) showed that the ultrastructure of the mitochondria in rat intestinal mucosa incubated *in vitro* showed marked changes related to the presence of amino acids in the incubation media. Mitochondria isolated from tissues under various physiological conditions have been shown to differ with respect to biochemical properties (Kafer & Pollak, 1961; Mintz *et al.* 1967) and buoyant density in sucrose density gradients (Goldhor, 1968).

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It has also been shown by means of histochemical techniques and electron microscopy that mitochondria differing enzymically and morphologically may be present in one tissue simultaneously (e.g. Novikoff & Shin, 1964; Pette & Brandau, 1966). Several attempts have been made to separate mitochondria isolated from such tissues into populations that differ chemically, enzymically or morphologically. Lusena & Depocas (1966) showed that mitochondria isolated from rat liver could be separated by differential centrifugation into five fractions which could be distinguished on the basis of their fragility. Gear (1965a,b) isolated 'heavy' and 'light' rat liver mitochondrial fractions by differential centrifugation and showed differences in their iron and cytochrome *a* contents and cytochrome oxidase activity. Pette & Brdiczka (1966), using differential and density-gradient centrifugation, also obtained two types of mitochondria from rat liver. The two types differed with respect to the activities of certain enzymes and also in their electrophoretic mobilities. Matile & Bahr (1968) separated mitochondria isolated from yeast into two bands by centrifugation on Urografin gradients; the mitochondria in the bands were thought to represent different populations occurring in the yeast cell, since they differed with regard not only to density but also to enzymic composition. On the other hand Beaufay & Berthet (1963) and Beaufay *et al.* (1964) attributed the bimodal distribution of rat liver mitochondria on sucrose density gradients to an artifact due to exposure to high sucrose concentrations and consequent dehydration of the mitochondrial proteins. In the present study two mitochondrial types were separated by isopycnic centrifugation and it was concluded that they differed with respect to the permeability of their inner membranes to sucrose. Mitochondria from the livers of embryonic and starved and normal adult rats were compared since it was thought that this might provide some insight into the mechanism of formation of the two types and the possibility of heterogeneity of the mitochondria *in vivo*.

MATERIALS AND METHODS

Materials. Cytochrome *c* was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and NAD⁺ from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Other chemicals were of A.R. grade whenever possible.

Animals. Female Wistar rats weighing 150–200 g were used throughout the investigation. The rats were provided with food until they were killed for the experiment. When starved rats were required, food was withdrawn between 9 and 11 a.m. for the requisite time. Water was provided *ad libitum*.

Embryonic rats whose age was known within about 16 h were obtained by keeping one male rat overnight in a

cage with three female rats; the female rats were then kept separate until the embryonic livers were required. The number of successful matings was low.

Preparation of mitochondrial fractions. Mitochondrial fractions were prepared essentially as described by McMurray & Dawson (1969). The rats were killed by cervical fracture and the livers were rapidly excised and chilled in 0.25 M-sucrose–1 mM-EDTA at pH 7.4 and 0°C. All subsequent operations were performed at 0–2°C. Usually one liver was used per experiment; the liver was minced with scissors and homogenized in a Teflon-glass homogenizer (Wesley-Coe Glass Co., Cambridge, U.K.) using eight up and down strokes for the livers from adults and four up and down strokes for those from embryos. A 10% homogenate was prepared in 0.25 M-sucrose–1 mM-EDTA, and the homogenate was centrifuged at 650 *g*_{av} for 10 min. Unless otherwise stated all centrifugations were carried out in a Sorvall RC-2 centrifuge with an SS-34 rotor. The supernatant was centrifuged at 4500 *g*_{av} for 8 min, and the force was then increased to 10000 *g*_{av} for 2 min. The mitochondrial pellet was resuspended by hand homogenization in the sucrose–EDTA medium and was washed three times by sedimenting and resuspending the mitochondria as described in the last step. The three-times-washed mitochondrial pellet was resuspended in 0.25 M-sucrose to give mitochondrial suspensions which varied in their protein content between 10 and 30 mg/ml.

Isopycnic centrifugation and the collection of the resulting bands. Sucrose gradients containing 1 mM-EDTA, pH 7.4, and extending from 0.9 to 1.9 M-sucrose, were prepared in 2 in × $\frac{1}{4}$ in SW39 Spinco tubes (nitrocellulose) from 11 portions of 0.4 ml of sucrose solutions (containing EDTA) which ranged from 1.9 to 0.9 in 0.1 M intervals; discontinuities were smoothed out usually by letting the gradients stand overnight at 2°C, or in a few instances by centrifuging them for 45 min at 132000 *g*_{av} in an MSE superspeed 65 centrifuge with a 59589 swing-out rotor (with the brake off). The distance from the bottom of the tube to the mid-point of each 0.4 ml of sucrose solution was noted and a curve relating distance to concentration of sucrose was plotted.

Mitochondrial suspensions in 0.25 M-sucrose were layered on top of such gradients and centrifuged for 90 min (total centrifugation time, with the brake off) at 132000 *g*_{av} in an MSE swing-out 59589 rotor. The buoyant densities of the resulting mitochondrial bands were determined from the curve relating distance from the bottom of the tube to density. The bands, which were easily visible, were separated by means of a tube-cutter (Randolph & Ryan, 1950) and the fractions were collected with Pasteur pipettes.

Analytical methods. Proteins were determined by a micromodification of the biuret method suitable for measuring 0.1–1.0 mg of protein (Pollak & Shorey, 1964). Phospholipids were extracted for 2 h at room temperature with 20 vol. of chloroform-methanol (2:1, v/v), and the extract was filtered. Then 0.2 vol. of 0.9% NaCl was added to the filtrate and the biphasic system was vigorously shaken and centrifuged to break the emulsion. The upper phase was discarded and the lower phase was washed three times by shaking and centrifuging, after adding each 0.2 vol. of chloroform-methanol–0.9% NaCl (3:48:47, by vol.). Suitable portions were evaporated to

dryness, and total phospholipid phosphorus was determined by the method of Chen, Toribara & Warner (1956). For the analysis of the individual phospholipids suitable portions of the chloroform-methanol extract were evaporated to dryness, treated with ethanolic NaOH, and the alcoholysate was subjected to paper chromatography followed by ionophoresis (Dawson, Hemington & Davenport, 1962). The areas on the paper containing water-soluble degradation products were detected with ninhydrin and phosphate spray reagents. The spots were cut out and digested with 72% HClO₄ and the P_i content was determined by the method of Bartlett (1959).

Enzyme assays. Cytochrome oxidase (EC 1.9.3.1) activity was determined by measuring the decrease in E_{550} of cytochrome *c* that had been previously reduced with NaBH₄ (Sottocasa, Kuylenskierna, Ernster & Bergstrand, 1967). The enzyme was assayed in a very dilute solution, such that the initial rate was linear with respect to enzyme concentration. Monoamine oxidase (EC 1.4.3.4) activity was determined with benzylamine as substrate by measuring the appearance of benzaldehyde by the increase in E_{250} (Tabor, Tabor & Rosenthal, 1954). Nonidet P40 (BDH Chemicals Ltd., Poole, Dorset, U.K.) was added to the enzyme suspension as described by McMurray & Dawson (1969). Malate dehydrogenase (EC 1.1.1.37) activity was determined by measuring the oxidation of 0.1 mM-NADH in the presence of 5 mM-oxaloacetate, 50 mM-tris-HCl buffer, pH 7.5, 0.015 M of 1% Nonidet P40 (total vol. 3 ml) at 340 nm. All the determinations of enzymic activities were carried out with a recording Unicam SP.800 spectrophotometer, fitted with a slave recorder with tenfold attenuation. Glucose 6-phosphatase (EC 3.1.3.9) was assayed by the method of Swanson (1955). Acid phosphatase (EC 3.1.3.2) assays were carried out essentially as those for glucose 6-phosphatase, but with 0.02 M-sodium β -glycerophosphate as substrate and 0.03 M-acetate buffer, pH 5, for the buffer system.

Fixation and electron microscopy. Mitochondria, whether resuspended in 0.25 M-sucrose or suspended in their isopycnic medium, were fixed with an equal volume of redistilled freshly diluted 4% glutaraldehyde in 0.25 M-sucrose containing 0.05 M-sodium phosphate buffer, pH 7.3, at 0°C for several hours. The samples were washed twice in 0.25 M-sucrose then placed in 2% osmium tetroxide solution in 0.05 M-sodium phosphate buffer, pH 7.3, for 2 h at 0°C, dehydrated in a graded series of acetone solutions and propylene oxide, and finally embedded in Araldite. Sections were cut on a Reichert OmU2 microtome, stained with lead acetate and examined in a Siemens Elmiskop I electron microscope.

RESULTS

Isopycnic fractionation of mitochondria. Suspensions of mitochondria, isolated from liver from normally fed, starved and embryonic rats and containing 2–10 mg of protein were layered on top of sucrose gradients. Isopycnic centrifugation produced a concentration of material in three distinct narrow bands designated B1, B2 and B3. The upper band (B1) from normal liver had a buoyant density of 1.162 and consisted mainly of broken

mitochondria and fragments of mitochondrial membranes (Plate 1a). The middle band (B2) from normal liver had a mean buoyant density of 1.184 and the B3 band had a mean buoyant density of 1.216. Both the B2 and B3 bands consisted of intact mitochondria, which when fixed in their equilibrium-density sucrose had electron-dense matrices (condensed forms) (Plate 1b and 1c). In embryonic rat liver the B1 band was barely detectable and, since electron microscopy and preliminary chemical and enzymic analyses established that fraction B1 of adults consisted predominantly of damaged and broken mitochondria, only the B2 and B3 fractions were studied further.

The buoyant densities of fractions B2 and B3 prepared from liver mitochondria from fed, starved and embryonic rats are presented in Table 1. The positions of bands B2 and B3 on the gradient were remarkably constant between experiments for each physiological state that was examined: the isopycnic equilibrium positions of bands B2 and B3 applied equally to mitochondria isolated from liver from fed, starved, neonatal and 2-day-prenatal rats, and was only slightly lower for 6- and 4-day-prenatal rats. The proportions of fractions B2 to B3 differed in each of the three physiological states examined, there being a preponderance of fraction B2 over fraction B3 in mitochondria from livers of fed adult rats, whereas in mitochondria from starved rats fractions B2 and B3 were present in approximately equal amounts (Table 1). Mitochondria isolated from embryonic rat livers showed a significant preponderance of the B3 type over B2, but the proportions altered progressively as development proceeded (Table 1).

Lipid content of fractions B2 and B3. Fractions B2 and B3 were virtually identical with respect to their protein/phospholipid ratios (Table 1) and their phospholipid composition (Table 2). They both contained the same small amount of neutral lipid, the fatty acids of which [obtained by the Dawson (1960) procedure] when analysed by g.l.c. were found to be almost identical, with respect to both the types and amounts of fatty acids present (P. Kemp & J. K. Pollak, unpublished work). Hence the difference in density between these two mitochondrial populations cannot be accounted for in terms of differences of protein and lipid content.

Enzymic activities of fractions B2 and B3. The original mitochondrial fraction and fractions B2 and B3 were assayed for three mitochondrial marker enzymes, cytochrome oxidase for inner membrane, malate dehydrogenase for matrix and monoamine oxidase for outer membrane. Fractions B2 and B3 were not significantly different from each other with respect to the specific activities of these enzymes, although for each the activity was higher in fraction B3 than in fraction B2 (Fig. 1).

Table 1. *Densities and protein/phospholipid ratios of B2 and B3 mitochondria obtained from rat liver at different physiological states*

For experimental details see the Materials and Methods section.					
	B2		B3		
Physiological state	Density	Protein/phospho- lipid ratio	Density	Protein/phospho- lipid ratio	B2/B3 protein ratio
Adult					
Fed	1.184	4.6	1.216	5.1	3.5
Starved for 2 days	1.180	4.4	1.216	4.4	1.2
Starved for 4 days	1.185	4.9	1.217	4.3	1.0
Embryonic					
6 days prenatal	1.172	—	1.200	—	0.1
4 days prenatal	1.176	—	1.209	—	0.3
2 days prenatal	1.182	4.0	1.215	4.3	0.7
1 day neonatal	1.183	3.3	1.215	3.7	2.6

Table 2. *Percentage distribution of the phospholipids from mitochondrial fractions B2 and B3*

For experimental detail see the Materials and Methods section.

Phospholipid	% of diacylphosphoglycerides	
	B2	B3
Phosphatidylcholine	40.0	41.6
Phosphatidylethanolamine	37.5	35.2
Phosphatidylserine	1.0	0.8
Phosphatidylinositol	7.5	7.8
Cardiolipin	12.7	13.4
Phosphatidic acid	1.3	1.2

The fact that fractions B2 and B3 showed somewhat lower specific activities than the original unfractionated mitochondria with respect to the two membrane-bound enzymes and a higher specific activity for the matrix enzyme is in agreement with the observations that fractions B2 and B3 contain fewer broken mitochondrial membranes than the original mitochondrial fraction (Plate 1a) and that these are accumulated in fraction B1. There was no significant difference in the cytochrome oxidase activity between fractions B2 and B3 obtained from embryonic rat liver mitochondria at any one development stage, though there was a slight increase in the cytochrome oxidase activity of fractions B2+B3 as development progressed.

Glucose 6-phosphatase and acid phosphatase are commonly used as marker enzymes for microsomes and lysosomes respectively. The original mitochondrial fractions had only low glucose 6-phosphatase and acid phosphatase activities (Fig. 1), their activities suggesting a 2–4% contamination with microsomes and lysosomes. The specific

Fig. 1. Enzymic activities of (a) cytochrome oxidase, (b) monoamine oxidase, (c) malate dehydrogenase, (d) glucose 6-phosphatase and (e) acid phosphatase in non-fractionated mitochondria from liver from fed adult rats and fractions B2 and B3 obtained therefrom expressed as a percentage of the original mitochondrial activity. □, Original mitochondria; ▨, fraction B2; ■, fraction B3; Abs. act., absolute activity of each enzyme in the non-fractionated mitochondria expressed as μmol of substrate transformed/min per mg of protein.

activities of these enzymes were even lower in fractions B2 and B3.

Structure of mitochondria. Mitochondria isolated from adult rats in 0.25M-sucrose (Plate 2a) are in the so-called condensed form (Hackenbrock, 1966). This structure was retained by the mitochondria in fractions B2 and B3 when they were fixed in their equilibrium-density sucrose (Plate 1b and 1c). The mitochondria were also fixed after they had been diluted with 12 vol. of 0.25M-sucrose, centrifuged at $10000g_{av}$ for 10min and resuspended in 0.25M-sucrose. The structure of the mitochondria from band B2 was unaltered by this procedure (Plate 3a), but the B3 mitochondria were now extensively swollen with diffuse dilated matrices (Plate 3b). When B3 mitochondria were suspended

EXPLANATION OF PLATE I

Electron micrographs of sections of liver mitochondria from adult rats fractionated by isopycnic density-gradient centrifugation and fixed in their equilibrium-density sucrose. (a) Fraction B1; (b) fraction B2; (c) fraction B3. The bar represents $0.5\mu\text{m}$.

EXPLANATION OF PLATE 2

Electron micrographs of sections of rat liver mitochondria isolated in 0.25M-sucrose-1mM-EDTA before fractionation by density-gradient centrifugation. Mitochondria were isolated from (a) fed adult, (b) starved adult and (c) embryonic rats. The bar represents 0.5 μ m.

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EXPLANATION OF PLATE 3

Electron micrographs of sections of rat liver mitochondria fractionated by isopycnic density-gradient centrifugation. (a) Fraction B2 and (b) fraction B3 from fed adult rats diluted rapidly with 0.25M-sucrose before fixation; compare with Plate 1(b) and 1(c) respectively. (c) Fraction B3 from embryonic rats fixed in equilibrium density sucrose. The bar represents 0.5 μ m.

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EXPLANATION OF PLATE 4

Electron micrographs of sections of adult rat liver mitochondria fractionated by isopycnic density-gradient centrifugation after induction of steady state 4. (a) Fraction B2; (b) fraction B3. The fractions were fixed in their equilibrium-density sucrose. The bar represents $0.5\mu\text{m}$.

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in their equilibrium-density sucrose solution and dialysed against 200 vol. of a sucrose solution the concentration of which was decreased from 1.6M to 0.25M in approx. 0.2M steps over 70min the great majority had the same condensed matrix structure as the B3 mitochondria that had been fixed while suspended in their isopycnic sucrose solution (Plate 1c). The apparent extinction at 550nm of the dialysed B3 mitochondria after sedimentation, resuspension and fixation was more than 80% of the extinction of the B3 mitochondria that had been fixed in their isopycnic sucrose solution and diluted to a comparable extent. The extinction of B3 mitochondria that had been diluted with 0.25M-sucrose, sedimented and resuspended in 0.25M-sucrose without the dialysis procedure had only 51% of the extinction of the B3 mitochondria fixed in their isopycnic sucrose solution.

As isolated, most of the embryonic liver mitochondria had condensed matrices, but a significant proportion had less condensed matrices (Plate 2c). The B2 mitochondria were all condensed, but the B3 bands obtained from embryonic mitochondria differed from the B3 bands isolated from adult rat liver in that, irrespective of the mode or time of fixation, they always contained a mixture of mitochondria with condensed and mitochondria with less electron-dense matrices (Plate 3c). Further, in these mitochondria the cristae were considerably better preserved than in the B3 mitochondria of adult rat liver, which had been subjected to rapid dilution in 0.25M-sucrose as described above (Plate 3b).

Stability of fractions B2 and B3 and effect of high sucrose concentration. The positions on the gradient of fractions B1, B2 and B3 obtained from liver mitochondria from adult rats did not alter whether the centrifugation time was 30, 60, 90, 120 or 150min. However, a minimum of 90min was required to obtain well-defined bands with negligible amounts of matter located between the bands. The amount of protein in fraction B3 increased by up to 15% at the expense of protein from fraction B2 during the longer centrifugation time.

When the isolated B2 fraction, after sedimentation and resuspension in 0.25M-sucrose, was layered again on an isopycnic gradient and centrifuged as before, 70% of the protein of the B2 fraction was recovered again in the buoyant density region of 1.184, whereas the remaining 30% now had a buoyant density of 1.216–1.220. On resuspending and recentrifuging the isolated B3 fraction, all of its protein was recovered in the gradient at a buoyant density of 1.202.

Mitochondria exposed for up to 90min to 1.6M-sucrose (density 1.216) at 0°C, and then fixed and resuspended in 0.25M-sucrose had the same iso-

pycnic properties as mitochondria that had been fixed after being suspended in 0.25M-sucrose for 90min (at 0°C) or mitochondria fixed at 0min (the boundaries of the bands were at densities of 1.186–1.226 and 1.194–1.230 for mitochondria exposed to 1.6 and 0.25M-sucrose respectively). Mitochondria isolated from adult liver and fixed with 2% glutaraldehyde did not segregate into distinct bands on isopycnic centrifugation, but gave rise to one broad band extending from the buoyant-density region 1.182–1.222. Glutaraldehyde-fixed mitochondria from embryonic rat liver, on the other hand, behave identically with unfixed mitochondria on an isopycnic gradient, giving rise to separate B1, B2 and B3 bands, the relative amounts of B2 and B3 depending on the developmental stage of the embryo (Table 1).

Conversion of B2 and B3 mitochondria by the addition of succinate and P_i . The original mitochondrial suspension or the isolated B2 fraction from normal rat liver (2–10mg of mitochondrial protein) were each incubated at 30°C for 15min with constant mixing in a medium containing 0.25M-sucrose, 10mM-sodium phosphate buffer, pH 7.0, 10mM-sodium succinate and 1mM-magnesium chloride in a total volume of 8ml. The mixtures were concentrated at the end of the incubation period by centrifuging for 10min at 10000g_{av}, and the resulting pellets were resuspended in 0.8ml of their own supernatant; on centrifugation in a continuous sucrose gradient containing 10mM-sodium phosphate and 10mM-sodium succinate, one main band was found with a buoyant density extending from 1.210 to 1.218 (mean value for fraction B3 1.216; see Table 1) with a small amount of material in the position of B2-type mitochondria. The B2-type mitochondria retained the condensed appearance (Plate 4a), but most of the B3-type mitochondria, fixed in their equilibrium sucrose, had the appearance of matrix (Plate 4b) characteristic of the so-called orthodox form (Hackenbrock, 1966, 1968). In controls that had been incubated in 0.25M-sucrose only the original mitochondrial suspension gave the usual B1, B2 and B3 bands, and the isolated B2 fraction gave a typical B2 band and a faint B3 band. Electron microscopy confirmed the conversion from the orthodox form (Plate 4b) into the swollen form (Plate 3b) typical of the B3 fraction on rapid dilution of the medium with 0.25M-sucrose. If the mitochondria were fixed with glutaraldehyde after incubation in the sucrose-phosphate-succinate mixture, and then placed on a continuous sucrose density gradient, the resulting bands were identical in buoyant density with those obtained from samples that had not been fixed before the isopycnic centrifugation.

Similar morphological changes were obtained by

isolating mitochondria in 0.25M-sucrose-1mM-EDTA, containing 10mM-sodium phosphate buffer, pH 7.0, and 10mM-sodium succinate. A liver from a normal adult rat was divided into approximately equal parts, one part was homogenized and the mitochondria subsequently isolated in the sucrose-EDTA-phosphate-succinate mixture, the other half being used as a control, and the mitochondria were isolated in 0.25M-sucrose-1mM-EDTA. On isopycnic centrifugation the sucrose-EDTA-phosphate-succinate-isolated mitochondria formed a band in the buoyant-density region 1.206-1.218. Identical results were obtained with sucrose-EDTA-phosphate-succinate-isolated mitochondria that had been fixed in glutaraldehyde before isopycnic centrifugation. The control mitochondria gave rise to the usual B2 and B3 bands when unfixed, and to a broad band extending from 1.182 to 1.222 when fixed.

DISCUSSION

The most reasonable explanation for the separation of the mitochondria into two populations, which, in the present study, differ only in respect of their buoyant densities is that there are two types of mitochondria present: one type having a matrix space that is normally totally inaccessible to sucrose, the other type having a matrix space readily accessible to sucrose. The buoyant density of the first type is the resultant of the densities of the proteins in the matrix, the inner and outer membranes, and the water in the matrix. The buoyant density of the second type is the resultant of that due to the matrix proteins and the membranes only.

The swelling of the matrix compartment of the second type (B3) when the concentration of the sucrose is rapidly decreased from 1.6 to 0.25M is in agreement with this interpretation. We conclude that the flow of water into the matrix space is more rapid than the flow of sucrose out, and this is confirmed by slowly equilibrating the mitochondria from the gradient (in 1.6M-sucrose) with 0.25M-sucrose by dialysis against intermediate concentrations of sucrose.

Beaufay & Berthet (1963) have separated rat liver mitochondria into two bands with equilibrium densities of approx. 1.19 and 1.22 by density-gradient centrifugation when the organelles were initially distributed homogeneously throughout the gradient. They found, however, that a single band with a mean density of 1.19 was obtained when the mitochondria were layered on top of the gradient. They equated the formation of the band with a density of 1.22 with an abnormal dense state, which they attributed to partial dehydration of the mitochondrial proteins due to exposure to high

concentrations of sucrose. Beaufay *et al.* (1964) showed that the critical concentration of sucrose was 37×10^{-3} mol fraction (density 1.195). In our experiments the mean buoyant density of the B2 mitochondria was 1.180 for adult rats and 1.172 to 1.183 for pre- and neo-natal rats (Table 1). The mitochondria isolated from the livers of prenatal rats have the highest proportion of B3 mitochondria. When mitochondria isolated from prenatal rats in 0.25M-sucrose were fixed before density-gradient centrifugation they behaved identically with mitochondria that had not been fixed before centrifugation, indicating that in this case the formation of the B3-type mitochondria did not result from exposure of the mitochondria to high concentrations of sucrose. The evidence for the mitochondria from adult rats is not so clear-cut, since the fixed mitochondria did not give distinct B2 and B3 bands, but a broad band extending over the range of the buoyant densities of the B2 and B3 mitochondria. However, when the original mitochondria were exposed to 1.6M-sucrose (density 1.216) for up to 90 min and then fixed and resuspended in 0.25M-sucrose, they had the same isopycnic properties as mitochondria fixed in 0.25M-sucrose directly.

This could be explained if some of the mitochondria in the original preparation have inner membranes with limited permeability to sucrose, and that under the influence of high sucrose concentrations they are rendered totally permeable to sucrose so that they are distributed between the positions of the B2 and B3 mitochondria when fixed before centrifugation but end up in the B3 band when non-fixed preparations are used.

For mitochondria from fed adult rats, at least, it appears that the permeability of the inner membrane of those mitochondria partially or completely impermeable to sucrose can thus be increased in three ways: (a) slightly, by incubation in 0.25M-sucrose at room temperature, (b) substantially, by incubation in 0.25M-sucrose containing 10mM-succinate and phosphate, and (c) by treatment with higher sucrose concentrations at 0°C; in this instance the increase in permeability is determined by the duration of exposure to the sucrose and its concentration. The act of centrifugation of the mitochondria through a sucrose gradient thus alters the permeability of the inner membrane of some potential B2-type mitochondria so that they become B3-type. This is in partial agreement with the conclusions of Beaufay & Berthet (1963) and Beaufay *et al.* (1964).

Leighton *et al.* (1968) examined adult rat liver mitochondria that were unimodally distributed by isopycnic density-gradient centrifugation about a peak at a density of 1.180 and fixed in their equilibrium sucrose by addition of glutaraldehyde. Over 80% of the mitochondria at a density of 1.180 were

in the condensed form, whereas less than 50% of the mitochondria equilibrating at a density of 1.211 were condensed, and most were swollen. In our experiments the mitochondria (from adult rats) equilibrating at a density of 1.21 (B3 mitochondria) were in the condensed form and indistinguishable from the B2 mitochondria (density 1.18).

The situation is somewhat more complex for mitochondria from embryonic rat liver, as these vary in at least two respects from adult rat liver mitochondria. First, a considerably greater proportion of embryonic rat liver mitochondria than adult rat liver mitochondria equilibrates in the B3 region. This is particularly true for the early stages of morphogenesis (Table 1). Secondly, a considerable proportion of mitochondria from embryonic liver are not in the condensed form but resemble rather more the form seen *in situ*, the orthodox form described by Hackenbrock (1966, 1968). Hackenbrock demonstrated that mitochondria isolated from mouse liver in 0.25M-sucrose were in the condensed form and that this changed to the orthodox form under steady state 4 (Chance & Williams, 1955) conditions. Under the conditions of incubation used by Hackenbrock (1968) for induction of steady state 4 (10mM-succinate, 10mM-phosphate and 1mM-magnesium chloride) liver mitochondria from normal adult rats are transformed from primarily B2-type mitochondria into B3-type mitochondria and this raises the possibility that the permeability of the inner membrane to other low-molecular-weight solutes including metabolites may vary with the metabolic state of the mitochondria.

It appears that the impermeability of the inner membrane to sucrose, which is a feature of mitochondria from the livers of fed adult rats, is developed progressively by mitochondria in embryos. The structure of the mitochondria in early embryonic livers fixed *in situ* differs slightly from that of adults and, as we have noted, some of the mitochondria isolated from embryonic livers differ in appearance from those from livers of adult rats. The appearance of the mitochondria is not necessarily a guide to their behaviour on the density gradients since some of them have a matrix space accessible to sucrose irrespective of their structural or metabolic state, but those mitochondria (either from embryos, or from adults and in steady state 4) that have diffuse matrices (and resemble somewhat in this respect mitochondria *in situ*) accumulate in fraction B3. At the present time no direct physiological significance can be attributed to the differences in the mitochondria from rats in different physiological states.

We are grateful to Miss A. Currell and Mr N. Clarke for their skilled technical assistance. The Siemens electron microscope used in this work was generously provided by the Wellcome Trust. J.K.P. is further indebted to the Wellcome Trust for a travel grant.

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