Changes in the Proportions of Two Mitochondrial Populations during the Development of Embryonic Chick Liver

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1. Two populations of morphologically intact mitochondria were isolated from embryonic, neonatal and adult chick liver by isopycnic centrifugation. 2. The protein/phospholipid ratio of the total mitochondrial fraction, the low-density mitochondria (B2, d 1.176) and the high-density mitochondria (B3, d 1.206) did not differ significantly. 3. During development there is a marked increase in the B2 fraction in relation to the B3 fraction. 4. Cytochrome oxidase and malate dehydrogenase activities as well as respiratory control increased during the embryonic development of the chick, though their rates of increase were not correlated. 5. In the three different embryonic stages that were investigated, as well as in the neonatal and adult chick, the protein/lipid as well as the protein/phospholipid ratio stayed constant and showed no progressive increase, as had been previously reported. 6. It was shown that forces greater than $18400g_{av}$ for 2 h have to be used before chick liver mitochondria reach isopycnic equilibrium. 7. As for rat liver mitochondria, the constant protein/phospholipid ratio of the B2 and B3 fractions and their apparent morphological intactness leads one to conclude that the matrix space of B2 mitochondria is inaccessible to sucrose, whereas B3 mitochondria possess an inner membrane that is permeable to sucrose.

In the study by Pollak & Munn (1970) rat liver mitochondria were separated into two populations by isopycnic centrifugation. Evidence was provided that the mitochondrial population equilibrating in the denser portion of the isopycnic sucrose gradient did not differ significantly in its protein/phospholipid ratio or neutral-lipid content from the less-dense mitochondria. Equilibration in the denser part of the sucrose gradient was presumed to be due to a greater permeability of the inner mitochondrial membrane to low-molecular-weight compounds such as sucrose.

It was also shown that the distribution of mitochondria between the two different equilibrium densities varied in different physiological states. In preparations from normally fed rats, the hepatic mitochondria equilibrating in the lower-density region (d 1.1816) were considerably in excess of the higher-density mitochondria (d 1.216). On the other hand a greater proportion of mitochondria isolated from the livers of starved rats and from embryonic rats equilibrated in the denser region of the sucrose gradient. During embryonic development from 16 to 20 days the ratio of low-density/high-density mitochondria changed from 0.1 to 0.7, whereas in neonatal (1-day-old) rats the ratio increased to 2.6.

While this work was still in progress a brief report was published (Goldhor, 1968) in which it was suggested that during the development of embryonic chick liver the mitochondrial protein/lipid ratio increased with a concomitant increase in mitochondrial density. The implication of these results was that during development the mitochondria accumulate protein.

Such a conclusion, although contrary to the findings with embryonic rat liver (Pollak & Munn, 1970), was in accord with the ultrastructural findings on embryonic chick liver (Pollak & Shorey, 1967). The hepatic mitochondria from chick embryos at the later stages of development were considerably more electron dense than were those from earlier developmental stages. In view of these results the work on the properties of embryonic chick liver mitochondria was repeated and extended.

The results of this investigation confirmed those obtained with rat liver mitochondria, namely that there was no significant change in the protein/lipid ratio during development, and that there was no or only slight change in the buoyant density of mitochondria throughout development. The proportion of mitochondria found in the lower-buoyant-density region increased throughout development. Other parameters, such as enzyme

activities, also changed progressively during development.

MATERIALS AND METHODS

Chemicals. Cytochrome *c*, oxaloacetic acid and cholesterol were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and synthetic L- α -phosphatidylcholine from Fluka A.G., (Buchs, Switzerland). Other chemicals were A.R. grade whenever obtainable.

Animals. Fertile eggs were obtained from the N.S.W. Government Experimental Station, Seven Hills. The eggs were derived from an inbred E-type broiler strain. The eggs were incubated at $38 \pm 0.5^\circ\text{C}$ in a Gamble incubator. When required the chick embryos were dissected out and their developmental age was spot-checked occasionally with Hamburger stages as described by Lillie (1952). As there was good agreement between the known times of incubation and the corresponding Hamburger stages, the ages of the chick embryos were equated with the duration of the incubation as a routine.

Preparation of mitochondrial fractions. Mitochondrial fractions were prepared essentially as described by Pollak & Munn (1970). After being dissected out of the egg, the embryonic chicks were dropped into ice-cold 0.25M-sucrose-1mM-EDTA (pH 6.8). The livers were then dissected out and placed into ice-cold 0.25M-sucrose-1mM-EDTA. All subsequent operations were performed at 2°C . In most experiments the centrifugations for the preparation of mitochondria were carried out in a Beckman-Spinco L2 65 centrifuge with a 50 rotor; in some of the later experiments the mitochondria were prepared in an International PR2 centrifuge with the high-speed attachment and a 296 rotor with adaptors for 10ml tubes. The average angular velocities used were the same as described previously (Pollak & Munn, 1970). Yields and quality of the isolated mitochondria did not differ significantly with either of these centrifuges.

Isopycnic centrifugation and the collection of the resulting bands. Sucrose gradients containing 1mM-EDTA and extending from 0.9 to 1.9M were prepared as previously described (Pollak & Munn, 1970); in some experiments a gradient maker based on the design of Britten & Roberts (1960) was employed to make the gradients. Mitochondrial suspensions (0.2–0.5 ml) in 0.25M-sucrose were carefully layered on top of the gradients and centrifuged for 90 min (including time allowed for acceleration to maximal speed) at $130\,000g_{av.}$. The rotor was then allowed to slow down with the brake off. For the centrifugations a SW50L swinging-bucket rotor was used in a Beckman-Spinco L2-65 centrifuge fitted with a stabilizer. The densities at various levels of the tube were checked by means of a refractometer and standard tables relating refractive index to density. The isopycnic mitochondrial bands were collected by means of a tube-cutter as previously described (Pollak & Munn, 1970).

Analytical methods. Proteins were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Phospholipids were extracted and determined as described previously (Pollak & Munn, 1970). The total lipid content of mitochondrial suspensions was determined by a micro-modification of the method of Bloor (1928) devised by Professor K. W. Cleland (personal communication).

The lipid sample in a chloroform-methanol (2:1, v/v) extract was evaporated to complete dryness in a test tube (6in $\times$ 1in) and 1ml of silver reagent (Bloor, 1928) and 0.6ml of $\text{M-K}_2\text{Cr}_2\text{O}_7$ were added. The tube was covered with close-fitting aluminium foil, which was held in place by a rubber band, and heated in a water bath at $90\text{--}92^\circ\text{C}$ for 90 min, the mixture being swirled around several times during the incubation. The contents of the tube were transferred quantitatively to an Erlenmeyer flask, the test tube then being washed with 3×5 ml of water. After addition of 2 ml of 10% (w/v) KI the sample was back-titrated with 0.1M- $\text{Na}_2\text{S}_2\text{O}_3$ with soluble starch as an indicator during the later stages of the titration. Standard samples of cholesterol gave 90–95% recovery and synthetic phosphatidylcholine gave better than 85% recovery. A total lipid-equivalent value was compiled on the assumption that mitochondrial lipid consisted of approximately 13% cardiolipin, 41% phosphatidylcholine, 37% phosphatidylethanolamine and 6% cholesterol (Pollak & Munn, 1970; Ashworth & Green, 1966), and it was assumed that the constituent fatty acids had an average chain length of 17.5 carbon atoms.

Enzyme assays. Cytochrome oxidase (EC 1.9.3.1) and malate dehydrogenase (EC 1.1.1.37) activities were determined as described previously (Pollak & Munn, 1970). Respiratory control was determined by means of a Rank (Rank Bros., Bottisham, Cambs., U.K.) oxygen electrode, by using a volume of 2ml, containing 25mM-tris-HCl buffer, pH 7.4, 5mM-sodium phosphate buffer, pH 7.4, 5mM- MgCl_2 , 50mM-KCl and adding $10\mu\text{mol}$ of sodium succinate and 100–500nmol of ADP as necessary to measure state III and state IV respiration and respiratory control (Chance & Williams, 1956).

Fixation and electron microscopy. Mitochondria were fixed, dehydrated and embedded essentially as described by Pollak & Munn (1970). Sections were cut on an LKB ultramicrotome, stained with uranyl acetate and examined in a Hitachi 7B electron microscope.

RESULTS

Separation of two mitochondrial populations by isopycnic centrifugation. Hepatic mitochondria were isolated from embryonic chicks at different developmental stages and also from neonatal and young adult chicks. As experience had shown that mitochondria from the embryonic stages investigated (7.5–17 days) were particularly prone to be damaged by the usual homogenization procedure, with a high-torque motor revolving at 1400 rev./min, livers from these developmental stages were hand-homogenized to prepare cell-free homogenates.

When mitochondria were layered on sucrose density gradients and centrifuged, the mitochondria equilibrated in at least two isopycnic regions corresponding to the B2 (d 1.176) and B3 (d 1.209) regions of embryonic rat liver (Pollak & Munn, 1970). The chick liver mitochondria differed from rat liver mitochondria in not giving rise to a B1 band (d 1.162), but occasionally they formed a B0 band (d 1.116) close to the top of the gradient. Both

Table 1. *Protein/phospholipid ratios and densities of total mitochondrial fractions and B2 and B3 fractions isolated from different developmental stages of chick liver*

For experimental details see the Materials and Methods section. Results represent mean values $\pm$ s.e.m. Numbers in parentheses indicate numbers of experiments.

Developmental stage of chick liver	Total mitochondria		B2		B3		B2/B3	
	Protein	ratio	Protein	ratio	Protein	ratio		
	phospholipid		phospholipid		phospholipid			
				<i>d</i>		<i>d</i>		
Embryonic								
7.5–9 days	3.4 ± 0.33	(8)	3.3 ± 0.41	(8)	1.171	3.3 ± 0.68	(8)	0.48 ± 0.059 (8)
9.5–11 days	4.0 ± 0.25	(6)	4.0 ± 0.41	(4)	1.175	4.2 ± 0.02	(4)	0.68 ± 0.081 (4)
14.5–15 days	4.0 ± 0.22	(4)	3.9 ± 0.04	(4)	1.173	3.9 ± 0.01	(4)	0.48 ± 0.60 (4)
17 days	3.6 ± 0.04	(8)	4.3 ± 0.28	(8)	1.176	4.3 ± 0.34	(8)	13.6 ± 2.68 (8)
Neonatal								
1–14 days	4.2 ± 0.17	(6)	4.6 ± 0.06	(4)	1.176	4.4 ± 0.23	(4)	4.7 ± 0.22 (4)
Adult								
9 weeks old or older	4.7 ± 0.38	(6)	4.2 ± 0.38	(4)	1.184	4.1 ± 0.36	(4)	6.7 ± 1.38 (4)

the rat liver mitochondrial fraction equilibrating in the B1 region and the chick liver mitochondrial fraction equilibrating in the B0 region consisted mainly of broken mitochondria and pieces of membranous material and hence were not further investigated. Occasionally a very faint high-density band B4 (d 1.220) was also observed, but in only three instances was there enough material to determine the protein/phospholipid ratio of band B4, and it was then found that it equalled the protein/phospholipid ratio of bands B2 and B3. Band B4 always made up less than 5% of the band B2 protein content.

The densities of B2 and B3 mitochondria at various development ages are presented in Table 1 together with protein/phospholipid ratios and the proportion of mitochondria found in the B2 and B3 regions at different developmental ages of the chick.

The results shown in Table 1 demonstrate that the densities of B2 and B3 mitochondria vary only slightly during development. The trend for this relatively small increase in the isopycnic density of both B2 and B3 mitochondria with developmental age is similar to that observed before in embryonic rat liver (Pollak & Munn, 1970). During the development from 7.5 days to 17 days there is a significant change in the B2/B3 ratio (Table 1). B3 mitochondria are the predominant form up to the fifteenth day of development, when there is a sharp reversal, and by 17 days B2 mitochondria outnumber B3 mitochondria by 14:1.

Since the liver weight of the earlier developmental stages is small, many livers have to be pooled and therefore the younger the embryos the more embryonic livers are required to obtain enough tissue mass to carry out the necessary experimental

work. The change in B2/B3 ratio could therefore be a function of anoxia or other deleterious effects which occurred while the livers were kept in the ice-cold 0.25M-sucrose-1mM-EDTA solution until completion of all the liver dissections. The possibility that increased dissection time was the contributing factor for low B2/B3 ratios was investigated by dividing 40 eggs, which had been incubated for 9.5 days, into two batches. One batch was dissected as quickly as possible, just over 10min elapsing between the dissection of the first embryo and homogenization of the pooled livers; the other batch was dissected deliberately slowly and subsequently left for a total time of 60min before homogenization began. The densities of the B2 and B3 fractions of both batches were identical and so also were the B2/B3 ratios. From this experiment it was concluded that the increased time to dissect early developmental stages could be eliminated as a contributing factor for low B2/B3 ratios.

Differences in isopycnic densities do imply differences in protein/phospholipid ratios, but there is no significant difference between the protein/phospholipid ratio of whole mitochondria, B2 and B3 mitochondria of all the different embryonic developmental stages. The neonatal and adult chick liver mitochondria showed a small but definite increase in their protein/phospholipid ratio when compared with embryonic liver mitochondria.

These results, although in agreement with previous work on rat liver mitochondria (Pollak & Munn, 1970), show a very different trend from the results obtained by Goldhor (1968) with embryonic neonatal and adult chick liver. Direct comparisons could, however, be carried out as Goldhor (1968)

Table 2. *Comparison of protein/phospholipid ratios and protein/total lipid ratios found in the present work with the protein/lipid ratios obtained by Goldhor (1968)*

For experimental details see the Materials and Methods section. Results are mean values; individual results are presented in parentheses.

Developmental age	Protein phospholipid ratio	Protein total lipid ratio	Protein lipid ratio (Goldhor, 1968)
Embryonic			
7.5–8.5 days	4.3 (5.2, 4.5, 3.2)	3.4 (4.4, 3.0, 2.7)	1.9
9–11 days	3.6 (4.0, 3.1, 3.7)	2.6 (3.1, 2.0, 2.7)	2.65
14–17 days	4.2 (3.7, 4.4, 4.5)	3.6 (3.4, 3.6, 3.7)	—
Neonatal			
1–14 days	4.4 (4.2, 4.2, 4.8, 4.3)	2.9 (3.1, 2.9, 2.9, 2.8)	4.73
Adult	4.9 (3.3, 5.0, 5.3, 5.5)	3.2 (2.8, 4.5, 2.3, 3.2)	3.2

measured protein/lipid ratios. Consequently in Table 2 the protein/total lipid ratios of the mitochondrial fractions isolated from five developmental stages were compared with each other as well as with their protein/phospholipid ratios and the values obtained by Goldhor (1968). The results of the present investigation demonstrate that mitochondria isolated from chick livers of different developmental stages show no progressive increase in the protein/total lipid ratios (Table 2).

Since the method of preparation and gradient centrifugation used in the present investigation differed somewhat from that used by Goldhor (1968), the isolation and centrifugation procedures were compared by placing mitochondria prepared by these two methods, either on a 0.9–1.9M-sucrose–1mM-EDTA (pH 6.8) gradient, as used in the present investigation, or on a 1.02–1.75M-sucrose–1mM-EDTA (pH 6.8) gradient as used by Goldhor (1968).

These results indicated that mitochondria isolated from 10-day-old embryonic chick liver or from adult fowl liver and placed on a gradient and centrifuged as described by Goldhor (1968) have not reached their equilibrium density, as further centrifugation for 90 min at 130 000g_{av.} produces a very definite B3 band, which was not apparent after the first centrifugation. This aspect was not further pursued, as the isopycnic density method used in the present investigation was deemed satisfactory from that point of view. It was also demonstrated that the washing procedure for mitochondria as carried out in the present investigation produced 'cleaner mitochondrial fractions'. This was quite apparent when fixed and stained sections of the fractions were examined with the electron microscope (Plate 1).

Having established in this investigation that the B2/B3 ratio increased during the embryonic development of the chick, we decided to find out if the mitochondria also showed concomitant changes in their metabolic activities. Accordingly three

parameters (cytochrome oxidase, malate dehydrogenase and the respiratory control of mitochondrial suspensions) were measured at five different developmental stages. The results are presented in Table 3 and show that all three metabolic activities increase during development; however, these increases were not synchronous. For example, between 7.5–8.5 days and 9–11 days embryonic age the respiratory control increased to approx. 130%, whereas cytochrome c oxidase activity increased to 300%, and malate dehydrogenase to 600–900%, of the original activity.

The change in the B2/B3 ratio, although also increasing with developmental age, again took place at a different rate. Since there exists at least a qualitative correlation between these different parameters, it is of some importance to establish the nature of B2 and B3 mitochondria. In Plate 2 the ultrastructures of B2 and B3 mitochondria isolated from 9.5-day-old embryonic chick liver are compared. B2 and B3 mitochondria differ from each other in matrix conformation and size (compare Plates 2a and 2b). B2 mitochondria (Plate 2a) have their matrix in a condensed form (Hackenbrock, 1966), whereas B3 mitochondria consist of a mixture of mitochondrial profiles, some with a condensed matrix, others in which the matrix is in the orthodox conformation (Plate 2b). This holds for all developmental stages except the earliest investigated; the B3 mitochondria obtained from livers of 8-day-old chick embryos were predominantly in the condensed form.

As previously demonstrated with rat liver mitochondria (Pollak & Munn, 1970), the addition of 10mM-sodium succinate and 10mM-sodium phosphate buffer, pH 7.4, either to the isolation medium or later to the isolated mitochondria, increased the proportion of B3 mitochondria, but only if succinate and P_i were also included on the isopycnic sucrose gradient. In the present series of experiments such an addition of succinate and P_i to mitochondria isolated from 17-day-old embryonic

EXPLANATION OF PLATE I

Electron micrographs of mitochondria isolated from adult and embryonic chick liver. (a) Structure of adult chick liver mitochondria and the degree of contamination when the isolation method of Pollak & Munn (1970) was used. (b) Structure of adult chick liver mitochondria and the degree of contamination when the isolation method of Goldhor (1968) was used. There is considerably more microsomal contamination in the preparation used for (b) than in that used for (a). (c) Profiles of hepatic mitochondria from 9.5-day-old embryonic chicks. These mitochondria were subsequently fractionated into B2 and B3 fractions (see Plates 2a and 2b). The bar represents $0.5\mu\text{m}$.

EXPLANATION OF PLATE 2

(a) Profiles of hepatic mitochondria from a B2 fraction of 9.5-day-old chick embryos. All the mitochondria are in a 'condensed' state. (b) Profiles of hepatic mitochondria from a B3 fraction of 9.5-day-old chick embryos. This fraction contains a mixture of 'orthodox' and 'condensed' mitochondria. The bar represents 0.5 μm .

Table 3. *Activities of mitochondrial enzymes during development*

For experimental details see the Materials and Methods section. Results are mean values; individual results are presented in parentheses.

Developmental age	Respiratory control	Cytochrome oxidase	Malate dehydrogenase
	(state 4 state 3 ratio)	activity ($\mu\text{mol/min per mg of protein}$)	activity ($\mu\text{mol/min per mg of protein}$)
Embryonic			
7.5–8.5 days	1.6 (1.3, 1.7, 1.8)	0.04 (0.04, 0.03, 0.02, 0.07)	0.5 (0.3, 1.1, 0.2)
9–11 days	2.1 (2.1, 2.1, 2.4, 1.9)	0.12 (0.08, 0.15)	3.8 (2.9, 4.6)
14–17 days	3.2 (3.2, 3.2, 3.4, 3.1)	0.33 (0.24, 0.41, 0.35)	2.7 (2.1, 2.5, 3.6)
Neonatal			
1–14 days	2.5 (2.4, 2.5, 2.3, 2.7)	0.31 (0.36, 0.26)	3.2 (3.1, 3.8, 2.7)
Adult	3.8 (3.6, 3.1, 5.1, 3.2)	0.46 (0.59, 0.38)	2.2 (1.5, 2.9, 2.1)

chick liver, as well as to the sucrose gradient, decreased the B2/B3 ratio to 0.77 (see Table 1 for comparison). As in rat liver, B3 mitochondria that have been obtained by transforming B2 mitochondria to steady-state 4 are mainly in the orthodox form (Pollak & Munn, 1970). This provides evidence that the routine isolation of B3 mitochondria is not due to a proportion of the mitochondria being in steady-state 4, as then one would expect to see only orthodox mitochondria in the B3 fraction. Further, the routine isolation of B3 mitochondria does not require the presence of succinate and P_i as part of the gradient, as is required for the isolation of steady-state 4 mitochondria in the B3 fraction.

DISCUSSION

The existence within one tissue of mitochondria that differ in their morphology and their enzyme content has been demonstrated by electron microscopy and by histochemical techniques (Pette & Brandau, 1966; Novikoff & Shin, 1964). Mitochondria have also been fractionated by differential centrifugation according to their fragility (Lusena & Depocas, 1966), or according to their iron and cytochrome *a* content and cytochrome oxidase activity (Gear, 1965*a,b*). More recently two mitochondrial populations (B2 and B3), thought to differ in the permeability of their inner membrane to sucrose, were isolated from normally fed, starved and embryonic rat liver by isopycnic centrifugation (Pollak & Munn, 1970); in embryonic rat liver the ratio of sucrose-impermeable (B2) mitochondria to sucrose-permeable (B3) mitochondria increased with developmental age.

In the present investigation it was shown that apart from the actual quantitative values of the ratios of B2/B3 mitochondrial fractions, a similar change from low B2/B3 ratios to high B2/B3 ratios takes place also during the development of the chick embryo (Table 1).

In a recent study using gradient centrifugation it was shown that in embryonic chick liver the protein/lipid ratio increased with increasing developmental age (Goldhor, 1968). In this respect these mitochondria seemed more similar to mitochondria isolated from *Neurospora crassa*, which exhibited a changing protein/phospholipid ratio when exposed to media containing different amounts of choline (Luck, 1965), than to embryonic rat liver mitochondria, which had a constant protein/phospholipid ratio throughout development (Pollak & Munn, 1970).

In gaining an understanding of the synthesis and maturation of mitochondria in vertebrates, whether the protein/lipid ratio is constant or is increasing is a matter of considerable importance.

The different results obtained with embryonic rat liver mitochondria (Pollak & Munn, 1970) and embryonic chick liver mitochondria (Goldhor, 1968) make it difficult to see any unifying concept; hence it was decided to repeat the isolation and fractionation of mitochondria from both embryonic chick and rat liver, so that a direct comparison could be carried out within the same laboratory and under the same conditions. The re-investigation of adult and embryonic rat liver mitochondria gave essentially the same results as those obtained by Pollak & Munn (1970) (J. K. Pollak & M. Woog, unpublished work). By using the same methods on embryonic chick liver it was found that the protein/phospholipid ratio changed only insignificantly during development (Table 1).

Goldhor (1968) suggested that mitochondria isolated from adult chick liver equilibrated in a sucrose gradient at a higher density than mitochondria isolated from 8-day embryonic chick liver.

We submit that the explanation of these differences in the results lies in the different centrifugation speeds that were being used in these two investigations. Goldhor (1968) centrifuged the mitochondria in a sucrose gradient (1.02–1.75M) at 18400*g*_{av.} whereas in the present study 130000*g*_{av.} was used.

Goldhor (1968) found that $18000g_{av}$. for 2h gave rise to an isopycnic equilibrium for mitochondria, as further centrifugation did not change the position of the bands. This was not confirmed in the present series of experiments. Additional centrifugation for 90min at $130000g_{av}$. separated the main band ($d1.1903-1.2000$) formed by the original centrifugation at $18400g_{av}$. into two bands corresponding to B2 and B3. This behaviour applied to both embryonic and adult chick liver mitochondria.

The constancy of the protein/lipid or protein/phospholipid ratio of B2 and B3 mitochondria coupled with their different isopycnic equilibria in a sucrose density gradient suggests that, as in rat liver mitochondria, B2 and B3 mitochondria of chick liver differ in their permeability to sucrose. This feature together with an increasing B2/B3 ratio during development indicates that in embryonic chick liver the impermeability of the mitochondrial inner membrane is progressively developed with embryonic age.

This concept of a relatively constant protein/lipid or protein/phospholipid ratio together with changes in the permeability of the inner mitochondrial membrane during development should be taken into consideration in any proposed mechanism of mitochondrial biosynthesis. It has been repeatedly shown that in embryonic liver, mitochondrial counts or yields of isolated mitochondria are considerably lower than those of the corresponding adult tissue (Schneider & Hogeboom, 1949-50; Mintz *et al.* 1967); during development there is thus a considerable change in the rate of mitochondrial biosynthesis.

The changes in the B2/B3 distribution ratio during development (Table 1) are due both to an increase in the number of B2 mitochondria and a decrease of B3 mitochondria. This trend is very definite up to the seventeenth day of development; after that stage the amount of the B3 fraction stays fairly constant and the amount of the B2 fraction varies. The B2/B3 ratios are more meaningful than the actual amounts of B2 and B3 mitochondria, since the actual amounts of B2 and B3 fractions that are obtained are dependent on the percentage yield of mitochondria, which is variable. The extent of mitochondrial yield may be calculated from the succinoxidase activities of the whole homogenates. Variations in mitochondrial yield were found not to affect the B2/B3 distribution ratios. It is tentatively suggested that the number of B3 mitochondria within the total mitochondrial population of embryonic liver tissue may reflect the number of newly formed mitochondria and hence give an indication of the rate of mitochondrial biosynthesis. The small proportion of B3 mitochondria, with a permeable inner membrane, present even in normally fed adult liver (Pollak & Munn, 1970, and

Table 1) is compatible with the normal rate of turnover of mitochondria. The considerable increase in the B3 mitochondrial fraction in starved rat liver (Pollak & Munn, 1970) may be best explained perhaps by assuming either a rapid turnover of mitochondria during starvation or the action of degradative processes that render the inner membrane permeable to low-molecular-weight compounds such as sucrose. It should be pointed out that this increase in B3 mitochondria is rapidly reversed by feeding the starved rat; normal B2/B3 ratios are restored 2-4h after feeding the starved rat.

In Table 3 it is shown that the specific activity of at least two enzymes as well as the respiratory control of mitochondria increase with developmental age. The constancy of the protein/lipid ratio implies, however, that no significant amount of protein can be added to the mitochondrion without the addition of a proportional amount of lipid. The change in the permeability of the inner mitochondrial membrane during development may then be considered to be brought about by a change in its conformation, which is due either to the exchange of specific proteins or lipids or to the addition of a new lipoprotein, or to allotropic changes of the existing membrane lipoproteins.

After this investigation was completed two reports relevant to the central theme of this study (the isolation of two mitochondrial populations) became available to us. Loeb & Kimberg (1970), in a paper dealing with the sedimentation properties of mitochondria obtained from normal and cortisone-treated rats, found that equilibrium sedimentation in a linear 1.0-2.0M-sucrose gradient at $95000g_{av}$. (calculated from Beckman-Spinco specifications) for 20h resulted in only one tightly packed band of mean density 1.215. Wattiaux & Wattiaux-De Coninck (1970) used higher g for isopycnic centrifugation in their investigation and described experiments in which centrifugation at $140000g_{av}$. (calculated from Beckman-Spinco specifications) resulted in one mitochondrial band ($d1.19$), whereas at $230000g_{av}$. (calculated from Beckman-Spinco specifications) three bands ($d1.165$, 1.181 and 1.220) were formed. These authors concluded that forces above $140000g_{av}$. caused damage to mitochondria and that the damaged mitochondria equilibrated at different density regions of the gradient.

The results of several investigations dealing with equilibrium sedimentation of mitochondria in sucrose gradients are difficult to reconcile. Thus Beaufay *et al.* (1964) obtained a single band of mitochondria with mean $d1.19$; the same workers also concluded that mitochondria with an abnormally dense state ($d1.22$) were formed if the sucrose concentration to which the mitochondria were

exposed exceeded $d1.195$. This aspect, as well as other findings of Beaufay *et al.* (1964), were discussed in some detail by Pollak & Munn (1970), who regarded their results to be compatible with those of Beaufay *et al.* (1964).

The findings of Wattiaux & Wattiaux-De Coninck (1970) that forces above $140\,000g_{av.}$ have a damaging effect on mitochondria is a new proposal, but does not really affect the results of the present investigation, as in all the experiments described only $130\,000g_{av.}$ was used; however, in several experiments that were not described it was found that $204\,000g_{av.}$ for 90 min gave very similar results to the $130\,000g_{av.}$ for 90 min used in the present investigation and by Pollak & Munn (1970). Further, the protein/phospholipid ratios of B2/B3 mitochondria were always of the same order and did not imply the separation of inner and outer membranes from the matrix as suggested by Wattiaux & Wattiaux-De Coninck (1970). Other indications of the intactness of B2 and B3 mitochondria were the facts that B2 and B3 mitochondria possessed very similar specific activities of cytochrome oxidase, malate dehydrogenase and monoamine oxidase, which represent inner membrane, matrix and outer membrane enzyme markers (Pollak & Munn, 1970). The equilibration of mitochondria in the B3 region ($d1.2$) after incubation with sodium succinate and P_i , when isopycnic centrifugation was carried out in the presence of succinate and P_i , cannot be ascribed to mitochondrial damage, particularly as the omission of succinate and P_i from the gradient causes a marked decrease in the B3 population and a concomitant increase of B2 mitochondria.

On the other hand we have experienced unusually high yields of aggregated B3 mitochondria, if perchance the temperature rose above 7°C (measured in the tube immediately after centrifugation) through failures in the refrigeration or vacuum systems. Strict temperature control seems to be essential for reproducible isopycnic centrifugation of mitochondria.

The present investigation as well as the study by

Pollak & Munn (1970) demonstrate the presence of two different types of hepatic mitochondria, the proportions of which vary in different physiological conditions.

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