The Maturation of the Inner Membrane of Foetal Rat Liver Mitochondria

AN EXAMPLE OF A POSITIVE-FEEDBACK MECHANISM

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1. A new method was devised for the isolation of foetal and neonatal rat liver mitochondria, giving higher yields than conventional methods. 2. During development from the perinatal period to the mature adult, the ratio of cytochrome oxidase/succinate-cytochrome *c* reductase changes. 3. The inner mitochondrial membrane of foetal liver mitochondria possesses virtually no osmotic activity; the permeability to sucrose decreases with increasing developmental age. 4. Foetal rat liver mitochondria possess only marginal respiratory control and do not maintain Ca^{2+} -induced respiration; they also swell in respiratory-control medium in the absence of substrate. ATP enhances respiratory control and prevents swelling, adenylyl imidodiphosphate, ATP+oligomycin or ATP+atractyloside enhance the R.C.I. (respiratory control index), Ca^{2+} -induced respiratory control and prevent swelling, whereas GTP and low concentrations of ADP have none of these actions. It is concluded that the effect of ATP depends on steric interaction with the inner mitochondrial membrane. 5. When 1-day pre-partum foetuses are obtained by Caesarean section and maintained in a Humidicrib for 90 min, mitochondrial maturation is 'triggered', so that their R.C.I. is enhanced and no ATP is required to support Ca^{2+} -dependent respiratory control or to inhibit mitochondrial swelling. 6. It is concluded that foetal rat liver mitochondria *in utero* do not respire, although they are capable of oxidative phosphorylation in spite of their low R.C.I. The different environmental conditions which the neonatal rat encounters *ex utero* enable the hepatic mitochondria to produce ATP, which interacts with the inner mitochondrial membrane to enhance oxidative phosphorylation by an autocatalytic mechanism.

During foetal development the contribution of cellular organelles to the overall metabolism of the cell varies considerably. This may be due to the changing environment within the differentiating cell, as well as to the fact that cellular organelles undergo a maturation process. It has been clearly demonstrated that during the perinatal period rat liver mitochondria undergo many significant changes with respect to both their morphology and their metabolism (reviewed by Pollak & Duck-Chong, 1973). Several investigators have studied the energy-conservation system of foetal and neonatal rat liver mitochondria (Mintz *et al.*, 1967; Hallman, 1971; Duck-Chong & Pollak, 1973; Nakazawa *et al.*, 1973). Although there are some discrepancies between the findings from different laboratories, the general consensus is that the energy-conservation system of rat liver mitochondria becomes truly functional only in the immediate post-natal period.

The present paper deals with the maturation of foetal rat liver mitochondria and is particularly concerned with the changes to the inner mitochondrial membrane resulting in altered osmotic properties, respiratory control, transport phenomena and changes in the activities of two associated respiratory enzymes.

The results of this investigation support the concept that during the perinatal period the inner mitochondrial membrane undergoes a maturation process which changes a relatively permeable membrane to the osmotically active membrane which one associates with the inner mitochondrial membrane of adult rat liver mitochondria. This maturation of the inner mitochondrial membrane appears to be dependent on a stimulus, normally provided by parturition, which activates a positive-feedback mechanism involving an initially slow production of ATP. The ATP reacts with the inner mitochondrial membrane

so that the efficiency of ATP production is increased, resulting in raised ATP concentrations in the liver parenchymal cells of the neonatal rat.

Experimental

Materials

Chemicals. All reagents used were of Analytical Reagent grade. The following fine chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; ATP, ADP, GTP, potassium atractyloside, oligomycin, Trizma base (Tris) and Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid]. Cytochrome *c* was obtained from Calbiochem (Australia) Pty., Carlingford, N.S.W., Australia, and adenylyl imidodiphosphate [AMP-P(NH)P] from Boehringer G.m.b.H., Mannheim, Germany.

Animals. Wistar rats from the University of Sydney Animal House, Castle Hill, were used throughout the investigation. For experiments involving adult rat liver, female Wistar rats weighing between 150 and 200 g were used. To obtain foetal rats of known age, four female rats were mated with two male rats for either 24 or 48 h. The foetal age ± 12 h was evaluated from the foetal weight and foetal liver weight obtained from the 24 h matings.

Methods

Preparation of mitochondrial fractions. Unless otherwise stated mitochondria from foetal and neonatal rats were prepared by a newly devised method based on a procedure described by Herzfeld & Green-gard (1971). Normal adult rats or pregnant rats were killed by cervical fracture. The liver of normal adult rats was rapidly excised and chilled at 2°C in medium A [0.25 M-sucrose–1 mM-EDTA–1 mM-Hepes buffer (pH 7.4)–0.2% bovine serum albumin]. Foetal rats were quickly dissected out from the freshly killed mothers, and the livers of foetal rats pooled and suspended in medium A at 2°C. Neonatal and suckling rats were decapitated and their livers dissected out and pooled as described for foetal rats. All subsequent preparative procedures were carried out at 2°C.

Adult rat liver was minced with scissors and machine-homogenized in a 50 ml glass-Teflon homogenizer-size C (A. H. Thomas Co., Philadelphia, Pa., U.S.A.) with a constant-speed motor at 1400 rev./min by using eight up-and-down strokes. Foetal and neonatal rat livers were homogenized by hand homogenization in a 10 ml PTG 2 glass-Teflon Jencons homogenizer, by using five up-and-down strokes. All stages of the preparation were monitored by phase-contrast microscopy. The homogenization procedure gave very good cell breakage, particularly with foetal rat liver. It was estimated that as a routine more than 90% cell breakage was obtained; only the occasional unbroken cell could be observed per field at a magnification of 300 diameters. Homogenates for all develop-

mental ages were prepared as 10% (w/v) suspension (ideally containing 2–3 g of liver). Foetal and neonatal homogenates were then diluted with medium A to give 5% homogenates. Portions (5 ml) of the homogenates were layered over 5 ml of 0.4 M-sucrose in a 15 ml glass Sorvall centrifuge tube and centrifuged at 270 $g_{av.}$ for 10 min in a 269 swing-out rotor of an International PR2 centrifuge. Adaptors were placed into the rotor buckets so that the midpoint of each tube was situated 15 cm from the centre of the rotor. During centrifugation all the nuclei and most of the erythrocytes sedimented, though sometimes, particularly when early foetal liver was used, a ring of erythrocytes aggregated at the 0.4 M-sucrose–medium A interface. The milky upper layer was syringed off, pooled and placed into 25 ml tubes of the high-speed rotor (296) of the International PR 2 centrifuge and centrifuged at 4500 $g_{av.}$ for 8 min; the force was then increased to 10000 $g_{av.}$ for 2 min. The supernatant was sucked off and the mitochondrial pellet was re-suspended by hand homogenization with a loose-fitting 10 ml glass-Teflon homogenizer in medium A.

The mitochondria were washed for the first time by sedimenting and resuspending as described in the last step. Two more washes were carried out by centrifugation at 4500 $g_{av.}$ for 8 min and resuspending the pellet by hand homogenization as described above. The three-times washed mitochondrial pellet was resuspended in medium A to give mitochondrial suspensions which varied in their protein content between 5 and 40 mg of protein/ml, the low values being unavoidable in suspensions derived from very young (4–5-day pre-partum) foetal rat liver.

In the latter part of the investigation the sedimentation of the nuclear pellet for the preparation of adult rat liver mitochondria was carried out in the high-speed rotor (296) of the International PR2 centrifuge by centrifuging at 650 $g_{av.}$ for 10 min, since for adult liver the more elaborate layering technique did not increase the yield (Table 1).

Enzyme assays. Cytochrome oxidase (EC 1.9.3.1) was measured by the spectrophotometric method described by Sottocasa *et al.* (1967), except that the final concentration of the sodium phosphate buffer was 50 mM and that of reduced cytochrome *c* 55 mM. The decrease in extinction was measured at 550 nm with a Unicam SP.800 spectrophotometer, by using a 10-fold scale expansion. Succinate-cytochrome *c* reductase (EC 1.3.99.1) was measured spectrophotometrically at 30°C, with the use of the 10-fold scale expansion of the Unicam SP.800 spectrophotometer, by following the reduction of cytochrome *c* at 550 nm. The assay mixture contained in 3 ml: 33 mM-sodium phosphate buffer, pH 7.5, 33 μ M-cytochrome *c*, 300 μ M-KCN, 0.1 ml of diluted enzyme suspension, and the reaction was commenced by adding 1 M-sodium succinate to a final concentration of 5 mM. Glucose 6-phosphatase (EC 3.1.3.9) was assayed

without detergent as described by Duck-Chong & Pollak (1973).

Measurements of mitochondrial swelling and respiratory control. Changes in the extinction at 546 nm (1 cm light-path), measured with a Unicam SP.800 spectrophotometer, were taken as an indication of changes in light-scattering or changes in the mitochondrial diameters (Packer, 1960); temperature was maintained at 30°C. Respiratory control, ADP/O ratios and Ca^{2+} -stimulated O_2 uptake were measured by means of a Rank (Bottisham, Cambs., U.K.) O_2 electrode. The 2 ml incubation mixture was maintained at 30°C and contained 25 mM-Hepes buffer, pH 7.4, 5 mM-sodium phosphate buffer, pH 7.4, 5 mM- MgCl_2 , 50 mM-KCl and 0.4 ml of medium A, including the volume of medium A containing the mitochondria.

Protein determination. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (fraction V; Sigma) as standard.

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 on a Hitachi 7B or a Philips 201c electron microscope.

Results

Recovery of foetal rat liver mitochondria

It is generally recognized that foetal and neonatal rat liver contain fewer mitochondria per unit cell volume than does adult rat liver (Ontko, 1966; Vergonet *et al.*, 1970; Herzfeld *et al.*, 1973; Pollak & Duck-Chong, 1973).

The morphometric data confirm the general impres-

sion gained by viewing electron micrographs of foetal and adult rat liver. The lower mitochondrial yield obtained from foetal rat liver by conventional differential-centrifugation techniques used for the isolation of adult rat liver mitochondria, together with the lower specific activities of several mitochondrial enzymes, has been generally accepted to represent one of the differences between foetal and adult rat liver. However, it has been pointed out that isolation techniques which result in the recovery of 45–55% of the mitochondrial protein of adult rat liver are nowhere near as effective when used to isolate foetal rat liver mitochondria (Pollak & Duck-Chong, 1973). Complete balance studies with marker enzymes indicated that the recovery of foetal rat liver mitochondria was only 24 and 34% (Pollak & Duck-Chong, 1973). Further, in foetal rat liver, microsomal contamination as determined by marker enzymes such as glucose 6-phosphatase or NADH-cytochrome *c* reductase has to be considerably more carefully measured than in adult rat liver, as these enzymes have only marginal activities in foetal rat liver.

The low recovery of mitochondria from foetal rat liver prompted us to devise a technique which now yields recoveries between 45 and 55% as a routine (Table 1). The method is based on a separation technique described by Herzfeld & Greengard (1971), but contains several important modifications. A comparison of yields of the isolation technique described under 'Methods' with those of a more conventional method (McMurray & Dawson, 1969) is presented in Table 1. In the latter method 0.25 M-sucrose–1 mM-EDTA (pH 7.4) was used as the isolating medium and the nuclei were sedimented at $650g_{av}$; the subsequent steps were essentially the same as those used in the modified method. Glucose 6-phos-

Table 1. Comparison of yields of foetal, neonatal and adult rat liver mitochondria isolated by a conventional and a modified method

Conventional method is the method of McMurray & Dawson (1969). Modified method is described under 'Methods'. Results are means $\pm$ S.E.M., for the numbers of experiments given in parentheses.

Developmental age	Protein in mitochondrial fraction (mg/g of liver)		Cytochrome oxidase (% of that in whole homogenate)	
	Conventional method	Modified method	Conventional method	Modified method
18–19-day foetal rats (3–4 days pre-natal)	2.0 $\pm$ 0.22 (5)	5.3 $\pm$ 0.24 (11)	24.0 (1)	—
20–21-day foetal rats (1–2 days pre-natal)	3.2 $\pm$ 0.41 (10)	7.9 $\pm$ 0.51 (15)	22.9 (1)	49.9 $\pm$ 3.29 (2)
22-day foetal rats (term)	—	12.3 $\pm$ 1.05 (4)	—	55.0 (1)
Neonatal rats (between 0 and 16 h)	6.2 $\pm$ 0.66 (8)	10.2 $\pm$ 0.35 (2)	27.4 $\pm$ 2.72 (7)	50.2 (1)
Adult rats	19.2 $\pm$ 1.60 (14)	25.4 $\pm$ 2.35 (13)	47.8 $\pm$ 1.60 (4)	48.7 $\pm$ 0.35 (2)

Table 2. *Changes in the ratio of the two inner-membrane enzymes, cytochrome oxidase and succinate–cytochrome c reductase, during the maturation of rat liver mitochondria*

For experimental detail see under 'Methods'. Results are means $\pm$ S.E.M., for the numbers of experiments shown in parentheses.

Developmental age	Enzyme activity of mitochondrial fraction				Cytochrome oxidase succinate–cytochrome <i>c</i> reductase ratio
	(μmol/min per g of liver)		(nmol/min per mg of protein)		
	Cytochrome oxidase	Succinate–cytochrome <i>c</i> reductase	Cytochrome oxidase	Succinate–cytochrome <i>c</i> reductase	
2–4 days prenatal	8.30 ± 1.55 (4)	2.72 ± 0.54 (4)	731 ± 60 (4)	241 ± 22 (4)	3.0 ± 0.14
4–12 h postnatal	16.43 ± 1.73 (3)	3.27 ± 0.77 (3)	1393 ± 116 (3)	259 ± 47 (3)	5.4 ± 0.91
Adult	24.28 ± 0.73 (4)	3.75 ± 0.53 (4)	1380 ± 193 (4)	224 ± 57 (4)	6.2 ± 1.07

Fig. 1. *Comparison of the osmotic properties of foetal and adult rat liver mitochondria in a series of sucrose solutions varying in concentration*

The sucrose solutions varied between 0.05 and 0.5 M. To 2.9 ml of the sucrose solutions at 30°C, 0.1 ml of mitochondria in medium A was added. Mitochondria for adult, 1-day prenatal and 3-day prenatal rats were added at a concentration of 1 mg of protein/0.1 ml; the mitochondria from 5-day prenatal rats were at a concentration of 0.5 mg/0.1 ml. Changes in extinction were observed at 546 nm at 30°C with a recording spectrophotometer until constant readings were obtained. (a) 5-day prenatal; (b) 3-day prenatal; (c) 1-day prenatal; (d) adult.

phatase assays of whole homogenates, nuclear, mitochondrial and postmitochondrial fractions indicated that the newly devised method yielded mitochondrial fractions from foetal rat liver which contained less than 4% of the whole homogenate glucose 6-phosphatase activity. Between 95 and 102% of the whole homogenate cytochrome oxidase activity could be accounted for in the combined nuclear, mitochondrial and postmitochondrial fractions.

A further indication of the low contamination of the foetal mitochondrial fraction by other cell components is the fact that the present study shows that foetal rat liver mitochondria actually have as great a specific activity for succinate-cytochrome *c* reductase as adult rat liver mitochondria (Table 2). The specific activities per mg of protein and the total activities of the whole homogenate per g of liver are also presented in Table 2.

Osmotic properties of foetal and adult rat liver mitochondria

In earlier studies it was shown that two distinct populations of mitochondria can be isolated from both foetal and adult rat liver (Pollak & Munn, 1970; Packer *et al.*, 1971; Duck-Chong & Pollak, 1973). In foetal rat liver mitochondria the inner mitochondrial membrane of the predominant population seemed to be permeable to low-molecular-weight solutes such as sucrose. Results shown in Fig. 1 confirm the earlier observations in the sense that mitochondria isolated from 17-day (5-day prenatal) foetal liver are shown to be virtually inactive osmotically,

and a definite correlation between osmotic activity and increasing developmental age is demonstrated (Fig. 1).

Maturation of respiratory control

In an earlier study we reported that foetal rat liver showed no respiratory control (Duck-Chong & Pollak, 1973); it has since been found, by using the new method of isolating foetal rat liver mitochondria, that some respiratory control is present even at the earliest developmental stages that were investigated (Table 3).

Hallman (1971) and Nakazawa *et al.* (1973) showed that pretreatment of foetal mitochondria with 2mM-ATP and Mg^{2+} increased the R.C.I. (respiratory control index) to a value of 2–3. These experiments have been repeated in the present study and it has been found that 100–400nmol of ATP (125–500 μ M) added to the respiratory-control medium just before or even during the respiratory-control experiment causes a significant increase in the R.C.I. as well as in state-3 respiration (Chance & Williams, 1956), although no change in the ADP/O ratio occurs (Fig. 2, Table 3). Repeated additions of up to 200nmol of ADP enhance each subsequent state-3 respiration only slightly, but give rise to a cumulative increase in the R.C.I. (Fig. 2a), confirming an observation originally made by Hallman (1971). In the present investigation it was also found that the addition of one large amount (3.5 μ mol) of ADP enhances state-3 respiration more rapidly, but under this condition it is not possible to demonstrate respiratory control as the system runs out of oxygen before all the added ADP has been

Table 3. Measurements, in the absence or presence of ATP, of the respiratory-control index, state-3 respiration and ADP/O ratios of rat liver mitochondria derived from different developmental stages

The measurements were carried out in an O_2 electrode as described under 'Methods'. To commence the reaction, 1.2–2.0 mg of mitochondrial protein was added; the substrate used was 50mM-sodium succinate; repeated additions of 200nmol of ADP were made. When addition of ATP is indicated, 400nmol of ATP was added to the medium before the addition of mitochondria. Results are means $\pm$ s.e.m., for the numbers of experiments given in parentheses. R.C.I. and ADP/O ratio were measured after the second addition of ADP. Student's *t* test was used to evaluate the statistical significance of the addition of ATP: ** indicates statistical significance at the 1% level; * indicates statistical significance at the 5% level.

Age of rats	R.C.I.		State-3 respiration (g-atoms of oxygen/min per mg of protein)		ADP/O ratio	
	No ATP	+ATP	No ATP	+ATP	No ATP	+ATP
18–19-day foetal rats (3–4 days prenatal)	1.2 $\pm$ 0.09 (7)	2.7 $\pm$ 0.30** (6)	97 $\pm$ 19.0 (2)	169 $\pm$ 14.4** (3)	1.7 $\pm$ 0.3 (3)	1.7 $\pm$ 0.10 (6)
20–21-day foetal rats (2–12 day prenatal)	1.2 $\pm$ 0.09 (9)	2.3 $\pm$ 0.10** (12)	71 $\pm$ 6.9 (6)	139 $\pm$ 16.4** (6)	1.4 $\pm$ 0.15 (5)	1.7 $\pm$ 0.08 (12)
22-day foetal rats (term)	1.4 $\pm$ (0.15) (4)	2.6 $\pm$ 0.06** (3)	77 $\pm$ 9.5 (2)	133 $\pm$ 27.0* (2)	1.3 $\pm$ 0.15 (4)	1.6 $\pm$ 0.20 (2)
Neonatal rats (2–4h)	3.0 (1)	3.1 (1)	190 (1)	188 (1)	1.5 (1)	1.8 (1)
Adult rats	4.7 $\pm$ 0.20 (16)	4.6 $\pm$ 0.31 (3)	171 $\pm$ 14.1 (8)	178 (8)	1.7 $\pm$ 0.07 (16)	1.6 $\pm$ 0.26 (3)

Fig. 2. Respiratory control of foetal rat liver mitochondria in the absence and presence of ATP

R.C.I. measurements were carried out as described under 'Methods'. (a) No ATP added; (b) ATP added before the addition of mitochondria; (c) ATP added as indicated in the Figure. Foetal mitochondria (Mito) (1-day prenatal) containing 1.1 mg of protein were added to respiratory-control medium; 10 μ mol of sodium succinate (Succ), 200 nmol of ADP and 400 nmol of ATP were added wherever indicated.

Fig. 3. Effect of atractyloside on state 3 respiration in foetal and neonatal rat liver mitochondria

R.C.I. measurements were carried out as described under 'Methods'. Foetal mitochondria (1.2 mg of protein) were added to (a), (b) and (c) and neonatal mitochondria (1.6 mg of protein) were added to (d) as indicated; 10 μ mol of sodium succinate, 200 nmol of ADP or 3.5 μ mol of ADP and 50 nmol of potassium atractyloside (Atract) were added wherever indicated.

converted into ATP and therefore the system does not return to state-4 respiration (Fig. 3a).

Effect of ATP on foetal rat liver mitochondria

The enhancement of the R.C.I. of foetal rat liver mitochondria by exogenous ATP was suggested to be due to the low endogenous adenine nucleotide content of foetal mitochondria (Nakazawa *et al.*, 1973). This concept implied a net uptake of ATP by mito-

chondria, but as the adenine nucleotide translocase is only an exchange system which cannot increase the adenine nucleotide concentration within the mitochondria it was decided to investigate if the ATP effect on the foetal mitochondria was inhibited by atractyloside.

After the addition of 3.5 μ mol of ADP to foetal rat liver mitochondria, the ensuing state-3 respiration is only partially inhibited by atractyloside (Fig. 3b). However, after the addition of ATP state-3 respiration of the foetal mitochondria is completely sensitive to atractyloside, irrespective of whether state-3 respiration is induced with 200 nmol or 3.5 μ mol of ADP (Fig. 3c). In a comparable experiment with neonatal rat liver mitochondria, it is shown that state-3 respiration is completely inhibited by atractyloside even in the absence of ATP (Fig. 3d).

In some preliminary experiments the extinction (at 546 nm) of foetal rat liver mitochondria was measured before and after the R.C.I. was determined. It became apparent that the extinction of foetal mitochondria in respiratory-control medium to which ATP had been added did not change, whereas the untreated mitochondria (no addition of ATP) had a decreased extinction at the end of the respiratory-control experiment. It has long been recognized that substrate oxidation by the respiratory chain causes mitochondrial swelling, which may be measured as a decrease in the extinction at 546 nm (Packer, 1960). Therefore further experiments were carried out in the absence of substrate (state-1 respiration).

As soon as foetal mitochondrial stock suspensions (10 mg of protein/ml) are diluted at 30°C with respiratory-control medium a decrease in the extinction at 546 nm occurs, representing mitochondrial swelling (Fig. 4a). When ATP is added to the respiratory-control medium before the addition of foetal mitochondria the extinction remains constant (Fig. 4b).

The morphological changes that take place during these experiments are particularly striking. Plate 1(a) shows profiles of foetal rat liver mitochondria fixed after isolation, and Plate 1(b) depicts the same mitochondria after 'swelling' in respiratory-control medium has taken place. These mitochondria were fixed with an equal volume of 2% glutaraldehyde in 0.075 M-sodium phosphate buffer, pH 7.4, at the time indicated by the arrow in Fig. 4(a). Mitochondria which were exposed to 100 nmol of ATP/ml of medium and fixed at the time-interval as indicated by the arrow in Fig. 4(b), are shown in Plate 1(c). The morphological changes are quite marked in the swollen foetal mitochondria, whereas in the ATP-treated foetal mitochondria only marginal changes in the ultrastructure are observed.

In comparable experiments with neonatal and adult rat liver mitochondria the addition of ATP neither enhanced respiratory control (Table 3), nor did these

EXPLANATION OF PLATE I

Electron micrographs of foetal rat liver mitochondria

(a) After isolation in medium A; (b) after suspension in R.C.I. medium (no substrate) and fixed as indicated by the arrow in Fig. 4(a); (c) after suspension in R.C.I. medium (no substrate) +200nmol of ATP, fixed as indicated by arrow in Fig. 4(b). The bar represents 1 μ m.

Fig. 4. Effect of ATP on the swelling of foetal mitochondria

Foetal rat liver mitochondria (2-day prenatal), containing 0.6 mg of protein/0.1 ml of medium A, were added to respiratory-control medium (total volume 1.0 ml) in a spectrophotometer cuvette in the absence (a) and the presence of 200 nmol of ATP (b). Incubation temperature was 30°C; changes in extinction at 546 nm were observed with a recording spectrophotometer. Arrow indicates time of fixation as described under 'Methods'.

mitochondria swell in the absence or presence of ATP (J. K. Pollak, unpublished work).

To investigate the mode of action of ATP on the foetal mitochondria, an alternative substrate of the mitochondrial ATPase* (GTP), an ATP analogue [AMP-P(NH)P] and a specific inhibitor of the ATPase (oligomycin) were used. GTP is hydrolysed by foetal rat liver mitochondria at a rate comparable with that of ATP hydrolysis in the absence or presence of 2,4-dinitrophenol (L. Olsen & J. K. Pollak, unpublished work). When 200 nmol of GTP/ml were added to the medium before the measurement of respiratory control, neither state-3 respiration nor the R.C.I. increased significantly (Fig. 5). For comparison the effect of ATP on the mitochondrial preparation is also presented in Fig. 5. GTP did not prevent the decrease in extinction of foetal rat liver mitochondria, whereas in a control experiment, ATP completely prevented the fall in extinction, which may represent swelling of foetal mitochondria (Fig. 5).

Oligomycin added at a concentration of 2 nmol/ml per mg of protein inhibited the ATPase activity of

* Abbreviation: ATPase, adenosine triphosphatase.

Fig. 5. Effects of AMP-P(NH)P, GTP and ATP+oligomycin on respiratory control and swelling of foetal rat liver mitochondria

Respiratory-control measurements were carried out as described under 'Methods.' The following were included in the R.C.I. medium wherever indicated in the Figure: 400 nmol of ATP; 400 nmol of AMP-P(NH)P; 400 nmol of ATP+4 nmol of oligomycin; 400 nmol of GTP. Foetal mitochondria (2 days prenatal) containing 1.1 mg of protein, 10 μ mol of sodium succinate and 200 nmol of ADP were added as indicated. Repetitive 200 nmol of ADP additions are indicated by unnamed arrows. Swelling experiments were carried out in 1 ml cuvettes at 30°C with a recording spectrophotometer, by using 10-fold scale expansion to measure changes in E_{546} . The same mitochondrial suspension was used as for the R.C.I. experiments above. The incubation mixture used was the same as that for the respiratory-control experiments, scaled down to 1 ml volume, with 0.5 mg of mitochondrial protein. The results of a typical experiment are presented in the Figure; mean values $\pm$ S.E.M. of four experiments for the R.C.I. after the second addition of ADP were as follows: control = 1.8 ± 0.13 , +ATP = 2.7 ± 0.14 , +AMP-P(NH)P = 2.8 ± 0.31 , +ATP+oligomycin = 1.8 ± 0.45 +GTP = 1.6 ± 0.04 . Decreases in E_{546} in the same four experiments after 4 min per mg of mitochondrial protein were as follows: control = 0.104 ± 0.003 , +ATP = 0.009 ± 0.009 , +AMP-P(NH)P = 0.049 ± 0.006 , +ATP+oligomycin = 0.002 ± 0.001 , +GTP = 0.122 ± 0.026 .

foetal rat liver mitochondria more than 50% in the absence and 74% in the presence of 2,4-dinitrophenol (L. Olsen & J. K. Pollak, unpublished work), but did not inhibit the effect that ATP had on the prevention of swelling of these mitochondria (Fig. 5).

AMP-P(NH)P is a potent inhibitor of the soluble oligomycin-insensitive and bound oligomycin-sensitive ATPase, but it has been shown to affect the phosphorylation of ADP only minimally (Melnick *et al.*, 1975). The addition of AMP-P(NH)P to the respiratory-control medium enhances the R.C.I. of foetal rat liver mitochondria (Fig. 5). The increases in the R.C.I. are comparable with those produced by an equivalent amount of ATP.

The decrease in the E_{s46} of foetal rat liver mitochondria in respiratory-control medium which is abolished by the addition of ATP is, however, only partially prevented by comparable amounts of AMP-P(NH)P.

These results therefore suggest that the effect of ATP on foetal rat liver mitochondria may not depend on energy production facilitated by the oligomycin-sensitive ATPase, as GTP cannot replace ATP and the presence of oligomycin does not interfere with the inhibitory effect of ATP on the swelling of the mitochondria. Further, the analogue AMP-P(NH)P which inhibits ATP hydrolysis, simulates the effects of ATP on foetal rat liver mitochondria. Thus ATP may be envisaged to act by means of an interaction between ATP and the inner mitochondrial membrane.

It has repeatedly been shown that rat liver mitochondria can accumulate large amounts of Ca^{2+} in the presence of a permeant anion such as phosphate (Lehninger, 1974; Lehninger *et al.*, 1967). This transport across the inner mitochondrial membrane into

the inner matrix compartment is considered to be energetically coupled to electron transport or to the hydrolysis of ATP. The capacity of a mitochondrial suspension to accumulate Ca^{2+} depends in the first instance on the presence of a 'non-leaky' inner membrane, therefore the repeated uptake of Ca^{2+} provides an excellent test system to establish the osmotic activity of mitochondrial suspensions. Foetal rat liver mitochondria were examined for their ability to take up Ca^{2+} as measured by the Ca^{2+} -dependent respiratory stimulation shown in Fig. 6. In the absence of ATP only one or at the most two 'respiratory jumps' occur and then the Ca^{2+} -stimulated respiratory control is lost (Fig. 6a), whereas in the presence of ATP the repetitive addition of Ca^{2+} stimulates respiration each time until the O_2 is depleted (Fig. 6b). The addition of oligomycin or atractyloside to such a system does not interfere with the effect that ATP has on the Ca^{2+} -stimulated respiration of foetal rat liver mitochondria (Figs. 6c and 6d). In control experiments using adult rat liver mitochondria Ca^{2+} stimulates respiration repetitively in the absence of ATP (Fig. 6e); the addition of ATP has no enhancing effects.

These results agree with those obtained by Nakazawa *et al.* (1973), who measured energy-dependent H^+ efflux on the addition of Ca^{2+} to foetal rat liver mitochondria in the absence and presence of ATP.

Since oligomycin inhibits ATP hydrolysis by bound mitochondrial ATPase, and since in the absence of ATP Ca^{2+} does not support the repetitive respiratory stimulation (Fig. 6a), it is concluded that the ATP effect on Ca^{2+} -stimulated respiratory control depends on an interaction between ATP and the inner mitochondrial membrane.

Fig. 6. Effect of ATP, oligomycin and atractyloside on Ca^{2+} -induced respiration of foetal rat liver mitochondria

The conditions for incubation at 30°C were as described under 'Methods'. A mitochondrial suspension (1-day prenatal) containing 2 mg of protein was used to commence the experiment in (a), (b), (c) and (d); 10 μmol of sodium succinate was added as indicated. Unnamed arrows represent successive additions of 200 nmol of CaCl_2 ; (a) control; (b) +400 nmol of ATP; (c) +400 nmol of ATP + 10 nmol of oligomycin; (d) +400 nmol of ATP + 100 nmol of potassium atractyloside; (e) contained control adult rat mitochondria (2 mg of protein) for comparison.

Table 4. Comparison between 1-day pre-partum rat liver mitochondria after Caesarean section with and without a 90 min 'maturation' period

For details see the Experimental section and the text. Four litters were used (A, B, C and D); results from littermates are designated by the same letter.

Treatment and conditions		R.C.I.	Decrease in $E_{346}/4\text{ min per mg of protein}$	Ca^{2+} -stimulated respiration respiration in absence of Ca^{2+}	Number of times Ca^{2+} could stimulate respiration
1-day pre-partum (control)	A	1.3	0.17	—	—
	B	—	0.19	—	—
	C	1.8	—	1.63	2
	D	1.3	0.11	1.67	1
1-day pre-partum + 200 μM -ATP in reaction mixture	A	2.1	—	—	—
	B	—	—	2.50	4*
	C	2.8	—	1.35	5*
	D	2.2	0.02	2.80	4*
1-day pre-partum, left for 90 min in Humidicrib for maturation	A	2.3	0.01	—	—
	B	2.1	0.00	—	—
	C	2.4	—	—	—
	D	2.2	0.03	1.38	4*
1-day pre-partum, left for 90 min in Humidicrib for maturation, +200 μM -ATP in reaction mixture	A	2.2	—	—	—
	B	2.0	—	—	—
	C	2.5	—	—	—
	D	2.22	0.01	—	—

* No further additions of Ca^{2+} could be made owing to exhaustion of O_2 .

Post-Caesarean maturation of mitochondria

Shortly after birth the mitochondrial ATP+ADP content and ATP/ADP ratio increases significantly and the R.C.I. increases to 2–3 (Nakazawa *et al.*, 1973; Hallman, 1971; Duck-Chong & Pollak, 1973; and Table 3). These alterations are thought to be related to other dramatic biochemical changes occurring during the perinatal period. These changes are a function of the altered nutritional status, the foetus being dependent on maternal nutrients, whereas the neonatal rat has an independent existence, rapidly depleting its carbohydrate reserves (Dawkins, 1959; Greengard, 1971). If the maturation of the inner membrane of foetal rat liver mitochondria depends on a changed environment with its concomitant biochemical changes, it should be possible to induce this maturation by exposing foetal rats obtained by Caesarean section on day 21 (1 day *pre partum*) to the same environment that the neonatal rat experiences.

Foetal rats obtained by Caesarean section were divided into two groups. From one group liver mitochondria were processed immediately after the completion of the Caesarean section. The other group was kept in a Humidicrib at 35°C in a water-saturated atmosphere for 90 min, and then these premature neonatal rats were killed and their liver mitochondria isolated. The R.C.I., the light-scattering characteristics in respiratory-control medium and the Ca^{2+} -stimulated respiration of these two groups were compared in the absence and presence of ATP (Table 4). The results show incisively that during the 90 min exposure to neonatal conditions (without feeding or

supplying water) the inner mitochondrial membrane has 'matured' so that the mitochondria now exhibit respiratory control, show virtually no changes in light-scattering when exposed to respiratory-control medium and are capable of several 'respiratory jumps' when Ca^{2+} is added without the addition of ATP. The addition of ATP to 'matured' mitochondria does not affect respiratory control or light-scattering.

Discussion

The perinatal period of the rat lends itself well to the study of the biogenesis of liver mitochondria, as a considerable increase in mitochondrial protein per g as well as per liver takes place (Table 1). Most investigations concerned with the changes in rat liver mitochondria during foetal and early neonatal development have placed no particular emphasis on the maximal recovery of mitochondria from the liver homogenates. It has been shown that conditions for the isolation of mitochondria that yield satisfactory recoveries from adult liver homogenates result in only low recoveries from foetal rat liver (Duck-Chong & Pollak, 1973; Table 1). The method devised in the present investigation increases the yield of foetal mitochondria by preventing gelatinous material present in the foetal liver from enmeshing the mitochondria in the whole homogenate and forming a plug which sediments about half of the mitochondria into the nuclear pellet. Subsequent washing of the nuclear pellet releases only a small proportion of the mitochondria that have sedimented with the nuclei and erythrocytes (Table 1). Another problem encountered

when working with foetal mitochondria is that the contribution of non-mitochondrial protein to the total protein of the mitochondrial fraction may be much higher in foetal than in either postnatal or adult mitochondrial preparations, because of the relatively lower mitochondrial protein content of foetal liver. The problem is aggravated when isolation procedures are used that give lower mitochondrial recoveries from foetal as compared with postnatal and adult liver.

Such facts must be taken into account when assessing reports indicating lower than normal specific activities (per mitochondrial protein) for mitochondrial enzymes in foetal liver compared with the corresponding postnatal and adult values (reviewed by Pollak & Duck-Chong, 1973).

The results in Table 2 show clearly that the specific activities of two mitochondrial inner-membrane enzymes, cytochrome oxidase and succinate-cytochrome *c* reductase, are at least as great in foetal and neonatal rat liver mitochondria as in adult rat liver mitochondria. The dynamic nature of the inner mitochondrial membrane during development is exemplified by the changing cytochrome oxidase/cytochrome *c* reductase activity ratio in foetal, neonatal and adult rat liver mitochondria.

In adult rat liver mitochondria, the inner membrane which surrounds the matrix space (sucrose-impermeable space) is considered to be an osmotically active membrane, exhibiting selective permeability, and responsible for mitochondrial swelling when mitochondria are exposed to hypo-osmolar solutions. The outer membrane, on the other hand, has been shown to be permeable to solutes with molecular weights of up to 10000 (Cereijo-Santaló, 1970).

The findings presented in Fig. 1 indicate that the osmotic activity of the inner mitochondrial membrane increases with developmental age and that 5-day prenatal foetal mitochondria may hardly be regarded as possessing a sucrose-impermeable space, confirming earlier observations made by entirely different experimental approaches (Pollak & Munn, 1970; Nakazawa *et al.*, 1973).

The permeability of the inner membrane of foetal rat liver mitochondria is correlated with their low R.C.I. (Table 3, Fig. 2). The virtual lack of respiratory control of foetal mitochondria (Mintz *et al.*, 1967; Hallman, 1971; Duck-Chong & Pollak, 1973; Nakazawa *et al.*, 1973) was regarded by Nakazawa *et al.* (1973) as being due to the low adenine nucleotide content of the isolated mitochondria. The lack of ATP in foetal rat liver mitochondria raises three important questions. (1) How do the mitochondria of the neonatal rat accumulate additional ATP? (2) What triggers off the increased production and accumulation of ATP? (3) By what mechanism does ATP convert 'foetal-type' mitochondria into 'adult-type' mitochondria?

The low adenine nucleotide concentration of foetal mitochondria (Nakazawa *et al.*, 1973), as well as the low adenylate charge $[(\text{ATP} + \frac{1}{2}\text{ADP})/(\text{ATP} + \text{ADP} + \text{AMP})]$ (Atkinson & Walton, 1967) is probably not an artifact of isolation, since it reflects the low adenine nucleotide content and adenylate charge in foetal liver parenchymal cells (Ballard, 1970, 1971; Knowles & Ballard, 1974). Within 60 min from the time of delivery the ATP/ADP ratio in the liver increases from 1.76 at 0 min to 7.33 at 60 min, with a corresponding change in the adenylate charge from 0.67 to 0.89 (Ballard, 1971). The concomitant increase of the R.C.I. of mitochondria isolated from neonatal liver, as compared with foetal liver mitochondria (Table 3), is presumably related to these changes.

Although the oxygen partial pressure of foetal blood of several species is known to be considerably lower than that of the maternal circulation (Dawes, 1969), there seems to be no direct information on the oxygen partial pressure within the foetal rat liver, though it is generally assumed that the metabolism of foetal rat liver is predominantly anaerobic (Dawkins, 1959).

There is also evidence in the literature, even if somewhat indirect, which implies that foetal rat liver mitochondria may exist in a relatively hypoxic situation. The high values obtained in experiments *in vivo* as well as *in vitro* measuring lactate/pyruvate ratios in the cytosol of foetal rat liver indicate a relatively reduced environment (Philippidis & Ballard, 1969). The lactate/pyruvate ratio fell from 213 in the foetal liver at term to 13.5 in the 1-day newborn (Philippidis & Ballard, 1969). In a different type of experiment the ATP/ADP ratio within the liver of 1 h-old rats was found by Knowles & Ballard (1974) to be 6.8, compared with 1.54 in foetal liver; this ratio changed to 4.23 in the 2-day-old rat liver. If a 2-day-old rat was exposed to an N₂ atmosphere for 15 min this ratio decreased to 1.12, implying that the ATP/ADP ratio of the foetal rat liver may also be the result of hypoxia (Knowles & Ballard, 1974). Thus high lactate values and the high lactate/pyruvate ratio, as well as the low adenylate charge and low ATP/ADP ratio mentioned above, all suggest that foetal rat liver provides its mitochondria with a hypoxic environment. The results of earlier studies (Hallman, 1971; Nakazawa *et al.*, 1973) as well as of the present study imply that foetal rat liver mitochondria require a threshold ATP concentration to increase their R.C.I. from about 1.4 to 2.6 and nearly double their state-3 respiratory rate. Therefore the foetal liver is faced with the difficult task of providing its mitochondria with sufficient ATP before they can themselves contribute effectively to the ATP production of the hepatocyte.

The results of the present investigation are compatible with a scheme which suggests that ATP synthesis and accumulation by mitochondria is

'triggered' by an extrauterine stimulus resulting from parturition.

On this basis it may be proposed that in foetal liver the mitochondria are not functional with respect to oxidative phosphorylation, owing to hypoxia. Under hypoxic conditions the foetal mitochondria, when isolated, are leaky, as indicated by the results of the osmotic experiments (Fig. 1), the atractyloside-insensitive ADP-stimulated respiration (Fig. 3b) and the Ca^{2+} -induced uncoupled respiration (Fig. 6a); these mitochondria have also only a marginal R.C.I. (Table 3).

The permeability of the inner mitochondrial membrane in the prenatal liver is considered to be essential, as the adenine nucleotide translocase is only an exchange system which cannot lead to any increase in adenine nucleotides within the mitochondria. As the adenylate charge increases (i.e. ADP and ATP increase at the expense of AMP), facilitated by adenylate kinase, which has low but sufficient activity in perinatal rat liver (Filler & Criss, 1971), the ATP content of the cell increases to a point where it affects the mitochondria so that their inner membranes become impermeable to adenine nucleotides. This may be confirmed *in vitro* by the addition of ATP to foetal mitochondria, conferring atractyloside-sensitivity to their state-3 respiration (Fig. 3c). Hence in the newborn the rate of oxidative phosphorylation increases as the adenylate charge increases.

The effect of ATP is thought to be due to an ATP-membrane interaction, rather than to the provision of energy by hydrolysis, as AMP-P(NH)P, which cannot be hydrolysed, substitutes for ATP (Fig. 5), and GTP, which can act as substrate for foetal mitochondrial ATPase, has no effect on foetal mitochondria (Fig. 5). ADP can replace ATP only at considerably higher concentrations (20-fold), presumably because it then cumulatively gives rise to sufficient ATP by oxidative phosphorylation or adenylate kinase action.

The cessation of the maternal blood-sugar supply makes it imperative for the neonatal rat to have a highly efficient energy-conversion system. The proposed mechanism of mitochondrial maturation requires only non-functional mitochondria, which contain all the necessary enzymes for oxidative phosphorylation, as well as a 'trigger', provided possibly by O_2 , to set off an autocatalytic or positive-feedback mechanism, involving ATP production.

The timing of the increased adenylate charge owing to the mitochondrial ATP formation from ADP is apt, occurring just before the synthesis of phosphoenolpyruvate carboxykinase, which is the last enzyme required for gluconeogenesis to commence (Yeung & Oliver, 1967). In addition the high adenylate charge limits glycolysis by inhibiting phosphofructokinase (Atkinson, 1966).

Finally it should be pointed out that foetal mito-

chondria may be used with considerable advantage for the study of the mechanism of oxidative phosphorylation, as these mitochondria have a permeable inner membrane, which may be rendered impermeable either by the addition of ATP or by a short period of maturation of the foetal rat.

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