Adenine Nucleotides in Foetal Rat Liver Cells

COMPARTMENTATION AND VARIATION WITH AGE

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The digitonin method for the separation of cytosolic and mitochondrial fractions was applied to liver cells isolated from foetal rats. The cytosolic [ATP]/[ADP] ratio approximately doubles during the last 4 days of gestation, whereas the mitochondrial ratio remains constant. In the presence of oligomycin and added glucose, the cytosolic [ATP]/[ADP] ratio does not increase with age, but is still considerably higher than the mitochondrial ratio. Without added glucose, and when the glycogen content of foetal liver is still very low (more than 3 days before birth), the cytosolic [ATP]/[ADP] ratio in the presence of oligomycin becomes very low and equal to the mitochondrial ratio. It is concluded that the increase in the cytosolic [ATP]/[ADP] ratio during the last 4 days of gestation is solely due to enhanced mitochondrial activity in this period. Atractyloside and bongkreic acid do not influence the O_2 consumption, nor the [ATP]/[ADP] ratios in either compartment of foetal liver cells. Respiration of isolated foetal mitochondria, however, is strongly inhibited by both compounds. The implications of these findings are discussed.

Considerable uncertainty exists about the phosphorylation state of adenine nucleotides in foetal rat liver. [ATP]/[ADP] ratios ranging from 1.5 to 5.7 have been reported (Ballard, 1970, 1971*b*; Philippidis & Ballard, 1970; Hommes, 1971; Knowles & Ballard, 1974; Kraan & Dias, 1975). These data were obtained by using the freeze-clamp technique, and therefore represent the ratio for total intracellular adenine nucleotides. The freeze-clamp technique has a number of pitfalls (Faupel *et al.*, 1972), especially where mother and foetus have to be anaesthetized and/or killed. The availability of preparations of cells isolated from foetal liver provides a good alternative to determine adenine nucleotides under standardized conditions.

The use of digitonin for separating rat liver cells into mitochondrial and cytosolic fractions within seconds has been described by Zuurendonk & Tager (1974*a,b*). This method offers the possibility of determining both metabolite concentrations in the two compartments and concentration gradients across the mitochondrial membrane [see, e.g., Zuurendonk *et al.* (1976) and Siess & Wieland (1976)]. These parameters had been estimated previously by the metabolite-indicator method (Williamson, 1969; Greenbaum *et al.*, 1971).

In the study reported in the present paper, the digitonin method was applied to foetal liver cells of different gestational ages to determine the adenine nucleotide content and the [ATP]/[ADP] ratio in the cytosolic and mitochondrial compartments under

defined conditions. A preliminary account of this work has appeared (Van Lelyveld & Berger, 1977).

Experimental

Except for experiments not related to foetal age, foetal ages were determined by dated matings. Male and female rats (200–250 g, Wistar, TNO strain) were caged overnight; the next day was regarded as day zero. Foetal age was also read from a curve of body weight against day of gestation (Gonzalez, 1932). Good agreement was observed between the two methods.

Foetal rat liver cells were prepared as described by Hommes *et al.* (1971), with some variations. One to four pregnant rats, each carrying 7–12 foetuses, were decapitated, the foetuses were quickly removed, weighed and decapitated, and their livers were rapidly removed. The foetal livers were cut into small pieces in 100 ml of a medium containing 3 mM-NaCl, 5 mM- KH_2PO_4 , 0.5 mM- $MgCl_2$, 24 mM- $NaHCO_3$, 2 mM-glucose, 5 mM-EDTA, 230 mM-sucrose and lysozyme (50 mg/100 ml; EC 3.2.1.17); the pH was 7.0. The livers were then incubated at 37°C for 30 min with vigorous shaking under O_2/CO_2 (19:1) and subsequently pressed very gently through two nylon cloths of mesh widths of 500 and 100 μm respectively. The cells were harvested by centrifugation at 50 *g* for 2 min, and washed three times at room temperature in a Krebs-bicarbonate buffer (Krebs & Henseleit,

1932), containing glucose in the amount indicated, but no Ca^{2+} . Total cell yield was 30–110 mg of protein, depending on foetal age and number of pregnant rats used.

Immediately after isolation, the cells (2–7 mg of protein/ml) were incubated in 25 ml vials in the Krebs-bicarbonate buffer supplemented with 2.5% dialysed bovine serum albumin. The vials were gassed with O_2/CO_2 (19:1) and the incubation temperature was 37°C. Samples were taken after a preincubation of at least 10 min.

During the washings separation occurs between parenchymal and haematopoietic cells, the latter remaining for the most part in suspension during the low-speed centrifugations. Throughout preparation, incubations and sampling, plastic apparatus was used.

The method for cell fractionation was as follows. A 13 ml polypropylene centrifuge tube was loaded with 1 ml of 1 M- HClO_4 , 4 ml of silicone oil (Wacker Chemie G.m.b.H., München, Germany) consisting of a mixture of types AR 20 and AR 200 (9:11, v/v). The upper layer consisted of 1.5 ml of a medium containing 0.25 M-mannitol, 3 mM-EDTA, 20 mM-Mops (4-morpholinepropanesulphonic acid) and, unless stated otherwise, 4.1 mM-digitonin (Merck, Darmstadt, Germany); the final pH was 7.0. The tube was kept at 0°C. A portion (1.5 ml) of the cell suspension was rapidly mixed with the upper layer by means of a Gilson Pipetman P 5000 with bent disposable tip, thus ensuring good mixing without contaminating the upper layer with silicone oil. Then 5–10 s after sampling, the tube was centrifuged for 30 s at 5000 g in a Janetzki centrifuge, model T32c. The time elapsed between sampling and fixation of the digitonin-treated cells never exceeded 15 s. Parallel with this separation an equal amount of cells was centrifuged without lysis in a second tube with silicone oil mixture AR 20/AR 200 (47:53, v/v) without the digitonin-containing upper layer. This tube was kept at room temperature (18–20°C) to avoid damage to cells occurring at lower temperatures.

The values obtained for whole cells minus those found in the digitonin-treated cell pellet were taken to represent the cytosolic compartment. In some cases samples of the supernatants were acidified with 6 M- HClO_4 , and, after removal of the denatured protein by centrifugation at 5000 g for 5 min, the pH was adjusted to 6.5–7 with 3 M-KOH plus 0.3 M-Mops. After removal of the silicone oil, the densely packed denatured protein in the pellet was resuspended by stirring and centrifuged again before neutralization. For the determination of marker enzymes the HClO_4 solution at the bottom of the tubes was replaced by 1 ml of 300 mM-sucrose. The calculation of relative enzyme activities in supernatant and pellet was performed either by direct measurement in both fractions (after treatment of the pellet fraction with Triton X-100), or by subtraction of values obtained from

the supernatants from those derived from whole cells plus medium in the presence of 1% Triton X-100.

Protein was measured by the method of Lowry *et al.* (1951), by using bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard. Cell suspension (50 μl) was mixed with 3 ml of water containing 2 mg of deoxycholate (Merck) and sonicated in a Branson sonifier for 10 s at 0°C. Deoxycholate did not interfere with the protein determination.

Metabolites were determined by standard enzymic procedures (Williamson & Corkey, 1969; Bergmeyer, 1970) on either an Eppendorf fluorimeter or an Aminco dual-wavelength spectrophotometer. Enzyme activities were measured as described by Bergmeyer (1970). Citrate synthase activity was measured as described by Ochoa (1955). Atractyloside was obtained from Sigma and dissolved in water. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide was a gift from Dr. P. G. Heytler (Dupont, Wilmington, DE, U.S.A.). Aurovertin was a gift from Professor Dr. J. M. Tager (University of Amsterdam, The Netherlands). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide, aurovertin and oligomycin were dissolved in ethanol. The final concentration of ethanol in the cell suspension did not exceed 0.15 M. Bongkreic acid was a gift from Professor W. Berends (Technical University, Delft, The Netherlands). The original preparation in 0.5 M- NH_3 was freeze-dried and redissolved in 0.1 M- NaHCO_3 . All inhibitors were added 5 min before sampling unless otherwise stated.

Foetal rat liver mitochondria were prepared by the method of Hogeboom (1962). O_2 consumption of isolated mitochondria or liver cells was measured with a Clark oxygen electrode.

Results and Discussion

Preparation and viability of foetal rat liver cells

As perfusion of foetal rat livers is not feasible, a combined enzymic-mechanical dispersion method was chosen to isolate parenchymal cells. The use of lysozyme/EDTA gave the best results (cf. Hommes *et al.*, 1971). The foetal liver cell preparation isolated by this method contained a high percentage of viable cells as judged by exclusion of 0.2% Trypan Blue (at least 85% of the cells). The relative numbers of haematopoietic cells in the foetal liver cell preparation were 30, 20, 10, 10 and 5% at days 17, 18, 19, 20 and 21 of gestation respectively. With these data, and those obtained by Greengard *et al.* (1972), we calculated that the volume percentages taken in by haematopoietic cells in the foetal rat liver cell preparation were about 10, 7, 2, 2 and 0.5 on days 17, 18, 19, 20 and 21 of gestation respectively.

Leakage of lactate dehydrogenase after 5–120 min incubations amounted to $10.7 \pm 0.6\%$ (mean $\pm$ S.E.M.;

$n=25$) of the total cellular amount. Less than 5% of the total adenine nucleotides were present outside the cells, mainly as AMP. The cells were able to maintain cell-medium concentration gradients of more than 90 for glutamate and α -oxoglutarate, and of about 2 for alanine (R. Everts, unpublished work). The cells show only a slight stimulation of respiration on addition of 1 mM-succinate: in three experiments, the ratio O_2 uptake in the presence of succinate/ O_2 uptake in its absence was 1.5, 1.7 and 1.8 respectively (cf. Baur *et al.*, 1975; Tager *et al.*, 1973).

Concentrations of adenine nucleotides did not change more than 10% during a 1 h incubation. A 10 min incubation of the cells with 25 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone did not seem to damage the cells: the leakage of lactate dehydrogenase into the medium was 14.1% of the total amount at the end of incubation in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, compared with 12.7% without the uncoupler.

Digitonin method

The percentage of the total lactate dehydrogenase found in the supernatant rapidly increased with increasing digitonin concentrations, from 11% in the absence of digitonin to about 90% in the presence of 0.8 mM-digitonin. Further increases in digitonin concentration up to 4.1 mM did not lead to any further change in the amount of lactate dehydrogenase found in the supernatant. The supernatant contained maximally 12 and 6% of the total amounts of glutamate dehydrogenase (EC 1.4.1.3) and citrate synthase (EC 4.1.3.7) respectively. The high percentage of total lactate dehydrogenase recovered in the supernatant of centrifuged, digitonin-treated foetal liver cells demonstrates that virtually complete rupture of the plasma membranes had been achieved. The low percentages of mitochondrial enzyme activities in the supernatant, on the other hand, demonstrate that little damage had occurred to the mitochondria. The separation of cytosol and mitochondria seems therefore to be satisfactory. Fig. 1 shows that the [ATP]/[ADP] ratio in the digitonin pellet decreases with increasing digitonin concentration. A similar curve can be drawn for the sum of [ATP] and [ADP] in the pellet; the amount of these nucleotides progressively decreases from 12.7 to 1.7 nmol/mg of protein when the digitonin concentration increases from 0 to 4.1 mM. Thus 13% of the total ATP+ADP is present in the mitochondrial fraction of cells isolated from foetuses at the end of gestation, which is substantially lower than the percentages found in this fraction of adult liver cells, where values of 25–40% have been observed (Zuurendonk & Tager, 1974a; Elbers *et al.*, 1974). Part of this difference between liver cells from foetal

Fig. 1. Correlation between the digitonin concentration and the [ATP]/[ADP] ratio in the pellet of digitonin-treated foetal liver cells

For details see the Experimental section. Foetal age was 21 days after conception; [glucose]=2 mM. In some cases S.E.M. (indicated by the bar) was calculated. The other points denote single observations; the numbers of observations are shown in parentheses.

and adult rats may be explained by the smaller volume occupied by the mitochondria in the foetal liver compared with the adult (Vergonet *et al.*, 1970; Herzfeld *et al.*, 1973).

Table 1 illustrates the distribution of adenine nucleotides in the various fractions. The total amount of nucleotides is reasonably constant on fractionation by digitonin: a slight increase is found on treatment with digitonin (112%), whereas centrifugation of whole cells yields a somewhat lower amount of adenine nucleotides (88%).

The contribution of adenine nucleotides present in the cell nuclei (which constitute a large part of the volume in foetal liver cells; Vergonet *et al.*, 1970) was neglected, as nuclei seem to lack any permeability barrier for these metabolites (cf. discussion in Siess & Wieland, 1976). Essentially no glucose 6-phosphate was found in the digitonin pellet.

[ATP]/[ADP] ratio in isolated foetal rat liver cells as a function of the age of the foetus

In Table 2 the [ATP]/[ADP] ratio in liver cells is shown at different gestational ages. At all ages, [ATP]/[ADP] ratios in foetal rat liver cells are generally higher than those reported by others for freeze-clamped foetal livers (Ballard, 1970, 1971b; Philippidis & Ballard, 1970; Hommes, 1971; Knowles & Ballard, 1974; Kraan & Dias, 1975). The large variation in [ATP]/[ADP] ratios (1.5–5.7) observed by these

Table 1. *Distribution of adenine nucleotides between supernatants and pellets of digitonin-treated cells and whole cells*
Foetal liver cells (foetal age 22 days) were fractionated as described in the Experimental section. As a reference, contents of adenine nucleotides in untreated, non-centrifuged cells are included (cells+medium). Mean values $\pm$ S.E.M., expressed as nmol/mg of protein, are shown, the numbers of samples being given in parentheses.

	Untreated cells				Digitonin-treated cells		
	Cells+medium	Pellet	Supernatant	Pellet+supernatant	Pellet	Supernatant	Pellet+supernatant
ATP	11.64 (2)	9.57 $\pm$ 0.54 (4)	<0.03 (4)	9.60	0.65 $\pm$ 0.04 (4)	12.10 $\pm$ 0.34 (4)	12.75
ADP	1.77 (2)	1.68 $\pm$ 0.10 (4)	0.07 $\pm$ 0.04 (4)	1.75	0.55 $\pm$ 0.02 (4)	1.30 $\pm$ 0.06 (4)	1.85
AMP	0.38 (2)	0.56 $\pm$ 0.11 (4)	0.20 $\pm$ 0.02 (4)	0.76	0.38 $\pm$ 0.02 (4)	0.53 $\pm$ 0.06 (4)	0.91
ATP+ADP+AMP	13.79 (2)	11.81 $\pm$ 0.48 (4)	0.30 $\pm$ 0.02 (4)	12.11	1.58 $\pm$ 0.04 (4)	13.93 $\pm$ 0.44 (4)	15.51

Table 2. *[ATP]/[ADP] ratios in foetal rat liver cells*
Rat liver cells, isolated from fetuses at different gestational ages, were incubated in the absence or presence of 10mm-glucose, and centrifuged as described in the Experimental section. Results are means $\pm$ S.E.M. for the numbers of experiments given in parentheses. Significance was calculated by Student's *t* test for comparison of two groups.
* *P* < 0.05 against day 18; ** *P* < 0.01 against day 17.

Age (days after conception)	[ATP]/[ADP] ratio	
	-Glucose	+Glucose
17	—	3.48 $\pm$ 0.66 (4)
18	4.46 $\pm$ 0.98 (5)	4.79 $\pm$ 0.83 (4)
19	4.82 $\pm$ 0.55 (5)	4.89 $\pm$ 0.47 (7)
20	5.18 $\pm$ 0.61 (6)	5.01 $\pm$ 0.37 (4)
21	6.18 $\pm$ 0.54 (7)*	6.20 $\pm$ 0.32 (10)**

authors probably stems from two factors: (1) the age dependency of this ratio, as shown in Table 2, and (2) the technique of freeze-clamping a foetal liver *in situ*, necessitating anaesthesia of the mother, which may affect the [ATP]/[ADP] ratio in the foetus.

As shown in Table 2, the [ATP]/[ADP] ratio is not influenced by addition of 10mm-glucose. This is consistent with previous observations (Berger & Hommes, 1974) that added glucose contributes little to the glycolytic flux in isolated foetal liver cells when glycogen is present. When glycogen is virtually absent (day 17) (Devos & Hers, 1974; Watts & Gain, 1976) and no glucose is added, no viable liver cells can be prepared.

[ATP]/[ADP] ratios in the mitochondrial and cytosolic compartments of isolated foetal rat liver cells as a function of the age of the foetus

In Table 3 the [ATP]/[ADP] ratio in the mitochondrial and cytosolic compartments as determined

by the digitonin method is shown as a function of the age of the foetus. The mitochondrial [ATP]/[ADP] ratio does not change with age, irrespective of the presence of 10mm-glucose, whereas the cytosolic [ATP]/[ADP] ratio approaches, with increasing age, the value observed in adult liver cytosol (Zuurendonk & Tager, 1974a,b; Siess & Wieland, 1976). Here, also, addition of glucose (Table 3) does not lead to significant changes in the [ATP]/[ADP] ratio in either compartment.

When oligomycin is added to the cell suspension, however, glucose affects the cytosolic [ATP]/[ADP] ratio. Table 3 shows that at day 17 the cytosolic [ATP]/[ADP] ratio is hardly influenced by oligomycin. This means that at this early stage, glycolysis plays a major role in the energy supply of the foetus. On the other hand, it should be noted that the addition of oligomycin causes a large Pasteur effect; the increased glycolytic flux becomes apparent in the lactate production, which is enhanced by a factor of 2 (at all ages studied) after a preincubation of 10min followed by 5min in the presence of oligomycin (cf. Berger & Hommes, 1974).

Table 3 also shows that the age-related increment in the cytosolic [ATP]/[ADP] ratio is not observed in the presence of oligomycin, which demonstrates that the increase of the cytosolic [ATP]/[ADP] ratio is solely due to enhanced mitochondrial activity (cf. De Vos *et al.*, 1968; Vergonet *et al.*, 1970; Jakovcic *et al.*, 1971; Herzfeld *et al.*, 1973).

In the absence of added glucose (Table 3), and when the glycogen content in foetal liver is low, i.e. before day 20 (Devos & Hers, 1974; Watts & Gain, 1976), the cytosolic [ATP]/[ADP] ratio in the presence of oligomycin becomes very low and equal to the mitochondrial ratio. Previous investigations (Berger & Hommes, 1975) have shown that towards the end of gestation the glycolytic flux increases, as shown by lactate production. However, the present results lead to the conclusion that in the early stages

Table 3. *Compartmentation of adenine nucleotides in foetal rat liver cells as a function of foetal age*

Rat liver cells, isolated from foetuses at different gestational ages, were incubated for 15 min in the absence or presence of 10 mM-glucose. After 10 min of incubation, oligomycin (1 µg/ml) was added where indicated. For details of the digitonin fractionation see the Experimental section. Results are means $\pm$ S.E.M. for the numbers of experiments given in parentheses. Significance was calculated by Student's *t* test for comparison of two groups. * 0.025 < *P* < 0.05 against day 18; ** *P* < 0.01 against day 17.

Age (days after conception)	[ATP]/[ADP] ratio							
	Cytosol				Mitochondria			
	-Glucose	+Glucose	-Glucose +oligo- mycin	+Glucose +oligo- mycin	-Glucose	+Glucose	-Glucose +oligo- mycin	+Glucose +oligo- mycin
17	—	4.53 $\pm$ 0.75 (5)	—	3.54 (3)	—	1.09 $\pm$ 0.19 (5)	—	0.33 (3)
18	7.13 $\pm$ 1.09 (4)	6.38 $\pm$ 1.14 (4)	0.68 (2)	2.10 (1)	0.86 $\pm$ 0.16 (4)	0.92 $\pm$ 0.10 (4)	0.59 (2)	0.17 (1)
19	6.93 $\pm$ 0.90 (5)	7.44 $\pm$ 0.88 (7)	2.17 (1)	2.88 (3)	0.75 $\pm$ 0.06 (5)	0.81 $\pm$ 0.08 (7)	0.20 (1)	0.22 (3)
20	7.19 $\pm$ 0.93 (6)	8.12 $\pm$ 0.79 (4)	2.22 (1)	—	1.13 $\pm$ 0.15 (6)	0.91 $\pm$ 0.11 (4)	0.20 (1)	—
21	11.84 $\pm$ 1.80* (6)	10.81 $\pm$ 0.89** (10)	1.42 (1)	3.60 (2)	1.00 $\pm$ 0.07 (6)	1.20 $\pm$ 0.07 (10)	0.17 (1)	0.36 (2)

Table 4. *Effect of some inhibitors on cytosolic and mitochondrial [ATP]/[ADP] ratios in foetal rat liver cells*

For details see the Experimental section. Foetal age was 20.5–22.0 days after conception. Mean values $\pm$ S.E.M., expressed as nmol/mg of protein, are given for the numbers of different cell preparations in parentheses. [Glucose]=2 mM; [Digitonin]=0.81 mM. Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

Inhibitor(s) added	Concn.	Concn. (nmol/mg of protein)					
		Cytosol			Mitochondria		
		ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
None		8.78 $\pm$ 0.77 (6)	1.21 $\pm$ 0.04 (6)	7.3	0.97 $\pm$ 0.07 (6)	0.70 $\pm$ 0.07 (6)	1.4
Atractyloside	0.12 mM	8.89 $\pm$ 0.43 (6)	1.58 $\pm$ 0.18 (6)	5.6	1.10 $\pm$ 0.12 (6)	0.72 $\pm$ 0.06 (6)	1.5
Oligomycin	1 µg/ml	5.74 (3)	1.85 (3)	3.1	0.36 (3)	1.17 (3)	0.3
Oligomycin+atractyl- oside		4.31 (3)	2.31 (3)	1.9	0.32 (3)	0.63 (3)	0.5
FCCP	25 µM	6.23 (2)	2.43 (2)	2.6	0.42 (2)	1.02 (2)	0.4
FCCP+atractyloside		7.32 (1)	2.14 (1)	3.4	0.70 (1)	1.14 (1)	0.6
Aurovertin	50 µg/ml	5.13 (1)	2.04 (1)	2.5	0.40 (1)	0.94 (1)	0.4
Aurovertin+atractyloside		5.05 (1)	2.13 (1)	2.4	0.35 (1)	0.85 (1)	0.4

of gestation glycolysis is largely responsible for the energy supply in the foetal liver cytosol, but from day 17 until birth, at day 22, the 'adult system', i.e. oxidative phosphorylation, almost completely takes over despite the increase in glycolytic capacity (cf. Woods & Krebs, 1971).

It is suggested that the values of [ATP]/[ADP] ratios as presented here, obtained with well-oxygenated liver cells, actually represent values *in vivo*, although direct evidence is, of course, lacking. During the process of birth, which is accompanied by hypoxia (Ballard, 1971a), this would not apply.

Effects of various inhibitors on cytosolic and mitochondrial [ATP]/[ADP] ratios in foetal liver cells

In contrast with adult liver cells, foetal cells exhibit hardly any sensitivity towards inhibitors of adenine nucleotide translocation. Atractyloside, as shown in Table 4, has virtually no effect on either the cytosolic or mitochondrial [ATP]/[ADP] ratio in foetal cells, even when the inhibitor is present at 1.2 mM during a 10 min incubation with the inhibitor (results not shown). Addition of bongkreikic acid, albeit at a very high concentration, leads to a decrease

Table 5. Effect of bongkreikic acid on cytosolic and mitochondrial [ATP]/[ADP] ratios in foetal rat liver cells
For details see the Experimental section. Foetal age was 21–22 days after conception. Mean values $\pm$ s.e.m., expressed as nmol/mg of protein, are given for the numbers of different cell preparations in parentheses. [Glucose]=10 mM.

Inhibitor(s)	Concn.	Concn. (nmol/mg of protein)					
		Cytosol			Mitochondria		
		ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
None		11.06 $\pm$ 0.99 (4)	0.95 $\pm$ 0.18 (4)	11.6	0.88 $\pm$ 0.11 (4)	0.82 $\pm$ 0.20 (4)	1.1
Bongkreikic acid	1 mM	9.63 $\pm$ 1.52 (4)	1.52 $\pm$ 0.29 (4)	6.3	0.60 $\pm$ 0.04 (4)	0.62 $\pm$ 0.13 (4)	1.0
Bongkreikic acid + oligomycin	1 μ g/ml	4.83 (1)	1.61 (1)	3.0	0.48 (1)	1.02 (1)	0.5

in the cytosolic ratio (Table 5), but no change in the mitochondrial ratio occurs. No further changes were observed during a 15 min incubation with bongkreikic acid. Addition of either atractyloside or bongkreikic acid together with either oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine or aurovertin (Tables 4 and 5) does not lead to changes in cytosolic or mitochondrial [ATP]/[ADP] ratios compared with the situation where solely the uncoupler or the inhibitors of mitochondrial ATP synthesis are added. Respiration of isolated foetal mitochondria is inhibited by 74 and 83% by 20 μ M-attractyloside and 10 μ M-bongkreikic acid respectively, whereas respiration of foetal liver cells is not affected by either inhibitor. Moreover, on addition of bongkreikic acid to foetal cells, no Pasteur effect is observed.

Two possible explanations exist for these observations. The fact that the isolated foetal mitochondria respond swiftly to the inhibitors, whereas isolated foetal liver cells do not, could be due to a low permeability of the plasma membrane to atractyloside and bongkreikic acid. An alternative explanation could be derived from the work of Pollak (1975) and Sandor & Pollak (1976) who reported a maturation at about the time of birth of adenine nucleotide translocation in foetal rat liver mitochondria by the action of extramitochondrial ATP.

However, extramitochondrial ATP is present in isolated foetal liver cells, as shown in the present paper. Furthermore, foetal mitochondria were found to be sensitive to inhibition of adenine nucleotide translocation by either atractyloside or bongkreikic acid in the absence of added ATP. Such an alternative explanation is therefore unlikely.

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