Maturation in liver mitochondria of Ruthenium Red-sensitive calcium-ion-transport activity and the influence of glucagon administration *in vivo* and *in utero*

Veronica PRPIĆ and Fyfe L. BYGRAVE

Department of Biochemistry, Faculty of Science, Australian National University, Canberra, A.C.T. 2600, Australia

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The maturation of Ca^{2+} transport in mitochondria isolated from rat liver was examined, from 5 days before birth. The mitochondria used were isolated from liver homogenates by centrifugation at 22 000 g-min. Ca^{2+} transport by mitochondria isolated from foetal liver is energy-dependent and Ruthenium Red-sensitive. The transmembrane pH gradient in these mitochondria is higher by about 7 mV and the membrane potential lower by about 20 mV than in adult mitochondria. The inclusion of 2 mM- P_i in the incubation medium enhances the protonmotive force by approx. 30 mV. The rate of Ca^{2+} influx in foetal mitochondria measured in buffered KCl plus succinate is low until about 2–3 h after birth, when it increases to about 60% of adult values; approx. 24 h later it has reached near-adult values. Higher rates of Ca^{2+} influx are observed in the presence of 2 mM- P_i ; 3–5 days before birth the rates are about one-third of adult values and decline slightly as birth approaches. By 2–3 h *post partum* they have reached adult values. The inclusion of 12.5 μM -MgATP with the P_i stimulates further the initial rate of Ca^{2+} influx in foetal mitochondria. The rates observed are constant over the prenatal period examined and are 50–60% of those observed in adult mitochondria. Mitochondria isolated from foetal livers 4–5 days before birth retain the accumulated Ca^{2+} for about 50 min in the presence of 2 mM- P_i . In the period 2 days before birth to birth, this ability is largely lost, but by 2–3 h after birth Ca^{2+} retention is similar to that of adult mitochondria. The presence of 12.5 μM -MgATP progressively enhances the Ca^{2+} retention time as development proceeds until 2–3 h after birth, when it becomes less sensitive to added MgATP. Glucagon administration to older foetuses *in utero* enhances both the rate of mitochondrial Ca^{2+} influx assayed in the presence of 2 mM- P_i and the time for which mitochondria retain accumulated Ca^{2+} in the presence of 12.5 μM -MgATP and 2 mM- P_i . Its administration to neonatal animals leads to an increase in mitochondrial Ca^{2+} retention similar to that seen in adult mitochondria. The data provide evidence that the Ruthenium Red-sensitive Ca^{2+} transporter is potentially as active in foetal mitochondria 5 days before birth as it is in adult mitochondria. They also show that foetal mitochondria have an ability to retain accumulated Ca^{2+} reminiscent of mitochondria from tumour cells and from hormone-challenged rat liver.

Some energy-linked reactions in mitochondria isolated from early- or late-foetal liver (Mintz *et al.*, 1967; Hallman, 1971; Nakazawa *et al.*, 1973; Pollak, 1975; Bygrave & Ash, 1977) are known to be low in activity, a feature that has been attributed to the low endogenous adenine nucleotide content in mitochondria at this time. By only a few hours after birth the adenine nucleotide content has increased significantly (Ballard, 1971; Nakazawa *et al.*, 1973; Sutton & Pollak, 1978; Pollak & Sutton, 1980),

concomitant with an onset in mitochondrial maturation. The maturation of foetal mitochondria may be induced *in vitro* by preincubating them with MgATP (Hallman, 1971; Pollak, 1975; Bygrave & Ash, 1977) or by the intrauterine administration of glucagon to the foetus (Sutton & Pollak, 1980); this hormone attains a high serum concentration at birth (Girard *et al.*, 1977).

Previously we examined aspects of the development of mitochondrial Ca^{2+} transport in a popu-

lation of 'light' mitochondria, i.e. those that sediment at relatively high forces of centrifugation (see, e.g., Satav *et al.*, 1976), isolated from neonatal rat liver (Bygrave & Ash, 1977). However, we subsequently observed that it is the 'heavy' mitochondrial population in adult liver, i.e. that obtained by sedimentation at relatively low forces of centrifugation, that is enriched in the ability to transport Ca^{2+} (Bygrave *et al.*, 1978). Moreover, it is the 'heavy' mitochondrial population that undergoes sudden changes in development soon after birth (Pollak, 1977). We considered it necessary therefore to examine mitochondrial Ca^{2+} influx and retention during development in rat liver by using the procedure for isolating the 'heavy' mitochondrial population. Particular emphasis has been placed in this examination on the period of development from 5 days before birth to only hours after birth, as it is within this period that many of the factors known to influence mitochondrial maturation begin to exert their effects (see, e.g., Pollak & Sutton, 1980).

Experimental

Animals and glucagon administration

The animals used were rats of a Wistar albino strain. A breeding colony was established by housing two adult female rats with one adult male for an unrestricted period. Females were checked each morning by a vaginal smear for the presence of sperms. Females that conceived were then housed in separate cages. The day sperms were detected was determined as day 1 of a gestation period of 22 days (Reinhart & Bygrave, 1981). For the experiment with untreated fetuses one to three females, matched for the same stage of pregnancy, were killed by cervical dislocation and the fetuses removed. The crown-to-rump body lengths were measured as a double-check for an indication of relative maturity. It was generally found that crown-to-rump body lengths of 18, 24, 30, 36, 42 and 44–48 mm corresponded to -5, -4, -3, -2, -1 days before birth and birth respectively.

In the experiments involving glucagon administration into fetuses, laparotomies were performed, with diethyl ether employed as a general anaesthetic. A proportion of the fetuses received subcutaneous injections of $10\mu\text{g}$ of glucagon dissolved in $5\mu\text{l}$ of a mixture containing equal volumes of 1 mM-NaOH and tricaprillin oil (glycerol trioctanoate) through the uterine wall. Adoption of this experimental approach enabled the control and treated fetuses to be of the same age and subject to the same maternal influences. At 1 h after injection of glucagon the mother was killed by cervical dislocation and the fetuses were removed. The entire operation procedure took no longer than 10–15 min.

The neonatal age of rats was determined by

recording the time of birth. For single experiments the entire litter was used. When glucagon was injected the litter was split and half (usually six) of the neonatal animals were injected with $2.5\mu\text{g}$ of glucagon/g body wt. in $5\mu\text{l}$ of a mixture containing equal volumes of 1 mM-NaOH and tricaprillin. The remainder of the neonatal animals were injected in the same way with $5\mu\text{g}$ of the NaOH/tricaprillin mixture but without glucagon. Then 1 h later neonatal animals were killed by decapitation.

Preparation of mitochondria

The livers from foetal and neonatal rats, killed by decapitation, were rapidly removed and placed in ice-cold isolation medium containing 250 mM-sucrose, 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and 0.5 mM-EGTA adjusted to pH 7.4 with KOH. They were minced with scissors and homogenized by hand with ten passes in a Thomas size C glass homogenizer. The resulting suspension was made up to 14 ml and centrifuged at 900 g for 5 min in a Sorvall RC-2B refrigerated centrifuge fitted with an SS-34 rotor. The pellets were resuspended in sucrose/Hepes medium similar to the above but free of EGTA, and centrifuged at 800 g for 5 min. The combined supernatants were centrifuged at 4500 g for 5 min. The mitochondrial pellets were washed once with EGTA-free isolation medium by centrifugation at 4500 g for 5 min and resuspended in this at a protein concentration of 40–50 mg/ml. Preparation of adult rat liver mitochondria was as reported previously (Prpić *et al.*, 1978).

Measurement of Ca^{2+} transport

Ca^{2+} transport was measured by using techniques described by Reed & Bygrave (1974, 1975). The incubation medium contained, in a final volume of 2 ml, 230 mM-sucrose, 10 mM-KCl, 5 mM-Hepes, 5 mM-sodium succinate, $1\mu\text{M}$ -rotenone and 2 mM-potassium phosphate (when used). Mitochondria (2 mg of protein) were added and the mixture was allowed to preincubate for 1 min, except when MgATP was present. In that situation mitochondria were preincubated for 45 s without and 15 s with $6.25\text{--}37.5\mu\text{M}$ -MgATP as indicated. The final pH was 7.4 and the temperature 25°C . CaCl_2 ($100\mu\text{M}$, and containing $0.5\mu\text{Ci}$ of $^{45}\text{CaCl}_2$) was added rapidly to initiate the reaction. Transport of Ca^{2+} was terminated by transferring, at times indicated in the legends to Figures, $100\mu\text{l}$ of the incubation mixture into $100\mu\text{l}$ of an ice-cold quench medium (150 mM-KCl, 0.5 mM-EGTA and $2\mu\text{M}$ -Ruthenium Red) contained in Eppendorf centrifuge tubes. These were centrifuged to pellet the mitochondria, and $100\mu\text{l}$ of the supernatant was transferred to vials containing 10 ml of scintillation fluid (Prpić *et al.*, 1978). The ^{14}C channel of a Packard Tri-Carb scintillation counter

was used for counting the radioactivity of the sample. Measurements of the effect of metabolic and Ca^{2+} -transport inhibitors on foetal mitochondria were performed under conditions similar to those for Ca^{2+} -transport studies as described above, but the inhibitors ($1\mu\text{M}$ -valinomycin, $1\mu\text{M}$ -antimycin, $1\mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazone, $1\mu\text{M}$ -atractyloside, $1\mu\text{M}$ -oligomycin or $1\mu\text{M}$ -Ruthenium Red) were preincubated with mitochondria before initiation of Ca^{2+} transport. Carbonyl cyanide *m*-chlorophenylhydrazone-induced Ca^{2+} efflux from Ca^{2+} -preloaded mitochondria was performed by addition of 100 nM -carbonyl cyanide *m*-chlorophenylhydrazone to the incubation mixture.

Measurement of respiratory behaviour

Mitochondrial respiration was measured, with a Clark oxygen electrode, in a medium that contained, in a final volume of 2.5 ml , 50 mM -KCl, 100 mM -sucrose, 15 mM -Tris/HCl, 10 mM -potassium phosphate, 2 mM - MgSO_4 , 1 mM -EDTA, $1\mu\text{M}$ -rotenone and 2.5 mg of mitochondrial protein; the pH was 7.4 . ADP ($120\mu\text{M}$) was added to initiate State-3 respiration (Chance & Williams, 1956) with or without a previous addition of $1\mu\text{M}$ -atractyloside.

Protonmotive force

The protonmotive force (Δp) was determined by using the ion-distribution technique of Nicholls (1974). The incubation medium contained 150 mM -LiCl, 0.5 mM -KCl, 3 mM -Hepes (pH 7.4 with Tris), 5 mM -sodium succinate, $10\mu\text{M}$ - $^{86}\text{RbCl}$ ($0.12\mu\text{Ci/ml}$), $50\mu\text{M}$ - $[^{14}\text{C}]$ methylamine ($0.3\mu\text{Ci/ml}$), $50\mu\text{M}$ -sodium $[^3\text{H}]$ acetate ($1.2\mu\text{Ci/ml}$), $1\mu\text{M}$ -rotenone and $0.5\mu\text{M}$ -valinomycin. When used, 2 mM -sodium phosphate was added.

The scintillation fluid contained 6 g of butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole]/litre in toluene/2-methoxyethanol ($3:2$, v/v) containing 10% Biosolv BBS-3 (Beckman Instruments, Fullerton, CA, U.S.A.). Radioactivity was counted by using the three channels of the Packard Tri-Carb scintillation counter. Corrections were made for cross-over and background. The three discriminator control settings were such that cross-overs of 21 and 3.6% were allowed from the ^{32}P channel into the ^{14}C and ^3H channels respectively, and 26.8% was allowed from the ^{14}C and ^3H channels. The components of Δp were calculated exactly as described by Nicholls (1974). A limiting matrix volume of $0.4\mu\text{l/mg}$ of mitochondrial protein was used for the calculations, as assumed by Nicholls (1974) and Mitchell & Moyle (1969).

Chemicals

Unless indicated otherwise, all chemicals were of analytical grade. All radiochemicals were from The

Radiochemical Centre, Amersham, Bucks., U.K. Glucagon and puromycin dihydrochloride were from Sigma Chemical Co., St. Louis, MO, U.S.A.

Reproducibility

Unless indicated otherwise, all experiments were usually performed three times and sometimes more often. A particular problem was encountered in expressing the results in Fig. 2. As the rates of Ca^{2+} influx in foetal mitochondria, especially in the absence of added P_i , were quite low, the incubation temperature used in the assay was 25°C . To maintain an identical assay throughout development and because of the limited quantity of foetal material available, we chose to adopt the 25°C incubation conditions. Unfortunately, this limited the accuracy with which the initial rates of Ca^{2+} influx could be measured *post partum*, and *pre-partum* when MgATP was present *in vitro*. For this reason the influx measurements do not always represent true initial rates, and the difference between the rates of Ca^{2+} influx immediately *pre-partum* and those *post partum* may be greater than that shown in Fig. 2.

Results

Ca²⁺ transport by mitochondria during development of liver

The ability of mitochondria to transport Ca^{2+} has been shown to be very low or absent in the perinatal period of liver development (Nakazawa *et al.*, 1973; Pollak, 1975; Bygrave & Ash, 1977). In these studies mitochondria usually were obtained by centrifugation of the homogenate at relatively high forces of centrifugation. Because of the finding that 'heavy' mitochondria, obtained at relatively low forces of centrifugation, are enriched in their ability to transport Ca^{2+} (Bygrave *et al.*, 1978), it was necessary to re-examine the maturation profile of Ca^{2+} transport over the developmental period with the use of preparations of 'heavy' mitochondria.

Fig. 1 shows the pattern of Ca^{2+} influx and retention by mitochondria isolated from livers of 3h-old neonatal rats. Fig. 1(a) reveals that, under the experimental conditions employed, Ca^{2+} transport was enhanced in terms of both the rate of influx and attainment of the steady state when 2 mM - P_i or 2 mM - P_i plus $12.5\mu\text{M}$ -MgATP was included in the reaction medium. In these latter situations the mitochondria already had accumulated up to 80 nmol of Ca^{2+}/mg of mitochondrial protein as early as 10 s after the reaction was initiated.

Fig. 1(b) illustrates the pattern of Ca^{2+} retention by the same mitochondria when incubated in the presence of 2 mM - P_i and various concentrations of MgATP. In the absence of the metal-ion-nucleotide complex, release of Ca^{2+} occurs soon after the

Fig. 1. Ca^{2+} influx and retention by mitochondria isolated from livers of 3 h-old neonatal rats

Mitochondria were isolated from livers of neonatal rats as described in the Experimental section. Ca^{2+} transport was measured under the following conditions: 230 mM-sucrose, 10 mM-KCl, 5 mM-Hepes buffer (pH 7.4), 5 mM-sodium succinate, 1 μM -rotenone, 2 mg of mitochondrial protein and 200 nmol of $^{45}\text{Ca}^{2+}$. The temperature was 25°C and the final volume 2.0 ml. P_i (2 mM) was added with or without MgATP as indicated 15 s before the initiation of Ca^{2+} transport. Data shown are representative of a series of experiments that gave similar results. (a) Ca^{2+} influx: Δ , no additions; $\circ$, 2 mM- P_i present; $\bullet$, 2 mM- P_i plus 12.5 μM -MgATP present. (b) Ca^{2+} retention; $\circ$, 2 mM- P_i present; $\bullet$, 2 mM- P_i plus 6.25 μM -MgATP present; Δ , 2 mM- P_i plus 12.5 μM -MgATP present; $\blacktriangle$, 2 mM- P_i plus 25 μM -MgATP present; $\square$, 2 mM- P_i plus 37.5 μM -MgATP present.

Fig. 2. Ca^{2+} influx by rat liver mitochondria during development

Mitochondria were isolated from livers of rats of various prenatal and postnatal ages as described in the Experimental section. Ca^{2+} influx was measured under the following experimental conditions: 230 mM-sucrose, 10 mM-KCl, 5 mM-Hepes buffer (pH 7.4), 5 mM-sodium succinate, 1 μM -rotenone, 2 mg of mitochondrial protein and 100 nmol of Ca^{2+} /mg of mitochondria. The temperature was 25°C and the final volume 2.0 ml. P_i (2 mM) was added without or with 12.5 μM -MgATP to the incubation medium as indicated 15 s before the addition of the $^{45}\text{Ca}^{2+}$. Samples (100 μl) of the incubation medium were removed at 10 s intervals for 60 s (see Fig. 1a) in order to follow the progress of Ca^{2+} influx by the mitochondria. Data shown (see the Experimental section) are means $\pm$ S.E.M. for three or more independent experiments in which ten to 20 foetal livers or ten neonatal livers were pooled. The exceptions are values for adult mitochondria, for which livers were not pooled before the isolation of mitochondria, and data for 5-day-prenatal foetuses that represent a single experiment in which mitochondria were isolated from 35 pooled livers. Δ , No additions; $\circ$, 2 mM- P_i present; $\bullet$, 2 mM- P_i plus 12.5 μM -MgATP present.

mitochondria have accumulated 80–90 nmol of Ca^{2+} /mg of mitochondrial protein in about 10 s (cf. Fig. 1a). By approx. 8 min the mitochondria have released 50% of the Ca^{2+} originally accumulated. However, as the concentration of MgATP initially added is increased, a corresponding increase in

ability of the mitochondria to retain the ion is seen. The concentrations of MgATP required to enhance retention are much lower than those previously employed to enhance energy-linked reactions in foetal liver mitochondria (Hallman, 1971; Nakazawa *et al.*, 1973; Pollak, 1975; Bygrave &

Ash, 1977). Unless indicated otherwise, Ca^{2+} retention was assessed by measuring the time for which 50% of the accumulated Ca^{2+} is retained by the mitochondria. Also, in most of the experiments, $12.5 \mu\text{M}$ -MgATP was employed, where indicated.

Ca^{2+} influx during development

Data in Fig. 2 show that, in the absence of added P_i , the rate of Ca^{2+} influx, as measured by the amount of Ca^{2+} accumulated in 10s, is low in mitochondria isolated from foetal liver, but that by 2–3h after birth it increases significantly to be approx. 60% of adult values; approx. 24h later it has reached near-adult values (see comment at the end of the Experimental section on the limitations to the quantitative measurement of Ca^{2+} influx during development).

When 2 mM - P_i is included in the incubation assay medium, higher rates of Ca^{2+} influx are observed at all stages of liver development examined. However, the initial rates of mitochondrial Ca^{2+} influx in the prenatal period of development still are only a relatively small fraction (approx. 25–40%) of those observed in liver mitochondria from post-natal rats. A pronounced increase in Ca^{2+} transport occurs immediately after birth, as in the absence of added P_i ; by 2–3h *post partum* the rates of Ca^{2+} influx are similar to those observed in mitochondria isolated from adult liver.

The inclusion of $12.5 \mu\text{M}$ -MgATP (see above) with 2 mM - P_i in the incubation medium leads to a further

stimulation of Ca^{2+} influx in foetal mitochondria at all ages. These rates, however, though constant over the 5 days before birth, are about 50–60% of those seen in mitochondria isolated from adult rat liver.

Ca^{2+} retention during development

In recent years the ability of mitochondria to retain accumulated Ca^{2+} has been recognized as a potential site for the action of physiological stimuli that may influence the redistribution of intracellular Ca^{2+} (McIntyre & Bygrave, 1974; Dorman *et al.*, 1975; Prpić *et al.*, 1978; Hughes & Barritt, 1978, 1979; Bygrave, 1978; Taylor *et al.*, 1980; Prpić & Bygrave, 1980). In the present study experiments were conducted in which mitochondria were isolated from rat livers at the indicated stages of development and allowed to accumulate Ca^{2+} *in vitro*, and the time was determined for which accumulated Ca^{2+} was retained by the mitochondria. The experiments were conducted with $12.5 \mu\text{M}$ -MgATP either absent from or present in the incubation medium.

Data in Fig. 3 reveal that, at the earliest stages of development examined (4–5 days before birth), mitochondria exhibit a marked ability to retain accumulated Ca^{2+} that exceeds by several-fold that seen in adult mitochondria. [A similar amount of Ca^{2+} was taken up by the foetal mitochondria, although the rate of influx was lower (see Fig. 2), especially when MgATP was absent.] The presence of $12.5 \mu\text{M}$ -MgATP has little effect on such reten-

Fig. 3. Ca^{2+} retention in rat liver mitochondria during development

Mitochondria were isolated from livers of rats of various prenatal and postnatal ages as described in the Experimental section. Ca^{2+} transport was measured as indicated in Fig. 1(b) under similar experimental conditions. The temperature was 25°C and the final volume 2.0 ml . P_i (2 mM) was present in all reaction mixtures. Assays were performed without (O) and with (●) a preincubation of the mitochondria with $12.5 \mu\text{M}$ -MgATP for 15s before the addition of $^{45}\text{Ca}^{2+}$. The amount of Ca^{2+} initially taken up by the mitochondria at all stages of development was approx. 80 nmol of Ca^{2+}/mg of protein. The data represent means $\pm$ S.E.M. for three or more independent experiments, except for data with 5-day-prenatal foetuses, which represent a single experiment in which mitochondria were isolated from pooled livers of 35 foetuses. In other independent experiments mitochondria were obtained from pooling the livers of ten to 20 foetuses or livers from ten neonatal rats.

tion. As the foetal liver matures, a marked decline in ability of the mitochondria to retain Ca^{2+} occurs; in the period 2 days before birth to birth, Ca^{2+} retention is much lower than at any stage of development, but is still enhanced by the presence of MgATP in the reaction medium. By 2–3 h after birth, the ability of mitochondria to retain Ca^{2+} is the same as that in adult liver mitochondria. This retention is enhanced considerably by the presence of MgATP. However, as development proceeds further, retention progressively becomes less sensitive to added MgATP.

Characteristics of energy-linked reactions in mitochondria isolated from foetal rat liver

Effects of inhibitors and uncouplers of oxidative phosphorylation. As shown in Figs. 2 and 3, mitochondria isolated from livers of foetuses 3–4

days before birth or earlier exhibit a remarkable capacity for Ca^{2+} influx and Ca^{2+} retention. It was therefore desirable to examine some characteristics in these mitochondria of the Ca^{2+} -transport system and other energy-linked reactions.

In experiments, the results of which are not shown, it was found that more than 80 nmol of Ca^{2+} /mg of mitochondrial protein could be accumulated by the mitochondria within 10 s (see Fig. 1a) and retained for at least 20 min. This accumulation was blocked by approx. 75% when $1 \mu\text{M}$ -Ruthenium Red was also present in the reaction medium. Moreover, the presence of a range of compounds known to prevent the generation of ATP or the protonmotive force completely inhibited any Ca^{2+} -transport activity. Addition of $0.1 \mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazone to mitochondria that already had accumulated Ca^{2+} induced a rapid efflux of the ion. By 1 min after the addition of the

Table 1. Measurement of the components of the protonmotive force in mitochondria isolated from livers of rat foetuses 4 days before birth

The components of the protonmotive force were measured exactly as described in the Experimental section, by assuming a matrix volume of $0.4 \mu\text{l}$ /mg of mitochondrial protein. Data represent the means $\pm$ S.E.M. of eight values obtained from two independent experiments.

Condition of medium	Membrane potential (mV)	Transmembrane pH gradient (mV)	Protonmotive force (mV)
P_i absent	134.4 ± 0.6	89.4 ± 1.9	224.0 ± 2.3
2 mM- P_i present	166.0 ± 0.2	87.2 ± 2.1	253.4 ± 2.3

Fig. 4. Effect of glucagon administration *in utero* on the initial rate of Ca^{2+} transport in the mitochondria subsequently isolated from rat liver

Glucagon ($10 \mu\text{g}$ /foetus) was injected *in utero* into foetuses of 1-day-prenatal age. Then 1 h later the mitochondria were isolated from the foetal livers and the rate of Ca^{2+} transport (b) was compared with that in the mitochondria isolated from control-injected foetuses (a). Ca^{2+} transport was measured in a medium similar to that described for Fig. 2. The incubation temperature was 25°C . $\blacktriangle$, No addition; $\bullet$, mitochondria plus 2 mM- P_i ; $\circ$ and $\triangle$, mitochondria preincubated for 15 s with $12.5 \mu\text{M}$ -MgATP before the addition of Ca^{2+} with ($\circ$) or without ($\triangle$) 2 mM- P_i .

uncoupler, approx. 50% of the accumulated Ca^{2+} had been released.

Protonmotive force and respiratory behaviour. Data in Table 1 show values for the components of the protonmotive force in 4-day-prenatal liver mitochondria. The membrane potential (134 mV), measured in the absence of added P_i , is approx. 20 mV less than that in adult mitochondria, and the transmembrane pH gradient (89 mV) is slightly greater (cf. Prpić *et al.*, 1978). Thus the protonmotive force is approx. 10 mV less than that measured in adult mitochondria.

The presence of 2 mM- P_i in the incubation mixture induces an increase of some 30 mV in the membrane potential, but the anion has no significant effect on the transmembrane pH gradient. Thus the protonmotive force is also enhanced by approx. 30 mV by the presence of P_i .

Concomitant with these findings, mitochondria isolated from foetal liver consistently exhibited acceptor control ratios greater than 2. As well, stimulation of respiration by ADP could be prevented by the prior addition to the medium of low concentrations of atractyloside (results not shown).

Influence of glucagon administration in utero and in vivo on Ca^{2+} transport

Data in Fig. 4 show that administration of glucagon *in utero* to foetuses 1 day before birth leads to a marked increase in the rate of Ca^{2+} influx when P_i is included in the reaction medium. It was

observed consistently that only when P_i was present did the rate of Ca^{2+} influx increase after glucagon administration. In all other situations, i.e. with MgATP or MgATP plus P_i in the incubation medium, glucagon administration had little effect on Ca^{2+} influx.

The ability of glucagon to influence Ca^{2+} retention by mitochondria isolated from liver during different stages of liver development is shown in Fig. 5. Little enhancing effect of glucagon is seen with mitochondria isolated from livers of 3-day-prenatal foetuses. However, within a day of birth, and subsequently, glucagon administration *in utero* and *in vivo* leads to very pronounced increases in mitochondrial Ca^{2+} retention, similar to that seen in adult 'heavy' mitochondria (see Prpić *et al.*, 1978).

Discussion

Data in this paper reveal some hitherto undescribed features of energy-linked reactions in foetal mitochondria, especially of Ca^{2+} transport, and provide some clues as to their mode of control. The 'heavy' mitochondria (Bygrave *et al.*, 1978) employed in the present study are enriched in Ca^{2+} -transport activity and may be more susceptible to hormone action *in vivo* than are 'light' mitochondria (Prpić *et al.*, 1978; Hughes & Barritt, 1978; Blackmore *et al.*, 1979).

Two sets of experiments provided evidence for the functional integrity of the foetal mitochondria. The

Fig. 5. Effect of glucagon administration *in vivo* on Ca^{2+} retention by mitochondria subsequently isolated from rat liver. Glucagon ($10 \mu\text{g}/\text{foetus}$ or $2.5 \mu\text{g}/\text{g}$ body wt. of neonatal animal) was injected. Then 1 h later the mitochondria were isolated from the liver and Ca^{2+} retention was measured under the conditions described in Fig. 3. ● and ▲, Hormone-treated animals; ○ and △, control animals. (a) Mitochondria from 3-day-prenatal (○ and ●) or 2-day-prenatal (△ and ▲) animals. (b) Mitochondria from 1-day-prenatal (△ and ▲) or 1-day-old neonatal (○ and ●) animals. All were preincubated with 2 mM- P_i and $12.5 \mu\text{M}$ -MgATP for 15 s before the initiation of Ca^{2+} transport.

first was that preparations of the organelle, isolated as early as 4 days before birth, exhibited acceptor control ratios of at least 2; the succinate-supported respiration was responsive also to repeated additions of ADP. Addition of $1\mu\text{M}$ -atractyloside completely blocked the stimulation of respiration by ADP in 2-day-prenatal mitochondria, providing evidence for the existence of the atractyloside-sensitive adenine nucleotide translocase in these mitochondria at this early stage of development (see Pollak, 1975). The second set of experiments indicating the intactness of the mitochondria was that involving measurement of the protonmotive force (Table 1), which gave values remarkably similar to those obtained for intact adult rat liver mitochondria (cf., e.g., Nicholls, 1974; Bygrave *et al.*, 1978).

Results from influx experiments (Fig. 2), especially those obtained in the presence of $12.5\mu\text{M}$ -MgATP, provide strong evidence that the Ca^{2+} uniporter in rat liver mitochondria is fully capable of transporting Ca^{2+} as early as 5 days before birth. As in fully mature mitochondria, the system already is sensitive to low concentrations of Ruthenium Red and exhibits a complete dependence on the membrane potential for translocation of the ion, as judged by the effectiveness of inhibitors and uncouplers of oxidative phosphorylation. A further feature of Ca^{2+} transport in foetal mitochondria concerns the large degree of stimulation by exogenous P_i . The reason for this is unclear at present. Nakazawa *et al.* (1973) have reported that the concentration of endogenous P_i is similar in foetal and adult mitochondria, and Bygrave *et al.* (1978) could not detect any correlation between the endogenous P_i concentration and Ca^{2+} -transport ability in various mitochondrial fractions isolated from rat liver. On the other hand, the values for Ca^{2+} transport in foetal mitochondria measured in the absence of added P_i are similar to the values obtained when Ca^{2+} transport in adult mitochondria was performed in the presence of *N*-ethylmaleimide to block P_i transport (cf. Fig. 2 with Prpić *et al.*, 1978). It is possible that foetal mitochondria may lack the P_i -cycling system operating in adult mitochondria (see, e.g., Ramachandran & Bygrave, 1978).

In this regard it was of some interest that the transmembrane pH gradients in mitochondria isolated from livers 4 days before birth are practically identical in the presence and in the absence of added 2mM - P_i (Table 1), a situation that markedly contrasts with that in adult mitochondria. In the latter mitochondria, the transmembrane pH gradient is decreased some 20–50% on the addition of 2mM - P_i to the incubation medium (see Nicholls, 1974; C. Ramachandran & F. L. Bygrave, unpublished work). The behaviour seen in the foetal mitochondria would preserve the alkalinity of the

mitochondrial matrix on P_i addition, and may be relevant to the greatly increased Ca^{2+} retention times (Fig. 3; Prpić *et al.*, 1978).

Results from the retention studies indicate that liver mitochondria isolated as early as 5 days before birth are more resistant to the uncoupling action of Ca^{2+} than at any other time of development. Indeed, they may be even more resistant than hormone-treated mitochondria (cf. Prpić *et al.*, 1978) to such effects, and this is a feature reminiscent of the resistance seen in tumour mitochondria (McIntyre & Bygrave, 1974). It is noteworthy, however, that this resistance appears not to exist in liver mitochondria at 1–2 days before birth to anywhere near the same degree.

The possibility should not be excluded that the lower ability for Ca^{2+} retention at 1–2 days before birth (i.e. increased Ca^{2+} efflux) contributes to the lower rates of Ca^{2+} influx seen at this time.

A second major point revealed in the present work is that a marked enhancement in Ca^{2+} influx and retention occurs immediately after birth. This coincides with the marked enhancement in Ruthenium Red-insensitive (i.e. non-mitochondrial) Ca^{2+} transport (Reinhart & Bygrave, 1981). Data in Fig. 2 showed this was so in respect of Ca^{2+} influx in the absence and in the presence of P_i , and data in Fig. 3 showed this to be so for Ca^{2+} retention in the absence and in the presence of $12.5\mu\text{M}$ -MgATP. It is clear that by 2–3 h after birth Ca^{2+} -transport activity has a similar capacity to that seen in mature adult mitochondria.

Influence of MgATP

It has been reported previously that addition of 2mM -MgATP to foetal mitochondria induces an enhanced ability in these organelles to perform a range of energy-linked reactions (see Hallman, 1971; Nakazawa *et al.*, 1973; Pollak, 1975; Bygrave & Ash, 1977). In initial experiments in the present work it was established that concentrations of MgATP as low as $12.5\mu\text{M}$ could induce prolonged retention of Ca^{2+} in foetal mitochondria (Fig. 1b).

MgATP produced different effects on the initial rates of Ca^{2+} influx and Ca^{2+} retention, and these also depended on the stage of mitochondrial development (Figs. 2 and 3); the substance had little effect at 4–5 days before birth. From 3 days before birth to birth its enhancing effects were much more pronounced. By 2–3 h after birth, $12.5\mu\text{M}$ -MgATP no longer enhanced the rate of Ca^{2+} influx (Fig. 2), yet it was precisely at this stage of development that it had its most pronounced effects on Ca^{2+} retention (Fig. 3). These latter effects diminished gradually, so that in the adult this concentration of MgATP had little effect on Ca^{2+} retention. Although these stimulatory effects of MgATP are produced when the compound is added as late as 15 s before the

start of the reaction, the mechanisms involved are almost completely unknown. However, it is clear from the above that MgATP may have at least two sites of action on the mitochondria that induce alterations that lead to an enhancement of mitochondrial Ca^{2+} transport, at least when incubated with neonatal mitochondria for brief periods. Whether these effects have any physiological significance remains to be assessed.

Influence of hormones

In the present work, glucagon administration *in vivo* had two major effects on Ca^{2+} transport in mitochondria subsequently isolated from livers of perinatal rats. The earliest time at which an enhancing effect of glucagon on the initial rate of Ca^{2+} transport was reproducibly observed was about 1 day before birth (Fig. 4). At this stage of development the effect was confined to the incubation system that included only 2 mM- P_i . The possibility exists that this in turn arises from a stimulation by glucagon of P_i transport in these mitochondria, as seen with adult rat liver mitochondria (Barritt *et al.*, 1978).

The other major action of glucagon administration *in vivo* was on mitochondrial Ca^{2+} retention, and this, like the effect on Ca^{2+} influx, was most pronounced at 1 day before birth. Only slight but significant enhancements were seen at an earlier stage of development (Fig. 5). By 1 day after birth the response of mitochondrial Ca^{2+} retention to glucagon administration was similar to that observed with adult mitochondria (cf. Prpić *et al.*, 1978; Hughes & Barritt, 1978).

Hormones other than glucagon may also be involved in the maturation process in the liver. For instance, catecholamines secreted at birth can initiate the maturation of energy transduction in foetal rat liver mitochondria (Sutton & Pollak, 1980). On the other hand the plasma concentration of glucocorticoids rises rapidly at birth (Dupouy *et al.*, 1975). They are thought to be responsible for the changes in liver before birth such as the disappearance of erythropoietic cells and appearance of glycogen synthesis (see Greengard, 1971). Relevant to this are reports that glucocorticoids and catecholamines *in vivo* can lead to changes in the ability of mitochondria to transport Ca^{2+} (Kimura & Rasmussen, 1977; Hughes & Barritt, 1978, 1979; Taylor *et al.*, 1980).

The question arises as to the possible nature of the mechanism(s) responsible for the maturation of mitochondrial Ca^{2+} transport, especially at the time of birth. Although it is perhaps premature at present to speculate in detail on this, we are cognizant of the merit and relevance to our work of the postulate by Sutton & Pollak (1980) (see also Pollak & Sutton, 1980) that the actions of adrenaline and glucagon

acting through cyclic AMP affect glycogenesis and glycolysis, giving rise to transient localized high concentrations of ATP. This ATP then initiates mitochondrial maturation through its action as a molecular trigger. Of particular significance to the present work is that the greatest rate of change in the mitochondrial adenine nucleotide content occurs during the first 2 h of the life of the neonatal animal (Sutton & Pollak, 1978). This coincides precisely with the greatest rate of change that we observe in mitochondrial Ca^{2+} influx and retention (Figs. 2 and 3) as well as with marked changes in the plasma concentration of glucagon and insulin (Girard *et al.*, 1977) and, as mentioned above, Ruthenium Red-insensitive Ca^{2+} transport (Reinhart & Bygrave, 1981). The importance of intramitochondrial ATP in the ability of the organelle to take up and retain Ca^{2+} is already widely recognized (see, e.g., Lehninger *et al.*, 1967; Bygrave, 1978).

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References

- Ballard, F. J. (1971) *Biochem. J.* **124**, 265–274
- Barritt, G. J., Thorne, R. F. W. & Hughes, B. P. (1978) *Biochem. J.* **172**, 577–585
- Blackmore, P. F., Dehaye, J. P., Strickland, W. G. & Exton, J. H. (1979) *FEBS Lett.* **199**, 117–120
- Bygrave, F. L. (1978) *Biol. Rev. Cambridge Philos. Soc.* **53**, 43–79
- Bygrave, F. L. & Ash, G. R. (1977) *FEBS Lett.* **80**, 271–274
- Bygrave, F. L., Heaney, T. P. & Ramachandran, C. (1978) *Biochem. J.* **174**, 1011–1019
- Chance, B. & Williams, G. R. (1956) *Adv. Enzymol. Relat. Subj. Biochem.* **17**, 65–134
- Dorman, D. M., Barritt, G. J. & Bygrave, F. L. (1975) *Biochem. J.* **150**, 389–395
- Dupouy, J. P., Coffigny, H. & Magre, S. (1975) *J. Endocrinol.* **65**, 347–352
- Girard, J. R., Ferré, P., Kervran, A., Pegorier, J. P. & Assan, R. (1977) in *Glucagon: Its Role in Physiology and Clinical Medicine* (Foa, P. P., Bajaj, J. S. & Foa, N. L., eds.), pp. 563–581, Springer-Verlag, New York, Heidelberg and Berlin
- Greengard, O. (1971) *Essays Biochem.* **7**, 159–205
- Hallman, M. (1971) *Biochim. Biophys. Acta* **253**, 360–372
- Hughes, B. P. & Barritt, G. J. (1978) *Biochem. J.* **176**, 295–304
- Hughes, B. P. & Barritt, G. J. (1979) *Biochem. J.* **180**, 291–295
- Kimura, S. & Rasmussen, H. (1977) *J. Biol. Chem.* **252**, 1217–1225

- Lehninger, A. L., Carafoli, E. & Rossi, C. S. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 259–320
- McIntyre, H. J. & Bygrave, F. L. (1974) *Arch. Biochem. Biophys.* **165**, 744–748
- Mintz, H. A., Yawn, D. H., Safer, B., Bresnick, E., Liebelt, A. G., Blailock, Z. R., Rabin, E. R. & Schwartz, A. (1967) *J. Cell Biol.* **34**, 513–523
- Mitchell, P. & Moyle, J. (1969) *Eur. J. Biochem.* **7**, 471–484
- Nakazawa, T., Asami, K., Suzuki, H. & Yukawa, O. (1973) *J. Biochem. (Tokyo)* **73**, 397–406
- Nicholls, D. G. (1974) *Eur. J. Biochem.* **50**, 305–315
- Pollak, J. K. (1975) *Biochem. J.* **150**, 477–488
- Pollak, J. K. (1977) *Biochem. Soc. Trans.* **5**, 341–348
- Pollak, J. K. & Sutton, R. (1980) *Trends Biochem. Sci.* **5**, 23–27
- Prpić, V. & Bygrave, F. L. (1980) *J. Biol. Chem.* **255**, 6193–6199
- Prpić, V., Spencer, T. L. & Bygrave, F. L. (1978) *Biochem. J.* **176**, 705–714
- Ramachandran, C. & Bygrave, F. L. (1978) *Biochem. J.* **174**, 613–620
- Reed, K. C. & Bygrave, F. L. (1974) *Biochem. J.* **140**, 143–155
- Reed, K. C. & Bygrave, F. L. (1975) *Anal. Biochem.* **67**, 44–54
- Reinhart, P. H. & Bygrave, F. L. (1981) *Biochem. J.* **194**, 541–549
- Satav, J. G., Katyare, S. S., Fatterpaker, P. & Sreenivasan, A. (1976) *Biochem. J.* **156**, 215–223
- Sutton, R. & Pollak, J. K. (1978) *Differentiation* **12**, 15–21
- Sutton, R. & Pollak, J. K. (1980) *Biochem. J.* **186**, 361–367
- Taylor, W. M., Prpić, V., Exton, J. H. & Bygrave, F. L. (1980) *Biochem. J.* **188**, 443–450