CELLULAR ENERGY METABOLISM DURING FETAL DEVELOPMENT

I. Oxidative Phosphorylation in the Fetal Heart

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INTRODUCTION

Mitochondria are known to be present during the early embryonic stages of a variety of organisms (1), including embryos of the toad *Xenopus laevis*, sea urchin eggs, as well as the embryo grasshopper *Melanoplus differentialis*. It has also been established by Mahler and his associates (2–4) and by others (5) that the early chicken embryo contains the mitochondrial enzymes required for aerobic oxidations. However, there have been few studies of the efficiency of phosphorylation coupled to mitochondrial oxidations during development of the mammalian fetus and no previous investigation of oxidative phosphoryla-

tion in the developing heart. This communication will describe studies of the efficiency of oxidative phosphorylation in mitochondria isolated from bovine fetal and chicken embryo hearts. The protective effect of bovine serum albumin on isolated fetal mitochondria will also be described.

METHODS

Bovine fetuses used in this investigation were obtained from a local slaughterhouse. Their fetal age was determined by measurements of crown-rump length and their weight by use of the scale reported by Winters et al. (6). The gestational age of the bovine fetuses ranged from 55 to 180 days. Hearts were also obtained from 6-wk-old calves. The full-term gestation of the cow is 270 days. Fertile White Leghorn chicken eggs were incubated to obtain 10-day chicken embryos, and the pooled hearts of 25 to 35 embryos were used in the experiments. Hearts from adult White Leghorn chickens were used as controls.

Mitochondria were isolated by a modification of the method of Chance and Hagihara (7) utilizing a bacterial protease (Nagarse). The hearts were rinsed several times in 0.25 M sucrose until the preparation was clear of blood and loose connective tissue. Heart weight was determined immediately after removal from the fetus, or the heart was gently blotted and weighed after rinsing in the sucrose solution. The hearts were finely minced with scissors before homogenization; or, in the case of the chicken embryo, the hearts were homogenized directly. 4 g of heart was the maximum weight used to prepare mitochondria from the large fetuses, and the smallest heart weight was 75 mg. After mincing, the hearts were suspended in a solution of 0.225 M mannitol, 0.075 M sucrose, 0.1 mM EDTA (MSE), and the suspension was poured into a Dounce glass homogenizer with a loose-fitting pestle. The sample was allowed to settle to the bottom of the homogenizer, and the supernatant was decanted as completely as possible. The protease was then added to the heart sample. This protease was prepared by dissolving 10 mg of Nagarse in 10 ml of the MSE and 0.1 ml of 1 M Tris base. 1 ml of the Nagarse solution per g of heart was added to the homogenizer; a minimum addition for small hearts was 1 ml. After addition of Nagarse, the heart sample was rapidly homogenized with five passes of the pestle and immediately diluted to 40 ml with MSE. The sample then was subjected to three or four additional passes in the homogenizer and was diluted further to a total volume of 80 ml. The suspension was then centrifuged at 7000 *g* for 10 min, and the supernatant containing the Nagarse was discarded. After being rinsed with 1–2 ml of MSE, the pellet was suspended in 80 ml of MSE and rehomogenized with two to three passes in the loose-fitting Dounce homogenizer. Centrifugation at 800 *g* for 10 min was carried out for removing the debris and nuclear fraction, and the resultant pellet was discarded. Mitochondria obtained by centrifugation of supernatant at 6000 *g* for 10 min were washed twice by suspension in 40 ml of MSE and centrifuged for 10 min at 6000 *g*. The final mitochondrial pellet was suspended in a small volume of MSE. The entire operation was carried out at 3°, and care was taken to avoid contamination with blood in the mitochondrial pellet. Mitochondrial yields obtained with the Nagarse were generally five to 10 times greater than those obtained by the procedure carried out without the Nagarse. Electron microscopic examination of mitochondria obtained by this method revealed a homogeneous mitochondrial preparation without contamination.

Oxidative phosphorylation was measured polarographically by the method of Chance (8). Measurements were carried out at 25° in a 1.5 ml reaction vessel containing 0.2 M sucrose, 50 mM Tris chloride, pH 7.5, 12 mM phosphate, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaCl, and 5 mM substrate (Na glutamate + Na malate or succinate). The respiratory control ratio was calculated as the ratio of ADP-stimulated respiration to the respiratory rate after ADP was exhausted. Crystalline bovine serum albumin was depleted of fatty acids by utilizing the method of Chen (9).

For electron microscopy, the whole heart of a 10-day chicken embryo was obtained and immediately placed in a prefixing solution of paraformaldehyde, cacodylate, and glutaraldehyde for 1 hr at 4° (10). The specimen was then rinsed in 0.1 M cacodylate buffer for 30 min at 4° and postfixed in OsO_4 in *s*-collidine buffer, pH 7.4, for 1 hr. For the adult control, a sample of left ventricle was obtained and

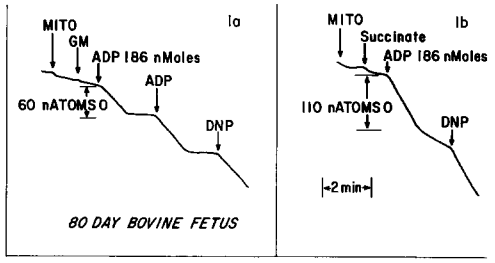

FIGURE 1 *a*, Polarographic tracing of fetal heart mitochondria with glutamate-malate (*GM*) as the substrate. Mitochondria were isolated from an 80-day bovine fetus. 0.6 mg of mitochondria was used in the experiment. Dinitrophenol (*DNP*) concentration was 2×10^{-5} M. Other conditions are as described in the text. *b*, Polarographic tracing of fetal heart mitochondria with succinate as the substrate. The conditions are the same as for Fig. 1 *a*.

immediately placed in 4% glutaraldehyde in phosphate buffer (11) for 1 hr at 4°. The specimen was kept overnight in phosphate buffer, pH 7.3, at 4°. Postfixation was carried out in 1% OsO_4 in phosphate buffer for 1½ hr at 4°. After fixation, the specimens were dehydrated in graded ethanols and embedded in Araldite 6005. Sections were cut with glass knives on an LKB microtome and were double stained with uranyl acetate and lead citrate or stained with lead citrate alone (12).

Protein was determined by a modification of the biuret method (13) and by the Lowry method (14). ADP was obtained from the Sigma Chemical Company, St. Louis, and the exact concentration was determined by using a μM extinction coefficient of 14.8 at 260 mμ. Nagarse was obtained from the Enzyme Development Corporation, New York, and other reagents were obtained from commercial sources.

RESULTS

Mitochondrial Yields and Oxidative Phosphorylation

A representative polarographic tracing with glutamate-malate and succinate as the substrate is shown in Fig. 1 *a* and 1 *b*, respectively. All preparations exhibited marked stimulation of oxidation with ADP and respiratory control values of over 5. Loss of respiratory control by the fetal mitochondria with dinitrophenol or dicumarol was the same as that observed with adult mitochondria. ADP/O ratios with succinate as the substrate showed no variation with age. Many values approached the generally accepted maximal value of 2 for succinate. When glutamate-malate was the substrate the values for the younger fetuses in the series equaled the maximal value of 3, the older fetuses giving somewhat lower ADP/O ratios of 2–2.3. As will be noted below, when bovine serum albumin was present in the assay medium, all values approached the generally accepted values. No age differences in oxygen consumption were noted with glutamate-malate or succinate as substrate.

Mitochondria isolated from the pooled hearts of 10-day chicken embryos had ADP/O ratios of 3.2 and 1.55 with glutamate-malate and succinate, respectively. These ratios were also similar to the ratios in the adult control. Respiratory rates of fetal chicken mitochondria with both substrates also approximated the values for the adult controls.

Mitochondrial yields from bovine fetal hearts and chicken embryo hearts were less than half of the yields of the adult controls. Average mitochondrial yields from fetal and adult cow hearts were 3.03 and 12.0 mg per g wet weight, respectively. The data on these yields for the chicken hearts were similar, with 4.84 mg per g wet weight and 14 mg per g wet weight for the embryo and adult samples, respectively.

Effect of Albumin on Oxidative Phosphorylation

Bovine serum albumin (BSA) restored to expected levels the efficiency of oxidative phosphorylation in heart mitochondria isolated from the older bovine embryos and the calf heart. As seen in Fig. 2 *a* and 2 *b*, albumin inhibited the endogenous rate of oxidative phosphorylation and also improved the respiratory control ratio from 5:1 to 9.4:1 when glutamate-malate was substrate. The ADP/O ratio of the calf heart mitochondria was restored to 3.0 from 2.4 following the addition of BSA to the assay medium. BSA inhibited the endogenous rate of oxidative phosphorylation of heart mitochondria isolated from both younger and older bovine fetuses, but did not affect the ADP/O ratio of mitochondria from young fetal hearts which already showed maximal efficiency. As noted above, ADP/O ratios with succinate as the substrate showed no variation with fetal age. However, if BSA was added to the assay medium, these values with succinate also approached the expected value of 2. No effect of

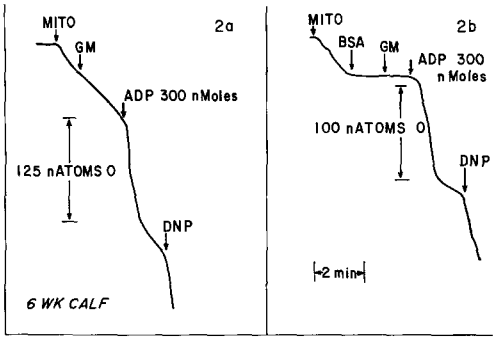

FIGURE 2 *a*, Polarographic tracing of 6-week-old calf heart mitochondria with glutamate-malate as substrate. 1.32 mg of mitochondria was used in the assay. Other conditions are as described in the text. *b*, Polarographic tracing of 6-week-old calf heart mitochondria with bovine serum albumin (5 mg of BSA) added to the assay. Other conditions are the same as for Fig. 2 *a*.

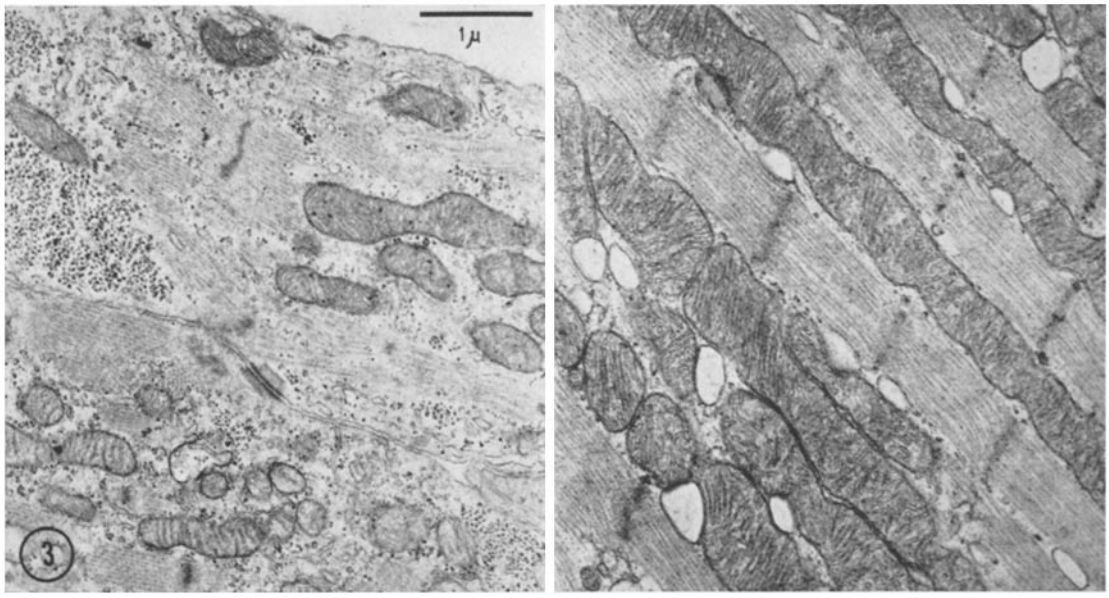

FIGURE 3. Electron micrograph of 10-day embryonic chicken myocardium (left) and of adult chicken myocardium (right). × 15,000.

albumin on the state 3 oxidative rate was observed in any of the specimens.

Fine Structure of the Fetal Heart

Fig. 3 compares the fine structure of heart obtained from a 10-day chicken embryo to that of heart from an adult chicken. In the fetal heart the mitochondria show no parallel arrangement with the myofibrils, which differs from the ordered arrangement seen in the adult heart. Also, the mitochondria in the fetal heart appear fewer in number than in the heart of the adult control. This is consistent with the lower mitochondrial yields from fetal heart as compared with the adult heart. Glycogen is more abundant in the fetal heart. The fine structure of the mitochondria in the fetal heart is similar to that of the mitochondria in the adult heart, but there is considerable irregularity in the size and shape of fetal mitochondria which may relate to the absence of an organizing effect of the mature myofibrils on mitochondrial morphology. The more characteristic parallel arrangement of mitochondria and myofibrils is seen in the adult heart. This picture of the ultrastructure of fetal chicken myocardium is consistent with previously reported studies of embryonic and fetal myocardium of the chicken and other species (15–17).

DISCUSSION

The enzymes of oxidative phosphorylation are known to be present in the early avian embryo (2–4). However, few studies of the functional efficiency of fetal mitochondria have been done, and those studies have been largely contradictory. Mitochondria isolated from newborn rat skeletal muscle have been reported to have a slower rate of oxidation and decreased P/O ratio compared to the adult controls (18). More recently, Henson (19) reported that with NADH-linked substrates the mitochondria isolated from 19-day avian embryonic muscle gave P/O ratios similar to those of the mitochondria of the adult, but that with succinate as substrate the mitochondria showed uncoupling. Mitochondria isolated from whole 5-day chicken embryos have been reported by Nowinski and Mahaffey (20) to have P/O ratios less than the expected values. Liver mitochondria isolated from fetal rats at 20 days of gestation exhibited P/O ratios similar to those of the adult control (21). Oxidative phosphorylation has also been investigated in the developing rat brain (22). In these studies, oxygen consumption and ADP/O ratios have been reported to increase with maturation of neonatal rats, 1–20 days old.

It has been established by the present investiga-

tion that mitochondria isolated from the fetal heart have the capacity for coupled oxidative phosphorylation with substrates entering both the succinate and NADH limbs of the respiratory chain. The P/O ratios with succinate approach the generally observed value of 2 in many of the samples. In the case of glutamate-malate, the values for the younger bovine fetuses in the series were closer to the expected value of 3 than were the values for fetuses over 100 days of gestational age. Those samples which gave a decreased P/O ratio with glutamate-malate showed no age variation with succinate as the substrate. The decreased ADP/O ratios observed with glutamate-malate as substrate may, therefore, reflect uncoupling or decreased stability of the first phosphorylation site in the older fetuses in the sample investigated. Albumin restores full coupling efficiency to mitochondria isolated from hearts of older bovine fetuses and from calf hearts. This effect of BSA may reflect restabilization of mitochondrial structure after the stresses of isolation, or BSA may be removing an endogenous uncoupler from the assay medium. As reported by others, albumin effectively binds fatty acids, thus preventing uncoupling of oxidative phosphorylation (23, 24). Therefore the albumin effect on fetal mitochondria may be due to an increase in fatty acid synthesis and to the presence of fatty acids in the older bovine fetal hearts.

Substrate requirements for the fetal heart have not been defined fully. It is recognized that anaerobic pathways such as the Embden-Myerhoff and the hexose monophosphate shunt play a major role in fetal metabolism (25–27). An impairment of fatty acid oxidation by fetal heart homogenates due to low levels of carnitine and carnitine acyl transferase has also been described by Wittels and Bressler (28). However, the results of the present investigation indicate that the final pathway of aerobic energy production by the fetal heart is functionally intact. Although the mitochondria appear well coupled in the present study, the relative contribution of energy production by aerobic and anaerobic pathways during fetal development is not known. The well recognized resistance of fetal and newborn heart to anoxia suggests that the Embden-Myerhoff pathway may be of considerable functional importance in the fetal heart.

The lack of organization seen in the electron micrograph of fetal chicken heart compared with the adult chicken heart (Fig. 3) suggests that maturation of the heart as previously suggested by Sippel (29) is paralleled by and may be primarily associated with structural integration of mitochondria with the myofibril and with an increase in mitochondrial number rather than with major developmental changes in energy-coupling pathways. As noted above, mitochondrial yields from fetal hearts were less than half the yields from the adult controls. Goldhor (30) has reported a higher lipid-to-protein ratio in fetal rat liver mitochondria that resulted in changing the sedimentation properties of fetal mitochondria during maturation. Therefore, the low yields of mitochondria from fetal heart observed in the present study may reflect both a decrease in mitochondrial number and a difference in the sedimentation properties of fetal mitochondria. Differences in the fragility of mitochondria isolated from the younger and older hearts may also be of importance.

Greenfield and Boell (31) have studied developmental changes in mitochondrial membrane isolated from chick heart and skeletal muscle. They find an increase in the amount of membrane per mitochondrion in the newly hatched chick as compared with that of the 9-day embryo. Thus, although there may be increased mitochondrial membrane with maturation, the present study indicates that heart mitochondria isolated from 10-day chick embryos exhibit functionally efficient oxidative phosphorylation.

SUMMARY

The efficiency of oxidative phosphorylation has been determined in mitochondria isolated from bovine fetal hearts of different gestational ages. With succinate as substrate, the ADP/O ratios approached the expected value of 2 throughout the gestational range. With glutamate-malate as substrate, the values in the older fetuses were lower than expected, but approximated the generally observed value of 3 in the younger fetuses in the sample. The addition of bovine serum albumin to the assay medium restored the ADP/O ratios with glutamate-malate to expected levels in the older fetal heart mitochondria. The improvement of ADP/O ratios in the older fetuses brought about by bovine serum albumin may reflect the binding of endogenous uncouplers of oxidative phosphorylation such as fatty acids. These data indicate that terminal aerobic energy production is functionally intact in the fetal heart

and that maturation is associated primarily with improved structural organization of mitochondria with myofibrils and with increased mitochondrial number rather than with changes in the efficiency of oxidative phosphorylation.

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