MORPHOLOGICAL AND BIOCHEMICAL STUDIES
OF ISOLATED MITOCHONDRIA FROM
FETAL, NEONATAL, AND ADULT LIVER
AND FROM NEOPLASTIC TISSUES

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ABSTRACT
A combined morphological and biochemical investigation of mitochondria from developing
and rapidly growing tissues (tumors, fetal, and very early neonatal rat liver) revealed mito-
chondria which were deficient in respiratory control, showed no valinomycin-induced K+
accumulation or spontaneous Ca++ uptake, and were unable to undergo a swelling-contrac-
tion cycle. Electron microscopic examination of fetal and neonatal livers and a mammary
tumor revealed mitochondria which differed from controls with respect to matrix density
and ability to undergo reversible structural changes. The importance of isolation and assay
media in interpretation of results is emphasized.

INTRODUCTION
Morphological and biochemical differences be-	ween mitochondria from some types of tumor
and from liver have been reported. Schneider and
Hogeboom observed diminished total nitrogen
content of hepatoma homogenates compared to
liver homogenates and suggested that the de-
ficiency resided in the mitochondrial fraction (1,
2). In further studies, these and other investigators
found that the tumors showed reduced activities of
succinoxidase, adenosine triphosphatase (ATP-
ase), and cytochrome oxidase, and an increase in
activity of NADH-cytochrome c reductase as com-
pared to liver tissues (3–5). In a more recent
investigation, Boxer and Devlin have demon-
strated that certain tumors lack specific “shuttle”
pathways for the transfer of NADH to mitochon-
dria (6). Allard et al. found that hepatoma mito-
chondria were smaller in size and number per cell
as compared to liver mitochondria (7). Yamamoto
et al. (8) and Miller and Goldfeder (9) reported
diminished rates of swelling of mitochondria iso-
lated from hepatomas.

The question arises whether some of these de-
scribed phenomena may be a common characteris-
tic of mitochondria from some tumors only or
from all rapidly growing tissues. This study, there-
fore, is designed to probe, in some depth, possible
functional and morphological similarities among
mitochondria from various tumors and fetal and
neonatal livers.

MATERIALS AND METHODS
Adult male rats (200–250-g) and 18–19-day preg-
nant rats of the Cheek-Jones strain (200-g) were
employed. Adult mice bearing transplanted tumors and untreated adult mice of the same inbred strains were obtained from the Kirschbaum Memorial Laboratory of Baylor University College of Medicine and from the Department of Radiology of the University of Texas M. D. Anderson Hospital and Tumor Institute. The designation, histological type, transplant generation, and latent period (time to reach 1.0-1.5 cm in size) are given in Table I.

All animals were killed by decapitation and bled. The liver or tumor tissue was removed and quickly washed in ice cold 0.25 M sucrose. Mitochondria were isolated in 0.25 M sucrose (with or without 1 mM EDTA) by the method of Schneider (10). The final pellet was suspended in cold 0.25 M sucrose in an approximate 1:1 ratio (original tissue weight to volume). The appropriate control tissue, either adult mouse or rat liver, was always fractionated simultaneously with the experimental tissue (fetal or neonatal rat liver and tumor). A number of different types of isolation media were employed with no significant differences in results. More recent studies, however, indicate that Mg++ and bovine serum albumin may be of importance in the assay procedure (manuscript in preparation). Some of the mitochondrial suspensions were stained with 0.01% Janus Green in 0.25 M sucrose and examined microscopically. This stain is relatively specific for mitochondria. The stained organelles exhibit a color transition from bluish-green to colorless to red (10 a).

Mitochondrial swelling was measured optically (using a Coleman Jr. or Spectronic 20 spectrophotometer at 520 m$\mu$) by the method of Tedeschi and Harris (11) as modified by Schwartz et al. (12), in a medium consisting of 20 mM Tris-HCl, 125 mM choline chloride ($\text{pH}$ 7.4), 3-6 mg of mitochondrial protein, water, and/or 1 mM Ca++. Protein was estimated by the Biuret procedure (13).

Valinomycin- and histone-induced K$^+$ transport were studied with a Beckman cationic electrode (No. 39137) connected to a Beckman research pH meter and a calibrated Sargent Polarograph, Model XV. The data were programmed and processed by the Computational Research Center of Baylor University College of Medicine. Spontaneous Ca$^{2+}$ flux was measured by means of Ca$^{2+}$-sensitive ion-exchange electrode (Orion Research Incorp., Cambridge, Mass.). The ion transport studies were usually carried out in the Tris-choline buffer, supplemented with inorganic phosphate, Tris-succinate, and/or Tris-glutamate. Tris-ADP for these studies was prepared by passing the disodium salt through a Dowex cation exchange resin (50W, H+X12).

Mitochondrial respiration was measured with a vibrating platinum electrode (Oxygraph, Gibson Medical Electronics, Middleton, Wis.). The ion transport studies were usually carried out in the Tris-choline buffer, supplemented with inorganic phosphate, Tris-succinate, and/or Tris-glutamate. Tris-ADP for these studies was prepared by passing the disodium salt through a Dowex cation exchange resin (50W, H+X12).

State 4 respiration is defined as the rate of oxygen consumption in the presence of substrate and absence of ADP; state 3 occurs when ADP is added (14). The temperature of all experiments was maintained between 23-25°C.

Mitochondria were examined by electron microscopy under three conditions: (1) in intact tissue, (2) in suspension in state 4 respiration, and (3) in suspension in state 3 respiration. In both intact tissue and suspensions, three groups were compared (maternal rat liver, fetal rat liver, and mouse mammary tumor). For each group, two specimens of intact tissue and three samples of each suspension were prepared; thus, a total of 24 specimens was examined by electron microscopy. Approximately five grids per specimen were studied.

Samples prepared for electron microscopic examination were taken as follows: 50-150 $\mu$g aliquots of freshly isolated mitochondrial suspensions and of suspensions in the oxygraph reaction chamber were centrifuged in a Coleman model 6-811 centrifuge (Herbach and Rademan, Incorp., Philadelphia).

### Table I

<table>
<thead>
<tr>
<th>Designation</th>
<th>Histological type</th>
<th>Transplant generation</th>
<th>Latent period</th>
</tr>
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<tbody>
<tr>
<td>C3H/f- S1</td>
<td>Fibrosarcoma</td>
<td>T-4, T-5, T-6</td>
<td>2</td>
</tr>
<tr>
<td>C3H/f- S2</td>
<td>Mammary carcinoma</td>
<td>T-3</td>
<td>4</td>
</tr>
<tr>
<td>CBA/S 170</td>
<td>Mammary carcinoma</td>
<td>T-11</td>
<td>7</td>
</tr>
<tr>
<td>DBA/2if 3709</td>
<td>Mammary carcinoma</td>
<td>T-1</td>
<td>8</td>
</tr>
<tr>
<td>C3H/f 2977</td>
<td>Facial-poorly differentiated carcinoma</td>
<td>T-54</td>
<td>4</td>
</tr>
<tr>
<td>Balb/c 39</td>
<td>Lung adenocarcinoma</td>
<td>T-160</td>
<td>4</td>
</tr>
<tr>
<td>CE 5649</td>
<td>Testicular carcinoma</td>
<td>T-95</td>
<td>2</td>
</tr>
<tr>
<td>C3H/f 2977</td>
<td>Hepatoma</td>
<td>T-5, T-6, T-7</td>
<td>8</td>
</tr>
</tbody>
</table>

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for 15 sec at 13,000 rpm. The specimens reached maximum rpm within 4 sec and came to rest within 5 sec. The polyethylene microcentrifuge tubes were then quickly removed. The tip bearing the mitochondrial pellet was cut off, split with a razor blade, and placed in cold 2.5% glutaraldehyde in phosphate buffer pH 7.2.

1-mm cubes of liver or tumor tissue, obtained immediately upon sacrifice of the animals, were also placed in cold 2.5% glutaraldehyde in phosphate buffer. From this point, all specimens (pellets and tissues) were treated identically. They were fixed for 2 hr and then washed and stored in the phosphate buffer at 4°C until final processing (up to 1 wk later). At that time, they were postfixed in 2% osmium tetroxide in cold veronal-acetate, pH 7.2, for 1 hr. The specimens were then dehydrated in increasing concentrations of ethyl alcohol and embedded in Ciba 502. They were polymerized for 12 hr at 35°C, 8 hr at 45°C, and 12 hr at 60°C. Sections were cut on a Porter-Blum ultramicrotome and were placed on carbon-coated 200-mesh grids. They were stained with lead citrate prior to viewing on an RCA EMU-3F electron microscope at 50 kv.

RESULTS

The tumor, neonatal, and fetal tissues used in this study consistently yield lower amounts of mitochondrial protein (per gram of original wet weight tissue) compared to control tissues (Fig. 1).

Mitochondria isolated from the rapidly growing tissues all undergo an increased rate of spontaneous swelling which approaches that of the Ca++-induced rate (Figs. 2 and 3). This swelling is incompletely reversed on the addition of ATP; ATP addition does not prevent swelling of the tumor or fetal mitochondria ("anti-swelling effect" of ATP) (15, 16). In control tissues, Ca++-induced swelling is significantly more rapid than spontaneous swelling; both types are usually partially or completely reversed by the addition of ATP ("contraction effect" of ATP); ATP effectively prevents swelling.

The mitochondrial preparations from the tumor, fetal, or early neonatal tissues appear to lack respiratory control (Figs. 4 and 5); i.e., the characteristic burst of respiration induced by ADP in normal mitochondria is absent, in the medium used for assay in the present study.

Valinomycin, a toxic antibiotic, in very low concentrations induces an accumulation of K+ under specific energy-dependent conditions (17, 18). A number of conditions or treatments reverse or prevent valinomycin-induced K+ influx. These include: absence of substrate, inhibitors of electron transport (antimycin A, rotenone, cyanide), in-
mitochondria are inactive with respect to spontaneous Ca\(^{2+}\) accumulation (Figs. 8 and 9), another property of normal mitochondria (20). It should be noted that the 18-24 hr neonatal liver yields mitochondria which do exhibit respiratory control (Fig. 5), swell and contract (Fig. 3), and transport K\(^+\) and Ca\(^{2+}\) (Figs. 7 and 9).

Electron microscopic examination of tissues taken from normal adult rat liver, normal fetal rat liver, and mouse mammary tumor reveals morphological characteristics as shown in Fig. 10 a-i. The adult mitochondria in situ exhibit the usual conformation of cristae and moderately

Inhibitors of oxidative phosphorylation such as oligomycin, and "true" uncoupling agents such as 2,4-dinitrophenol (Figs. 6 and 7). ADP in concentrations which produce a burst of respiration (state 4-3-4 transition) in control liver mitochondria causes a transient K\(^+\) efflux (after the valinomycin-induced influx), which is then followed by a reaccumulation of K\(^+\) to approximately the original level. This "cycle" appears to be characteristic of normal mitochondria (manuscript in preparation). Histone-induced K\(^+\) efflux, another energy-dependent characteristic of normal mitochondria (19), is also a parameter in the present studies (data not shown). None of the above phenomena related to K\(^+\) transport are, however, observed in the mitochondria derived from the rapidly growing tissues. Similarly, these

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Figure 3: Swelling and contraction of mitochondria from adult, fetal, or 1-day neonatal, and 2-4-day neonatal rat liver. The medium was the same as in Fig. 2. Spontaneous, ■; 1 mM CaCl\(_2\), ○. A, Adult rat liver mitochondria. B, Fetal or 1-day rat liver mitochondria. C, 2-4-day rat liver mitochondria.

Figure 4: Respiratory control of mitochondria from adult mouse liver and tumor tissue. Reaction chamber initially contained 2.5 mM glutamate and 2.5 mM phosphate in a total of 2 ml of the choline-Tris medium defined in Fig. 2. A, Adult mouse liver mitochondria. B, Testicular tumor mitochondria.

Figure 5: Respiratory control of mitochondria from adult, fetal, or 1-day neonatal, and 2-4-day neonatal rat liver. The medium was the same as in Fig. 4. A, Adult rat liver mitochondria. B, Fetal or 1-day rat liver mitochondria. C, 2-4-day rat liver mitochondria.

Figure 6: Potassium ion flux in adult mouse liver and tumor tissue. The medium contained 2.5 mM Tris-gluatmate, 2.5 mM Tris-phosphate, and 2.5 µg valinomycin in 8 ml of the choline-Tris medium (see Fig. 2). The following additions were made: 70 µM Tris-ADP, 2 µg rotenone, 2.5 mM Tris-succinate, 70 µM Tris-ADP. A, Adult mouse liver mitochondria. B, Mammary carcinoma 84 mitochondrial.
Suspendions of mitochondria fixed after 10-15 min of incubation in state 4 are shown in Figs. 11 a, 12 a, and 12 b. In each group (adult, fetal, and tumor) the in vitro mitochondria resemble those of intact cells except that in vitro they are uniformly round or oval and no elongated forms, as seen in the intact cells, are present. Most of these state 4 mitochondria exhibit loose matrices with narrow platelike or tubular cristae; this conformation is termed "orthodox" as described by Hackenbrock (21). The matrices of the mitochondria of both the rat fetal liver (Fig. 12 a) and mouse mammary tumor (Fig. 12 b) in general are less dense than those of the mitochondria of adult rat livers (Fig. 11 a). Tumor mitochondria in vitro (Fig. 12 b) are smaller than the mitochondria of adult and fetal rat liver preparations.

Suspensions of control mitochondria fixed during state 3 respiration are shown in Fig. 11 b. These adult liver mitochondria undergo a distinct morphological alteration when changing from state 4 to state 3 respiration. In state 3, their matrices are quite dense, the inner membrane shows marked infolding, and the volume of the outer compartment is apparently increased at the expense of the inner or matrical compartment (compare Fig. 11 b with 11 a). This appearance of state 3 mitochondria (Fig. 11 b) is termed "condensed" as described by Hackenbrock (21). Upon resumption of state 4 respiration (i.e., when the ADP is completely converted to ATP), mitochondria return to the orthodox conformation. Fetal

Figure 7 Potassium ion flux in adult, fetal, or 1-day neonatal, and 2-4-day neonatal rat liver. The conditions were the same as in Fig. 6. The additions were also the same as in Fig. 6. A, Adult rat liver mitochondria. B, Fetal or 1-day rat liver mitochondria. C, 2-4-day rat liver mitochondria.

Figure 8 Calcium ion flux in adult mouse liver and tumor tissue. The medium contained 2.5 mM glutamate, 1.25 mM succinate, and .5 mM phosphate in 10 ml of choline-Tris (see Fig. 2). A, Adult mouse liver mitochondria. B, Mammary carcinoma 3709 mitochondria.

Figure 9 Calcium ion flux in adult, fetal, or 1-day neonatal, and 2-4-day neonatal rat liver. The conditions were the same as in Fig. 8. A, Adult rat liver mitochondria. B, Fetal or 1-day rat liver mitochondria. C, 2-4-day rat liver mitochondria.
or tumor mitochondria in vitro do not exhibit any condensed forms when changing from state 4 to state 3 respiration. Their appearance under state 4 conditions, as shown in Figs. 12a and b, is unchanged on addition of ADP to the suspension.

DISCUSSION

The low mitochondrial yield from fetal, neonatal, and tumor tissues is in agreement with the results of Schneider and Hogeboom (2), Pigareva (22), and Kit and Griffin (23) which indicated that the number of mitochondria per unit weight of tissue is reduced in tumor tissue as compared to normal tissues. Furthermore, Ontko has reported that the total mitochondrial volume and nitrogen are decreased in embryonic and early neonatal rat livers (24).

The observations on the swelling of tumor mitochondria, however, differ from those reported by Miller and Goldfeder on both slowly growing epithelial cell tumors and rapidly growing spindle cell tumors (9). These investigators observed an initial low optical density and reported a low rate of spontaneous swelling of the tumor mitochondria. Isolation procedures were similar to those used in this laboratory. The discrepancy may be explained either by variability of swelling characteristics of mitochondria from different tumor types or more probably by the very rapid (almost instantaneous) rate of spontaneous swelling to a near "maximally swollen" state in mitochondria from some tumor types. The latter could be overlooked.

A relationship exists between functional and morphological activity of mitochondria. As suggested by Packer and others, ADP may be a key regulator of both morphological state and biochemical function (14, 25). Very recent electron micrograph studies by Hackenbrock (21) clearly demonstrate, e.g., a definite correlation between ultrastructure and specific metabolic states in purified mitochondrial suspensions. In the present study, the mitochondria of tumor, fetal, and early neonatal tissues all differed from those of adult controls with respect to ion transport, respiratory control, and the "swelling-contraction cycle." Moreover, mitochondria of mouse mammary tumor and rat fetal and neonatal livers lacked the ability to assume a condensed morphology. Studies of the mitochondria of other tumors are in progress to determine whether they, too, lack this ability.

Our findings support the hypothesis of Hackenbrock that respiratory control and ADP phosphorylation are dependent on conformational flexibility of the matrices and inner membranes of the mitochondria. The data further suggest that other characteristic activities of mitochondria, such as K+ and Ca++ transport, reversal of electron transport, and swelling and contraction, may similarly be interrelated through morphology.

The phenomenon of the ADP-stimulated "cycle" of K+ transport (extrusion and reaccumulation) in the valinomycin-treated mitochondria, which is currently being investigated in this laboratory, may involve a competition for high energy intermediates available for ion transport and for phosphorylation of ADP to ATP. This "cycle" as well as valinomycin- or histone-induced K+ extrusion is absent or "repressed" in the early neonatal, embryonic, and tumor mitochondria. Azzi and Azzone have shown that a loss of K+ in the presence of valinomycin is associated with a concomitant shrinkage of the mitochondrial membrane (26). Thus, there may be a relationship between the inability to reverse or prevent swelling in these mitochondria and their inability to transport K+. Similarly, reversible changes in the morphology of the mitochondrial membrane system may be essential to mitochondrial respiratory control.

Lack of Ca++ transport in mitochondria from the tumor tissues examined in this study is consistent with the recent work of Bygrave (27) whose data show that Ehrlich ascites tumor cells are unable to accumulate calcium, a factor which may

FIGURE 10  Electron micrographs of liver and tumor mitochondria, in situ. a, Control rat liver; portion of cytoplasm from hepatocyte. The mitochondria have tubular cristae and moderately dense matrices. × 25,000. b, Fetal rat liver; portion of cytoplasm from hepatocyte. Mitochondria have mixed tubular and plate-like cristae and loose matrices that appear less dense than in mitochondria of control liver (Fig. 10a). × 25,000. c, Mouse mammary tumor; portion of cytoplasm from tumor cell. Mitochondria have mixed tubular and plate-like cristae and loose matrices that appear less dense than in mitochondria of control liver (Fig. 10a). × 25,000.
Figure 11 Electron micrographs of liver mitochondria, in vitro. a, Control rat liver mitochondrial suspension, fixed after 15-min incubation in state 4. The majority of the mitochondria are similar to those seen in tissue (orthodox conformation). An occasional condensed form is present (arrow). X 25,000. b, Control rat liver mitochondrial suspension, fixed during state 3 respiration. All mitochondria have condensed morphological appearance. X 25,000.
FIGURE 12 Electron micrographs of liver and tumor mitochondria, in vitro. a, Fetal rat liver mitochondrial suspension, fixed after 15-min incubation in state 4. These mitochondria are markedly swollen, but most have an orthodox conformation. Their appearance was unchanged after addition of ADP to the suspension. X 25,000. b, Mouse mammary tumor mitochondrial suspension, fixed after 15-min incubation in state 4. These mitochondria have an orthodox conformation. Their appearance was unchanged after addition of ADP to the suspension. X 25,000.
contribute to their high rate of glycolysis. Reference is also made to some preliminary experiments which indicate that mitochondria isolated from the tumor cells accumulate calcium to a significantly lesser degree than mitochondria from rat kidney (27). Since calcium may be involved in the control of energy metabolism in mitochondria (20), the present results and those of Bygrave may have relevance to alterations in respiratory control. Other differences indirectly involving mitochondria from tumor and normal tissues have been reported. Boxer and Devlin, e.g. (6), found that malignant tissues were unable to oxidize NADH via “shuttle” systems. Busch (28) indicated that tumor mitochondria, in contrast to normal tissues, converted pyruvate primarily to lactic acid. Goldfeder and Selig (29) recently reported significantly lower levels of ATP and hexose monophosphate in rapidly proliferating tumors as compared to slowly growing tumors or normal livers. This may be of importance in considering deficiencies of respiratory control observed in mitochondria of rapidly growing solid tumors reported in the present study and other studies (30) as opposed to “normal” respiratory control and P:O ratios observed in mitochondria from other types of neoplastic tissues (5, 30–33). A phospholipid-dependent, Ca ++-activated ATPase has been found in sarcoma cell mitochondria, but not in mitochondria from heart, kidney, liver, or brain (34).

Of prime importance is the observation that 2-, 3-, and 4-day neonatal livers yield mitochondria which resemble those from the adult livers with respect to respiration, swelling, and ion transport. This might suggest that the mechanism(s) involved in these processes is (are) repressed in the embryonic tissues.

The interpretation of the data in this study may be complicated somewhat by the presence of considerable areas of extramedullary hematopoiesis in the fetal and neonatal livers. However, this population of hematopoietic cells has been shown to contain decidedly fewer mitochondria than the parenchymal cells and may thus be assumed to contribute a relatively insignificant amount of mitochondria to the total yield from these tissues (24, 35).

It should be emphasized that the results reported in this communication were obtained on isolated preparations under specific conditions of measurement. No attempt, therefore, to draw specific conclusions concerning in situ characteristics is made. Recent studies, e.g., indicate that Mg ++ and serum albumin included in the assay medium appear to reestablish respiratory control and K ++ transport in the mitochondria of late fetal and early neonatal liver (manuscript in preparation). “Normal” mitochondria, however, do not require the addition of albumin plus Mg ++. Some types of tumor mitochondria isolated in a sucrose medium containing serum albumin demonstrate respiratory control. Other mitochondrial characteristics in the presence of albumin have not yet been studied. These preliminary findings suggest the possible importance of serum albumin in obtaining functioning mitochondria from rapidly developing tissues. Possible diminution or absence of critical cofactors or enzymes is being considered (5).

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522 THE JOURNAL OF CELL BIOLOGY • VOLUME 34, 1967