Activity and Ultrastructure of Mitochondria from Mouse Mammary Gland and Mammary Adenocarcinoma 1

Charles W. Mehard, Lester Packer, and S. Abraham

Department of Physiology-Anatomy, University of California, Berkeley, California 94720 [C. W. M., L. P.]; Physiology Research Laboratory, Veterans Administration Hospital, Martinez, California 94553 [L. P., S. A.]; and The Bruce Lyon Memorial Research Laboratory, Children's Hospital Medical Center of Northern California, Oakland, California 94609 [S. A.]

SUMMARY

Mitochondria isolated from normal mammary glands taken from lactating and nonlactating C3H mice were compared with those isolated from mammary adenocarcinomas. Functional mitochondria could be obtained from the normal mammary glands only when milk was not present in the tissue prior to homogenization and when bovine serum albumin was added to the isolation medium. Albumin also protected tumor mitochondria from destructive factors released into the homogenate by disruption of the tumor cells.

Although normal mammary glands contained more mitochondria per g of tissue than did the mammary adenocarcinomas, no major differences were observed in ultrastructure, phosphorylation, rate of substrate oxidation, phosphate ion transport, passive swelling, or cytochrome content between functional mitochondria from normal and neoplastic tissues. These studies suggest that there is no impairment in the functioning of mitochondria in the mammary adenocarcinoma.

INTRODUCTION

From their studies of the conversion of the various carbons of lactate to carbon dioxide and fatty acids by normal mammary glands and by adenocarcinomas excised from lactating C3H mice, Abraham and Chaikoff (1) suggested that lactate utilization by the cancer was impaired. Since succinate oxidation by tumor slices was not lower than that observed with normal gland slices, these workers further localized this defect to the mechanisms involved in the entry of lactate carbon into the Krebs cycle at the level of pyruvate decarboxylation and the condensation of the resulting acetyl-CoA with oxaloacetate-forming citrate. As these reactions are restricted to the mitochondrial compartment of the cell, these investigators inferred that an impairment in the mitochondrial function exists in the adenocarcinoma prior to entry of pyruvate carbon into the terminal oxidative pathway.

Evidence for defective mitochondria in neoplastic tissue is not unique to mammary adenocarcinomas, for many investigators have demonstrated that mitochondria isolated from a variety of hepatomas exhibit a lower respiratory capacity than do mitochondria isolated from normal liver (5, 10, 15, 18, 25, 36, 45). Arcos et al. (4, 5), for example, reported reduced pyruvate and glutamate oxidation in mitochondria isolated from rat hepatomas induced by 3'-methyl-4-dimethylaminoazobenzene and, in addition, demonstrated that these particles from the neoplasms had reduced capacities for swelling compared to those isolated from normal rat livers. Some workers (32, 45) have suggested that mitochondria isolated from mammary tumors were less active than those from normal livers. However, for valid conclusions to be drawn, comparisons between tumors and normal tissues should be restricted to the tissue of origin, for it is well known that mitochondrial activity varies from tissue to tissue. Therefore, this report was restricted to a comparison between the activity and ultrastructure of mitochondria from mammary gland and from mammary adenocarcinoma.

Because the mammary gland is composed of a mixed population which contains both adipose and parenchymal elements, one must select a condition in which the parenchymal cells predominate. The most advanced stage of mammary gland development occurs during the lactational period, and it is during this time that parenchymal cells are most abundant. Thus we initially sought to isolate mitochondria both from the normal mammary gland during lactation and from a well-characterized mammary tumor. The separation of these intracellular particles from the lactating gland in a high state of purity proved to be difficult due to the presence of milk. Therefore, as additional controls a study was made of mitochondria isolated from the mammary glands of mice (a) in the prelactating condition (i.e., during the last trimester before parturition) and (b) in the lactating condition during which milk production in one-half of the glands of the animal was arrested by closing the nipples.

MATERIALS AND METHODS

Animals and Their Treatment

Mice. Female mice of the C3H strain were fed an adequate stock diet (Purina laboratory chow) and given water ad libitum. Mice of this strain are infected with the mammary tumor virus and show a high incidence of spontaneous adenocarcinomas (8) in both virgin and parous females.

Normal Mammary Tissues. Mammary gland tissue was excised from C3H mice following sacrifice by cervical
dislocation between the 15th and 18th day of lactation or the 15th and 20th day of gestation. The glands from lactating mice were removed immediately after the early morning suckling (average litter, 6 pups), or 2 hr after i.p. injection with 2 units of oxytocin (Pitocin, Parke, Davis & Co., Detroit, Mich.) to facilitate the removal of milk by suckling.

Some pregnant mice, 1 to 6 days before parturition, were isolated from the colony. All nipples on the left side of the mouse were closed by electrocautery, and the nipples on the right side were left undisturbed. The animals delivered their young normally and supplied sufficient milk to allow at least 6 pups to grow at a normal rate for 14 to 18 days. When the mice were sacrificed, the mammary glands on the right side of the animal were full of milk and had a normal appearance, whereas those on the left side were fully developed, were somewhat smaller than the undisturbed normal lactating gland, and were devoid of any milk-like fluid. This tissue is designated here as the nonsuckled gland taken from lactating mice.

Mammary tissues, unless otherwise specified, were placed immediately after removal from the animals into an ice-cold solution of isolation medium composed of 0.33 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl at pH 7.4, containing 1% BSA. Glands from nonlactating mice or from lactating, nonsuckling mice were homogenized in a Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle (0.015-in clearance) with 10 volumes of the isolation solution.

Mammary tissues from lactating mice were sliced with a McIlwain-Buddle tissue chopper (30) to a thickness of 0.4 mm and washed for 1.5 to 4 hr with constant stirring at 0° in several changes of the isolation solution until milk was no longer visible in the wash. At this time, the lactating tissue lost its white color and had a pink appearance. The washed slices were then blotted dry on filter paper, weighed, and homogenized in 10 volumes of the isolation solution as given above.

Mammary Neoplasms. Mammary tumors which arose in hyperplastic alveolar nodular outgrowths carried by virgin mice were selected at random from our colony (27). To provide sufficient material for our studies, these tumors were also propagated in C3H virgin mice by s.c. transplantation. The morphology of these tumors was typical of the mouse mammary adenocarcinomas described by Dunn (11). Tumor tissue, between 3 and 8 g, excised from the mice was freed of blood clots and necrotic areas, washed in ice-cold isolation medium, blotted dry on filter paper, minced, and homogenized with 10 volumes of fresh isolation medium containing 1% BSA as given above.

Mouse Milk. Samples of milk were obtained from mice which had lactated for 12 to 18 days in the following manner. Dams, after being separated from their pups overnight, were given injections of 2 units of oxytocin i.p. They were then milked with a pulsating suction apparatus, and the milk was collected in a vessel immersed in an ice bath. The milk was kept in the ice bath until used.

Rats. Male rats of the Long-Evans strain were used as a source of liver. They were fed Purina laboratory chow ad libitum and had free access to water. They were sacrificed by cervical fracture, and their livers were removed and immediately placed in ice-cold isolation medium without BSA. After the liver was washed free of blood, weighed, and minced, it was homogenized as described above with 10 volumes of isolation medium without BSA. All preparative procedures were carried out at 0°–4°.

Isolation of Mitochondria for Functional Studies

Immediately after preparation, the homogenates were centrifuged at 4,500 X g for 3 min which served to sediment cell debris, unbroken cells, and nuclei. The infranatant fraction was carefully removed from below the upper fat layer and subjected to centrifugation at 10,000 X g for 10 min, which sedimented the mitochondria. After removal of the supernatant fraction, the mitochondrial pellet was resuspended in 50 ml of isolation medium per 10 g of original tissue used. Resedimentation was accomplished by centrifugation at 8,700 X g for 10 min. This washing procedure was repeated, and the final twice-washed mitochondrial pellet was resuspended in a minimum of isolation medium, yielding a suspension containing between 20 and 30 mg of mitochondrial protein per ml.

Determination of the Amount of Mitochondria in Tissues

The total mitochondrial content of normal and neoplastic mammary gland tissues was determined by the following procedure. The tissues were weighed and homogenized in a loosely fitting Potter-Elvehjem tissue grinder with 10 volumes of 0.33 M sucrose-1 mM HCl-1 mM EDTA at pH 7.4. The resulting suspension was centrifuged at 700 X g for 10 min. The supernatant fraction was combined with a wash (10 to 1) of the 700 X g pellet and centrifuged at 8700 X g for 10 min. The mitochondrial pellet was then suspended in 5 volumes of medium and reisolated by centrifugation at 8700 X g for 10 min. Protein was determined by the biuret method of Gornall et al. (20). BSA was used as standard.

Respiratory Measurements

Oxygen utilization was monitored with a Clark electrode at 25° and automatically recorded (16). The assay medium consisted of 0.25 M sucrose, 10 mM Tris-HCl, and 10 mM sodium phosphate buffered at pH 7.4. The final concentrations of the sodium salt of the oxidizable substrate and ADP were 1.4 and 0.14 mM, respectively. Each assay contained 3.5 mg mitochondrial protein in a total volume of 3.5 ml.

Upon addition of substrate to the assay medium containing mitochondria, State 4 respiration (the resting state) was measured. After the addition of ADP, State 3 respiration (the active state) was recorded. RCR was calculated as the ratio between State 3 and State 4 and represents a measure of the coupling of oxidation and phosphorylation. ADP:O ratios were determined by calculating the μmoles of ADP added per μatom of oxygen consumed (7).

Cytochrome oxidase activity was measured by the enzymatic assay procedure of Schreiber et al. (43).
Measurements of Volume Changes

Active Swelling. Energy-dependent phosphate transport was measured photometrically as the change in percentage of transmission of unfiltered light, with the use of a tungsten lamp as light source at 25° in a medium consisting of 120 mM sucrose, 40 mM sodium phosphate, 5 mM Tris-HCl, 11 μg rotenone, and 4.4 mg mitochondrial protein per 5.5 ml of total volume at pH 7.8 (34). Upon the addition of 3.0 mM sodium succinate, the low-amplitude swelling response of the mitochondria was automatically recorded for periods up to 7 min.

Passive Swelling. Volume changes were measured at 520 nm with a Cary Model 14 recording spectrophotometer in 2 ml of medium containing 20 mM Tris-HCl at pH 7.4 and either 0.125 M KCl or 0.3 M sucrose and enough mitochondrial protein to give an initial absorbance reading between 0.5 and 0.6 at room temperature (about 22°). Swelling was initiated by the addition of 4 × 10^{-4} M sodium phosphate or 5 × 10^{-5} M digitonin to both the ionic medium and the sucrose medium and was measured for 60 min.

Determination of Cytochromes

Difference spectra (oxidized minus reduced) of mitochondrial preparations solubilized with deoxycholate were obtained by the method of Williams (49) with the Cary Model 14 spectrophotometer. Cytochrome concentrations were then calculated from these spectra (49).

Electron Microscopic Studies

Samples selected from 5 different areas of each tissue were fixed for 20 min at room temperature in a mixture of 1% formaldehyde and 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, by the procedures outlined previously (37). Mitochondrial suspensions were fixed in 2% glutaraldehyde (42). All samples were postfixed with 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4 (35), dehydrated in ethanol, washed in propylene oxide, and embedded in Spurr's low-viscosity embedding medium (46). Sections, prepared on an MT-2 ultramicrotome with a diamond knife, were mounted on uncoated copper grids and poststained with lead citrate (40) and uranyl acetate (48). These sections were examined in an RCA-EMU-3H electron microscope.

RESULTS

Mitochondrial Fine Structure in Intact Tissue. The ultrastructure of mitochondria in mammary glands of pregnant and lactating mice as well as in mammary adenocarcinomas was studied (Fig. 1). Mitochondria from different regions of all tissues when fixed in glutaraldehyde showed varying degrees of pleomorphism, as seen in cross-section, from sausage-shaped (Fig. 1, a and e) to ovaloid (Fig. 1, b, c, and f) to circular forms (Fig. 1, a to f). All structural forms occurred with regularity in the tissues examined. In both normal and neoplastic tissues, the inner and outer mitochondrial membranes were clearly evident. In addition, all mitochondria contained numerous cristae and had dark-staining matrices. We have therefore concluded that, when examined in situ, there are no apparent differences in ultrastructure of mitochondria between normal tissue, whether the gland was rapidly growing (pregnant) or in an actively secreting state (lactating), and neoplastic tissue.

Yields of Isolated Mitochondria. Mitochondrial yields of 7.7 mg of protein per g, wet weight, of lactating mammary gland tissue and 4.2 mg of protein per g, wet weight, of mammary adenocarcinoma tissue are averages of 9 and 6 separate experiments, respectively.

In order to obtain a more precise estimate of the comparative amounts of mitochondria in each tissue, we examined the cytochrome oxidase activities of lactating mammary glands and tumors by the enzymatic assay method of Schreiber et al. (43). In preliminary experiments, we showed that almost all of the cytochrome oxidase activity present in these tissues was contained in the mitochondrial fraction when these particles were isolated in the BSA-containing medium. In addition, we could demonstrate that the supernatant fractions obtained after centrifugation at 700 × g for 10 min yielded 50% more activity for this enzyme in the experiments with normal lactating mammary glands than in those with tumors. These findings were substantiated by inspection of electron micrographs of sections of these tissues, since the mammary gland cell sections contained almost twice the number of mitochondria observed in the tumor cell sections. These results are in agreement with previous reports that neoplasms contain fewer mitochondria than their normal cells of origin (2, 3, 18, 39, 43, 44).

Mitochondria Isolated from Mammary Glands of Lactating Mice. Mitochondria (from the mammary glands of mice that were actively lactating) isolated in medium containing 0.33 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl at pH 7.4, did not exhibit respiratory control nor did they show any osmotic swelling response when placed in 0.06 M KCl or 0.06 M sucrose. In addition, particles isolated with this medium failed to give the low-amplitude swelling which is characteristic of energized phosphate transport (Chart 1, Curve a).

Neither the use of a rapid isolation technique which involved the sedimentation of the mitochondria with the debris pellet and subsequent isolation of the mitochondria nor the addition of 2% BSA, from 1 to 10 mM EDTA or from 1 to 10 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid, provided a means for the isolation of mammary gland mitochondria which would exhibit good coupling of oxidation to phosphorylation. Electron micrographs showed that these mitochondria appeared swollen or broken and that such preparations contained few intact particles (Fig. 2a). Because intact mitochondria were observed in situ (Fig. 1a) prior to homogenization, it was apparent that damage occurred during the isolation procedure. It seemed possible that damage was due to the high concentration of milk still present in the tissue which would readily mix with mitochondria after disruption of the gland. Therefore, an attempt was made to remove as much of the milk as possible before homogenization by washing thin slices of the tissue.

Mitochondria isolated from homogenates of washed slices exhibited energized ion transport only when isolated in the...
presence of BSA (Chart 1, curves c and d). However, respiratory control and ADP:O ratios were low (Table 1). Ultrastructural examination showed contracted and swollen mitochondria (Fig. 2b) even when the isolation medium contained BSA. Mitochondria were not much better than those obtained from the unwashed tissues (Fig. 2a).

Mitochondria Isolated from Mammary Glands of Pregnant Mice. To determine whether the presence of milk in the gland prevented the isolation of intact functional mitochondria, we made an examination of the activity of these particles isolated from a gland which was almost fully developed but which did not produce milk. Therefore, mitochondria were isolated from the glands obtained from pregnant animals between the 15th and 20th day of gestation.

These mitochondria when isolated in the presence of 1% BSA showed energized phosphate transport (Chart 1, Curves e and f) and oxidized substrates at a faster rate than those from the lactating glands. They also showed higher ratios for respiratory control and ADP:O than those of lactating tissue (Table 1). Intact, contracted mitochondria (Fig. 2c) with a dark-staining matrix similar to normal liver mitochondria (Fig. 2d) were obtained from this tissue when taken from the pregnant animal.

Effect of Mouse Milk on Mitochondrial Integrity. For a test of the hypothesis that damage to mitochondria resulted from the interaction with milk, the effect of mouse milk on isolated mitochondria was examined. Since rat liver mitochondria are easy to isolate in a good functional condition and are thoroughly characterized, they were chosen as the test system. When milk was added to the assay medium used to measure respiratory control, as little as 1 μl/mg mitochondrial protein produced a rapid decrease in control (Chart 2). In addition, State 4 respiration was completely uncoupled after only a 5-min exposure of the liver mitochondria to milk (Chart 2).

To demonstrate that milk damage to mitochondria is not restricted to liver, its effect on mitochondria isolated from the glands of pregnant mice was examined. To this end, an investigation was made of the effects of milk on energized phosphate transport of rat liver and pregnant mammary gland mitochondria. Complete suppression of oscillation and reduction in the initial swelling response (Charts 3 and 4) of both types of mitochondria suggested that milk not only produced an inhibition of the respiratory-linked ion transport but also caused a structural alteration. These findings were

Table 1
Respiratory activity of mitochondria isolated from mammary glands of pregnant and lactating mice

Mitochondria were assayed for oxygen utilization as given in the text. The substrate was 1.4 mM sodium succinate, and ADP was added to give a final concentration of 0.14 mM. The figures reported below are averages of 3 experiments each.

<table>
<thead>
<tr>
<th>State of: Mouse</th>
<th>Tissue</th>
<th>Treatment of tissue</th>
<th>Activity</th>
<th>$Q_{O_2}$</th>
<th>ADP:O</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactating</td>
<td>Lactating</td>
<td>None</td>
<td></td>
<td>20</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lactating</td>
<td>Lactating</td>
<td>Washed, 4 hr</td>
<td></td>
<td>82</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Lactating</td>
<td>Nonlactating</td>
<td>None</td>
<td></td>
<td>92</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>Pregnant</td>
<td>Nonlactating</td>
<td>None</td>
<td></td>
<td>77</td>
<td>1.4</td>
<td>2.5</td>
</tr>
</tbody>
</table>

*a Nanoatoms oxygen/min/mg mitochondrial protein.
*b μmoles of ADP added per μatom of oxygen consumed during State 3.
*c Ratio between State 3 and State 4 respiration.
*d Glands taken from lactating mice, 14 to 18 days postpartum, in which the nipples on the left side had been closed by electrocautery 1 to 6 days before parturition. The normal, undisturbed nipples on the right side of the animals delivered enough milk to sustain at least 6 healthy pups per dam for the entire postpartum period.
supported by an examination of the ultrastructure of isolated rat liver and pregnant mammary gland mitochondria before and after exposure to milk (Fig. 2, c to f). Similar damage is seen in the mitochondria isolated from lactating mammary gland tissue (Fig. 2a). Although these latter sections contain a few mitochondria with contracted inner membranes and a typical dense matrix, most mitochondria exposed to milk exhibited varying degrees of swelling and disruption.

The importance of BSA in the isolation medium is emphasized from the results of experiments in which energized swelling by mitochondria was studied (Chart 4). Here the addition of BSA to the medium protected the mitochondria isolated from the glands of pregnant mice from the damaging effects of added milk.

Mitochondria Isolated from Mammary Glands of Lactating Mice in Which Nipples Were Closed by Electrocautery. During lactation, the gland has the greatest number of parenchymal cells, and therefore it is during this state that the tissue is best suited for comparative studies with the adenocarcinoma. However, the presence of milk in the gland at this stage of development does not permit the isolation of intact organelles. Thus, an attempt was made to obtain mitochondria from mammary glands of lactating mice which were devoid of milk. Closing the nipples on only one side of the animal before parturition made it possible to obtain this type of mammary tissue after parturition. Mitochondria isolated in a medium

Chart 2. Effect of mouse milk on succinate oxidation by rat liver mitochondria. Milk (1 μl/mg mitochondrial protein) was added to the reaction medium following the addition of mitochondria and incubated for the periods indicated. Assay medium and experimental conditions are given in text and Table 1.

Chart 3. Effect of mouse milk on rat liver mitochondrial phosphate transport. Mitochondria were equilibrated for 5 min with milk before the addition of 3 mM sodium succinate. The amount of milk added in each assay is given above each trace as μl milk/mg mitochondrial protein. Assay medium and conditions are given in the text.

Chart 4. The protective effect of BSA on prelactating mammary gland mitochondrial phosphate transport. When added, milk was present at 4 μl/mg mitochondrial protein and BSA at 0.1% final concentration. Traces were obtained from experiments with (a) mitochondria; (b) mitochondria + milk; (c) mitochondria + BSA, and (d) mitochondria + BSA + milk. Milk was added to the mitochondrial preparations in each assay medium 5 min before the addition of substrate to initiate volume changes.
Mammary Gland and Enocarcinoma Mitochondria

containing BSA from such tissue showed higher activities for succinate oxidation (Table 1) than those prepared from mammary glands of pregnant mice or from washed mammary glands of lactating mice. Phosphate transport and ultrastructural features of these particles obtained from the non-milk-containing glands from the lactating mice were also similar to those parameters observed from organelles from the mammary glands of pregnant mice.

Mitochondria Isolated from Mammary Adenocarcinomas. Only in the presence of BSA did mitochondria isolated from tumor tissue exhibit good respiratory control, show proper ADP:O ratios (Table 2), exhibit energized phosphate transport (Chart 5), and appear to have intact ultrastructure (compare Fig. 3d with Figs. 3e and 3f). This protective effect of BSA on tumor mitochondria seems to be related to a factor present in the postmitochondrial supernatant fraction for, when intact liver mitochondria (Fig. 3a) were added to this supernatant fraction and reisolated, they no longer exhibited control activities for \( Q_{O_2} \), ADP:O, and RCR (Table 3, Experiment C) and exhibited ultrastructural damage (compare Fig. 3a with Figs. 3b and 3c). The results of additional experiments with liver and tumor tissues shown in Table 3 led to the same conclusion. The initial rate of swelling exhibited by rat liver mitochondria reisolated from the postmitochondrial supernatant fraction of the tumor was reduced (Chart 6), a finding which strongly suggests damage.

A Comparison between the Properties of Mitochondria from Mammary Gland and Mammary Adenocarcinoma. When mitochondria were isolated from normal and neoplastic tissues under conditions which would yield intact particles, no major differences in the rate of substrate oxidation (Table 4), phosphorylation (Table 5), or phosphate ion transport (Charts 1 and 5) could be demonstrated between them. In addition, when mitochondrial swelling in KCl or sucrose was tested in the presence of digitonin and phosphate, no differences could

**Table 2**

<table>
<thead>
<tr>
<th>BSA</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( Q_{O_2} )</td>
</tr>
<tr>
<td>Absence</td>
<td>30</td>
</tr>
<tr>
<td>Presence</td>
<td>80</td>
</tr>
</tbody>
</table>

**Table 3**

<table>
<thead>
<tr>
<th>Tissue homogenized</th>
<th>Fraction added</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Liver mitochonria</td>
<td>( Q_{O_2} )</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

\( ^a \) Livers taken from Long-Evans rats fed on adequate stock diet ad libitum.  
\( ^b \) Unwashed rat liver mitochondria isolated as given in text.  
\( ^c \) Supernatant fraction obtained from mammary tumor homogenates centrifuged at 10,000 \( \times g \) for 15 min.  
\( ^d \) In this column, + indicates that 1% BSA was added to isolation medium as given in text; - indicates the absence of BSA.
be detected (Table 6). The cytochrome content of these intact organelles from normal and tumor tissues was determined (Table 7). No significant differences could be demonstrated. This latter finding is in accord with our observation of similar respiration rates between the normal and neoplastic mitochondria.

DISCUSSION

The examination of mitochondrial ultrastructure in situ revealed no differences between mitochondria in mammary gland and tumor tissue. Following isolation in sucrose medium, however, ultrastructural damage was observed, and the mitochondria had low respiration rates and exhibited no phosphate ion transport. Therefore an effort was made to develop a procedure that would yield functional particles by controlling the damage occurring during isolation. Intact particles were obtained from mammary tumors when BSA was added to the isolation medium. This is in agreement with reports in which BSA was used for isolation of mitochondria from tumors of other tissues (9, 10, 17, 26, 33, 36, 45). The rates of oxidation of succinate and glutamate by these particles is in agreement with the mammary adenocarcinoma mitochondrial oxidation rates reported by Sordahl et al. (45).

Isolation of functional mitochondria from the normal mammary glands of lactating mice was found to depend upon the removal of milk from the tissue, as well as upon the addition of BSA to the isolation medium. Free fatty acids have been shown to uncouple phosphorylation and induce swelling in mitochondria (13, 38, 47, 50). Lipoprotein lipase activity in mammary tissue increases greatly during lactation (21, 29, 41) and has also been demonstrated in milk (21, 22, 28, 41). The ultrastructure and functional damage observed when intact mitochondria are exposed to milk may therefore result from the free fatty acids released by the action of the hydrolytic enzyme.

Because of the difficulties in removing milk, glands from lactating animals rendered milk-free by severing half of the nipples during the last week of pregnancy and nonlactating glands of pregnant animals were used to obtain active mitochondrial preparations.

The $Q_{O_2}$ value reported for malate oxidation by mitochondria isolated from lactating guinea pig mammary glands (24) and that shown here by lactating mouse mammary gland mitochondria are very similar, although the values with succinate as substrate in our experiments are somewhat greater. Respiration with glutamate as substrate by lactating

![Graph](image)

Chart 6. Effect of tumor supernatant fraction on liver and tumor mitochondrial phosphate transport. Rat liver mitochondria (unwashed 8700 x g pellet) were reisolated after mixing with (a) the mitochondria-free liver supernatant fraction or (b) the mitochondria-free tumor supernatant fraction. Tumor mitochondria (c) were isolated in the absence of BSA. Assay conditions are given in text.

### Table 4

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pregnant</th>
<th>Lactating&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lactating (NS) gland</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>15</td>
<td>7</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>Malate</td>
<td>12</td>
<td>10</td>
<td>26</td>
<td>22</td>
</tr>
<tr>
<td>Aspartate</td>
<td>17</td>
<td>10</td>
<td>20</td>
<td>26</td>
</tr>
<tr>
<td>Glutamate</td>
<td>43</td>
<td>11</td>
<td>29</td>
<td>39</td>
</tr>
<tr>
<td>γ-Hydroxybutyrate</td>
<td>50</td>
<td>15</td>
<td>33</td>
<td>31</td>
</tr>
<tr>
<td>Succinate</td>
<td>79</td>
<td>75</td>
<td>92</td>
<td>72</td>
</tr>
<tr>
<td>Ascorbate + tetramethylphenylenediamine</td>
<td>91</td>
<td>93</td>
<td>91</td>
<td>90</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lactating glands were washed for 4 hr as given in Table 1, before homogenization and isolation of mitochondria.
Table 5

Mouse mammary gland and tumor mitochondrial phosphorylation and RCR

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pregnant ADP:O</th>
<th>RCR</th>
<th>Lactating (NS) ADP:O</th>
<th>RCR</th>
<th>Tumor ADP:O</th>
<th>RCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>2.5</td>
<td>4.6</td>
<td>2.2</td>
<td>3.6</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Malate</td>
<td>1.5</td>
<td>1.8</td>
<td>1.5</td>
<td>3.1</td>
<td>2.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Aspartate</td>
<td>2.0</td>
<td>3.5</td>
<td>1.8</td>
<td>2.5</td>
<td>2.5</td>
<td>3.3</td>
</tr>
<tr>
<td>Glutamate</td>
<td>1.7</td>
<td>2.2</td>
<td>1.8</td>
<td>2.0</td>
<td>2.3</td>
<td>3.5</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>2.6</td>
<td>3.1</td>
<td>2.7</td>
<td>3.1</td>
<td>2.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.6</td>
<td>3.2</td>
<td>1.7</td>
<td>2.6</td>
<td>2.0</td>
<td>3.4</td>
</tr>
<tr>
<td>Ascorbate + tetramethylphenylenediamine</td>
<td>0.7</td>
<td>1.5</td>
<td>0.7</td>
<td>1.6</td>
<td>1.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 6

The response of mammary gland and mammary tumor mitochondria to swelling agents

Mitochondria were incubated at 22° for 60 min in 0.3 M sucrose or 0.125 M KCl each containing 20 mM Tris-HCl, pH 7.4. Additions of either 4 × 10⁻⁴ M sodium phosphate or 5 × 10⁻⁵ M digitonin, at pH 7.4, were made to induce swelling. The change in absorbance at 520 nm was measured with a Cary Model 14 recording spectrophotometer. Enough mitochondria were added to each cuvet to give an initial absorbance between 0.5 and 0.6.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Agent added</th>
<th>% absorbance change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactating (NS)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>None</td>
<td>3</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Digitonin</td>
<td>24</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Phosphate</td>
<td>5</td>
</tr>
<tr>
<td>KCl</td>
<td>None</td>
<td>16</td>
</tr>
<tr>
<td>KCl</td>
<td>Digitonin</td>
<td>22</td>
</tr>
<tr>
<td>KCl</td>
<td>Phosphate</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 7

Concentration of cytochromes in mouse mammary gland and tumor mitochondria

The cytochrome content of mitochondria isolated from prelactating mammary glands, glands from lactating mice in which the nipples were closed as given in Table 1, and tumors were determined with a Cary Model 14 spectrophotometer as given in the text. The results are presented as nmoles of cytochrome per mg of mitochondrial protein and are given as the average of 3 preparations.

<table>
<thead>
<tr>
<th>State of</th>
<th>Tissue</th>
<th>Cytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Tumor</td>
<td>a b c₁ c</td>
</tr>
<tr>
<td>Virgin</td>
<td>Tumor</td>
<td>0.158 0.125 0.059 0.059</td>
</tr>
<tr>
<td>Pregnant</td>
<td>Nonlactating</td>
<td>0.151 0.152 0.065 0.091</td>
</tr>
<tr>
<td>Lactating</td>
<td>Nonlactating</td>
<td>0.159 0.139 0.044 0.079</td>
</tr>
</tbody>
</table>

a Glands taken from mice on the 15th to 20th day of gestation.
b Glands taken from lactating mice in which the nipples were closed as given in Table 1.

The ultrastructure of tumor mitochondria isolated without BSA closely resembled that of liver mitochondria reisolated from the postmitochondrial tumor tissue homogenate fraction, also without BSA. In these experiments active swelling, while absent in the tumor mitochondria, was reduced in the liver mitochondria to two-thirds of that found with normal liver mitochondria (Chart 6). These damaged mitochondria exhibited a reduced QO₂, and were similar in ultrastructure to mammary gland tumor mitochondria also isolated without BSA (45).

Emmelot (14) was able to correlate the amount of free long-chain fatty acids in tissue homogenates with a reduction in the activities of mitochondria from different types of hepatomas. The long-chain fatty acids obtained from rat hepatoma homogenates uncoupled phosphorylation and inhibited substrate oxidation by normal rat liver mitochondria. He also showed that human serum albumin would protect against such damage and that this protein could partially restore phosphorylation to mitochondria which were uncoupled by the free fatty acids. Other workers have shown that BSA will also protect mitochondrial activity from fatty acid damage (13, 38, 50). BSA can inhibit the hydrolytic activity of lysosomes (31) and of phospholipase A (12, 23) and the hydrolysis of olive oil by pancreatic lipase (19). Thus, since mouse mammary gland and adenocarcinoma mitochondria when viewed in situ were undamaged and BSA was shown to protect mitochondrial activity, it is tempting to suggest that damage which occurs during isolation is the result of hydrolytic enzymes and free fatty acids.

The study comparing the activities of mitochondria from mammary gland and mammary adenocarcinoma revealed no differences in enzymatic activity (QO₂, ADP:O, RCR), energized phosphate transport (volume changes), cytochrome content, or ultrastructure. However, the amount of mitochondrial protein obtained per g, wet weight, of tumor tissue was about one-half that of normal tissue. This disparity between neoplastic and normal tissue is well known (2, 3, 18, 39, 43, 44). Since normal mammary gland and guinea pig mitochondria (6) and lactating mouse mammary gland mitochondria are in good agreement.

When milk was removed and normal mammary gland mitochondria were isolated in the presence of BSA, no structural damage was observed. Under these conditions, the ultrastructure of normal mammary mitochondria closely resembled tumor mitochondria. These active, undamaged mitochondria were similar to those isolated by Sordahl et al. (45) from mammary tumors and by Pedersen et al. (36) and Nakamura et al. (33) from hepatomas, in that the particles showed active swelling, good respiratory control, and coupled phosphorylation.

The ultrastructure of tumor mitochondria isolated without BSA closely resembled that of liver mitochondria reisolated from the postmitochondrial tumor tissue homogenate fraction, also without BSA. In these experiments active swelling, while absent in the tumor mitochondria, was reduced in the liver mitochondria to two-thirds of that found with normal liver mitochondria (Chart 6). These damaged mitochondria exhibited a reduced QO₂, and were similar in ultrastructure to mammary gland tumor mitochondria also isolated without BSA (45).

Emmelot (14) was able to correlate the amount of free long-chain fatty acids in tissue homogenates with a reduction in the activities of mitochondria from different types of hepatomas. The long-chain fatty acids obtained from rat hepatoma homogenates uncoupled phosphorylation and inhibited substrate oxidation by normal rat liver mitochondria. He also showed that human serum albumin would protect against such damage and that this protein could partially restore phosphorylation to mitochondria which were uncoupled by the free fatty acids. Other workers have shown that BSA will also protect mitochondrial activity from fatty acid damage (13, 38, 50). BSA can inhibit the hydrolytic activity of lysosomes (31) and of phospholipase A (12, 23) and the hydrolysis of olive oil by pancreatic lipase (19). Thus, since mouse mammary gland and adenocarcinoma mitochondria when viewed in situ were undamaged and BSA was shown to protect mitochondrial activity, it is tempting to suggest that damage which occurs during isolation is the result of hydrolytic enzymes and free fatty acids.

The study comparing the activities of mitochondria from mammary gland and mammary adenocarcinoma revealed no differences in enzymatic activity (QO₂, ADP:O, RCR), energized phosphate transport (volume changes), cytochrome content, or ultrastructure. However, the amount of mitochondrial protein obtained per g, wet weight, of tumor tissue was about one-half that of normal tissue. This disparity between neoplastic and normal tissue is well known (2, 3, 18, 39, 43, 44). Since normal mammary gland and
Charles W. Mehard, Lester Packer, and S. Abraham

adenocarcinoma mitochondria oxidized pyruvate at about the same rate when compared per mg of mitochondrial protein, the difference in lactate utilization observed in slices prepared from these tissues (1) is likely to result from the lower number of mitochondria per g of tumor rather than from a defect within the mitochondrion itself.

ACKNOWLEDGMENTS

We are grateful to Miss Susan Tinsley for her expert technical assistance with the electron microscopy.

REFERENCES


Fig. 1. Mammary gland tissue of lactating (a, b) and pregnant mice (c, d) and mammary adenocarcinoma (e, f). Two different areas of each tissue are shown to demonstrate the wide variation in mitochondrial size and shape within each tissue sample.

Fig. 2. Isolated mitochondria from mammary glands of lactating (a, b) and pregnant mice (c, d) and from rat liver (e, f). a, lactating mammary gland mitochondria isolated in the absence of BSA; b, lactating mammary gland mitochondria isolated from tissue which was sliced and washed for 4 hr in medium containing 1% BSA prior to homogenization; c, nonlactating mammary gland mitochondria from 15- to 20-day pregnant mice isolated in 1% BSA-containing medium; d, rat liver mitochondria isolated in the absence of BSA; e, nonlactating mammary gland mitochondria (isolated as in c, mixed with milk (2 μl/mg protein) 5 min before fixation with glutaraldehyde); f, rat liver mitochondria (isolated as in d), mixed with milk (2 μl/mg protein) 5 min before fixation with glutaraldehyde.

Fig. 3. Mitochondria from rat liver (a to c) and mammary adenocarcinoma (d to f). a, isolated rat liver mitochondria resuspended in the mitochondria-free liver supernatant fraction and resuspended; b, rat liver mitochondria suspended in the mitochondria-free tumor supernatant fraction and resuspended; c, fluffy layer obtained from the resuspension of rat liver mitochondria as described in b; d, tumor mitochondria isolated in the absence of BSA; e, tumor mitochondria isolated in the presence of BSA; f, tumor mitochondria isolated as in e with the exception that the final wash was made with no BSA in the medium and then resuspended in isolation medium without BSA.

DECEMBER 1971 2157
Activity and Ultrastructure of Mitochondria from Mouse Mammary Gland and Mammary Adenocarcinoma

Charles W. Mehard, Lester Packer and S. Abraham


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/31/12/2148

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.