A Comparison of Some Ultrastructural and Biochemical Properties of Mitochondria from Morris Hepatomas 9618A, 7800, and 3924A

Peter L. Pedersen, John W. Greenawalt, T. L. Chan, and Harold P. Morris

Department of Physiological Chemistry, Johns Hopkins School of Medicine, Baltimore, Maryland 21205 [P. L. P., J. W. G., T. L. C.], and Department of Biochemistry, Howard University School of Medicine, Washington, D. C. 20001 [H. P. M.]

SUMMARY

The ultrastructural and biochemical properties of freshly isolated mitochondria from host livers and from hepatomas 9618A, 7800, and 3924A were examined in some detail. Similar to host liver mitochondria, mitochondria from the two "minimal deviation" hepatomas, 9618A and 7800, have condensed densely staining matrices surrounded by intact inner and outer membranes, and acceptor control ratios near 5 with $\beta$-hydroxybutyrate as substrate. Mitochondria from the rapidly growing hepatoma 3924A, in contrast to those from hepatomas 9618A and 7800 and from host liver, have diluted-appearing matrices, fail to oxidize $\beta$-hydroxybutyrate, and have low acceptor control ratios in the presence of succinate as substrate. Addition of 2,4-dinitrophenol enhances the respiratory activity of mitochondria from all three hepatomas.

Assays for cytochrome oxidase, malate dehydrogenase, adenylate kinase, and monoamine oxidase, showed that only the specific activity of the latter enzyme is low in mitochondria isolated from all hepatomas studied. The specific activity of adenylate kinase is low in mitochondria from hepatomas 7800 and 3924A, but near normal in mitochondria from hepatoma 9618A. The specific activities of cytochrome oxidase and malate dehydrogenase are about as high in hepatoma mitochondria as in mitochondria from host livers. With respect to those properties examined, these studies clearly show that mitochondria from the most rapidly growing heptoma, 3924A, bear the least resemblance to host liver mitochondria, whereas mitochondria from hepatomas 9618A and 7800 are very similar to but not identical with control mitochondria.

INTRODUCTION

Although some properties of mitochondria freshly isolated from a number of transplantable hepatomas have been studied, little attention has been focused on a study of the ultrastructural, energy-coupling, and general enzymatic properties of mitochondria isolated from Morris hepatomas. In a brief communication, Devlin and Pruss (9) reported that mitochondria isolated from Morris hepatomas 5123, 3924A, and 3683 can catalyze coupled oxidative phosphorylation. More recently, Sordahl et al. (24) reported that mitochondria isolated from mouse and rat tumors of varying growth rate can also catalyze coupled oxidative phosphorylation. However, they also observed considerable variability in the ultrastructure of all preparations examined by electron microscopy. Thus the mitochondria of some tumors exhibited highly dense matrices, whereas mitochondria isolated from other tumors revealed less dense matrices and were in the orthodox configuration as described by Hackenbrock (10).

In the experiments described below, the biochemical and ultrastructural properties of mitochondria isolated from Morris hepatomas 9618A, 7800, and 3924A and from corresponding host livers are compared. Since the growth rates of these hepatomas range from very slow (9618A) to very rapid (3924A), the differences in mitochondrial properties reported below may prove useful in assessing the effect of growth rate on the overall biochemistry and ultrastructural features of transplantable hepatomas.

MATERIALS AND METHODS

Tumors. Tumor-bearing rats were obtained from Dr. H. P. Morris, Howard University, Washington, D. C. They were housed no more than 5 to a cage and given food and water ad libitum. Morris hepatomas 9618A (slow growth rate), 7800 (intermediate growth rate), and 3924A (rapid growth rate) were used. Hepatomas 9618A and 7800 have a normal chromosome number (42 chromosomes), and are considered to be of the "minimal deviation" variety (14–16, 19). Hepatoma 3924A has a chromosome number of 72 and is not of the minimal deviation variety. Hepatomas from the following transplant generations were used: 9618A (Generation 5), 7800 (Generations 39, 42, 43, and 49), 3924A (Generations 254, 260, and 262).

Preparation of Mitochondria. Mitochondria were prepared essentially as previously described by Schnaitman and Pedersen (23) and Schnaitman and Greenawalt (22). When the hepatomas grew to appropriate size (0.5 to 1 cm in diameter), the animals were killed by a blow to the head. Tumors were carefully dissected free of any hemorrhagic,
necrotic, and nontumorous material and immediately placed on ice. (It is perhaps important that rats used in this study were always sacrificed well before significant amounts of hemorrhagic area appeared.) When tumors from 10 to 15 rats had been excised and trimmed, they were rinsed with a small amount of ice-cold H medium (220 mM D-mannitol, 70 mM sucrose, 2.0 mM HEPES\(^3\) buffer, and 0.5 mg/ml crystalline bovine serum albumin, pH 7.4). The tumors were minced with a pair of scissors, suspended in a small Potter-Elvehjem tissue grinder (15-ml capacity), and homogenized with 4 passes of a radially serrated Teflon pestle in the case of the 9618A and 3924A mince and 2 passes when the 7800 mince was homogenized. The homogenate was diluted with H medium such that the volume of tissue was equal to approximately 10% of the final volume. Centrifugation was then carried out at 660 \(\times\) g for 15 min in a Sorvall RC-2B refrigerated centrifuge (0–4\(^\circ\)C, SS-34 rotor). The fluffy layer was carefully discarded, and the pellet was washed twice by resuspension in one-half and one-fourth the original volume of H medium followed by centrifugation at 9770 \(\times\) g for 15 min.

Mitochondria from the livers of the tumor-bearing animals were also identically prepared.

**Respiration Measurements.** All respiration studies were carried out at 25\(^\circ\)C in a closed 3.0-ml reaction vessel equipped with a Clark oxygen electrode. The respiration medium contained, in a final volume of 3.0 ml: 220 mM D-mannitol; 70 mM sucrose; 2.5 mM potassium phosphate, pH 7.4; 0.5 mM EDTA; 2.0 mM HEPES; 2.0 mg defatted bovine serum albumin; 10.0 mM sodium D-\(\beta\)-hydroxybutyrate (or 5.0 mM sodium succinate); and, where indicated, 142 \(\mu\)M ADP; 16.7 \(\mu\)M 2,4-dinitrophenol; 0.04 \(\mu\)M rotenone; 0.14 \(\mu\)M antimycin A; and 0.45 \(\mu\)M potassium cyanide. Acceptor control and ADP:O ratios were calculated as described by Chance and Williams (5).

**Enzyme Assays.** Cytochrome oxidase, malate dehydrogenase, adenylate kinase, and monoamine oxidase were assayed as described by Schnaitman et al. (21) and Schnaitman and Greenawalt (22). Prior to assay, mitochondrial samples were treated with 160 \(\mu\)g Lubrol WX/mg protein for 20 min to expose maximally all latent enzymatic activities (22).

**Protein Determinations.** Protein was estimated by the biuret procedure (11). Crystalline bovine serum albumin served as standard.

**Electron Microscopy.** Samples of the mitochondrial fractions (50 mg/ml) in H medium were rapidly fixed, dehydrated, and embedded as previously described (21). Small volumes (5 to 20 \(\mu\)l) were placed on top of about 200 \(\mu\)l of 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.1, in a Beckman Microfuge tube, centrifuged, and fixed with fresh glutaraldehyde for 2 hr. The micropellets were washed to remove glutaraldehyde, then postfixed with 2% osmium tetroxide, in 0.1 M phosphate buffer, pH 7.2, plus 0.05% calcium chloride. Following dehydration by rapid passage through a cold ethanol series, the pellets were embedded in Epon 812 as described by Luft (13). Thin sections were cut with a diamond knife on an LKB ultratome and stained with saturated uranyl acetate in 1% sodium borate for 15 min at 60\(^\circ\)C, and then with lead citrate for 10 min at room temperature. Samples were examined in a Siemens-Halske Elmskop I operated at 80 kV.

**Materials.** A Rockland rat diet was purchased from Teklad, Inc., Winfield, Iowa. All nucleotides were products of P-L Biochemicals, Milwaukee, Wis. Cytochrome c (Sigma type III) and HEPES were products of Sigma Chemical Company, St. Louis, Mo. Lubrol WX was obtained from I.C.I. Organics, Inc., Providence, R. I. D(+)-Mannitol was a product of Mann Research Laboratories, New York, N. Y. Sucrose was obtained from J. T. Baker Chemical Company, Phillipsburg, N. J. Crystalline bovine plasma albumin was purchased from Pentex, Inc., Kankakee, Ind. All other reagents used in these studies were of the highest commercial purity available.

**RESULTS**

**Morphology and Ultrastructure.** Mitochondria freshly isolated from host liver and from hepatomas 9618A, 7800, and 3924A are compared in the electron micrographs presented in Fig. 1. Host liver mitochondria from all 3 tumor-bearing animals, as illustrated in Fig. 1A with mitochondria isolated from the liver of an animal bearing the 9618A hepatoma, show essentially the morphological and ultrastructural features of mitochondria isolated from normal rat livers. Each exhibits a distinct, intact outer membrane and a condensed, densely staining matrix similar to that described by Hackenbrock (9). Some variability in the degree to which the matrices of host liver mitochondria are condensed was observed in the different preparations.

Freshly isolated mitochondria from the hepatomas also contain intact outer membranes (Fig. 1, B to D). In addition, the mitochondria from hepatomas 9618A and 7800 contain densely staining condensed matrices. However, mitochondria isolated from the rapidly growing hepatoma, 3924A, (Fig. 1D), in contrast to those from hepatomas 9618A and 7800 and those from host livers, have lightly staining, diluted-appearing matrices similar to the orthodox mitochondrial conformation described by Hackenbrock (9). As shown more clearly at higher magnifications (cf. Fig. 1, E and F), both the inner membranes and the outer mitochondrial membranes appear not to be ruptured. This observation is in agreement with the finding that the specific activity of malate dehydrogenase, an enzyme marker for the matrix (4, 12), is not reduced in mitochondria isolated from this rapidly growing tumor (see below). Thus, both biochemical and ultrastructural evidence suggest that the diluted appearance of the matrix is not the result of gross damage to the mitochondria. The presence of an apparently intact outer membrane is of further interest since, as shown below, the mitochondria from hepatoma 3924A do not contain monoamine oxidase activity which in normal rat liver mitochondria is found in the outer membrane (21).

\(^3\)The abbreviation used is: HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
Control and Inhibition of Respiration. Representative polarographic traces which summarize the general respiratory properties of freshly isolated mitochondria from host livers and from hepatomas 9618A, 7800, and 3924A are presented in Charts 1 to 3. In the presence of the substrate β-hydroxybutyrate, which enters the electron transport chain near the first energy-conserving site, mitochondria prepared from host livers and from hepatomas 9618A and 7800 catalyze a low rate of respiration (State 4 rate). Addition of ADP to any one of these slowly respiring mitochondrial preparations causes a marked increase in respiratory activity (State 3 rate) which soon subsides to the State 4 rate. A similar but continuous stimulation of respiration is observed in all cases upon addition of 50 μmole of the uncoupling agent 2,4-dinitrophenol. Acceptor control and P:O ratios calculated from polarographic traces of 9618A and 7800 mitochondria were only slightly lower than those of host mitochondria (see Charts 1 and 2). These findings were reproduced with 5 other preparations of host liver and tumor mitochondria.

In contrast to mitochondria isolated from hepatomas 9618A and 7800, 3924A mitochondria failed to exhibit significant respiratory activity in the presence of β-hydroxybutyrate as substrate. For this reason, succinate, which enters the electron transport chain near the second energy-conserving site, was used. Results presented in Chart 3 clearly show that in the presence of succinate, 3924A mitochondria have higher State 3, State 4, and uncoupled respiratory rates than host mitochondria. However, these tumor mitochondria appear to be loosely coupled since their acceptor control and P:O ratios as determined from polarographic traces are significantly lower than those of host mitochondria (Chart 3).

Respiration supported by β-hydroxybutyrate or succinate could be almost completely inhibited in all hepatoma mitochondria by cyanide, which acts near the last energy-conserving site of electron transport chain, and by antimycin A, which acts near the second energy-conserving site. Respiration supported by β-hydroxybutyrate in mitochondria from hepatomas 9618A and 7800 could also be inhibited by rotenone, which blocks respiration near the first energy-conserving site.

Results of studies summarized above suggest that mitochondria isolated from the “minimal deviation” hepatomas 9618A and 7800 are very similar in their electron transport...
and energy-coupling properties to liver mitochondria. Mitochondria isolated from the rapidly growing 3924A hepatoma, on the other hand, appear to have somewhat different respiratory characteristics than liver mitochondria and a less efficient mechanism for coupling oxidation to phosphorylation.

**Enzymatic Activities.** For additional information about the biochemistry of mitochondria isolated from hepatomas 9618A, 7800, and 3924A, these mitochondria were assayed for cytochrome oxidase, malate dehydrogenase, adenylate kinase, and monoamine oxidase. These enzymes were chosen for assay since they differ in submitochondrial localization as well as in function (18, 21–23). Results of assays summarized in Table 1 show that the specific activity of only monoamine oxidase, an outer membrane enzyme, is low in all tumor mitochondria studied. The specific activity of this enzyme is markedly reduced in mitochondria from hepatomas 9618A and 7800 (<40% of host activity), and completely absent in mitochondria isolated from hepatoma 3924A. The specific activity of adenylate kinase, an enzyme localized between the inner and outer mitochondrial membranes is near normal in mitochondria isolated from the slowly growing hepatoma 9618A but is reduced to less than 70% of the host value in mitochondria isolated from the more rapidly growing hepatomas 7800 and 3924A.

In contrast to monoamine oxidase and adenylate kinase, neither the specific activity of cytochrome oxidase, an inner membrane enzyme, nor malate dehydrogenase, a matrix enzyme, are markedly reduced in any of the tumor mitochondria studied. In general, the specific activities of these two enzymes are about as high as values obtained on host liver mitochondria and in some cases actually higher (Table 1).

**DISCUSSION**

Studies carried out by Aisenberg and Morris (1, 2) on tissue slices of the minimal deviation hepatomas, 5123 and 7800, in earlier generations led them to conclude that the energy metabolism of these tumors is very similar to that of normal liver. Results of experiments summarized here are in essential agreement with these conclusions since the general respiratory characteristics of isolated mitochondria from the minimal deviation hepatomas 7800 and 9618A bear a very close resemblance to those isolated from host liver (Charts 1 to 3). Whether or not the content of functional mitochondria in these minimal deviation hepatomas is as high as in host liver, however, has yet to be established.

Failure to find significant respiratory activity in mitochondria isolated from hepatoma 3924A in the presence of β-hydroxybutyrate as substrate is not surprising since Ohe et al. (17) and Weinhouse (25) have reported that the β-hydroxybutyrate dehydrogenase activity of the 3924A hepatoma is less than 1% of that found in host liver. Failure to find high acceptor control and ADP:O ratios in these mitochondria in the presence of succinate may be of considerable interest in regard to the energy metabolism of some rapidly growing tumors. It is possible that mitochondria from the rapidly growing 3924A tumor cells have a rather fragile membrane system that is broken or fragmented during the isolation procedure. This is rendered unlikely, however, both by ultrastructural and enzymatic data. Electron micrographs of 3924A hepatoma mitochondria (Fig. 1, D and F) clearly show the appearance of continuous inner and outer membranes. Moreover, malate dehydrogenase, a marker enzyme for the mitochondrial matrix, is not reduced in these mitochondria (Table 1), as would be predicted if the inner membrane were damaged. Also, despite the differences in morphology, mitochondria from hepatomas 7800 and 3924A have similar amounts of adenylate kinase, an enzyme which is easily lost when mitochondria are damaged (12). The low acceptor control and ADP:O ratios of the 3924A mitochondria may be related to the diluted appearance of the matrix space (Fig. 1, D and F). It may be that the inner membrane of these mitochondria may be more permeable to water than the inner membrane of host liver mitochondria. The presence of water at the site of ATP formation during a respiratory ADP jump, could result in significant hydrolysis of the ATP formed, in which case an incomplete return of respiration from State 3 to State 4 would occur. Sordahl et al. (24) have also noted that certain tumor mitochondria, which have an orthodox ultrastructure and lightly staining matrices, exhibit a reduced capacity to couple phosphorylation to oxidation, i.e., have a lower ADP:O ratio.

It would be of considerable interest to know whether the general morphological and ultrastructural properties of the 3924A mitochondria are different from those of the 7800 and 9618A mitochondria in situ. In general, however, only orthodox conformations have been observed in situ, not only

<table>
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<td>Some enzymatic activities of mitochondria freshly isolated from host rat livers and tumors</td>
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| Specific activity (nmoles product formed/min/mg protein) |
| Host | 9618A | Host | 7800 | Host | 3924A |
| Cytochrome oxidase | 686 ± 135 | 819 ± 142 | 626 ± 130 | 656 ± 155 | 973 ± 282 | 1004 ± 145 |
| Malate dehydrogenase | 2970 ± 666 | 3262 ± 801 | 2384 ± 318 | 1678 ± 182 | 2847 ± 1150 | 4895 ± 1960 |
| Adenylate kinase | 220 ± 12 | 196 ± 13 | 260 ± 11 | 129 ± 12 | 293 ± 103 | 168 ± 54 |
| Monoamine oxidase | 14.6 ± 2.6 | 4.6 ± 1.5 | 14.0 ± 3.3 | 5.4 ± 1.1 | 12.4 ± 3.0 | 0 |

*Mean ± S. D. on 5 to 8 tissues.*
in normal cells but in Morris hepatomas of widely different growth rates as well (7). It has been shown only recently by Hackenbrock (10) that mitochondria in situ can exist in the condensed conformation, the form in which freshly isolated mitochondria are usually observed. However, special metabolic conditions are necessary to produce the condensed conformation in situ, conditions which have not been established for Morris hepatomas. Thus, at this time such an in situ comparison of the mitochondria studied here would have little significance without a knowledge of the conditions required in situ to trigger the conversion from an orthodox to a condensed morphology.

The very low activity of monoamine oxidase in mitochondria from hepatomas 9618A and 7800 and the complete absence of this activity in mitochondria from hepatoma 3924A is of special interest (cf. Table 1). Since the 9618A hepatoma is karyotypically the least deviated hepatoma from normal liver (15, 16), the deficiency of monoamine oxidase activity in mitochondria from this tumor may be a reflection of an early stage in the carcinogenic process. It is possible that the synthesis of monoamine oxidase may be repressed in these hepatomas; however, the presence of an irreversible inhibitor of the enzyme or a defect in the mechanism for incorporating the enzyme into the outer mitochondrial membrane could also account for the reduced activity. In addition, the question of a multiplicity of monoamine oxidases in rat liver mitochondria differing in substrate specificity (18, 20) has not yet been resolved. Therefore, it is possible that the predominant form of monoamine oxidase in hepatoma mitochondria is one that shows little activity with benzylamine, the substrate used in these studies.

The finding that adenylate kinase activity is lower in mitochondria from the more rapidly growing hepatomas 3924A and 7800 than in mitochondria from hepatoma 9618A (cf. Table 1) is qualitatively similar to the findings of Criss et al. (6) on tumor homogenates. Although it would be premature to attach any special significance to these results at this time, it should be kept in mind that adenylate kinase probably plays an important role in regulating the relative intracellular levels of ATP, ADP, and AMP and, moreover, that these adenine nucleotides function as allosteric modifiers of a number of important regulatory enzymes involved in energy metabolism of the cell (3).

With respect to the biochemical and ultrastructural properties studied here, it can be clearly concluded that mitochondria isolated from the rapidly growing hepatoma 3924A, under conditions which yield structurally and functionally intact mitochondria from host and normal livers and from less rapidly growing tumors, bear much less resemblance to host liver mitochondria than do mitochondria from hepatomas 9618A and 7800. Further studies are necessary to provide definitive conclusions about the effect of growth rate on the biochemical and ultrastructural characteristics of mitochondria from the transplantable Morris hepatomas.

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REFERENCES

Fig. 1. Electron micrographs of freshly isolated mitochondria from host livers and Morris hepatomas. A, liver mitochondria of an animal bearing 9618A hepatomas. B, mitochondria from hepatoma 9618A. C, mitochondria from hepatoma 7800. D, mitochondria from hepatoma 3924A. E, mitochondria from host liver at high magnification. F, mitochondria from hepatoma 3924A at high magnification. Note the condensed, densely staining matrix of all mitochondria except those from hepatoma 3924A. In the micrograph taken at high magnification (F), the outer and inner membranes of the 3924A mitochondria are seen to be continuous and to show no signs of structural damage; however, the matrix space has a diluted appearance. Preparations were fixed with glutaraldehyde and OsO₄ and stained with uranyl acetate and lead citrate. C and D, X 15,000; E and F, X 70,000. The micron marker shown on D also applies to A, B, and C, and that on F applies also to E.
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