Oxidative Phosphorylation in Ascites Tumor Mitochondria*

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SUMMARY

A modified method for the cytochemical fractionation of an ascites tumor is described. The cells are pretreated in 10⁻³ M EDTA in 0.25 M sucrose before homogenization. Particulate fractions are obtained by the usual centrifugation procedures.

The capacity of the isolated mitochondrial fraction to undergo oxidative phosphorylation was investigated. Mitochondria were found to be active with the substrates succinate, malate, and glutamate, and P/O ratios approaching the theoretical were obtained. The use of malonate in conjunction with glutamate oxidation was necessary to obtain maximal P/O ratios. No exogenous DPN was necessary for optimal oxygen uptake. Added DPN usually inhibited respiration and phosphorylation, especially the latter. A possible explanation for this effect is given.

The oxidative and phosphorylative capacity of mitochondria isolated from mouse ascites tumors has been the subject of numerous studies (1, 5, 9, 12–14). In these investigations a variety of cytochemical techniques has been employed to obtain the mitochondrial fraction. The P/O ratios, especially those obtained when DPN-linked substrates were used, have been somewhat lower than those obtained with rat liver mitochondria. This has suggested to some (9, 18) that tumor mitochondria are deficient in DPN and consequently show an impairment of respiratory control.

In a previous report (17) we described a method for the cytochemical fractionation of Lettré-Ehrlich ascites tumor; we felt this technic to be less injurious to the cells than other procedures. The cells were pre-stressed in dilute Ringer's solution before homogenization in sucrose. Recently, we found that low concentrations of EDTA condition the cells for homogenization, thereby eliminating treatment in dilute salt solution and avoiding the possibility of removing soluble co-factors.

This procedure also reduced the time required for isolation.

We used mitochondria prepared by these two techniques to study oxidative phosphorylation. The results show that both mitochondrial fractions can carry out oxidative phosphorylation, with the use of succinate as well as DPN-linked substrates. Contrary to the findings of others (9, 18), we found that DPN was not required for optimal P/O ratios; indeed, data are presented to show that exogenous DPN can depress oxidation and phosphorylation. The results obtained indicate that there is no impairment of respiration which could be attributed to a deficiency of DPN. These tumor mitochondria are capable of providing for their own respiratory needs and when carefully prepared do not require added DPN for optimal rates of oxidation. These latter results are in agreement with recent results of Borst and Colpa-Boonstra (2).

MATERIALS AND METHODS

Tumor transplantation and harvest.—No changes were made from the method previously reported (17) except that tumor samples of age 12 days or younger were used. In those experiments with DPN-linked substrates, tumor cells from four rather than two mice were pooled to obtain a more concentrated preparation.

Reagents.—Succinate, L-malate, malonate, EDTA, and p-methylaminophenol sulfate (Elon) were purchased from the Eastman Kodak Co. L-Glutamate was purchased from the California Corporation. Hexokinase was obtained from the Sigma Chemical Co., as Type III, and from the

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1 Abbreviations are used as follows: DPN, diphosphopyridine nucleotide; EDTA, ethylenediamine tetraacetic acid disodium salt; ATP and ADP, adenosine tri- and diphosphate; TCA, trichloroacetic acid; and Tris, tris (hydroxymethyl) aminomethane.
Pabst Chemical Co. ADP, ATP, DPN, DPNH, Tris, and nicotinamide were purchased from Sigma Chemical Co.

Measurement of oxidative phosphorylation.—Oxygen consumption was measured by the Warburg apparatus. Contents of the flasks were essentially those described by Witter et al. (19) for use with rat liver mitochondria, with the following in a total volume of 3.0 ml.: ATP, 6 umoles; Mg++, 24.4 or 12.2 umoles; KH2PO4, 30 umoles; glucose, 100 umoles; hexokinase, 0.64 mg.; NaF, 20 umoles; Tris, 24 umoles adjusted to pH 7.4; substrates, 80–200 /¿moles; DPN, 0–20 /¿moles, and malonate, 10–90 /¿moles. Glucose and hexokinase were contained in the side-arm, and 0.2 ml. 20 per cent KOH (or NaOH) was placed in the center well. The mitochondrial suspension, 0.5 ml., was the last addition. During the pipetting, the flasks were kept cold by immersion in a tray of crushed ice.

Measurement of oxygen uptake was begun when the contents of the side-arm were tipped in after a 6- to 8-min. period of temperature equilibration at 30° C. Zero time flasks and experimental flasks were removed immediately after incubation, placed in an ice bath, and the reaction was stopped with 1.0 ml. of cold 20 per cent TCA.

The time of incubation of experimental flasks varied from 20 to 60 min., depending upon the activity of the particular preparation.

Phosphate determinations on the cold TCA supernatants were done by the method of Harris and Popat (8) as modified by Conover (6). Oxygen values are expressed as /µl O2/mg dry wt/hr. Dry-weight determinations were done as described previously (17). Qo, values were obtained from straight-line graphs of oxygen consumption measured over a 20- to 60-minute period.

Cytotoxicity fractionation.—The simplified method, with EDTA and nicotinamide, differs from that reported previously (17) mainly in the preparation of the homogenate. Once the homogenate is obtained the isolation of the nuclear, mitochondrial, and microsomal + “soluble” fractions is the same as in the first method. Material obtained by the previously described technic will hereafter be called the Ringer's preparation, and that obtained by the simplified method, the EDTA preparation. Mitochondrial fractions obtained from each will be designated in the same way.

Four mice with tumors at age 8–12 days were killed by cervical fracture, and the tumor was harvested by syringe and transferred to a 50-ml. round-bottom centrifuge tube in an ice bath. The cells were centrifuged in an International Refrigerated Centrifuge Model PR-9 (temperature setting at –2.5° C.) with the low-speed head No. 22S at 2200 r.p.m. (960 X g) for 5 minutes. The ascites fluid was discarded. Moderately to grossly bloody cell samples were cleared of erythrocytes by “osmotic shock” as follows: the cell pellet was slurried in 20 ml. of cold one-fourth strength ascites Ringer’s solution (17) for 1–2 min. with a rubber policeman. Twenty ml. of cold, double-strength ascites Ringer’s solution were added, and the suspension was recentrifuged as above. The supernatant containing the hemoglobin was discarded and the process repeated if necessary. Tumor samples which were cream-colored or only slightly bloody were not so treated.

The tumor cells were then suspended in 40 ml. cold 0.25 m sucrose containing 10–4 m EDTA and 0.05 m nicotinamide (hereafter called EDTA mixture) and centrifuged as above. The pellet was suspended in 20 ml. of cold EDTA mixture and transferred to a pre-cooled Dounce all-glass “ball and cylinder” homogenizer (7) with an additional 25 ml. of cold EDTA mixture. Twenty-five to 30 passes with the tight-fitting rod (clearance, 0.0005 in.) were then made, and the homogenate was examined microscopically for unbroken cells, and more passes made if necessary. No adjustment of pH was necessary. The homogenate was centrifuged at 2200 r.p.m. (960 X g) for 20 min., and the supernatant fluid containing the mitochondria and microsomes was decanted and maintained at 0°–4° C. The crude nuclear fraction pellet was resuspended in 30 ml. of cold EDTA mixture, homogenized, and centrifuged as above, and the supernatant was combined with that previously obtained. Usually the yield from the second homogenization was small. The mitochondria were isolated as previously described (17).

RESULTS

Table 1 shows a comparison of the Qo, and P/O ratios obtained with the mitochondrial fractions isolated by the two procedures with succinate, malate, and glutamate as substrates.

As can be seen, the Qo, values, although highest for succinate, generally showed close correlation within each substrate group, except for one unexplained high recording with succinate. Qo, values with malate were consistently low and required concentrated mitochondrial suspension for any measurable oxygen consumption. Qo, values with glutamate were intermediate in rate. There was no significant difference between the Qo, values obtained from the two mitochondrial preparations.

No DPN was added in any of these experiments. It will be noted that the substrate concentrations used are higher than those generally employed, since we have found maximal rates of oxygen uptake at these elevated levels. There was no en-
dogenous uptake of oxygen noted with mitochondria made by either method, in contrast to results frequently obtained with liver mitochondria (15). The P/O ratios obtained from these two types of preparations are in agreement. Maximum P/O values obtained with succinate were 1.77 and 1.83; malate, 2.89; and glutamate, 3.02.

**TABLE 1**

<table>
<thead>
<tr>
<th>Substrate (µmoles)</th>
<th>RINGER'S MITOCHONDRIA</th>
<th>EDTA MITOCHONDRIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q₀*</td>
<td>P/O*</td>
</tr>
<tr>
<td>Succinate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>6.1 1.45</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>41.4 1.55</td>
<td>43.0 1.47</td>
</tr>
<tr>
<td>90</td>
<td>53.3 1.77</td>
<td>40.1 1.60</td>
</tr>
<tr>
<td>100</td>
<td>48.5 1.58</td>
<td>46.3 1.33</td>
</tr>
<tr>
<td>100</td>
<td>100 1.93</td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>100</td>
<td>3.4 2.89</td>
</tr>
<tr>
<td>200</td>
<td>6.1 1.56</td>
<td>2.9 1.20</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>13.0 2.27</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>16.5 2.75</td>
<td>5.1 2.87</td>
</tr>
<tr>
<td>100</td>
<td>12.8 2.59</td>
<td>29.6 1.61</td>
</tr>
<tr>
<td>100</td>
<td>200 1.67</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>19.0 2.27</td>
<td>7.9 2.21</td>
</tr>
<tr>
<td>200</td>
<td>18.6 1.90</td>
<td>16.6 3.02</td>
</tr>
</tbody>
</table>

* These values represent averages of either two or three assays per mitochondrial preparation. Conditions for the assays as described in the text; no exogenous DPN or malonate present. Measurement of oxygen consumption varied from 20 to 40 min. for succinate and 35 to 60 min. for the others.

The effect of exogenous DPN on the oxidative capacity of mitochondrial preparations is shown in Table 2. It can be seen that, upon the addition of DPN to the EDTA or the Ringer's mitochondrial preparations, there was either a lack of effect or an inhibition of respiration; a stimulation of oxygen consumption was not observed until the mitochondria were lysed. Treatment of the mitochondria for 3 minutes with distilled water resulted in the stimulation of oxygen uptake upon the addition of DPN. It was observed that the depression of respiration by DPN was dependent on the amount of nucleotide added, greater amounts causing the greatest inhibition. It was noted, however, that the extent of inhibition by DPN varied from preparation to preparation.

The effect of DPN on phosphorylation was measured with the EDTA preparation. It can be seen that DPN depressed phosphorylation, even in those cases where there was no effect on oxygen consumption. The same inhibitory effect was shown by different DPN preparations.

Malonate was employed to obtain P/O values from glutamate oxidation which were maximal and dependent solely on DPN oxidation. As shown in Chart 1, oxygen consumption decreased markedly in the presence of 10 µmoles of malonate during the oxidation of glutamate, and the P/O ratio increased. In the presence of malonate, there was an appreciable lag period before the commencement of oxygen uptake. With malate as substrate, 90 µmoles of malonate (approximately equimolar with substrate) did not affect oxygen uptake but did depress phosphorylation. This resulted in the use of lower concentration of malonate for glutamate oxidation.

In addition to general studies of oxidative phosphorylation with DPN-linked substrates, we have examined the requirements for a phosphate acceptor, magnesium, and fluoride. These results, obtained with Ringer's mitochondria and succinate, are summarized in Table 3. The requirement for a phosphate acceptor (ATP) for phosphorylation is shown. The marked inhibition of oxygen consumption with resulting loss of oxidation in the absence of ATP is indicative of the tight coupling of oxidation to phosphorylation.

The requirement of Mg²⁺ for phosphorylation is shown. Lack of exogenous Mg²⁺ decreased respiration only slightly. The requirement of Mg²⁺ for phosphorylation is in agreement with results obtained with the use of liver mitochondrial fragments (19).

No specific requirement for fluoride was demon-
strated, although at amounts greater than 20 

µmoles oxygen consumption decreased slightly and phosphorylation was inhibited markedly. Consequently, 20 µmoles of fluoride were used routinely.

DISCUSSION

Our initial studies with mitochondria isolated by the Ringer's technic repeatedly showed very low oxidation rates with DPN-linked substrates, although pilot studies on the same preparation yielded appreciable rates of O2 uptake with succinate. We were unable to enhance this respiration with added DPN, even at very high levels. However, mitochondria homogenized in phosphate buffer did oxidize glutamate and malate with added DPN, demonstrating the presence of dehydrogenase. This led us to consider either that these mitochondria had been depleted of their bound DPN during the soaking process and that the sites were not available to exogenous DPN or that the preparations contained an active DPNase (12).

We consequently sought a better method for breaking the cells, one that would eliminate the necessity of pretreatment with a hypotonic solution, and a method of inhibiting DPNase. The latter could be accomplished using nicotinamide, and the former condition was satisfied by using EDTA.

Several things became important when using this new technic. Bloody tumor samples had to be cleared of erythrocytes by "osmotic shock" or the mitochondrial pellet was stained with hemoglobin, which often led to erratic results. Best results were found with tumors 8–12 days of age. Older tumors were occasionally erratic in their capacity to phosphorylate, generally in the direction of partial to complete uncoupling. By using the trypan blue stain method of King et al. (11) for cell viability, we found 6.2 and 4.8 per cent of cells nonviable for tumors of age 7 and 11 days, respectively, and 39.5 per cent nonviable for a 14-day-old tumor sample. The soaking process usually at least doubles the percentage of nonviable cells as estimated by this method. The clearance of the homogenizer became crucial. Poor breakage of the cells resulted when the clearance was too great because of loss of shear, or where the clearance was too small because of insufficient force generated manually to give the necessary shear.

Oxidation rates recorded with DPN-linked substrates continued to be low even with mitochondria made by the EDTA method. Only by using more tumor and therefore concentrating the preparation could accurately measurable rates of oxidation be obtained. Both the Ringer's and EDTA mitochondrial preparations proved satisfactory under these conditions.

Our findings with exogenous DPN are in contrast to those of Wenner and Weinhouse (18) and Hawtrey and Silk (9), who found a DPN requirement for oxygen consumption. Mitochondria prepared by either of the methods discussed in this paper showed better oxidation and phosphorylation in the absence of added DPN; when DPN was included in the reaction mixture both oxidation and phosphorylation were depressed in proportion.

<table>
<thead>
<tr>
<th>DETERMINATION</th>
<th>ATP (µmoles)</th>
<th>Mg⁺⁺ (µmoles)</th>
<th>F⁻ (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µl. O₂</td>
<td>37</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P/O</td>
<td>1.68</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Each value represents an average of two experiments. Different mitochondria preparations were used for each category investigated. Conditions as described in the text unless otherwise indicated. 80 µmoles of succinate was used.

† No measurable phosphorylation.
to the amount of nucleotide added. Apparently, there is no requirement for added coenzyme when ascites mitochondria prepared by these technics are used.

The mechanism of inhibition of oxidation by DPN is unknown but probably is nonspecific. In a few experiments the inhibition was prevented by increasing the concentration of Mg"++ in the reaction media, suggesting a chelation of Mg"++ or some other factor that was necessary for oxidative phosphorylation. Indeed, a requirement for Mg"++ has been demonstrated. A permeability barrier to DPN (4) probably accounts for the lack of stimulation of oxidation. Mitochondria prepared in 0.44 M sucrose by the Ringer's method, which consistently showed inhibition of oxidation with added DPN, were treated with distilled water in an attempt to destroy any permeability barrier and centrifuged to remove any liberated DPN. When these treated mitochondria were incubated in the presence of 10 μmoles DPN, a twofold stimulation of oxidation over that in the absence of DPN was recorded. This strongly suggests that ascites mitochondria prepared by methods utilizing lysis of the cells in distilled water may have an altered permeability. This altered permeability could lead to the entrance and stimulation of oxidation by added DPN as reported by Hawtrey and Silk (9).

In the study of glutamate oxidation, malonate was included in the assay media to limit the reactions studied to those of glutamate and a-ketoglutarate oxidation. Since it was found that high concentrations of malonate drastically decreased phosphorylation, perhaps by chelation of Mg"++ (16), a concentration of malonate (10 μmoles), calculated to be in excess of the amount of succinate formed, was used. The results show that respiration was depressed in the presence of malonate and that the P/O ratio was elevated, as would be expected when the contribution due to succinate oxidation had been repressed.

Quite unexpected was the increased time delay before the start of oxygen consumption when malonate and glutamate were used. This delay was not observed when malonate and malate were used. It is possible, as suggested by Borst and Slater (3), that the major pathway for glutamate oxidation is via a glutamate-oxaloacetate transaminase, the required oxaloacetate being accumulated during respiration.

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