Metabolism of Neoplastic Tissue

III. Diphosphopyridine Nucleotide Requirements for Oxidations by Mitochondria of Neoplastic and Non-neoplastic Tissues*

CHARLES E. WENNER† AND SIDNEY WEINHOUSE

(Lankenau Hospital Research Institute and the Institute for Cancer Research, and the Department of Chemistry, Temple University, Philadelphia, Pa.)

In previous publications we reported that oxidative processes of the citric acid cycle in tumor homogenates (29) or mitochondria isolated therefrom (30) are limited by the loss of diphosphopyridine nucleotide activity and that oxygen consumption can be restored by the addition of this coenzyme to the medium in relatively high concentration. The present report represents a further study of the effect of DPN on the oxidative behavior of tumor mitochondria, together with a comparison with mitochondria of various non-neoplastic tissues in regard to oxidative activity toward pyruvate and components of the citric acid cycle.

METHODS

Transplanted tumors were maintained by subcutaneous implantation in adult mice of the C3H, Swiss, or A strain. After reaching a diameter of approximately 1 cm., they were dissected from the decapitated, exsanguinated animals, and mitochondria from these tissues were prepared exactly according to the procedure of Schneider (22). The washed mitochondria were finally suspended in cold, isotonic sucrose unless otherwise specified. All operations were carried out at <5° C. The basic medium consisted of the following substances in the designated final concentrations: MgSO₄, 3 × 10⁻³ M; sodium fumarate ("sparker") 7 × 10⁻⁴ M; ATP, 2 × 10⁻³ M; cytochrome c, 4 × 10⁻⁴ M; phosphate buffer (pH 7.4), 6 × 10⁻³ M; KCl, 6.7 × 10⁻² M; DPN, 1.9 × 10⁻⁴ M. To this there was added substrate (0.006 M) and 0.4 ml. of the mitochondrial suspension, representing 200 mg. of the original tumor tissue or 133 mg. of original normal tissue, to a final volume of 1.6 cc. in chilled Warburg vessels. The flasks were shaken at 38° C., with air as the gas phase. Oxygen uptake measurements were begun after an initial 7 minutes of equilibration.

Substrates and other material.—Citrate, a-ketoglutarate, succinate, maleate, fumarate, pyruvate (all added as the sodium salts), cytochrome c, adenosine triphosphate (ATP), and diphosphopyridine nucleotide (DPN) were commercial products. The DPN was obtained either from Sigma Chemical Co. or the Pabst Laboratories, and assayed from 40 per cent to 90 per cent pure by the enzymatic procedure of Racker (21). Reduced DPN was prepared by the procedure of Ohlmeyer (17), and the contaminating sulfite and sulfate were removed according to Lehninger's modifications (11). This material was found to be 75 per cent pure by measurement of the absorption at 340 mμ with use of the molar extinction coefficient, 6.82 × 10⁶ sq cm/mole (7). Triphosphopyridine nucleotide, assaying 40 per cent pure by the Zwischenferment assay (28), was prepared by the method of LePage and Mueller (14). Nitrogen determinations on the tissues were made by the micro-Kjeldahl procedure of Ma and Zuazaga (15).

RESULTS

Pyruvate oxidation by hepatoma-cell components.—Previous investigations have demonstrated that
the enzymatic equipment for oxidation of fatty acids and citric acid cycle components in normal tissues is restricted to the cytoplasmic granules known as mitochondria (5, 6, 9, 23, 25). Results of a study of the distribution of pyruvic oxidation activity among the centrifugally separated cell components, as shown in Table 1, demonstrate that be assumed that the low activities observed in the nuclear, microsomal, and supernatant fractions are due to contamination by mitochondria. The whole homogenate displays an appreciable endogenous oxygen uptake, which is greatly augmented by addition of DPN, with or without substrate. This activation of the oxidation of endogenous substrates by DPN has been found to be characteristic of whole homogenates of a variety of tumors. The sum of the oxygen uptakes of the separate fractions is approximately two-thirds that of the whole homogenate, indicating that considerable inactivation occurs during separation of the fractions.

**Effect of pyruvate concentration.**—The characteristic oxidative behavior of DPN-fortified mitochondria of neoplastic tissue is shown in Chart 1, in which pyruvate oxidation was studied in particles obtained from mouse hepatoma 98/15. At high substrate concentrations oxygen consumption proceeded at linear rates for approximately 2 hours and was observed to be maximal at 0.005 M. It seems highly probable that oxidation of pyruvate goes to completion in this system, since the oxygen uptake at 0.001 M pyruvate leveled off at a value close to that calculated for complete oxidation of the substrate. Addition of DPN consistently caused an appreciably increased oxygen consumption in the absence of substrate, which is probably due to stimulation of the oxidation of the "priming" agent or of endogenous carbon. This pattern of oxidative activity was displayed for citric acid cycle components as well as pyruvate, and by mitochondria from non-neoplastic as well as neoplastic tissue.

**Specificity of DPN activation.**—In the absence of DPN, pyruvate oxidation in mitochondria of hepatoma and rhabdomyosarcoma was not activated by addition of TPN, flavin adenine nucleotide, coenzyme A, or any of the adenine nucleotides. Addition of fluoride, which Potter and Lyle (19) found to stimulate respiration of the Flexner-Jobling tumor, had no effect on oxygen consump-

<table>
<thead>
<tr>
<th>TISSUE FRACTIONS</th>
<th>TOTAL N†</th>
<th>WHOLE NOG.</th>
<th>OXYGEN CONSUMPTION</th>
<th>OXYGEN CONSUMPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.08</td>
<td>100</td>
<td>46</td>
<td>105</td>
</tr>
<tr>
<td>Nw1</td>
<td>0.506</td>
<td>24.4</td>
<td>2.8</td>
<td>14</td>
</tr>
<tr>
<td>Mw1</td>
<td>0.275</td>
<td>13.2</td>
<td>2.3</td>
<td>14</td>
</tr>
<tr>
<td>Pw</td>
<td>0.222</td>
<td>10.7</td>
<td>1.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Sn</td>
<td>1.150</td>
<td>55.3</td>
<td>5.8</td>
<td>41</td>
</tr>
</tbody>
</table>

* Notation is that of Schneider (21).
† Mg nitrogen per 100 mg. fresh tissue.

**TABLE 1**

**PYRUVATE OXIDATION IN CELLULAR CONSTITUENTS OF MOUSE HEPATOMA 98/15**

---

**Chart 1.**—Effect of substrate concentration on the oxidation of pyruvate by mouse hepatoma mitochondria. Ordinate represents the µL of oxygen consumed by mitochondria equivalent to 200 mg. of original tissue (wet wt.).

the hepatoma 98/15 also has essentially all this activity localized in the mitochondrial fraction. In agreement with previous fractionation data published by Schneider (23), the mitochondrial fraction contained about 13 per cent of the total nitrogen, and this was the only one of the fractions showing high oxidative activity toward pyruvic acid. Since the separations are not absolute, it can
tion, either with or without DPN, when tested with our tumors. The only substance having a similar effect was reduced DPN (Table 2). It was expected that differences in activation might have been observed as the result of differences in permeability of the mitochondrial membrane for the two forms of the coenzyme. However, as shown in Table 2, activation was the same at comparable concentrations. In accord with expectations, hepatoma mitochondria readily oxidized reduced DPN; as shown in Table 2, the oxygen uptake cor-

**TABLE 2**

<table>
<thead>
<tr>
<th>DPN (×10⁻⁴ M)</th>
<th>DPN-H⁺ (×10⁻⁴ M)</th>
<th>Pyruvate (0.01 M)</th>
<th>O₂ uptake (µl/mg N/30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18.7</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>55</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>30</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>46</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>+</td>
<td>146</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>146</td>
</tr>
</tbody>
</table>

*In this experiment DPN-H⁺ equivalent to 17.5 µM of the pure material was incubated for 3 hours at 38°C with mitochondria representing 200 mg of original tissue. The observed oxygen uptake compared favorably with the theoretical oxygen uptake of 193 µl.*

responded closely to the theoretical amount required for oxidation to DPN.

**Relationship between oxidative activity and mitochondrial content.**—Since the reactions of the citric acid cycle seemed to be localized in the mitochondria, it appeared probable that many of the quantitative differences in oxidative activity between different tissues could be referable to variability in mitochondrial content. Evidence for the correctness of this postulate has already been reported (20, 23, 30), and these observations have been extended in the present report. Data on pyruvate oxidation and mitochondrial nitrogen content for a number of neoplastic and non-neoplastic tissues are shown diagrammatically in Chart 2. A marked difference in mitochondrial content between the two types of tissue is immediately evident; indeed, it was surprising to observe the extreme variability displayed by the various tissues. In view of the generally low mitochondrial content of tumor tissues, one can readily understand why such tissues have been reported as deficient in such mitochondria-bound factors as succinic dehydrogenase and cytochrome c (4, 24).

A rather good correlation is displayed in Chart 2 between the mitochondrial content and the activity toward pyruvate oxidation, emphasizing again the quantitative as well as qualitative similarity of mitochondria of different tissues in their capacity for oxidation of pyruvate.

**Oxidation of citric acid cycle components.**—These studies have been further extended to include comparisons between normal and neoplastic mitochondria in the oxidation of citric acid cycle components and the effect thereon of the addition of DPN. The results of this survey are collected in Table 3. To aid in these comparisons, all oxygen uptake values are on a per milligram nitrogen basis. In agreement with the data of other investigators (8, 9), all the substrates were readily oxidized by mitochondria of liver and kidney, and little or no enhancement occurred with addition of DPN. In those instances in which DPN increased the oxygen uptake, the effect was due to better maintenance of the oxygen consumption rather than to an increase of the initial rate. In contrast

![Chart 2](chart2.png)

Chart 2.—Pyruvate oxidation versus nitrogen content of mitochondria of normal and neoplastic tissues. The unshaded bars on the left of each set of columns represent the mg. mitochondrial nitrogen per gram of original whole tissue. The shaded bars on the right side of each set of columns represent the net respiratory activity in µl. of O₂ consumed per gram fresh tissue per 30 minutes. Conditions of assay are outlined in the experimental section. Each column represents an average of two or three determinations. Normal tissues: column A, rat liver; B, mouse liver; C, mouse kidney; D, rat brain. Neoplastic tissues: column A, mouse hepatoma (A strain); B, mouse hepatoma (CSH strain); C, sarcoma 37; D, rhabdomyosarcoma; E, rat hepatoma.

1 The validity of basing mitochondrial content on the nitrogen content without knowledge of their composition may be questioned; however, in the absence of other easy methods, this may be regarded as a fair approximation for the purpose intended.
with liver and kidney, oxygen uptake was relatively low in the unfortified brain mitochondria in the presence of all substrates except succinate but was enhanced considerably with DPN addition. In this respect, brain mitochondria resemble those of the neoplastic tissues. All four tumors listed in Table 3 exhibited essentially the same oxidative pattern. In the absence of added DPN, oxygen uptake was invariably and consistently high; the only substrate whose oxidation was not increased by DPN addition was succinate. The activation of citrate oxidation by DPN was somewhat surprising, since the first dehydrogenase to act on this substance is isocitric dehydrogenase, which is generally considered to be specific for TPN (16). It is recognized, of course, that DPN would be required for the further oxidation of the products of isocitrate oxidation through the citric acid cycle, hence DPN should be expected to enhance the over-all oxidation of citrate. On the other hand, oxidation of citrate to ketoglutarate should proceed unimpeded in the absence of DPN, or should require TPN. Since this does not occur, we can only conclude either that DPN can function as electron carrier for isocitric dehydrogenase in mitochondria or that DPN is exerting some activating effect other than in electron transport.

**DISCUSSION**

It has been known for many years that slices of tumor tissue exhibit respiratory activities, which, though not generally so high as those of such highly metabolically active tissues as kidney, heart, and liver, are of similar magnitude to these and are within the range of any representative tumor tissues with the same order of magnitude as in non-neoplastic tissues (31). The present findings supplement these studies by establishing the ability of mitochondria of neoplastic tissues to carry out the complete oxidation of pyruvate and citric acid cycle components. The approximately equal activities of “neoplastic” and “non-neoplastic” mitochondria on a per milligram nitrogen basis emphasize that mitochondria from various cells are fundamentally similar in oxidative activities and indicate also that at least some of the reported differences in oxidative enzymes are due to variations in mitochondrial content rather than to differences in the enzymatic equipment of the mitochondria. That tumor mitochondria are able to carry out citric acid cycle oxidations has recently been shown also by other investigators. Potter and Lyle (19) reported that whole homogenates of the Flexner-Jobling carcinoma can oxidize a mixture of pyruvate and fumarate when fluoride is added to depress organic phosphate breakdown; and they attributed the previous failure of such systems to oxidize oxalacetate to an inability of phosphorylating processes to keep pace with the rapid dephosphorylation of ATP. More recently, Siekewitz, Simonson, and Potter (26) reported that different tumor homogenates display different rates of phosphate “turnover” and that good oxidation can be observed in tumor homogenates when these opposing activities are balanced by the addition of dinitrophenol or fluoride. In a study of oxidative phosphorylation, Kielley (10) found that phosphorylative oxidation of succinate, glutamate, and α-ketoglutarate occurred in a transplantable mouse hepatoma; and, in agreement

**TABLE 3**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mouse liver</th>
<th>Rat liver</th>
<th>Mouse kidney</th>
<th>Rat brain</th>
<th>Hepatoma 98/15</th>
<th>Hepatoma 97</th>
<th>Sarcoma 97</th>
<th>Rhabdomyosarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN</td>
<td>37</td>
<td>43</td>
<td>45</td>
<td>55</td>
<td>91</td>
<td>40</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>DPN + DPN</td>
<td>45</td>
<td>154</td>
<td>154</td>
<td>279</td>
<td>249</td>
<td>38</td>
<td>146</td>
<td>50</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>150</td>
<td>145</td>
<td>159</td>
<td>154</td>
<td>279</td>
<td>38</td>
<td>146</td>
<td>50</td>
</tr>
<tr>
<td>Citrate</td>
<td>178</td>
<td>915</td>
<td>154</td>
<td>239</td>
<td>296</td>
<td>19</td>
<td>116</td>
<td>14</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>146</td>
<td>140</td>
<td>115</td>
<td>212</td>
<td>355</td>
<td>45</td>
<td>116</td>
<td>100</td>
</tr>
<tr>
<td>Succinate</td>
<td>145</td>
<td>165</td>
<td>147</td>
<td>230</td>
<td>254</td>
<td>114</td>
<td>104</td>
<td>152</td>
</tr>
<tr>
<td>Fumarate</td>
<td>150</td>
<td>156</td>
<td>154</td>
<td>95</td>
<td>220</td>
<td>35</td>
<td>101</td>
<td>15</td>
</tr>
<tr>
<td>Malate</td>
<td>160</td>
<td>164</td>
<td></td>
<td>69</td>
<td>127</td>
<td>35</td>
<td>176</td>
<td>35</td>
</tr>
</tbody>
</table>

Lyle (19) reported that whole homogenates of the Flexner-Jobling carcinoma can oxidize a mixture of pyruvate and fumarate when fluoride is added to depress organic phosphate breakdown; and they attributed the previous failure of such systems to oxidize oxalacetate to an inability of phosphorylating processes to keep pace with the rapid dephosphorylation of ATP. More recently, Siekewitz, Simonson, and Potter (26) reported that different tumor homogenates display different rates of phosphate “turnover” and that good oxidation can be observed in tumor homogenates when these opposing activities are balanced by the addition of dinitrophenol or fluoride. In a study of oxidative phosphorylation, Kielley (10) found that phosphorylative oxidation of succinate, glutamate, and α-ketoglutarate occurred in a transplantable mouse hepatoma; and, in agreement...
with our findings, observed a more definite requirement of DPN in hepatoma than in liver mitochondria for the two latter substrates whose oxidation requires this co-factor.

In our hands, the DPN requirement for oxygen uptake was the most consistent and characteristic feature of the tumor mitochondria. It was observed in every experiment with all tumors, whether rapid or slowly growing, and with DPN from commercial sources, as well as with material made in our laboratory. We recognize, however, that the methods of preparation of mitochondria are largely empirical; hence, in view of the many obvious differences in experimental conditions among different investigators, such as the totality of medium, speed of centrifugation, concentrations of substrates and co-factors, temperature of incubation, etc., it is not too surprising that Potter et al. (26) found that certain tumor homogenates consume oxygen without DPN addition or that Kielley observed a pronounced DPN requirement only after short aging periods. Since a DPN requirement can be created for liver and kidney mitochondria by such mild treatments as freezing and thawing, hypotonicity, or aging at 0° (8), it seems probable that the DPN requirement of tumor mitochondria does not represent a fundamental difference in the metabolism of neoplastic tissue but is due rather to structural characteristics of the mitochondria which are manifested by a loosening of the binding of DPN.

The reasonable assumption that mitochondria of different cell types may vary in their ability to bind DPN or other coenzymes may serve to explain certain observed differences in metabolism among various tissues. For example, a weakness in the binding of DPN by tumor mitochondria, such as is suggested by the present study, may provide a simple, rational explanation for one of the most perplexing features of the metabolism of tumor cells—namely, their high aerobic and anaerobic glycolysis. The extensive studies of LePage (12, 18) have clearly established the fact that whole tissue homogenates, when fortified with DPN and other co-factors, can carry out glycolysis of hexose diphosphate at rates which are far higher than those observed for tissue slices acting on glucose, and have further shown that similar glycolytic levels are displayed by such fortified homogenates of both neoplastic and non-neoplastic tissues. These findings suggest that the potential glycolytic capacities of these cells are similar, but that the rates of these processes may be controlled and regulated in the intact cell by the intracellular distribution of these co-factors. Inasmuch as the glycolytic enzymes are in the soluble portion of the cytoplasm, it would be expected that glycolysis would be higher in tumor cells, in which DPN is loosely bound, and, hence, would be in correspondingly higher concentrations in the soluble portion of the cell where the process occurs. Conversely, glycolysis would be expected to be low in cells in which DPN is more strongly bound and, hence, not available for maximal activity of the electron-transferring steps of glycolysis. It is noteworthy in this connection that rat brain, whose mitochondria displayed a DPN requirement in this study, is one of the small number of non-neoplastic tissues which has a high rate of glycolysis (27). The correlation of glycolytic activity with the intracellular distribution of DPN is now under further investigation.

SUMMARY

The oxidation of pyruvate and citric acid cycle components occurs readily and consistently in mitochondria of neoplastic tissues when these are fortified by the addition of DPN in rather high concentration. This DPN requirement cannot be replaced by other known coenzymes or adenine nucleotides. On a per milligram nitrogen basis, oxidations by such fortified mitochondria of neoplastic tissues were quantitatively similar to their non-neoplastic counterparts. The suggestion is made that mitochondria of neoplastic tissues do not bind DPN as strongly as certain normal tissues, and that this may result in a correspondingly higher level of this coenzyme in the soluble portion of the cell cytoplasm. It is further suggested that this phenomenon may account for the characteristically high glycolysis rates of intact tumor cells.

REFERENCES


WENER AND WEINHOUSE—Metabolism of Neoplastic Tissue. III


causes must be sought in such aspects of the environment as are common to the majority of the population in countries in which a high incidence of the disease has been established.

Environmental factors other than those mentioned, but as yet unknown, may, however, enter the human body through ingestion, skin contact, etc. to exert a carcinogenic influence on the lung, although in so far as is known, the entry of environmental carcinogens into the human lung is by inhalation. Lung tumors induced in animals by materials introduced by other routes do not appear to have counterparts in human experience, but such possibilities must be borne in mind. Experimental studies should be conducted with special reference to inhalation techniques and to sites of deposition of inhaled particles in the respiratory tract; also the susceptibility of a variety of species of animals should be tested. Certain simpler procedures may be useful for preliminary investigations of suspect substances. These simpler procedures include (1) intratracheal injections, (2) skin painting, (3) subcutaneous injection, (4) the tissue transplant technic. Experiments may also be conducted to examine the effect of (5) material deposited in the nose and oropharynx. It must, however, be remembered that it is man with whom we are ultimately concerned and that animal experiments should be interpreted in the light of observations made on the occurrence of the disease in man. This need not preclude the taking of immediate precautions against hazards to man suggested by animal tests.

Variations in susceptibility of the individual exposed to the influence of environmental factors should not be ignored and would seem to deserve further investigation; for example, it is not known to what extent sex differences in incidence of cancer of the lung can be explained by differences between the two sexes with regard to smoking habits.

Co-action of etiologic factors should also be taken into consideration with a view to the possibility that inhaled substances may have a cocarcinogenic influence or that conditions within the lung may have such an effect, which ultimately may influence a possible latent period for carcinoma of the lung.

Studies of the gross and histological types of tumors may be significant in the evaluation of results from other kinds of study, which should be carried out with full attention to the type of lesion. Thus, studies on histological changes in the respiratory tract, which may result from the influence of tobacco smoking, and similar studies on individuals exposed to industrial hazards, and not developing carcinoma of the lung may give significant results. In view of these considerations the Symposium adopted the following recommendations:

Recommendation 3.—The Symposium on the Endemiology of Cancer of the Lung recommends:

3.1.—That the Committee on Geographical Pathology of Cancer, under the International Cancer Research Commission, should stimulate the organization and execution of further studies on the following subjects with a view to the etiology and pathogenesis of primary cancer of the lung.

3.1.1.—The extent of the increase in incidence of primary cancer of the lung observed particularly among males in various countries and areas, or, where such increase is not found, the conditions of its absence.

3.1.2.—The association between the smoking of tobacco, especially cigarettes, and cancer of the lung.

3.1.3.—The atmospheric pollution in urban and industrial areas.

3.1.4.—The occupational or industrial exposure to hazards associated with an increased incidence of cancer of the lung.

3.1.5.—Environmental factors yet unknown including any that may act through ingestion, skin contact, etc.

3.1.6.—Variations in the susceptibility of individuals exposed to environmental factors causing cancer of the lung.

3.1.7.—Co-action of factors causing cancer of the lung.

3.1.8.—Histology of lung lesions caused by any of the categories of factors mentioned.

3.2.—That such studies be carried out as international cooperative and correlated studies in populations with high and low incidence of cancer of the lung with a simultaneous view to as many of the factors mentioned as possible.

Erratum

The following correction should be made in the abstract by B. Grad, A. Oberleitner, and J. Berenson ("The Effect of Thyroxine and Thiouracil on the Incidence of Lymphogenous Leukemia in Akr Mice") in the Proc. Am. Assoc. Cancer Research, 1:20, 1958. The last sentence of the second paragraph on page 21 reads: "Hence, the thyroxine mice were hyperthyroid, but nevertheless their survival was significantly lowered." It should read: "... was not significantly lowered."
Metabolism of Neoplastic Tissue: III. Diphosphopyridine Nucleotide Requirements for Oxidations by Mitochondria of Neoplastic and Non-neoplastic Tissues

Charles E. Wenner and Sidney Weinhouse


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/13/1/21

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.