Intracellular Hydrogen Transport in Ehrlich Ascites Tumor Cells

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SUMMARY
The high rate of aerobic glycolysis of Ehrlich ascites tumor cells has been attributed to an ineffective mechanism for the mitochondrial oxidation of reducing equivalents synthesized in the extramitochondrial compartment. The present experiments provide additional support for this conclusion. Inhibition of the tricarboxylic acid cycle with arsenite or fluorocitrate, or of the respiratory chain with rotenone or Amytal, restricted the metabolism of endogenous substrate with consequent inhibition of respiration. Addition of glucose or fructose to the inhibited system resulted in considerable lactate production, but failed to initiate respiration. However, in the presence of vitamin K₃, a shunt was established via DT diaphorase. Under these conditions, hexose addition led to a marked stimulation of respiration and diminished lactate production as a result of the transfer of hydrogen from extramitochondrial reduced pyridine nucleotides to the mitochondrial respiratory chain. Thus, hexose metabolism contributes reducing equivalents for mitochondrial oxidation in the presence of the “K₃-shunt,” but not in its absence.

INTRODUCTION
It seems clear that extramitochondrial reduced pyridine nucleotides are not directly oxidized by the mitochondrial respiratory chain of animal cells. Lehninger (18) first observed that intact mitochondria isolated from liver are incapable of oxidizing added DPNH. Similar observations have been made with mitochondria of ascites tumor cells (1, 3). The slow rate of penetration of pyridine nucleotides into liver mitochondria in the intact animal (12, 22) confirms the conclusion drawn from the in vitro studies. Schemes suggesting an indirect mechanism for the introduction of extramitochondrial reducing equivalents to the mitochondrial respiratory chain have been proposed (2, 16). Reduced metabolic intermediates which are capable of being oxidized by mitochondria could theoretically serve to transport electrons to the mitochondrial respiratory chain; the oxidized product is released to the cell sap where it can be reduced in the presence of the appropriate enzyme and reduced pyridine nucleotide. Boxer and Devlin (3) have suggested that this indirect mechanism is not available to malignant cells. In accordance with the suggestion of Weinhouse (27), they believe that the high rate of aerobic lactate production by malignant cells is a consequence of the absence of an effective mechanism for the mitochondrial oxidation of extramitochondrial reduced pyridine nucleotides, although this hypothesis has been questioned by Borst (2).

In the present experiments the contribution of hexose metabolism to mitochondrial respiration has been assessed in the intact cell. This evaluation is made possible through the use of (a) specific inhibitors of endogenous respiration and (b) a vitamin K₃-mediated DT diaphorase pathway (10). The results are compatible with the suggestion of Boxer and Devlin (3) and Weinhouse (27), namely that the high rate of lactate accumulation under aerobic conditions is a consequence of the inefficiency of these cells to transfer electrons from extramitochondrial reduced pyridine nucleotides to the respiratory chain.

MATERIALS AND METHODS
Ehrlich ascites tumor cells of the hyperdiploid strain were harvested from the peritoneal cavity of white mice 8–12 days after inoculation. The tumor cells were collected in a Krebs-Ringer phosphate buffer (without calcium), pH 7.4, at ice bath temperature and separated from the ascitic fluid by centrifugation at 40 × g for 5 minutes in the cold. The tumor cells were resuspended in the medium and collected by centrifugation once or twice in order to remove the bulk of contaminating erythrocytes and leukocytes. After a final centrifugation at 250 × g for 5 minutes, the cells were collected and resuspended in two volumes of buffer.

Rotenone, antimycin, and vitamin K₃ were made up in ethanol or dioxane and added to the incubation mixture in 5- to 15-μl volumes; neither ethanol nor dioxane in these concentrations had a demonstrable effect on the metabolism of the cells. Aqueous solutions of fluorocitrate and fluorocitrate were prepared fresh daily as previously described (11). All other substrates and inhibitors were prepared in aqueous solution and were adjusted to neutral pH prior to their introduction into the incubation mixture.

Suspended cells were incubated in 3.0 ml of phosphate buffer at 37°C. Oxygen uptake was measured with the Clark O₂ electrode. Analysis of glucose with glucose oxidase (23), pyruvate with lactic dehydrogenase and DPNH (4), and lactate with lactic dehydrogenase and acetyl-DPN (15) were performed on perchloric acid filtrates. The protein concentration of the cell suspension was determined by a biuret method (14).

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3 The following abbreviations are used: DPN, DPNH, oxidized and reduced diphosphopyridine nucleotides; TPNH, reduced triphosphopyridine nucleotide; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine.

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RESULTS

Inhibition of Endogenous Respiration

Restriction of endogenous respiration was accomplished with a number of inhibitors of mitochondrial metabolism (Chart 1). Addition of either Amytal or rotenone, inhibitors of the respiratory chain, resulted in a prompt decline in respiration to about 15 percent of the initial level. Interruption of the tricarboxylic acid cycle with either arsenite, fluorocitrate, or fluoroacetate led to a gradual but progressive decline in oxygen uptake. The rate and extent of respiratory inhibition varied from one lot of cells to another and from one inhibitor to the other. In general, arsenite and fluorocitrate had comparable inhibitory activities and were more effective than fluoroacetate; in some experiments almost complete inhibition of respiration was observed 10–15 minutes after addition of arsenite or fluorocitrate.

Restoration of Respiration

Addition of glucose to cells which were inhibited with arsenite failed to restore respiration (Chart 2, Curves A and B). However, the reduced pyridine nucleotides generated during the oxidation of glucose were capable of transmitting electrons to the mitochondrial respiratory chain upon addition of vitamin K₃ to the cell suspension. In this manner an electron transfer pathway was established from extramitochondrial pyridine nucleotide via DT diaphorase and vitamin K₃ to the mitochondrial respiratory chain at the level of cytochrome b. Addition of vitamin K₃ alone to this system resulted in a slight and inconsistent increase in respiration (Chart 2, Curves C and D); a rapid restoration of the respiratory rate to values which often exceeded the initial, inhibited rate were again observed upon addition of glucose to the incubation mixture. Previous studies from this laboratory demonstrated the inhibitory effects of low concentrations of Dicumarol on DT diaphorase activity in cell-free preparations (10), and in intact ascites tumor cells (8). Curve C of Chart 2 confirmed these observations in intact cells with negligible endogenous respiration. Cyanide addition to respiring cells resulted in a prompt, essentially complete cessation of oxygen uptake; in contrast, cyanide failed to inhibit DT diaphorase-induced respiration completely (Chart 2, Curve B). A similar finding has been reported by Wenner (28), and can be explained by observations which indicate that reduced menadione is autoxidizable in the presence of oxygen (7, 26). Rotenone-resistance and antimycin A sensitivity seen in Chart 2, Curve A, were characteristic of DT diaphorase-mediated respiration. In other experiments the DT diaphorase bypass of a portion of the respiratory chain was demonstrated by inhibiting respiration with Amytal and rotenone. Restoration of respiration was rapidly accomplished by transmitting electrons from extramitochondrial reduced pyridine nucleotides into the mitochondrial respiratory chain beyond the site inhibited by Amytal and rotenone.

Respiration of the intact ascites tumor cells was also supported by establishing a bypass with TMPD (Chart 3). Packer and Mustafa (20) have recently made similar observations with antimycin-A-inhibited cells. In the presence of TMPD, electrons were introduced into the respiratory chain at the level of cytochrome c (17). Antimycin A resulted in an almost complete

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CHART 1. Inhibition of endogenous respiration. The final concentrations of inhibitors were: Amytal \(10^{-3}\) M, rotenone \(10^{-4}\) M, fluorocitrate (FCit) \(5 \times 10^{-4}\) M, arsenite \(2 \times 10^{-4}\) M, and fluoroacetate (FAc) \(10^{-4}\) M.

CHART 2. Restoration of arsenite-inhibited respiration via the "K₃ shunt." Agents were added in the following final concentrations: arsenite \(2 \times 10^{-4}\) M, glucose \(10^{-3}\) M, vitamin K₃ \(1.5 \times 10^{-5}\) M, rotenone (Rot.) \(10^{-4}\) M, antimycin A (A.A.) \(3.3 \times 10^{-4}\) M, KCN \(10^{-3}\) M, and Dicumarol \(2.6 \times 10^{-4}\) M.
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Chart 3. Restoration of antimycin A (A.A.)-inhibited respiration with N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). Agents were added in the following final concentration: antimycin A 3.3 X 10^-6 M, TMPD 10^-6 M, rotenone 10^-6 M, and KCN 10^-4 M.

respiratory inhibition which was restored by TMPD. Rotenone addition only partly inhibited the TMPD-mediated respiration, indicating that both intramitochondrial DPNH oxidation and transfer of electrons from the extramitochondrial compartment took place (Chart 3); ascorbate was without effect. Complete inhibition of TMPD-induced respiration with cyanide indicated that the oxygen uptake under these conditions proceeded through cytochrome oxidase.

Stoichiometry of the Glucose-Vitamin K3-mediated Respiration

Although glucose did not restore respiration after inhibition of the tricarboxylic acid cycle with arsenite (Table 1) or the respiratory chain with rotenone (Table 2), there was a vigorous metabolism of the glucose to lactate in each case. Addition of vitamin K3 to inhibited cells metabolizing glucose not only restored the oxygen uptake, but also diminished the conversion of glucose to lactate (Table 1, Experiment 3; Table 2, Experiment 2). The presence of 5 x 10^-3 M iodoacetate completely inhibited metabolism of glucose via the Embden-Meyerhof pathway as manifested by the absence of lactate (Table 1, Experiments 4 and 5; Table 2, Experiments 3 and 4) and pyruvate (Table 2, Experiment 4) accumulation. Despite inhibition of glucose metabolism via the Embden-Meyerhof pathway, respiration was reestablished when vitamin K3 was present. This indicated the presence of significant hexose monophosphate shunt activity, in agreement with the experiments of Wenner (28); electrons from TPNH were transferred to the mitochondrial respiratory chain via the DT diaphorase-vitamin K3 system.

When fructose was added to the arsenite-inhibited cells, similar findings were observed (Table 1). Fructose was unable to support respiration except in the presence of vitamin K3. A moderate restoration of respiration with fructose and vitamin K3 was observed after inhibition of the tricarboxylic acid cycle with arsenite, and the glycolytic pathway with iodoacetate. The fructose was converted to glucose-6-phosphate and metabolized through the hexose monophosphate shunt, but at a slower rate than was glucose. Lactate production from fructose was increased in the arsenite-inhibited cells and decreased in the presence of vitamin K3.

In the rotenone-inhibited system, the glucose was almost quantitatively converted to lactate (Table 2). In the presence of vitamin K3, the stoichiometry was altered. If all the glucose taken up in the presence of vitamin K3 were metabolized via the Embden-Meyerhof pathway, then the following relationships should hold:

\[
\text{Glucose} + 2 \text{DPN}^{+} \rightarrow 2 \text{pyruvate} + 2 \text{DPNH} + 2 \text{H}^{+}
\]

\[
2 \text{Pyruvate} + 2 \text{DPNH} \rightarrow 2 \text{lactate} + 2 \text{DPN}^{+}
\]

\[
\text{O}_{2} + 2 \text{DPNH} + 2 \text{H}^{+} \rightarrow 2 \text{H}_{2} \text{O} + 2 \text{DPN}^{+}
\]
TABLE 2
Glucose Metabolism in Rotenone-Inhibited Cellsa

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>mmol/mg protein/min</th>
<th>O2 uptake</th>
<th>Glucose consumption</th>
<th>Pyruvate accumulation</th>
<th>Lactate accumulation</th>
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<td>12.0</td>
<td>17.2</td>
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<tr>
<td></td>
<td>Rot. + glucose</td>
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<td>10.8</td>
<td>9.1</td>
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<tr>
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<tr>
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<td>Rot.</td>
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<td>0.9</td>
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<tr>
<td></td>
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<tr>
<td>3</td>
<td>IAA</td>
<td>2.9</td>
<td>0.2</td>
<td>0.9</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>IAA + Rot.</td>
<td>0.1</td>
<td>0.9</td>
<td>0.0</td>
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<tr>
<td></td>
<td>IAA + Rot. + glucose</td>
<td>0.2</td>
<td>0.9</td>
<td>0.0</td>
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<tr>
<td>4</td>
<td>IAA</td>
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<td></td>
<td>IAA + Rot.</td>
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<td>0.9</td>
<td>0.0</td>
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<tr>
<td></td>
<td>IAA + Rot. + K3 + glucose</td>
<td>2.4</td>
<td>1.3</td>
<td>0.0</td>
<td>0.0</td>
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</table>

a The final concentrations of the additions were: glucose, 10^{-4}; rotenone (Rot.), 10^{-4}; iodoacetate (IAA), 5 \times 10^{-4}; vitamin K3, 1.5 \times 10^{-4}. Cells containing 18.6 mg protein were present in each incubation. Ten minutes after addition of glucose, the incubation was terminated with perchloric acid.

The DPNH formed at the triose phosphate dehydrogenase step could have been oxidized by O2 or pyruvate. The moles of glucose utilized less the moles of oxygen taken up would be equivalent to half the molar accumulation of lactate; this stoichiometry was found (Table 2, Experiment 2). However, these equations predict that an amount of pyruvate equivalent to the oxygen uptake should accumulate—this was not observed. Therefore, it is likely that an appreciable, but indeterminate, quantity of the glucose was metabolized via the hexose monophosphate shunt. The data of Experiment 4, Table 2 supported this claim. For complete inhibition of glucose oxidation via the Embden-Meyerhof pathway resulted in substantial O2 uptake. The molar rate of O2 uptake under these conditions exceeded the molar rate of glucose utilization which indicated a recycling of glucose through the hexose monophosphate shunt pathway.

In contrast to the results obtained at 1 mm glucose concentration, there was complete stoichiometry at very low glucose concentrations. Addition of 0.15 μ mole glucose to arsenite-inhibited cells in the presence of vitamin K3 led to precisely 0.15 μ mole of O2 taken up (Chart 4). A second addition of 0.15 μ mole glucose led to an identical response. This relationship between glucose addition and O2 uptake suggested that all of the glucose was metabolized via the Embden-Meyerhof pathway, and that the generated DPNH was used exclusively to support respiration.

Table 3 compares the effects of vitamin K3 on the O2 uptake, glucose consumption, and lactate accumulation of cells incubated without a respiratory inhibitor, and in the presence and absence of 2,4-dinitrophenol. In the absence of K3 and 2,4-dinitrophenol glucose caused a depression of the endogenous respiration (Crab,

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_Incorporated into the hexose monophosphate shunt pathway._

**TABLE 3**
Glucose Metabolism in the Absence of Respiratory Inhibitors*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition</th>
<th>mmol/mg protein/min</th>
<th>O2 uptake</th>
<th>Glucose consumption</th>
<th>Lactate accumulation</th>
</tr>
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<td>Control</td>
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<td>1.47</td>
<td>9.7</td>
<td>10.2</td>
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<td>8.7</td>
<td>7.9</td>
</tr>
<tr>
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<td>Control</td>
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<td>1.47</td>
<td>9.7</td>
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<td>1.47</td>
<td>9.7</td>
<td>10.2</td>
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<tr>
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<td>4.02</td>
<td>14.3</td>
<td>18.8</td>
</tr>
<tr>
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<td>Control</td>
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<td>4.02</td>
<td>14.3</td>
<td>18.8</td>
</tr>
<tr>
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<td>4.02</td>
<td>4.02</td>
<td>14.3</td>
<td>18.8</td>
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<td>DNP + K3 + glucose</td>
<td>5.75</td>
<td>12.4</td>
<td>16.3</td>
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</table>

* The final concentration of the additions were: glucose, 10^{-4}; vitamin K3, 1.5 \times 10^{-4}; 2,4-dinitrophenol (DNP), 10^{-4}. Cells containing 18.6 mg protein were present in each incubation. Ten minutes after addition of glucose, the incubation was terminated with perchloric acid.
tree effect). About one-half of the glucose consumed accumulated as lactate. The oxygen consumption did not account for the balance of the glucose consumed; the unaccounted for carbons probably accumulated as glycolytic intermediates in accordance with previous observations (6, 29). Addition of vitamin K₃ resulted in an abolition of the Crabtree effect (8, 9), and in virtually no change in glucose consumption. Lactate accumulation decreased and O₂ consumption increased, in a manner that would be expected if the "K₃ shunt" were operating as has been described by Wu and Racker (30). Addition of 2,4-dinitrophenol in the absence of K₃ abolished both the Crabtree effect and the Pasteur effect, as revealed by a lack of inhibition of respiration by glucose, and by an increased glucose utilization and lactate production. When K₃ was added to the 2,4-dinitrophenol-containing system, a further increase in O₂ uptake was observed, with a slight decrease in glucose utilization and lactate production. The operation of the "K₃ shunt" under these conditions may be limited by the rate of electron flow through the respiratory chain.

**DISCUSSION**

The continuous, steady rate of respiration of Ehrlich ascites tumor cells does not require exogenous substrate. It is likely that endogenous fatty acids provide the substrate under these conditions. However, when glucose is added to these cells, there is appreciable conversion of the glucose carbons to lactate. The DPNH formed in the triose phosphate dehydrogenase reaction is reoxidized to DPN during the reduction of pyruvate to lactate. Thus, the DPNH generated during the oxidation of glucose via the Embden-Meyerhof pathway would not be available to support mitochondrial respiration. The high rate of lactate production can be viewed in still another way. Boxer and Devlin (3) suggested that the tumor cells lack a mechanism for the introduction of reducing equivalents generated in the extramitochondrial compartment to the mitochondrial respiratory chain. The rapid conversion of pyruvate to lactate could then be looked upon as a mechanism to provide oxidized pyridine nucleotides for the continuous operation of glucose oxidation via the Embden-Meyerhof pathway. This interpretation is consistent with the finding that TPNH generated in the hexose monophosphate shunt pathway can be used for the reduction of pyruvate to lactate (28).

Although it is clear that extramitochondrial reduced pyridine nucleotides cannot be used directly to support mitochondrial respiration, they may contribute electrons to the respiratory chain indirectly. A number of mechanisms have been proposed which could facilitate the transfer of extramitochondrial reducing equivalents into the mitochondria (2), but their importance in cellular metabolism remains to be elucidated (24). At present, experimental evidence to indicate that Ehrlich ascites tumor cells possess these mechanisms is lacking (1, 3), except under very special conditions (5, 19).

Assessment of the contribution of extramitochondrial reduced pyridine nucleotides to respiration in the intact cell has been difficult. Although the Ehrlich ascites tumor cell is an attractive tissue in which to study these mechanisms because of the high rate of aerobic glycolysis, the preferential use of endogenous substrate and the pronounced Crabtree effect complicate interpretation of data. In the present experiments, specific inhibitors of the tricarboxylic acid cycle were used to block endogenous respiration. Arsenite, a dithiol inhibitor has been shown to block the metabolism of pyruvate and α-ketoglutarate (25). Both fluorocitrate and fluorocitrate block the tricarboxylic acid cycle at the aconitase level (21). Under conditions of complete or almost complete inhibition of respiration with each of these inhibitors, glucose is vigorously metabolized to lactate. However, the reducing units formed during glucose oxidation could not be used to support respiration. In the presence of vitamin K₃ and glucose, respiration is restored via the DT diaphorase pathway and lactate production is diminished. These results demonstrate the intactness of the respiratory chain from the level of cytochrome b to cytochrome oxidase. A similar response to the presence of vitamin K₃ and glucose is observed when the respiratory chain is blocked with rotenone or Amytal. Wenner (28) found appreciable glucose oxidation by Ehrlich ascites tumor cells via the hexose monophosphate shunt. The present experiments with arsenite- or rotenone-inhibited cells support this conclusion. The DT diaphorase-mediated respiration is observed in the presence of glucose and fructose when the Embden-Meyerhof pathway is inhibited with iodoacetate.

The present experiments indicate that the reduced pyridine nucleotides generated in the extramitochondrial portion of the cell as a result of glucose oxidation are unable to serve effectively as electron donors for the mitochondrial respiratory chain. Thus, in the ascites tumor cell, the high rate of pyruvate conversion to lactate serves to oxidize the reduced pyridine nucleotides which are generated in the course of glucose oxidation. Other tissues which utilize glucose without appreciable lactate production possess other mechanisms for the reoxidation of reduced pyridine nucleotides.

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