The Role of Glycolysis in the Growth of Tumor Cells

I. EFFECTS OF OXAMIC ACID ON THE METABOLISM OF EHRlich ASCITES TUMOR CELLS IN VITRO*

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It is now well known that tumor tissues, either in the form of solid tumors or as ascites cells, have a high rate of aerobic and anaerobic glycolysis. A theory on the origin of the tumor cell as proposed by Warburg (1, 2) states that a normal cell becomes malignant as the result of impairment to its respiratory processes. Warburg has stated (1, 2) that in order for these cells to survive they must derive the energy that was once provided by respiration from other metabolic processes. Thus, he proposes that the increased rate of glycolysis in the tumor cell serves to provide energy for its survival. However, it has never been shown experimentally that tumor cells are dependent on glycolysis for survival. In the experiments to be reported, we have sought to establish conditions in which the glycolysis of tumor cells is specifically inhibited, whereas the respiration of the cells remains unaffected. These experiments were based on the view that glycolysis can be inhibited specifically only by inhibition of lactic dehydrogenase, since the latter is the only enzyme of the Embden-Meyerhof pathway of glycolysis which plays no role in the oxidation of carbohydrate via pyruvic acid.

Schwert et al. (3–6) have shown that, of a series of compounds structurally related to pyruvic acid, oxamic acid (H₂N·CO·COOH) is the most potent competitive inhibitor of crystalline beef heart lactic dehydrogenase. The present experiments were designed to test whether oxamic acid could also inhibit lactic dehydrogenase in the intact Ehrlich ascites tumor cell and whether it is a specific inhibitor of this enzyme in the intact cell. The effect of the inhibition of lactic dehydrogenase on anaerobic glycolysis, aerobic glycolysis, oxygen uptake, and the Crabtree effect is reported below. A preliminary account of this work has appeared (7). Other studies of oxamate effects on glycolysis have also appeared (8, 9).

EXPERIMENTAL PROCEDURE

Chemicals—Potassium oxamate was generously donated by Dr. George Schwert. Other samples of potassium or sodium oxamate were prepared by partial ammonolysis of diethyl oxalate (10), followed by hydrolysis of the ethyl oxamate with potassium or sodium hydroxide and crystallization from ethanol. Sodium pyruvate was obtained from the Mann Research Laboratories, Inc. Heparin was obtained from the California Corporation for Biochemical Research. Zinc di-lactate, used as a standard for lactic acid determinations, was prepared according to the method of Carmien and Dunn (11); DPNH was made according to the enzymatic method of Rafter and Colowick (12), and was stored as the barium salt. Before use, the barium salt was converted to the sodium salt. The DPN was obtained from the Pabst Laboratories, and in later experiments, the sodium salt of DPNH was also obtained from this company.

Manometric Studies—Anaerobic glycolysis and respiration experiments were done in the conventional Warburg respirometer. Anaerobic conditions were obtained by gassing the manometer flasks with a mixture of 95% N₂:5% CO₂ for 10 minutes during the initial incubation period at 37°.

Aerobic glycolysis studies were done both manometrically and colorimetrically. The manometric method is based on the assumption that the difference in respiration in bicarbonate buffer and in phosphate buffer is negligible. The manometric experiments were done by the use of a three flask technique. Flasks 1 and 2 contained Krebs-Ringer phosphate buffer, pH 7.4, with and without KOH in the center well, and were used to measure oxygen uptake and respiratory CO₂ evolution, respectively, according to the direct method of Warburg (13). The third flask contained Krebs-Ringer bicarbonate buffer, pH 7.4, and was gassed with 95% O₂:5% CO₂ for 10 minutes during the initial incubation period. The pressure changes in the third flask are due to O₂ uptake, the evolution of respiratory CO₂, and CO₂ liberation by lactic acid production, so that the latter value may be calculated by difference. The manometric method of measuring aerobic glycolysis was confirmed by the colorimetric measurement of lactic acid by the method of Barker and Summerson (14).

Since the resultant pressure change in flask 2, due to oxygen uptake and respiratory CO₂ evolution, is very close to zero, it is assumed that this is also the case in flask 3 and that the large positive pressure observed in flask 3 is almost completely due to CO₂ evolution by lactic acid production. Therefore, the degree of aerobic glycolysis can be calculated from the uncorrected resultant pressure changes in flask 3 alone (15). This one-flask method was used for following the time course of aerobic glycolysis in most experiments.

Preparation of Whole Cells for Glycolysis and Respiration Studies—Both male and female Swiss Albino mice (Albino Farms, Redbank, New Jersey) were used to carry the Ehrlich ascites tumor cells. The original supply of Ehrlich ascites tumor cells was generously donated by Dr. Abraham Goldin and Dr. Morris
Belkin of the National Institutes of Health. Other strains of ascites cells obtained from these laboratories were S-37, Hepatoma-137, Hepatoma-P, Krebs-2, and SH-60. The tumor cells were propagated, harvested, and washed free of red blood cells with 0.9% NaCl solution in the usual manner (16). After washing, the cells were centrifuged at 600 \( \times g \) in a calibrated 12-ml centrifuge tube, to determine the packed cell volume, and resuspended in 3 times their volume of Krebs-Ringer bicarbonate buffer or Krebs-Ringer phosphate buffer. The cells were then uniformly resuspended in a loosely fitting TenBroeck homogenizer. The cells are resistant to breakage even by a closely fitting homogenizer of this type.

**Preparation of Ascites Tumor Cell Mitochondria**—The tumor cells were washed free of red blood cells with 0.9% NaCl solution as above. However, before determining the packed cell volume, the cells were washed three times in 0.25 \( \mu \)M sucrose at 0–3\(^\circ\). The cells were then resuspended in 4.5 times their packed cell volume with 0.25 \( \mu \)M sucrose and disintegrated in the ball-type ground glass homogenizer described by Dounce et al. (17). The homogenate was centrifuged at 800 \( \times g \) for 5 minutes in a refrigerated centrifuge to eliminate the unbroken cells, nuclei, and other large particles. The supernatant, containing mitochondria and microsomes, was centrifuged at 8,500 \( \times g \) for 10 minutes. The supernatant was discarded and the mitochondria were washed twice by suspending them in 5 to 10 ml of 0.25 \( \mu \)M sucrose and centrifuging at 12,000 \( \times g \) for 10 minutes. The washed mitochondria were then resuspended in 1.5 times their packed cell volume with 0.25 \( \mu \)M sucrose, in a loosely fitting TenBroeck homogenizer.

**LDH Measurements**—The crystalline rabbit muscle LDH\(^1\) and beef heart LDH used in the kinetic studies were obtained from the Worthington Biochemical Corporation. The Ehrlich ascites tumor LDH used in the kinetic studies was obtained from a homogenate of the tumor cells. The homogenate was prepared by suspending the cells in 3 times their volume of 0.9% NaCl and subjecting them to sonic vibration for 10 minutes at 0.9 amperes in a Raytheon 10 kc model DF101 magnetostrictive oscillator. The homogenate was then centrifuged for 30 minutes at 1,600 \( \times g \) in the International refrigerated centrifuge.

The kinetic studies on rabbit muscle, beef heart, and Ehrlich ascites tumor LDH were made with a Beckman model DU spectrophotometer. The initial rate of reaction was determined by following the rate of disappearance of DPNH at 340 nm during the first minute.

Pyruvic acid was determined by enzymatic techniques. By the use of \( \beta \)-lactic dehydrogenase from *Leuconostoc mesenteroides*, which was found by us to be relatively insensitive to oxamic acid inhibition, pyruvic acid was determined by following the extent of disappearance of DPNH at 340 nm in the Beckman model DU spectrophotometer. The \( \beta \)-lactic dehydrogenase used was an ammonium sulfate fraction kindly donated by Dr. Don Dennis.

The glucose determinations were made by the method of Nelson (18).

### Results

**Effect of Potassium Oxamate on LDH from Various Sources**—The ability of varying concentrations of potassium oxamate at a given pyruvate concentration to inhibit the enzymatic activity of rabbit muscle, beef heart, and Ehrlich ascites tumor LDH is shown in the curves in Fig. 1. A kinetic analysis of the inhibition of these enzymes by potassium oxamate at varying pyruvate concentrations is shown in Fig. 2.

Schwert et al. (3–6) have shown that potassium oxamate is a competitive inhibitor of crystalline beef heart LDH. The data shown in Fig. 2 confirm the observations of Schwert et al. and show that potassium oxamate is also a competitive inhibitor of rabbit muscle and Ehrlich ascites tumor LDH. From the data shown in Figs. 1 and 2, it can also be concluded that the beef heart and ascites tumor enzymes are equally sensitive to oxamate, whereas the rabbit muscle enzyme is somewhat less sensitive. A survey of the effect of oxamate on the LDH from S-37, Hepatoma-137, Hepatoma-P, Krebs-2, and SH-60 ascites cells, under the same conditions as for Fig. 1, showed that the sensitivity of LDH of these different types of tumors lies in the same range of oxamate concentration as the LDH from the above mentioned sources. The effect of oxamate on the \( \beta \)-lactic dehydrogenase from *L. mesenteroides* was also studied under the same conditions.

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\(^{1}\) The abbreviation used is: LDH, lactic dehydrogenase.
Fig. 3. A. The effect of varying concentrations of potassium oxamate on aerobic and anaerobic glycolysis of Ehrlich ascites tumor cells. The anaerobic glycolysis system was composed of 0.5 ml of cell suspension (1:4 dilution), 0.2 ml of 0.3 M glucose and was made up to 5.0 ml with Krebs-Ringer bicarbonate buffer, pH 7.4, and varying concentrations of potassium oxamate (0.154 M). The glucose was tipped in from the side arm to start the reaction after a 10-minute period of equilibration with the gas phase at 37°. In experiments in which potassium oxamate was not used, KCl (0.154 M) was added. The anaerobic conditions were 95% N2-5% CO2. The percentage of inhibition is based on initial rates of glycolysis. The aerobic glycolysis system was the same as the anaerobic system except the flasks were gassed with 95% O2-5% CO2. All experiments were carried out at 37°. The percentage of inhibition is based on the colorimetric determination of lactic acid after a 90-minute incubation period. B. The effect of potassium oxamate on the aerobic glycolysis of Ehrlich ascites tumor cells incubated at 37° in Krebs-Ringer phosphate buffer, pH 7.4, and in Krebs-Ringer bicarbonate buffer, pH 7.4.

and it was found that this enzyme is very much less sensitive to oxamate inhibition than the mammalian lactic dehydrogenases. The n-lactic dehydrogenase was found to be inhibited 50% by 7 × 10⁻³ M oxamate. This concentration of oxamate is 100 times greater than the concentration giving 50% inhibition of Ehrlich ascites tumor LDH under the conditions in Fig. 1.

Inasmuch as Ehrlich ascites tumor cells were found to be less contaminated with red blood cells than the other tumor strains examined, these ascites cells were chosen to study the effect of oxamate on the glycolysis of the whole cell.

**Oxamate Effects on Anaerobic and Aerobic Glycolysis of Ehrlich Ascites Tumor Cells**—The effect of varying concentrations of potassium oxamate on anaerobic and aerobic glycolysis was studied, and the data from these experiments are shown in Fig. 3. It was found that a concentration of 8 × 10⁻³ M oxamate gives 50% inhibition of the initial rate of glycolysis under anaerobic conditions. Aerobic glycolysis was found to be equally sensitive to inhibition by potassium oxamate. This is shown also in Table I, in which the colorimetric and manometric methods are compared. The corrected manometric results (three-flask method) agree well with the colorimetric results. It may be seen that even the uncorrected manometric results (one-flask method) reflect accurately the effect of oxamate on aerobic glycolysis.

A comparison of the data presented in Fig. 1 for the ascites tumor LDH and in Fig. 3A for the intact ascites cells shows that the cells are 100 times less sensitive to oxamate than the extracted LDII under the conditions selected. For possible explanations for this difference in sensitivity, see "Discussion." It should also be noted that the sensitivity of the intact cells varies with the incubation medium. Oxamate is less effective as an inhibitor of aerobic glycolysis in Krebs-Ringer phosphate buffer than in Krebs-Ringer bicarbonate buffer (Fig. 3B).

It was found that there is a tendency toward reversal of oxamate inhibition with time under anaerobic conditions. The recovery from inhibition, shown in the anaerobic glycolysis curves in Fig. 4, is more obvious in the presence of the lower concentration of oxamate (1 × 10⁻⁴ M).

The results from the aerobic glycolysis studies are also shown in Fig. 4. Aerobic glycolysis is shown here to be just as sensitive to oxamate inhibition as anaerobic glycolysis, in agreement with the results in Fig. 3A. The aerobic glycolysis data shown in Fig. 4 indicate that there is no recovery from oxamate inhibition with time, in contrast to the results found under anaerobic conditions. The recovery of anaerobic glycolysis from oxamate inhibition could be explained by either of the following phenomena:

1. Potassium oxamate may be metabolized by the cells.

**Table I**

Effect of oxamate on aerobic glycolysis of Ehrlich ascites cells

A comparison of the colorimetric and manometric methods of lactic acid determination. The lactic acid was produced by Ehrlich ascites tumor cells in Krebs-Ringer bicarbonate buffer, pH 7.4, 37°, under aerobic conditions (95% O2-5% CO2).

<table>
<thead>
<tr>
<th>Oxamate concentration</th>
<th>Lactic acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colorimetric</td>
</tr>
<tr>
<td>0</td>
<td>3.16</td>
</tr>
<tr>
<td>0.01</td>
<td>3.08</td>
</tr>
</tbody>
</table>

![Fig. 4. Time course of oxamate effect on anaerobic and aerobic glycolysis by Ehrlich ascites tumor cells.](http://www.jbc.org/)

The pressure changes due to lactic acid production in Krebs-Ringer bicarbonate buffer, pH 7.4, were measured under aerobic and anaerobic conditions. The aerobic glycolysis experiment shows the recovery from oxamate inhibition. The data for aerobic glycolysis (one-flask method) are uncorrected for the relatively small pressure changes due to respiratory oxygen uptake and CO2 evolution. The experimental conditions are the same as described in Fig. 3.
2. The inhibition of LDH may cause an accumulation of pyruvic acid under anaerobic conditions. Since oxamate is a competitive inhibitor of LDH the accumulation of pyruvate would therefore, cause a reversal of the oxamate inhibition. Aerobically, pyruvate would not accumulate, since it would be removed by the pyruvic oxidase system.

The first possibility was ruled out in two ways: (a) Preincubation of oxamate with the cell suspension anaerobically for 90 minutes before addition of glucose resulted in no loss in the effectiveness of the oxamate as an inhibitor of glycolysis. (b) Analysis for oxamate in the complete system after anaerobic recovery from inhibition showed that no oxamate had disappeared. Oxamate was determined in neutralized trichloroacetic acid filtrates by measuring its inhibitory effect on beef heart LDH.

It was concluded from these studies that oxamate is not destroyed by the Ehrlich ascites tumor cells. Experiments were performed, therefore, to determine whether there is any pyruvic acid accumulation in the presence of oxamate during anaerobic and aerobic glycolysis under the conditions described in Fig. 4. The data in Table II show the correlation between oxamate concentration and the amount of pyruvate accumulation anaerobically. The higher concentrations of oxamate were found to cause a greater inhibition of lactic acid production and a greater accumulation of pyruvic acid. In the absence of oxamate there was a 4-fold increase in pyruvate, whereas when oxamate was present, there was a 10-fold increase in pyruvate content during the course of incubation anaerobically, thereby accounting readily for the reversal of inhibition with time.

Table II also shows that pyruvic acid does not accumulate under aerobic conditions in the presence of oxamate. These data are in agreement with the conclusions drawn from the time course of inhibition of aerobic glycolysis (Fig. 4), which gave no indication of a pyruvate accumulation. The fact that there was no pyruvate accumulation under aerobic conditions indicates indirectly that pyruvic oxidase is not strongly inhibited by potassium oxamate. To test directly whether oxamate will inhibit pyruvic oxidase, the following experiment was performed.

**Table II**

**Accumulation of pyruvic acid under aerobic and anaerobic conditions by Ehrlich ascites tumor cells in presence of potassium oxamate**

The cells were incubated in Krebs-Ringer bicarbonate buffer. Experimental conditions were as in Fig. 4. Pyruvic acid was determined in filtrates after fixing cells plus medium with trichloroacetic acid.

<table>
<thead>
<tr>
<th>Oxamate concentration</th>
<th>Pyruvic acid concentration</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anaerobic</td>
<td>Aerobic</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 min</td>
<td>90 min</td>
<td>0 min</td>
</tr>
<tr>
<td>M</td>
<td>2.4 X 10^-5</td>
<td>9.8 X 10^-5</td>
<td>5.6 X 10^-5</td>
</tr>
<tr>
<td>0.02</td>
<td>1.7</td>
<td>14.4</td>
<td>5.6</td>
</tr>
<tr>
<td>0.04</td>
<td>2.5</td>
<td>25.4</td>
<td>5.6</td>
</tr>
</tbody>
</table>

* Concentration expressed on assumption that pyruvate is distributed equally through medium and cell water. Since 0.125 ml of packed cells was used per 3 ml of reaction mixture, these values must be multiplied by 24 if all of the pyruvate is assumed to be intracellular.

Effect of Potassium Oxamate on Pyruvic Oxidase in Ehrlich Ascites Tumor mitochondria—The oxygen uptake data shown in Table III indicate there is little or no effect of oxamate on pyruvic oxidase. The data in Fig. 1 have shown that LDH from Ehrlich ascites tumor homogenate is inhibited 50% by 8 X 10^-2 M oxamate, with 3.3 X 10^-3 M pyruvate. At the lowest concentration of pyruvate used in the mitochondrial experiments (1 X 10^-2 M) there is only 18% inhibition of pyruvic oxidase with 1 X 10^-2 M oxamate. It was concluded from these experiments, therefore, that pyruvic oxidase is relatively insensitive to oxamate inhibition in comparison to LDH. That oxamate has little or no inhibitory effect on oxidative metabolism is substantiated by the studies in the following section.

**Figure 5.** The Crabtree effect and its reversal by oxamate. The cells were suspended in Krebs-Ringer phosphate buffer, pH 7.4. Glucose concentration, 0.02 M; potassium oxamate, 0.041 M.

**Effect of Potassium Oxamate on Pyruvic Oxidase in Ehrlich Ascites Tumor Cell Mitochondria**

The reaction mixture in Experiment 1 was composed of 0.2 ml of MgCl₂, 0.1 M; 0.1 ml of malate, 0.007 M; 0.1 ml of DPN, 10 mg per ml; 0.3 ml of sodium pyruvate, 0.1 M; 0.1 ml of 5'-AMP, 0.03 M; 1.7 ml of mitochondria, 0.2 ml of potassium oxamate, 0.154 M; and potassium phosphate buffer, 0.1 M, pH 7.4, to give a final volume of 3 ml. In Experiments 2 and 3, 0.1 ml of 5'-AMP, 0.01 M, and 1.2 ml of mitochondria were used. The temperature was 37° for Experiments 1 and 2, and 30° for Experiment 3.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Initial pyruvate concentration</th>
<th>Oxygen uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>μl/10 min</td>
</tr>
<tr>
<td></td>
<td>No oxamate</td>
<td>Oxamate (1 X 10^-2 M)</td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>0.0007</td>
<td>63.9</td>
</tr>
<tr>
<td>3</td>
<td>0.001</td>
<td>61.4</td>
</tr>
</tbody>
</table>

**Table III**

**Effect of potassium oxamate on pyruvic oxidase of Ehrlich ascites tumor mitochondria**

The reaction mixture in Experiment 1 was composed of 0.2 ml of MgCl₂, 0.1 M; 0.1 ml of malate, 0.007 M; 0.1 ml of DPN, 10 mg per ml; 0.3 ml of sodium pyruvate, 0.1 M; 0.1 ml of 5'-AMP, 0.03 M; 1.7 ml of mitochondria, 0.2 ml of potassium oxamate, 0.154 M; and potassium phosphate buffer, 0.1 M, pH 7.4, to give a final volume of 3 ml. In Experiments 2 and 3, 0.1 ml of 5'-AMP, 0.01 M, and 1.2 ml of mitochondria were used. The temperature was 37° for Experiments 1 and 2, and 30° for Experiment 3.

**Oxamate Effects on Respiration of Ehrlich Ascites Tumor Cells**—In 1929, Crabtree (19) showed that tumor cells, in the presence of glucose, fructose, or mannose, have a rate of respiration lower than the endogenous. This phenomenon, now well known as the Crabtree effect, and reported by many laboratories (20-22), is illustrated in Fig. 5. However, in the presence of potassium oxamate and glucose, it was found that the cells could respire at the endogenous rate, i.e. oxamate (4 X 10^-2 M) was found to cause a complete reversal of the Crabtree effect.
The observed values for pmoles of glucose accounted for from lactate formed were divided by 2.

† The observed values for pmoles of oxygen uptake were divided by 6.

In Fig. 6, is shown the effect of concentration of potassium oxamate on endogenous respiration and respiration in the presence of glucose. There is only a slight effect of oxamate on endogenous respiration at concentrations up to $6 \times 10^{-2} \text{m}$. In the presence of glucose it can be seen that as the oxamate concentration increases, the rate of oxygen uptake correspondingly increases and approaches the rate of oxygen uptake under endogenous conditions. The effect of oxamate on aerobic glycolysis (in phosphate buffer) is included for comparison (see Fig. 3A and 3B). The concentrations required for reversal of the Crabtree effect, parallel roughly those needed for inhibition of glycolysis.

Glucose Uptake in Presence and Absence of Oxamate—In an attempt to determine the mechanism of the reversal of the Crabtree effect, the rate of utilization of glucose, the rate of lactic acid production, and the rate of oxygen uptake were studied simultaneously in the presence and in the absence of oxamate.

The data shown in Table IV indicate that glucose uptake and lactic acid production are inhibited by oxamate, and that oxamate reverses the Crabtree effect. The inhibition of glucose utilization was found to vary from 39 to 42%, and lactic acid formation was inhibited by 68 to 72%. It can be seen that within each individual experiment it was consistently observed that the percentage of inhibition of lactic acid formation is greater than the percentage of inhibition of glucose uptake. However, when expressed in micromoles, the inhibition of glucose uptake is essentially equal to the inhibition of lactic acid formation. It should be noted that these data are for the phosphate buffer medium, in which oxamate is relatively ineffective as an inhibitor.

Carbon balances have also been calculated from the glucose uptake, lactic acid production, and oxygen uptake data. For purposes of calculation, it is assumed that all oxygen taken up is used for glucose oxidation to CO$_2$ and H$_2$O. It can be seen from these balances that in the absence of oxamate, the amount of glucose going to lactic acid varies from 60 to 70%. Also, the amount of glucose unaccounted for either by lactic acid or by oxygen uptake in the absence of oxamate ranges from 14 to 30%.

The carbon balances for the utilization of glucose in the presence of oxamate show that the percentage of glucose utilized which goes to lactic acid is significantly less and that thepercentage of glucose utilized that remains unaccounted for is greater. This phenomenon is considered further in the discussion.

**DISCUSSION**

Specificity of Oxamate as Inhibitor of LDH—Attempts have been made in these experiments to demonstrate the specificity of oxamate for inhibition of LDH. Because of the similarity in structure between oxamate and pyruvate, it is necessary to consider the possibility that oxamate could affect other enzyme systems, especially those for which pyruvate or a related compound acts as substrate. Pyruvate kinase is the first enzyme of the Embden-Meyerhof pathway for which pyruvate is a substrate. It may be concluded that the enzyme is relatively insensitive to oxamate because of the observation that under anaerobic conditions in the presence of oxamate, pyruvate accumulates to 10 times its initial concentration. However, the possibility remains that the reverse reaction catalyzed by this enzyme, namely the conversion of pyruvic acid to phosphoenol pyruvate, is oxamate-sensitive.

The pyruvic oxidase system also was found to be insensitive to concentrations of oxamate which significantly inhibit LDH. This failure to inhibit the complex pyruvic oxidase system of tumor mitochondria, indicates that oxamate was not inhibiting any of the enzymes of the Krebs tricarboxylic acid cycle or of the electron transport chain to an extent sufficient to suppress the over-all activity of the system. The same conclusion is reached from the failure of oxamate to inhibit oxidative processes in the intact ascites tumor cell.

Several systems which form D-lactate instead of L-lactate are relatively insensitive to oxamate. These include the D-lactate dehydrogenase of *L. mesenteroides*, *Leuconostoc plantarium* (23), and the glyoxalase of normal human leukocytes (9). In this connection, it is interesting to note that a D-lactate dehydrogenase was recently reported in kidney mitochondria (24).

The occurrence of such an enzyme may explain the observation that lactate formation accompanying pyruvate oxidation in tumor mitochondria was not very oxamate-sensitive. Thus,
in Experiments 2 and 3 of Table III, the values for lactate formation (not shown) were identical without and with 0.01 M oxamate. In another case, under the conditions of Experiment 1 of Table III, 0.01 M and 0.04 M oxamate caused only 10 and 23% inhibition of lactate formation, respectively. The absolute amount of lactate formed (0.2 to 0.6 umole per 30 minutes for mitochondria from about 1 ml of packed cells) is very small relative to the total lactate-producing capacity of glycolyzing intact ascites tumor cells (around 120 umoles per 30 minutes per ml of packed cells). However, rates of lactate production from glucose by the mitochondria were not measured, so the quantitative significance of this apparently oxamate-resistant glycolytic pathway remains to be assessed.

The question remains as to why oxamate competes with pyruvate in the case of lactic dehydrogenase but does not compete with pyruvate or related substances in other enzyme systems. The explanation may lie in the fact, observed by Schwert, that an interaction of oxamate with DPNH takes place during formation of the inactive LDH-DPNH-oxamate complex (6, 25, 26). This interaction with DPNH may account for the increased binding of oxamate to LDH, relative to its binding to pyruvic kinase, pyruvic oxidase, or glyoxalase.

Relative Sensitivity of LDH in Intact Cells and in Cell-free Extracts—The data on oxamate inhibition of LDH and of glycolysis in the whole cell show that the latter is 100 times less sensitive to oxamate under the conditions selected. One possible explanation for this lower sensitivity exhibited by the cell may be the presence of an excess of intracellular LDH. Our calculations indicate that enough enzyme is extractable to account, when saturated with substrate, for 30 times the actual glycolytic rate of the cell. However, if it were true that the cell has an excess of LDH, then the curve in Fig. 3 on the effect of oxamate on aerobic and anaerobic glycolysis should have been S-shaped, inasmuch as oxamate should not inhibit glycolysis at low concentrations where LDH activity is not rate limiting. The actual curves suggest that LDH is not in great excess and that some other factor is responsible for the lowered sensitivity of the intact cell.

Another explanation for this relative insensitivity of the cell to oxamate may be a high intracellular pyruvate concentration. The fact that pyruvate accumulation under anaerobic conditions does cause a reversal of the oxamate inhibition of glycolysis is experimental evidence that an increase in intracellular levels of pyruvate can decrease the effect of oxamate. From the data in Table II, one can calculate that at zero time the maximal intracellular pyruvate level is 0.6 to 1.4 \times 10^{-3} M (see footnote to Table II). In this range of pyruvate concentration, 1 \times 10^{-3} M oxamate was able to produce 70% or more inhibition of cell-free LDH. These data indicate that the pyruvate level in the cell is not great enough to account for the high oxamate concentration (8 \times 10^{-3} M) required to get 50% inhibition of glycolysis. This cellular insensitivity to oxamate, therefore, cannot be explained entirely either by the presence of an excess of LDH or by a high intracellular pyruvate level. It is possible that permeability factors are also involved in this effect.

Effect of Oxamate on Carbon Balance—The data with Ehrlich ascites tumor cells (Table IV), showing a significant amount of glucose unaccounted for in terms of lactic acid production and oxygen uptake, are in agreement with the experiments of Wenner and Weinhouse (27). Villavicencio and Barron (28) showed that Ehrlich ascites tumor cells in the presence of glucose form significant amounts of ribose, ribulose, and fructose, as well as lactic acid. Kvamme (29, 30) was able to show that in the presence of glucose ascites cells are able to accumulate an appreciable amount of barium insoluble fructose ester which he assumed to be fructose diphosphate.

When oxamate is present, the block in lactate production is reflected almost stoichiometrically by the diminished glucose utilization. On the other hand, the amount of glucose going to unidentified products remains unchanged by oxamate, so that the fraction of glucose unaccounted for becomes larger in the presence of the inhibitor. However, this does not mean that oxamate causes any additional accumulation of intermediates. The reason for the strong inhibition of glucose utilization by oxamate is not entirely clear. Analyses for pyruvate or for DPNH at the end of the experiments revealed no accumulation of these substances.\(^2\)

Mode of Reversal of the Crabtree Effect by Oxamate—Block-Frankenthal and Weinhouse (31) have shown that when the endogenous respiration of ascites tumor cells, due mainly to fatty acid oxidation, is inhibited by glucose, a large fraction of the respiratory CO2 is derived from the glucose. In the present experiments, when oxamate restores the glucose-inhibited respiration to the endogenous rate, the question remains open as to what fraction of the respiratory CO2 is still derived from glucose. Although glucose utilization is markedly inhibited by oxamate, there is still more than sufficient utilization to account for the total oxygen uptake as pointed out above (Table IV). One explanation for the apparent reversal of the Crabtree effect by oxamate may therefore be simply that more pyruvate is made available for oxidation when LDH is inhibited, and that the resulting extra oxygen consumption masks the Crabtree effect.

An alternative explanation for the reversal of the Crabtree effect by oxamate would be that the Crabtree effect depends on an intact glycolytic mechanism to limit the concentration of ADP and Pi, available for respiratory activity (20, 21, 32-34). Certain "pseudo-Crabtree" effects have been obtained in the absence of an intact glycolytic system, e.g. with glucose in the presence of iodoacetate (21), or with 2-deoxyglucose (22). However, these effects presumably have a different basis from the classical Crabtree phenomenon.

Possible Applicability of Oxamate as a Tool for Growth Studies—The present experiments suggest that oxamate may be a useful tool for inhibition of glycolysis in tumor cells without inhibition of respiration or other metabolic processes. It might, therefore, serve to inhibit the growth of cells dependent on aerobic glycolysis without interfering with the growth of cells which depend solely on oxidative metabolism. In this sense, oxamate would serve the opposite purpose to that served by another pyruvate analogue, namely fluoropyruvate (35). The latter compound inhibits oxygen uptake by nonglycolyzing cells, but is relatively ineffective on the respiration of cells exhibiting aerobic glycolysis because it is then rapidly detoxified to fluorolactate (36).

The use of oxamate in studies of the growth of glycolyzing cells is described in the paper which follows.

SUMMARY

1. The finding of Schwert and collaborators that potassium oxamate is a competitive inhibitor of beef heart lactic dehydrogenase has been confirmed and extended to the lactic dehydrogenase of rabbit skeletal muscle and of the Ehrlich ascites cells.

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\(^2\) The DPNH analyses were done by Miss Margaret Childs.
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tumor. Oxamate also inhibits the lactic dehydrogenase from S-37, Hepatoma-137, Hepatoma-1', Krebs-2, and SH-60 ascites cells.

2. Aerobic and anaerobic glycolysis by the Ehrlich ascites tumor cells are equally inhibited by potassium oxamate. The ascites cells were found to be inhibited 50% by $8 \times 10^{-4}$ M oxamate. This concentration of oxamate is 100 times greater than the amount giving 50% inhibition of the lactic dehydrogenase extracted from the ascites cell under the conditions of assay.

3. There is a reversal of oxamate inhibition with time under anaerobic conditions. This reversal was found to be due to the accumulation of pyruvic acid. Under aerobic conditions there is no accumulation of pyruvate and no reversal from inhibition by oxamate.

4. There is only a slight effect by oxamate ($6 \times 10^{-2}$ M) on the endogenous respiration of Ehrlich ascites tumor cells. The inhibition of endogenous respiration by glucose (Crabtree effect) is reversed by oxamate.

5. In the absence of oxamate it was found that not all of the glucose utilized can be accounted for as lactic acid or as CO$_2$. In the presence of oxamate, glucose uptake is inhibited to the same extent as lactate formation, but the amount of glucose unaccounted for remains the same as in the absence of oxamate.

6. The possible usefulness of oxamate as a specific inhibitor of glycolysis in mammalian cells is discussed.

REFERENCES

The Role of Glycolysis in the Growth of Tumor Cells: I. EFFECTS OF OXAMIC ACID ON THE METABOLISM OF EHRLICH ASCITES TUMOR CELLS IN VITRO

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