The Role of Glycolysis in the Growth of Tumor Cells

III. LACTIC DEHYDROGENASE AS THE SITE OF ACTION OF OXAMATE ON THE GROWTH OF CULTURED CELLS*

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(Received for publication, January 18, 1965)

Otto Warburg demonstrated the high rate of aerobic glycolysis of tumors in vitro and postulated this as a unique requirement for neoplastic growth. (For a recent review see Eisenberg (1).) There is evidence that this metabolic anomaly is not due to a fundamental difference between normal and tumor cells but merely reflects their particular growth rates. For example, some years ago O'Connor (2,3) demonstrated a linear correlation between glycolysis of chick embryo midbrain and mitotic index (which is a good index of growth in this tissue). More recently the Morris hepatoma, a very slowly growing tumor, was found to have low glycolytic activity (4, 5). It is well established that cells derived from normal tissue that have adapted to an environment in vitro and grow rapidly have a high glycolytic rate (6).

The question has not been satisfactorily answered as to whether the glycolysis of rapidly dividing cells is essential to their growth. Thus, in those experiments the case was probably at lactic dehydrogenase. To test the requirements of glycolysis for growth, a fast growing tumor cell in vitro, HeLa S3, was used. Oxamate inhibited growth, glycolysis, and glucose uptake of these cells (9). All of these effects of oxamate were reversed when pyruvate was also present in the medium, indicating that the site of action of oxamate in this case was probably at lactic dehydrogenase.

To test the requirement of glycolysis for growth, a fast growing tumor cell in vitro, HeLa S3, was used. Oxamate inhibited growth, glycolysis, and glucose uptake of these cells (9). All of these effects of oxamate were reversed when pyruvate was also present in the medium, indicating that the site of action of oxamate for these inhibitions in HeLa S3 cells was at lactic dehydrogenase.

Two main objections to this conclusion remained to be overcome. First, the previous experiments with HeLa S3 cells were done with small inocula which were allowed to grow about 6-fold. Thus, in those experiments the rate of glycolysis was not determined, and the small amount of lactate formed or glucose consumed in the presence of oxamate might just reflect the small amount of growth under those conditions. Second, these experiments did not establish the absolute specificity of oxamate. Since oxamate is a structural analogue of pyruvate, it might also inhibit some essential reaction other than lactic dehydrogenase for which pyruvate is required. In this report, then, we will endeavor to establish that oxamate lowers the rate of glycolysis in growing tumor cells, and also we will present more evidence that lactic dehydrogenase is the primary site in the inhibition of growth by oxamate.

Preliminary accounts of this work have been published (10, 11). The use of inhibitors of lactic dehydrogenase for inhibition of tumor growth has also been proposed by several other investigators (12-15).

MATERIALS AND METHODS

Special Chemicals—Sodium oxamate was prepared in the early work by partial ammonolysis of diethyl oxalate (16), followed by hydrolysis of the isolated crystalline ethyl oxamate with NaOH and crystallization from ethanol. In the later work, oxamic acid was obtained from the Aldrich Chemical Company and was found to be 96% pure by titration. It was neutralized with NaOH and used after two recrystallizations from hot water.

α-Keto acids were obtained commercially from the California Corporation for Biochemical Research or from the Sigma Chemical Company in the purest available grade, and were used without further purification.

Cells—HeLa S3 cells were maintained as stock cultures in milk dilution bottles and were grown under sterile conditions at 37.5°C in a CO₂-air atmosphere in Eagle’s minimal essential medium (17) with 10% human serum, except that the concentrations of Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻, SO₄²⁻, and PO₄³⁻ were according to Hanks and Wallace (18). Hanks’ salt solution containing only these salts, titrated with NaOH to pH 7.1 to 7.4, was also used for washing the cells on various occasions. Streptomycin sulfate, 67 units per ml; sodium penicillin G, 147 units per ml, free of citrate buffer and sulfite preservative (obtained as a gift from Chas. Pfizer and Company, Inc.); and phenol red (5 × 10⁻⁴%) were also used in the medium.

The α-keto acids were neutralized with NaOH after addition to the medium. When these were present, or when sodium oxamate was added, an equivalent amount of NaCl was omitted from the medium.
Trypsinization—Cells were trypsinized as required in order to transfer them either for stock maintenance or for use in experiments. The trypsin solution was 0.05% in a Tris-buffered salt solution containing 8 g of NaCl, 0.38 g of KCl, 0.10 g of Na2HPO4·7H2O, and 3.0 g of Tris in 1 liter of solution, and was titrated to pH 7.2 with HCl. The old medium was poured off the cells, and the cells were incubated for ½ hour, bathed in trypsin solution. An equal volume of medium was added to stop the trypsinization, and after pipetting up and down several times to break up clumps of cells, they were replated.

Typical Experimental Procedure—In large inoculum experiment a trypsinized suspension of cells is counted in a hemacytometer and diluted to about 10⁶ cells per ml with medium. Portions (5 ml) are precisely pipetted into plastic Petri dishes (50 mm in diameter) (Falcon Products). The cells are incubated for 12 to 20 hours to allow time for the cells to adhere to the bottom of the dish. The medium is aspirated off, the experimental medium is added, and the cells are incubated. At appropriate times the plates are removed from the incubator (each determination is the average of at least three replicate plates), and the medium is poured off and frozen until analysis. The dish with cells is washed four times with Hank’s salt solution. After the salt solution is drained from the inverted Petri dishes, they are frozen until analysis for protein.

Determination of Intracellular Concentrations of Pyruvate and Oxamate—Approximately 10⁶ trypsinized cells were allowed to attach to the surface of either Roux bottles or large (100-mm diameter) Petri dishes. At the appropriate times the plates are removed from the incubator and the cells were washed three times with Hank’s salt solution of an equal or greater amount than the medium. Three washes were sufficient to remove all the medium protein detectable by measurement at 280 mμ. After draining, 5% perchloric acid was added (10 ml per Roux bottle or 5 ml per Petri dish) to extract the oxamate and pyruvate remaining in the cells. This was decanted, leaving the cellular protein adhering to the dish for subsequent determination. The 5% perchloric acid supernatant fluid was then neutralized with KOH, and the KClO4 was removed. This supernatant fluid was lyophilized, and 0.5 or 1.0 ml of water was added prior to analysis for oxamate and pyruvate.

Chemical Analyses—Lactate in the media was determined according to the method of Barker and Summerson (19), by-passing the barium-zinc filtration when glucose was to be determined by a method other than that of Nelson (20). Lactate determination of replicate plates generally had a precision of better than 4% standard deviation. Protein was determined by the Lowry method as modified by Oyama and Eagle (21) with a hexokinase-glucose-6-P dehydrogenase assay used in these cases. The extent of the reaction was determined by measuring the reduction of TPN at 340 mμ in a spectrophotometer. The reaction mixture (1.0 ml) contained 0.24 μmole of TPN, 1.0 μmole of ATP, 2.0 μmoles of MgCl₂, 50 μmoles of Tris, pH 8.0, 10 μl of medium sample, and sufficient yeast hexokinase (first crystals as prepared by Darrow and Colowick (23)) and yeast glucose-6-P dehydrogenase (purified, C. F. Boehringer and Sons) to complete the reaction in 90 sec. This method was found to be both accurate and reproducible to 1% standard deviation.

Glucose analysis by Nelson’s method (20) was not found to be precise enough to measure the small differences in glucose uptake reliably. The glucose oxidase method (22) was then used on a 50-μl portion of medium directly, and the results were reproducible to 1% standard deviation. For experiments with α-keto acids in high concentrations, however, this method was not satisfactory. α-Keto acids compete with the dye for H₂O₂ produced in the reaction. Therefore a hexokinase-glucose-6-P dehydrogenase assay was used in these cases. The extent of the reaction was determined by measuring the reduction of TPN at 340 mμ in a spectrophotometer. The reaction mixture (1.0 ml) contained 0.21 μmole of TPN, 1.0 μmole of ATP, 2.0 μmoles of MgCl₂, 50 μmoles of Tris, pH 8.0, 10 μl of medium sample, and sufficient yeast hexokinase (first crystals as prepared by Darrow and Colowick (23)) and yeast glucose-6-P dehydrogenase (purified, C. F. Boehringer and Sons) to complete the reaction in 90 sec.

α-Hydroxybutyrate was assayed essentially by the Barker and Summerson method, but the absorption was determined with a red filter instead of a green filter in a Klett-Summerson colorimeter. The absorption spectrum of the α-hydroxybutyrate chromogen determined on a Cary recording spectrophotometer is compared with that of lactate in Fig. 1. The peak for the lactate product is at 575 mμ, but its absorption is small at 660 mμ, where the α-hydroxybutyrate product absorbs well. Therefore, lactate and α-hydroxybutyrate were determined in a mixture of the two after measurement of each sample with the red filter and the green filter in the colorimeter. The reproducibility of analysis of replicate plates was usually about 5% standard deviation.

Oxamate was determined by measuring the inhibition of the
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GLYCOLYSIS

GROWTH

INCUBATION TIME (HRS.)

FIG. 2. The effect of sodium oxamate on the growth and glycolysis of HeLa S3 cells.

rate of oxidation of DPNH in the presence of rabbit muscle lactic dehydrogenase and low pyruvate concentrations. As little as 0.01 amole of oxamate could be detected in this way in a 1.0-ml reaction mixture.

Pyruvate was determined in two ways: first, by an enzymatic lactic dehydrogenase assay with excess enzyme so that oxamate did not slow the reaction too much; second, by the 2,4-dinitrophenylhydrazone method of Friedemann and Haugen as modified by Wormser and Pardee (24). This determined total α-keto acids.

RESULTS AND DISCUSSION

Evidence for Inhibition of Glycolytic Rate by Sodium Oxamate—Fig. 2 shows the effect of sodium oxamate on the growth and glycolysis of HeLa S3 cells in a large inoculum culture. The figure illustrates the reproducibility of triplicate determinations of protein and thus the feasibility of these relatively short term growth experiments. For a given amount of cell protein the rate of glycolysis in the presence of oxamate is much smaller than in the absence of oxamate. This could not have been the case if glycolytic inhibition were only a reflection of growth inhibition. Therefore we conclude that the rate of glycolysis per cell, as well as the rate of growth of HeLa S3, is inhibited by oxamate.

Fig. 3 shows the effect of various oxamate concentrations on growth, glycolysis, and glucose uptake. It can be seen that the inhibition of lactate production and that of glucose uptake increase in parallel up to 0.08 M oxamate. Thus, inhibition of lactic dehydrogenase stops glucose utilization, as described earlier for ascites tumor cells (7). The mechanism of this phenomenon will be clarified later. In several experiments like the one illustrated, the growth at 6 hours is inhibited to a smaller extent than at 12 hours, while glycolysis and glucose uptake are maximally inhibited. This might be due to a lag of the action of glycolytic inhibition on growth inhibition. The cells may be more dependent on glycolysis at a certain stage of the growth cycle.

Fig. 3 shows that even at 0.08 M oxamate neither the growth rate nor the glycolytic rate is completely inhibited. The lactic dehydrogenase in crude extracts of HeLa S3 is inhibited 50% by 1.5 × 10^{-4} M oxamate in the presence of 10^{-4} M pyruvate, which corresponds roughly to the intracellular pyruvate concentration. Thus the requirement for such high oxamate concentrations for inhibition of glycolysis in the whole cell (either ascites or HeLa S3) is still unexplained. To see if this discrepancy was due to either the rate or the extent of transport of oxamate into the cell, we studied the accumulation of oxamate in the cells with respect to time. In 15 min the oxamate concentration reached 0.016 ± 0.003 M (standard deviation for three measurements) and was not significantly higher at later periods (0.021 ± 0.015 M for eight measurements at various times from 1 to 20 hours). These are minimal values since some of the oxamate inside may have been bound to proteins or other macromolecules.

Throughout this report, the sodium oxamate concentration in the medium used is 0.08 M unless otherwise stated.

The large difference in starting points of the growth curves, amounting to 14% in this experiment, is not typical. In other experiments, in which initial values were essentially the same without or with oxamate, similar results were obtained. The possibility was considered that cells adherent to the Petri dish before the addition of oxamate would stay on and reproduce at the normal rate but that the newly formed cells would not stick as well, resulting in apparent growth inhibition. This has been ruled out by counting cells in the medium.

Complete inhibition was observed previously (9) under different growth conditions (small inoculum).
have come out during washing and draining. The intracellular pyruvate concentration remains roughly constant during this time and is about $10^{-4}$ M as stated above. Therefore the lactic dehydrogenase in the cell should be inhibited over 99%. Transport, then, cannot be the factor accounting for the low sensitivity to oxamate.

Evidence That Site of Action of Oxamate Is Lactic Dehydrogenase—Fig. 4 shows the effect of various $\alpha$-ket acids in preventing the oxamate inhibition of growth of HeLa S3 cells. In this figure the reversibility by pyruvate (which was already shown for conditions of small inoculum (9)) is shown for large inoculum cultures. The possibility that increased pyruvate oxidation was responsible for the increased growth was not ruled out. It is known that $\alpha$-ketoglutarate or oxaloacetate, which are Krebs cycle intermediates but not lactic dehydrogenase substrates, can substitute for pyruvate in promoting the growth of Walker carcinoma cells (25). Fig. 4 also shows that there was no relief of the oxamate inhibition of HeLa S3 by $\alpha$-ketoglutarate up to 0.03 M. Thus, if $\alpha$-ketoglutarate does penetrate these cells, it does not appear likely that pyruvate is reversing the oxamate inhibition of growth by Krebs cycle oxidation.

In order to show that it is actually the reduction of pyruvate to lactate via lactic dehydrogenase which is un inhibited by oxamate in HeLa S3, $\alpha$-ketobutyrate was used. This compound is not a known metabolite for oxidation by HeLa cells. It is, however, a good substitute for pyruvate in the lactic dehydrogenase reaction, and in this reaction it forms $\alpha$-hydroxybutyrate. Since an $\alpha$-hydroxy acid is the normal end product of glycolysis which is excreted into the medium, it was thought that this compound might serve as a substitute for pyruvate on this pathway. Herzenberg and Roosa (26) have in fact shown that $\alpha$-ketobutyrate can substitute for pyruvate when the latter is required for clonal growth of lymphoma cells. It cannot substitute for pyruvate in the Krebs cycle, since Stern (27) has shown that propionyl-CoA is only 0.1% as active as acetyl-CoA for mammalian condensing enzyme. Thus $\alpha$-ketobutyrate can be used as a tracer for the lactic dehydrogenase reaction.

Preliminary studies of the kinetics of lactic dehydrogenase in extracts of HeLa S3 cells showed that the $K_m$ values for $\alpha$-ketobutyrate and pyruvate were $1.2 \times 10^{-3}$ and $1.4 \times 10^{-3}$ M, respectively. These values are similar to those for crystalline beef heart lactic dehydrogenase (28). The ratio of maximum velocities, $V_{max}$ (pyruvate):$V_{max}$ ($\alpha$-ketobutyrate), is 3.4 for the HeLa enzyme, compared with 1.3 for the beef heart enzyme (28). Pyruvate at high concentrations showed substrate inhibition as it does with lactic dehydrogenase from other sources (29). $\alpha$-Ketobutyrate did not show this phenomenon at concentrations up to 0.036 M. These data indicated that $\alpha$-ketobutyrate could serve to reverse oxamate inhibition of lactic dehydrogenase.

Fig. 4 and Table I show the effect of $\alpha$-ketobutyrate in reversing the inhibition, due to sodium oxamate, of both growth and glycolysis. The concentration of $\alpha$-ketobutyrate required to reverse the inhibition of growth due to oxamate is an order of magnitude higher than that required for pyruvate reversal. This reflects their relative Michaelis constants.

Table I also shows that $\alpha$-ketobutyrate at high concentrations inhibits both growth and "glycolysis" in the absence of oxamate. The reasons for this inhibition are not understood. This inhibition is not due to an increase in Na+ or to an increase in osmolarity (see References 30 and 31) since the sodium $\alpha$-ketobutyrate replaces NaCl in the medium. Neither is it due to a lack of Cl- ions since the Cl- concentration never reached 50 mM or below (see Reference 39).

Table I shows further that added $\alpha$-ketobutyrate completely displaces pyruvate in the cell, substituting $\alpha$-hydroxybutyrate formation for lactate production. In fact, if we were to retain our original definition of glycolysis, we could infer from the data at high $\alpha$-ketobutyrate concentrations that cells do not need to glycolyze in order to grow, since they produce no lactate. These data show that lactate itself is not the important product of the lactic dehydrogenase reaction required for growth. The significance of this reaction will be discussed in the following paper of this series (33). The point to be stressed here is that $\alpha$-ketobutyrate in the presence of oxamate increases the rate of lactic dehydrogenase and probably thereby increases the growth rate.

![Fig. 4. Reversal by lactic dehydrogenase substrates of oxamate-inhibited growth of HeLa S3 cells.](http://www.jbc.org/)
This is the most direct evidence we have that the site of oxamate inhibition of glycolysis is at lactic dehydrogenase. It also indicates that this is the only reaction needed to restore growth, since this is the only path we know in mammalian cells in which \(\alpha\)-ketobutyrate participates.

**Dependence of Glucose Utilization on Lactic Dehydrogenase Activity**—Table I shows the inhibitory effect of oxamate on glucose utilization and its reversal by \(\alpha\)-ketobutyrate. At concentrations of \(\alpha\)-ketobutyrate which reverse the inhibition of glucose uptake maximally, the oxamate-induced inhibition of "glycolysis" is reversed because of the greater reduction of \(\alpha\)-ketobutyrate to \(\alpha\)-hydroxybutyrate. This shows that the site of reversal of inhibition of glucose uptake under these conditions is lactic dehydrogenase. Therefore the inhibition of glucose uptake must be a secondary phenomenon dependent upon the action of oxamate on lactic dehydrogenase. The mechanism of this phenomenon will be considered in the following paper of this series.

**SUMMARY**

1. The glycolytic activity of HeLa S3 cells has been studied in short term experiments during which increments of growth as small as \(20\%\) could be accurately measured. Under these conditions, the inhibition of growth by oxamate is accompanied by essentially complete inhibition of glycolysis, showing that the glycolytic activity per cell has been inhibited.

2. The site of inhibition of glycolysis is localized at lactic dehydrogenase by experiments showing that \(\alpha\)-ketobutyrate, a substrate for the enzyme, permits both growth and glucose utilization in oxamate-treated cultures; the product of glycolysis in this case is \(\alpha\)-hydroxybutyrate. \(\alpha\)-Ketoglutarate, which substitutes for pyruvate in other systems, but which is not a substrate for the enzyme, does not restore growth.

3. A method was developed to analyze for \(\alpha\) hydroxybutyrate and lactate together in the medium by utilizing the difference in spectra of the respective Barker-Summerson reaction products.

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The Role of Glycolysis in the Growth of Tumor Cells: III. LACTIC DEHYDROGENASE AS THE SITE OF ACTION OF OXAMATE ON THE GROWTH OF CULTURED CELLS
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