# THE HIGH RATE OF AEROBIC GLYCOLYSIS OF TRANSFORMED 3T3 CELLS PERSISTS AFTER CELL HOMOGENIZATION

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SUMMARY: The high rate of lactic acid production by 3T3 cells which have been transformed by simian virus 40 or by polyoma virus as compared to confluent untransformed 3T3 cells persists after cell homogenization. This difference is also reflected by increased phosphofructokinase activity in the viral transformed cells. The findings imply that the increased aerobic glycolysis in the transformed cells results from changes in the glycolytic pathway rather than changes in sugar transport.

Rapidly proliferating cells, including tumour cells, exhibit a high rate of aerobic glycolysis (1-4). This may result from an increased rate of sugar transport and/or from an increase in the activity of the glycolytic pathway itself. The determination of the rate-limiting step in this process has attracted much attention but is still controversial. Measurements of the intracellular concentrations of glycolytic intermediates and of glycolytic enzymes in chick embryo fibroblasts have led to opposite conclusions regarding the cause of the high rate of glycolysis in the transformed derivatives of these cells (5-7). Some studies with mouse 3T3 cells and their transformed derivatives indicate that the increased sugar uptake associated with transformation is not caused by increased transport but by enhanced sugar phosphorylation, reflecting an alteration in the control of glycolysis (8). However, other studies in 3T3 cells (9,10) and chick embryo fibroblasts (11-13) seem to contradict this conclusion.

In the present paper we report that the high rate of lactic acid production by cultures of SV40 virus transformed 3T3 cells (SV40-3T3) and





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polyoma virus transformed 3T3 cells (Py 3T3) cells, as compared to confluent 3T3 cells, persists after cell homogenization, i.e. when a permeability barrier for sugar no longer exists. Furthermore, we found that the specific activity of phosphofructokinase is markedly enhanced in homogenates of the transformed cells. Our results are consistent with the possibility that glycolysis rather than sugar transport is primarily altered in rapidly growing, virus-transformed 3T3 cell lines.

## MATERIALS AND METHODS

Cell Culture Cells were maintained in 90 mm Nunc Petri dishes in Dulbecco's modified Eagle's medium (DEM), 10% fetal bovine serum, 100 unit/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 10% $CO_2$ and 90% air at 37°. This medium contains 25 mM glucose. The medium was changed two days after sub-culturing 3T3, SV40-3T3, Py 3T3 cells, and the cultures were used at the confluent state. The proportion of cells synthesizing DNA under these conditions is shown in Table 1.

Measurement of lactic acid production by intact cells Before each experiment the cells were washed twice and incubated in serum-free DEM which was further modified to be pyruvate-free and contain a ten-fold reduction in phenol red ($1.5 \times 10^{-4}$%). The changes in medium were made to reduce the blank in the lactic acid determinations (see below) and did not affect the generation of lactic acid by the cultured cells. Lactic acid was measured in the medium by a modification of the procedure of Hohurst (14).

Preparation of cell-free homogenates Cell cultures were washed three times with cold 0.14 M NaCl, scraped off the dishes with a rubber policeman, pelleted by centrifugation, suspended in 10 mM Tris-HCl (pH 7.2), 10 mM KCl, 2 mM Mg $SO_4$, and disrupted by 100 strokes in a Dounce homogenizer. The homogenate was centrifuged at 20,000 g x 20 min at 4° and further measurements were made in the supernatant solution.

Measurement of lactate in cell-free supernatant Lactic acid was determined immediately following preparation of the supernatant by incubating 90 µl of this solution with 10 µl of reaction mix to give a final concentration of 2 mM Mg $SO_4$, 2 mM ATP, 5 mM potassium phosphate, 1 mM NAD, 50 mM Hepes buffer (pH 7.5) and 100 mM glucose at 37°. After a one hour incubation the tubes received 0.9 ml of water and lactic acid was measured as described above (14). The production of lactic acid was proportional to the amount of homogenate and the time of incubation.

Assay of phosphofructokinase The enzyme was assayed spectrophotometrically (15) at 340 nM at room temperature. The conditions were 2 mM Mg $SO_4$, 2 mM ATP, 5 mM $(NH_4)_2$ $SO_4$, 50 mM Hepes buffer (pH 7.4), 1 unit aldolase, 3 units $\alpha$-glycerophosphate dehydrogenase, 7 units triosephosphate isomerase, 0.15 mM NADH and the indicated concentration of Fru-6-P in a final volume of 0.4 ml. The auxilliary enzymes were obtained from Sigma and were dialyzed for 6 hours against two changes

TABLE 1

Lactic acid production by normal and transformed 3T3 cells.

Experimental details are as described in Materials and Methods

| Cell Type | Labelling Index | Lactic Acid Production | |
|---|---|---|---|
| | | Intact Cell | Cell-Free Supernatant |
| | % | μmole/h/mg protein | |
| 3T3 | 0.3 | 0.17 | 0.26 |
| SV40-3T3 | 66 | 0.83 | 1.4 |
| Py 3T3 | 66 | 0.62 | 1.1 |

of 1,000 volumes of 0.02 M Tris-HCl buffer (pH 7.5). Dialyzing the enzymes for 24 hours or using five times as much aldolase did not affect the enzyme activity obtained. For studies of initial rates, the fall in optical density was plotted for the first minute after the addition of supernatant. In initial studies with 3T3 cells it was observed that the reaction rate was not linear, but decreased with time, tending toward linearity after five minutes. Accordingly, in some assays the cellular supernatant was incubated with the reaction mixture without substrate at room temperature for ten minutes before the sequential addition of increasing amounts of substrate. Blanks were estimated by omission of the substrate or of the supernatant, and reaction rates were calculated from the linear sections of the recorded curves. One unit of phosphofructokinase phosphorylates one μmole Fru-6-P per minute per mg supernatant protein in the conditions described above. Initial rates of aldolase activity were measured in the same conditions, but using Fru-1,6-$P_2$ as substrate.

Other methods and materials The percentage of nuclei incorporating tritiated thymidine (labelling index) was determined by exposing cultures to [$^3$H] methyl thymidine (Amersham, 4 μCi/ml, 0.2 μM) for 4 hours and fixing the cells for autoradiography. Protein was determined colorimetrically (16) in saline-washed cells or in homogenates. All reagents were obtained from Sigma.

## RESULTS AND DISCUSSION

In a series of experiments with confluent cultures of 3T3 cells and their polyoma and SV40 virus-transformed derivatives we measured lactic acid formation by intact cells and by cell-free supernatants.

Table 1 illustrates a typical experiment in which SV40-3T3 or Py 3T3 show a 3.5 to 5 fold increase in their rates of lactic acid production as compared to confluent cultures of 3T3 cells. The rate of glycolysis by intact cells was determined in medium containing 25 mM glucose, a saturating concentration for lactic acid production (unpublished results). The striking feature shown in Table 1 is that the high rate of aerobic glycolysis of the transformed cells persists after homogenization and centrifugation. This finding indicates that the differences in glycolytic activity do not depend critically on the integrity of the cell membrane, but may be the consequence of a change in the glycolytic pathway itself.

In order to further study this possibility we measured the activity of phosphofructokinase, a key point of metabolic control in the glycolytic pathway (17). At 5 mM Fru-6-P, the initial rate of phosphofructokinase activity was approximately twice as great in the transformed cells than in the 3T3 cells. The time course of the reaction was not linear, however, declining much more rapidly in the homogenate of the untransformed 3T3 cells. Since the reaction rates became linear after five minutes, the phosphofructokinase activity was measured in homogenates prepared from 3T3, SV40-3T3, and Py 3T3 cells after a short preincubation in the assay mixture lacking Fru-6-P (see Materials and Methods). Assayed in this manner, Fig. 1 shows the dependence of enzyme activity as a function of Fru-6-P concentration in homogenates prepared from these cells, and demonstrates a substantial increase in the specific activity of phosphofructokinase in SV40-3T3 and Py 3T3 cells as compared to 3T3 cells. In contrast to the striking differences seen in the activity of phosphofructokinase, the specific activity of aldolase at 2.5 mM Fru-1,6-$P_2$ in homogenates of 3T3 as compared to SV40-3T3 cells was nearly identical (76.3 vs. 71.5 nmole Fru-1-6-P cleaved per min per mg protein respectively). In the same homogenates, at 2.5 mM Fru-6-P,

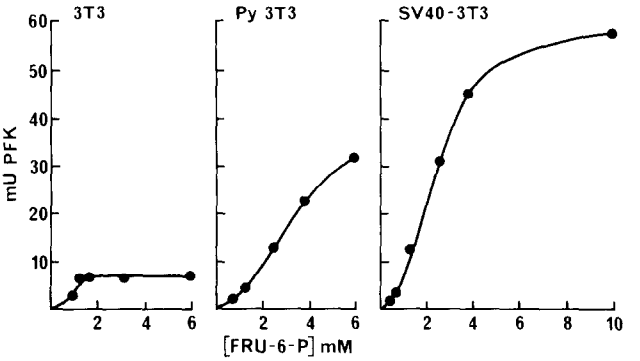


Fig. 1 Phosphofructokinase (PFK) activity in 3T3, Py 3T3 and SV40-3T3 cells is plotted as a function of Fru-6-P concentration. The cellular supernatant was preincubated with the incubation mixture for ten minutes before the addition of substrate. See Materials and Methods for details.

the phosphofructokinase activity from 3T3 cells was 12% that from SV40-3T3 cells (5.6 vs. 47 nmole Fru-6-P phosphorylated per min per mg protein). It is known that the influence of the many allosteric effectors of phosphofructokinase is markedly reduced at alkaline pH (17). Although the fibroblastic enzymes show a 20-fold increased in apparent affinity for Fru-6-P as the pH increases from 7.0 to 8.2, the large increase in specific activity of the extracts from transformed as compared to untransformed cells remains unchanged (Fig. 2). Furthermore, no evidence of a diffusible activator or inhibitor of enzyme activity in homogenates from 3T3 or SV40-3T3 cells was clearly detected in mixing experiments (Fig. 3). Thus, if such effectors play a role in this system they are tightly bound to the enzyme or they have induced a stable modification of the protein.

Taken together, our results provide direct evidence that an alteration in glycolysis, rather than in sugar transport, accounts for the high rate of lactic acid formation by transformed 3T3 cells. These findings do not negate changes in sugar transport following transformation (7,11-13). In this study, however, transport could not explain the

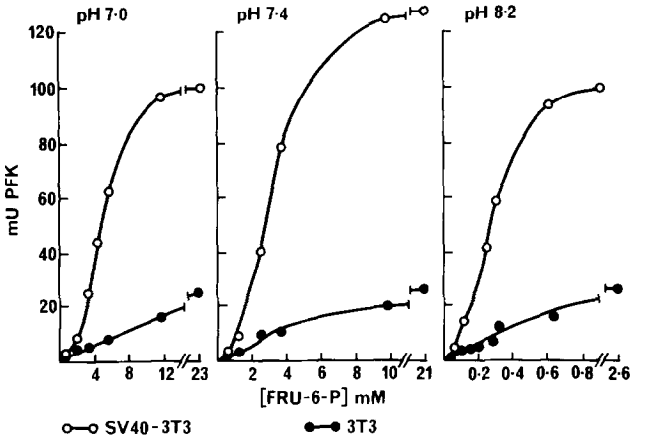


Fig. 2 Phosphofructokinase activity in 3T3 and SV40-3T3 cells is assayed at different pH values. The experimental conditions were as described in the legend to Fig. 1 except that Hepes buffer pH 7.4 was replaced by 50 mM MES buffer pH 7 or 50 mM Tris-HCl buffer pH 8.2. Note the scale change along the abscissa.

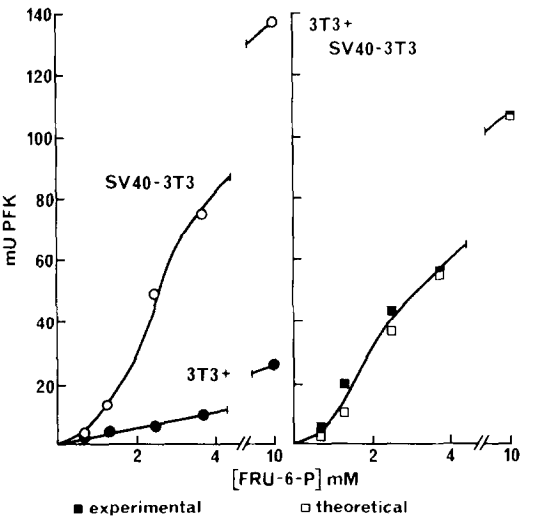


Fig. 3 Phosphofructokinase activity in 3T3 and SV40-3T3 cells. In the left panel, the enzyme activities are plotted as in Fig. 1, but in an independent experiment. In the right panel, cellular extracts from these cells were mixed at the start of the preincubation. The experimental results are plotted as closed squares, and the theoretical values as open squares. The experimental and theoretical activities are derived by dividing the combined enzyme activities by the total protein of the two extracts.

increase in lactic acid formation since this increase persists in the cell-free homogenate. Neither do our studies distinguish whether increased aerobic glycolysis is primarily linked to the increased rate of cell growth or to viral transformation. Indeed, the autoradiographic data in Table 1 show that the confluent cultures of virus-transformed 3T3 cells were growing rapidly. The essential point of this study is that changes in the rate of lactic acid formation in intact cells are faithfully reflected by the rate of lactic acid formation and the phosphofructokinase activity of homogenates of these cells. Recently, we found that serum and purified growth-promoting factors rapidly stimulate lactic acid production in quiescent cultures of 3T3 cells (unpublished results). The experimental approach described here may allow us to define how glycolysis increases after growth stimulation of cells arrested in the $G_1/G_0$ phase of the cell cycle.

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