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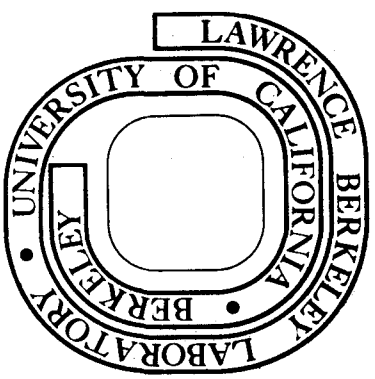

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Glycerol Phosphate Shuttle in Virus-Transformed Cells in Culture

Abstract. The glycerol phosphate shuttle is shown not only to be present and functional in virus-transformed cells, but its level is higher than in normal cells in culture. The increased aerobic glycolysis which has been demonstrated for these cells after transformation, therefore, is not due to an impairment of hydrogen transfer pathways.

The mechanism of increased aerobic glycolysis of tumor cells, when it occurs, is as yet not understood. One of the more widely accepted explanations is that the enzymes necessary for generation of NAD^+ are lacking in malignant cells (1). Of the three NADH shuttles proposed for transport of electrons to the mitochondria (2), glycerol phosphate (GP) shuttle is the most important. During the catabolism of one molecule of glucose, two molecules of NAD^+ are reduced to NADH when glyceraldehyde 3-phosphate (GAP) is converted to 1,3-diphosphoglycerate. The cytoplasmic NADH is used, in turn, either to convert dihydroxyacetone phosphate (DHAP) to ∝-glycerol phosphate (GP) or to convert pyruvate to lactate. The affinity of glycerol phosphate dehydrogenase (EC 1.1.1.8; GPDH) for NADH is greater than that of lactate dehydrogenase (EC 1.1.1.27; LDH) (3). Thus, under aerobic conditions, little lactate is produced by normal cells in vivo. However, many tumor cells were reported to lack, or have drastically reduced levels of, cytoplasmic GPDH (1,2). Boxer and Devlin proposed that LDH could now compete favorably for the cytosolic NADH with a resulting increase in lactic acid production (2). The NAD^+ thus produced would allow the continuous degradation of glucose.

The general applicability of this postulate has been questioned occasionally (4), although the postulate itself has gained general acceptability (5). More recently, in one kind of Ehrlich Ascites tumor cells with a high rate of aerobic glycolysis, the shuttle was shown to be absent. However, in another strain with equally high rate of glycolysis, the shuttle was fully operative (6). Ascites tumor cells do not have a normal counterpart in culture. A comparison between normal and malignant cells under comparable environmental conditions has not been reported previously and was therefore called for.

While normal chick embryo fibroblasts grown in tissue culture do produce an appreciable amount of lactic acid (7,8), Rous sarcoma virus (RSV) transformed cells have been shown to produce even more lactate (8,9). The increase was demonstrated under steady-state conditions and was shown to be in excess of the changes due to growth rates (8,9). An examination of the extent of the glycerol phosphate shuttle in these cells was undertaken to determine whether a decrease in shuttle activity accompanies the increased lactic acid production.

Primary cultures were prepared from 10-day old chick embryos free of resistance-inducing factor essentially as described (10). The single cells were plated in 100 mm culture dishes at 8×10^6 cells per plate in medium 199 supplemented with tryptose phosphate broth (2%) and 1% each of chick and calf serum. The use of fungizone as a fungicide was eliminated entirely, as harmful side effects have been observed in this laboratory (11). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at the desired cell concentration in 35-mm tissue culture dishes. The concentration of glucose and calf serum was doubled (11 mM and 2% respectively) at the time of secondary seeding.

For experiments with transformed cells, Schmidt-Ruppin strain of Rous sarcoma virus, subgroup A, was added at a multiplicity of 0.2 infectious units to half the cells from a single embryo at the time of primary seeding.

A comparison of GPDH and LDH enzyme activities indicated that the level of GPDH was low, but comparable in normal and RSV-transformed cells (Table I). The total level of enzyme fluctuated after the cells were grown in culture for 24 hours and was slightly lower for both normal and

transformed secondary cultures. The activity of LDH, on the other hand, was increased after the cells were grown in culture and after transformation (Table I;12).

Since the measurement of enzyme activities in extracts does not give indications of the actual activity of the enzyme in the cells, the rate of ^{14}C-carbon flow from U-^{14}C-glucose into glycerol phosphate was compared in normal and virus-transformed cells. Forty-eight hours after secondary plating, the cells were placed in the steady-state apparatus and labeled with U-^{14}C-glucose for various periods of time as described (9,13). The killed cells were analyzed for the distribution of label among various pools by two-dimensional paper chromatography and autoradiography (9,13). The initial rate of ^{14}C-carbon flow from glucose into the pool of GP, as well as the final level of ^{14}C label in GP, was 3- to 6-fold higher in transformed cultures than in normal cells (Fig. 1), with the magnitude of the increase being dependent on the degree of transformation. If one assumes that the flow of ^{14}C-carbon into GP is via DHAP, which is the most likely possibility, the initial rate of ^{14}C-flow into the pool of GP (Fig. 1) would indicate that the enzyme is not only active in transformed cells, but its activity is higher than in normal cells.

There are no indications that the mitochondrial glycerol phosphate dehydrogenase is deficient in malignant cells (2). Nevertheless, while the above data indicates that GPDH is active in transformed cells, the rate of electron transfer via the GP shuttle cannot be determined by the measurement of the pool sizes. To gain further information about the extent of the shuttle, we took advantage of the stereospecificity of dehydrogenases with respect to the hydrogen atom transferred during NADH reduction and oxidation. GAP-dehydrogenase transfers the C-1 hydrogen of GAP to the "B" position of NADH during the conversion of 1,3-diP glycerate

(Scheme I). Whether or not this hydrogen remains on the subsequently oxidized NAD^+ depends on which enzyme attacks the NADH molecule. While GPDH removes the "B" hydrogen, LDH removes the hydrogen from the "A" position. If the hydrogen on the C-1 position of GAP is labeled with tritium, the NAD^+ produced by the action of LDH may be distinguished from that produced by GPDH. Such labeling of the C-1 hydrogen of GAP is accomplished by allowing the cells to metabolize in HTO for 1 hour (14). In order to locate the metabolic products on the paper chromatogram, ^{14}C-U-glucose was also added to the medium. During the aldolase and triose phosphate isomerase reactions, there is an exchange with the protons of the medium which results in labeling the C-1 position of GAP with tritium. The fact that other positions on the carbohydrate moiety of NAD^+ may also be labeled by other reactions does not affect the interpretation of the results, since the hydrogen transfer via the dehydrogenases is very specific (15). The NAD^+ was isolated from paper chromatograms and the ratio of $^3H/^{14}C$ was determined as described (14). The ^{14}C-radioactivity in the isolated NAD^+ was comparable in normal and transformed cells under the conditions chosen. If one assumes that the level of ^{14}C labeling is proportional to the total level of nucleotides present, then the ratio of $^3H/^{14}C$ gives the specific tritium radioactivity in NAD^+. The results (Table II) indicate not only that GPDH is active in transformed cells, but that the ratio of the activity of the enzymes of the two NAD^+ producing reactions, i.e., LDH and GPDH, are the same in normal and transformed cells.

There have been many postulates regarding possible reasons for increased aerobic glycolysis of tumor cells, some of which are no longer accepted (16). Boxer and Devlin's postulate was based on measurement of enzyme activities alone and is no longer tenable as a general

explanation for increased aerobic glycolysis. There are, of course, changes in many enzyme levels after transformation, such as the rise in LDH, which have been reported in the literature. Whether these changes are the result of rapid growth and nutritional conditions of the culture or whether they are direct consequences of transformation needs careful scrutiny.

We have proposed previously (9) that the initial increase in glycolysis which occurs after virus-transformation is the result of altered glucose uptake in these cells. An increase in the rate of glucose transport after virus-transformation has been demonstrated for chick cells in culture (17). The changes in glycolytic enzyme patterns and levels as well as in glycogen synthesis and pentose shunt are then seen as secondary events which may also be produced in normal cells if the level of glucose uptake is varied (9). This possibility is under investigation.

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Table 1. LDH and GPDH levels in normal and virus-transformed chick cells.

Stage of cell preparation	GPDH * mg protein	LDH * mg protein
Embryo before trypsin	0.013**	1.20
Cells after trypsin	0.020	1.33
Primary cells after 24 hrs	0.022	1.59
Normal secondary cells	0.014	2.81
Transformed secondary cells	0.012	5.50

*one unit of activity = absorption change of 0.1 at 340 nm per min. LDH was measured by the rate of NADH oxidation following the decrease in absorbance at 340 nm in the presence of pyruvate. GPDH was assayed following the rate of NADH oxidation in the presence of dihydroxy acetone phosphate.

**values are average of 3 independent experiments.

Table 2. The ratio of tritium to ^{14}C in NAD^+ after metabolism in H^3HO and ^{14}C-glucose.

	Spec. Act. H^3HO (mCi/ml)	$^3H/^{14}C$	
		Normal	Transformed
Exp. 1	200	0.086	0.082
Exp 2	600	0.250	0.277
Exp. 3	600	0.298	0.265

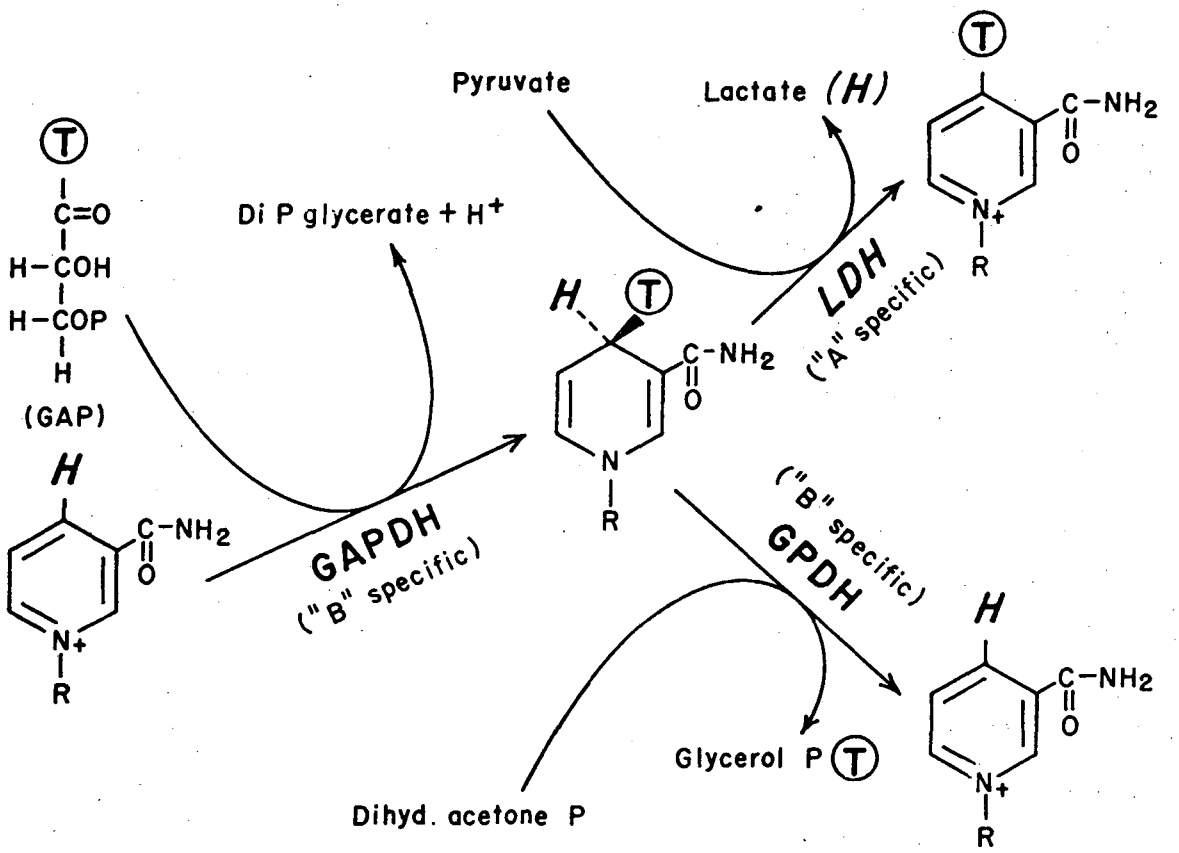
Specific activity of ^{14}C-glucose was 70 mCi/mM. Normal and transformed cells were brought into a closed system containing 5% CO_2 in air at 38-39°C. The entire apparatus was placed in a large glove box with pressure below atmospheric level for safe handling of H^3HO. The procedure was then as described for Fig. I. The isolated NAD^+ spot was cut out of the chromatograms and combusted and tritium and ^{14}C content of the products were counted in a scintillation counter. The error in determination of $^3H/^{14}C$ for individual compounds on the paper chromatogram (based on triplicate samples of the same material) is less than 1%.

Legend to Figure I.

^{14}C-pool level of glycerol 3-phosphate in normal and transformed cells. Secondary cultures were placed in the steady-state apparatus. After one hour of incubation in serum-free medium, medium containing uniformly labeled glucose (536 Ci/mole; 5·5mM) was added. At intervals medium was removed, the cells were washed rapidly and killed. After scraping and sonication, an aliquot was applied to filter paper for analysis by two dimensional chromatography and autoradiography (9,13). GP and DHAP run as a single spot under these conditions. The spot was eluted and rerun on DEAE cellulose paper in the first direction in phenol:water: acetic acid (84:16:1) for 48 hr which separated the two components. The spots were counted as described (13).

Scheme I

TRANSFER OF TRITIUM FROM THE "I" POSITION OF GAP TO THE "B" POSITION OF NAD^+



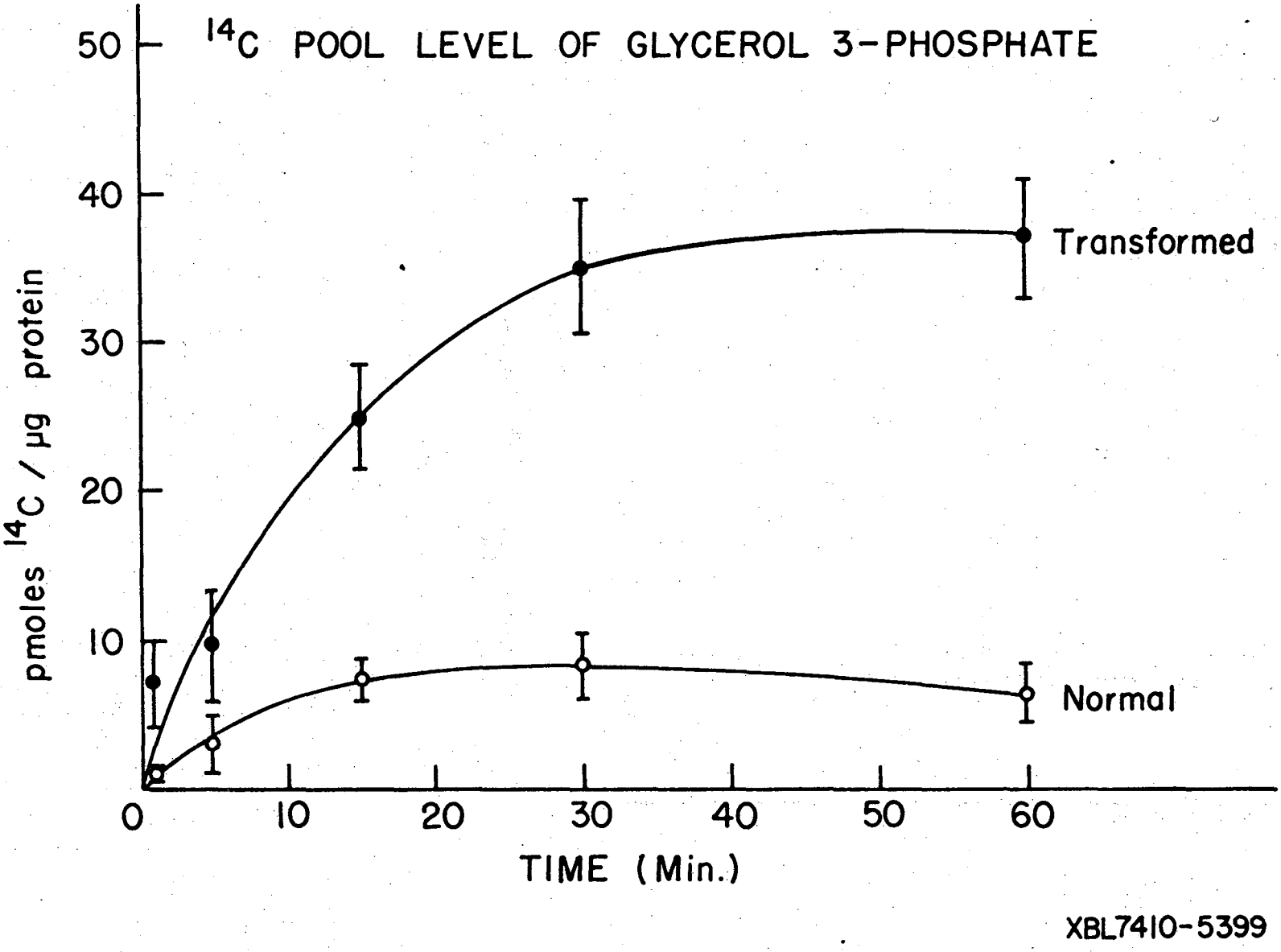


Fig. 1

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