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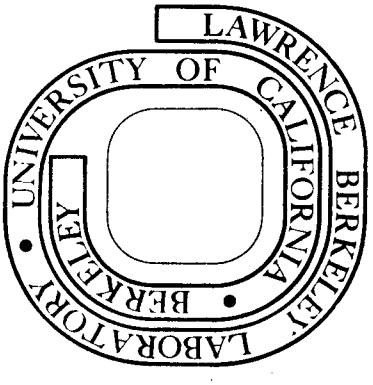

DISCLAIMER

TRITIATED- AND ^{14}C-GLUCOSE IN NORMAL AND TRANSFORMED CHICK CELLS: UPTAKE, PENTOSE SHUNT AND ISOTOPE EFFECTS

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(Received)

SUMMARY

Glucose metabolism in normal and transformed chick embryo fibroblast cells has been observed by allowing the cells to metabolize ^{14}C-U-glucose plus glucose labeled with tritium in the C-1, C-3, and C-6 positions. Similarities and differences between normal and transformed cells have been observed and measured. Both normal and transformed cells are found to metabolize more than half of all glucose-6-phosphate molecules formed from glucose via the oxidative pentose phosphate cycle. However, the ratio of glucose metabolized via oxidative pentose cycle to the net flow of that metabolized directly to fructose-6-phosphate is about the same in normal and transformed cells, with the rates being about twice as much for transformed cells as for normal cells under the chosen conditions.

Several other aspects of transport and metabolism of tritium labeled glucose, including isotope effects, were observed.

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INTRODUCTION

Hydrogen isotopes may be used in metabolic studies either in double-label experiments (with ^{14}C) or to investigate reactions in which the carbon skeleton does not change, but the hydrogen atoms do change (for example, in isomerization reactions). Such studies have been described for plants[1], yeasts and bacteria[2,3], and animal organs[4], but not with normal and transformed animal cell cultures (although Rose[5] employed 1D-^{14}C-glucose to determine the rate limiting step in the pentose phosphate cycle in ascites tumor cells).

During glycolysis via the phosphofructokinase mediated step the metabolic fates of the different carbon-bound hydrogen atoms of glucose are quite different:

C-1 Tritium in glucose becomes C-1 tritium in fructose-6-P and later is in the C-3 position in triosephosphates and lactate. In fructose-6-phosphate the C-1 bound tritium may be subject to some exchange with the medium due to the action of phosphomannose isomerase[6].

C-2 Tritium becomes C-1 tritium in fructose-6-P.

C-3, C-4, and C-5 tritium are lost during aldolase and triose phosphate isomerase reactions into the medium.

C-6 Tritium becomes the tritium in the C-3 position of the triose-phosphates as does tritium from C-1, since the triosephosphate isomerase equilibrates the two triose phosphate molecules. Therefore, a C-1 bound tritium is diluted twofold by the C-6 bound hydrogen in the trioses and vice versa.

Different metabolic fates occur for tritium when glucose is metabolized via the oxidative pentose phosphate pathway. The C-1 bound tritium

of glucose is lost from the molecule during the glucose-6-phosphate dehydrogenase reaction, forming tritium-labeled NADPH. The C-3 bound tritium is also transferred to NADP in the 6-phosphogluconate dehydrogenase reaction. The resulting NADPT is used to a large extent for the fatty acid synthesis or other reductive biosynthetic steps. Thus the amount of radioactivity lost from 3T glucose into the medium is a measure of how much glucose was metabolized via glycolysis[7] as compared to metabolism via the oxidative pentose phosphate pathway and other pathways which retain tritium in metabolites.

In our experiments ^{14}C was used to determine the amounts of some intermediate metabolites. For example, intracellular pools of glycolytic intermediates and lactate may be determined from their content of ^{14}C following a period of steady-state metabolism with ^{14}C-U-glucose[8]. For the intermediate compounds of the tricarboxylic acid cycle and the related amino acids only the rate of ^{14}C-glucose carbon flow may be followed, because in these pools constant specific radioactivity is not reached during 1 h of metabolism with ^{14}C-U-glucose.

MATERIALS AND METHODS

1) Tritiated glucoses and ^{14}C-U-glucose were obtained from New England uclear and Amersham. Specific activities are given in the Results.

2) Growth of Cell Cultures. Primary cultures were prepared from 10-day old C/O or C/B type chick embryos, free of resistance-inducing factor as described previously[9,10]. The cells were seeded at 6-7 x 10^6/100 mm plate in medium 199 (Grand Island Biological) which was supplemented with tryptose phosphate broth (2%), calf serum (1%), and heated chicken serum (1%)

(Microbiological Associates). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at 1 x 10^6 in 35 mm tissue culture dishes (Falcon). The concentration of tryptose broth, calf and chick serums was 10%, 4% and 1%. An additional 1 mg/ml of glucose was added to the medium at this time. For studies with transformed cultures, half the cells of a single embryo were usually infected 4 h after the primary seeding with 10^6 foci forming units of Schmidt-Ruppin strain of Rous-sarcoma virus per plate. Secondary cultures were prepared as above. Assays of virus stock have been described[11].

3) Determination of ^{14}C and ^{3}H Content of Isolated intermediates.

a) 1-T and 6-T experiments: After 48 h the medium of secondary cultures was changed to fresh medium 199 (containing no tryptose phosphate or serum). After 1-1/2 h (which was shown to be sufficient to reach steady-state conditions for glycolytic intermediates[8]), the plates were washed 3 times with glucose-free Hank's buffer. One ml of medium 199 containing high specific activity ^{14}C-glucose (New England Nuclear, final specific activity 26.8 mc/mM) and selectively labeled ^{3}H-glucose (New England Nuclear, specific activities described in the text) were added to each culture. The cells were permitted to take up the radioactive substrates for various lengths of time. The medium was then removed and frozen for later analysis, the cells were washed rapidly with cold unlabeled glucose containing Hank's buffer and killed by addition of cold 80% methanol (less than 15 sec after removal of medium).

The killed cells were then scraped with a rubber policeman, disrupted by sonic oscillation, and applied to filter paper for analysis

by two-dimensional chromatography[12]. The samples were first run with phenol:water:acetic acid (84:16:1) for 24 h. After drying, the paper was turned 90° and run with butanol:water:propionic acid (50:28:22) for another 24 h and then dried. The location of the labeled metabolites on the paper was detected by radioautography. Further details and identification procedures have been recently described[8]. The isolated spots were then cut out and combusted in a Packard Automatic Combustion Apparatus to give CO_2 and water. The tritium and ^{14}C content of these products were determined separately in a Packard Scintillation Counter. The error in determination of $T/^{14}C$ for individual compounds on the paper chromatograms (based on triplicate samples of the same material) is less than 1%. However, the variation in $T/^{14}C$ ratio in the pool of a given metabolite from one experiment to another is $\pm$5% for large pools such as lactate and glutamate, and as high as $\pm$10% for smaller pools. The values given in the tables are the averages from three experiments.

b) 3-T experiments: After the cells were killed, the glucose concentration in an aliquot of the medium was determined by the glucostat method (Worthington). Another aliquot was lyophilized and the tritium radioactivity was measured before and after the freeze-drying. The volatile radioactivity lost as HTO corresponds to the loss of tritium from 3T-glucose during the triose phosphate isomerase reaction in glycolysis.

RESULTS

The ^{14}C specific radioactivity of carbon skeletons derived from administered ^{14}C-labeled glucose is essentially unchanged from that of the added glucose for carbohydrates and closely related compounds of

glycolysis and glycogen synthesis. Thus T/^{14}C is a measure of specific tritium radioactivity. The T/^{14}C ratio for the administered glucose is normalized to 1.00.

Metabolism of 6-T-U-^{14}C-Glucose. Actually, no glucose molecules contain both ^{14}C and T, but rather the double-labeling is due to mixing of 6T-U-^{12}C-glucose with U-^{14}C-glucose. 6T-U-^{14}C-glucose is taken up by normal and transformed cells without a visible change in the T/^{14}C ratio (Table IA). The T/^{14}C values for intracellular glucose itself remain constant with time of incubation and equal to T/^{14}C of administered glucose within experimental error (Table IA).

The three-carbon compounds, glycerol phosphate and dihydroxyacetone phosphate (not separated in this experiment), contain only one-half as much ^{14}C per mole (since they have only three carbon atoms per molecule). The tritium radioactivity is also reduced 50% since in triose phosphates the tritium derived from the 6T position of glucose is diluted by an equal number of triose molecules derived from C-1, C-2, and C-3 of glucose which are not tritium labeled. We thus might expect T/^{14}C = 1.0 for these compounds in the absence of isotope effects. The T/^{14}C ratio for glycerophosphate (GP) plus dihydroxyacetone phosphate (DHAP) is slightly lower than the ratio in the applied glucose. This may result from incomplete equilibration of the two trioses (C-1 to C-3 equilibrates with C-4 to C-6) that would lead to a slightly lower tritium content than one-half of the 6T-glucose[13,2]. The T/^{14}C ratios for GP-DHAP from transformed cells are 4 to 10% higher than the same pools isolated from normal cells.

There is no difference between normal and transformed cells for the T/^{14}C ratios in UDPG and ATP. UDPG has ratios similar to glucose, at least in the first 30 min (Table IA).

The T/^{14}C ratio in the origin of the paper chromatogram, which contains mostly glycogen and proteins, goes up slowly as a result of the increasing T/^{14}C values of the amino acids. The pools of the intermediates of the TCA-cycle and the amino acids deriving from them show a marked change during the first hour (Table IB). In general, however, no significant differences between normal and transformed cells could be demonstrated in these experiments with 6T-U-^{14}C-glucose.

Metabolism of 6T-U-^{14}C-Glucose Compared to Metabolism of 1T-U-^{14}C-Glucose. For several reasons, the T/^{14}C ratios for metabolites obtained following metabolism of 1T-U-^{14}C-glucose are subject to changes which make precise interpretation difficult, and conclusions unreliable. First, if phosphomannose isomerase is present, the C-1 hydrogen exchanges with protons in the medium, as has been demonstrated by studies of incorporation of tritium from HTO into hexose phosphate[6]. Second, when G6Pis metabolized via the oxidative pentose phosphate cycle, the G6P dehydrogenase reaction removes the tritium from the C-1 position completely.

After 1 hour of metabolism by normal and transformed cells of 6T-U-^{14}C- and 1T-U-^{14}C-glucose, T/^{14}C ratios of the intracellular glucose pool are the same in all 4 cases as for the administered labeled glucose (Table II). The T/^{14}C ratio for lactate in normal and transformed cells is lower than that of glucose with both kinds of labeled substrate. The T/^{14}C ratio for lactate from 1T-U-^{14}C-glucose is about 15% lower than that from 6T-U-^{14}C-glucose for the reasons given above. Similar results with 1T and 6T glucose were obtained during aerobic fermentation in yeasts[2].

It might seem that the loss of tritium from 1T-glucose could be used as a measure of the oxidative pentose phosphate cycle. However,

considerable isotopic discrimination against tritium occurs in the glucose-6-phosphate dehydrogenase reaction, so that, in fact, there is a preferred oxidation of unlabeled G6P, while the 1T-G6P is mostly metabolized via glycolysis[14].

Metabolism of 3-T-Glucose. The isotopic discrimination which affected the conversion of 1-T-glucose-6-phosphate via the oxidative pentose phosphate cycle is not paralleled by a similar effect in the case of 3-T-glucose-6-phosphate because glucose-6-phosphate is at a branching point of the metabolic pathways, while 6-phosphogluconate is an intermediate in a straight metabolic sequence[15]. Thus, even if 3-T-6-phosphogluconate accumulates as a result of isotopic discrimination in the oxidation of 6-phosphogluconate, eventually a steady state will be reached in which the proportion of labeled to unlabeled 6-phosphogluconate being oxidized will be the same as that of the 6-phosphogluconate being formed. When 3-T-glucose is metabolized via glycolysis, tritium is lost to water in the medium during the triose phosphate isomerase reaction. During metabolism via the pentose phosphate cycle, the tritium in the 3 position of 6-phosphogluconate is quantitatively transferred to NADP, and the resulting NADPT transfers tritium nearly quantitatively to fatty acids during fatty acid biosynthesis. Tritium from NADPT would be transferred into water only if there were a high activity of transhydrogenase. The tritium in the 3 position of glucose is retained during glycogen synthesis also. The retention of tritium in the cells is therefore a measure of the sum of oxidative pentose phosphate cycle and glycogen (and other polysaccharide) synthesis. The small intracellular pools of glucose, G6P, UDPG, and 6-phosphogluconate also contribute to the tritium retained in the cells.

Except for very short times of metabolism, these pools are negligible compared to the effects of further metabolism.

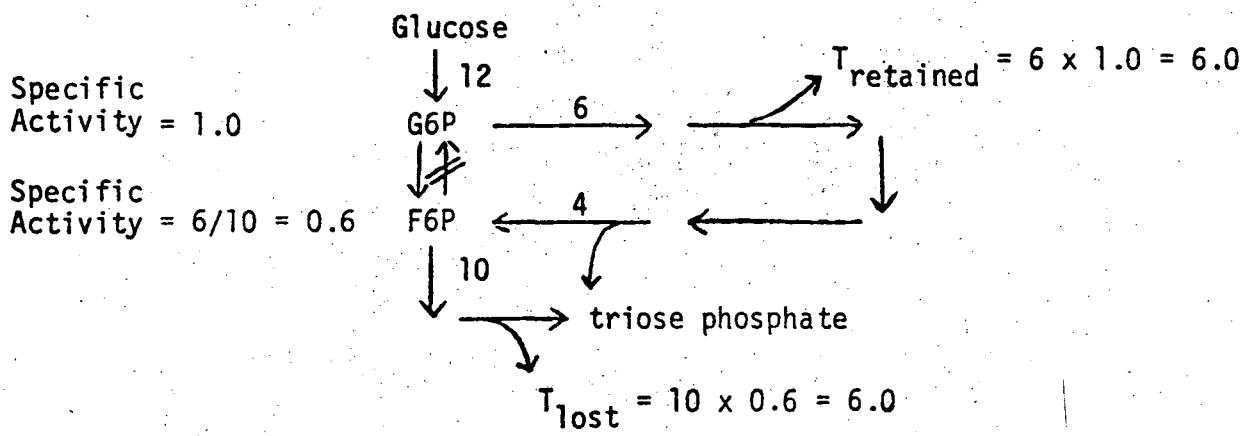
The glucose metabolized via pathways leading to retention of tritium in the cells (line B, Table III) divided by the total glucose used during metabolism (line A) thus gives an approximate proportion of 3-T-glucose metabolized via the oxidative pentose phosphate cycle/(line C). (OPP)

It should be noted that once the tritium label is transferred to fatty acids by the oxidation of 3-T-6-phosphogluconate, recycling of unlabeled sugar occurs, since the pentose phosphate cycle produces one triose phosphate and two fructose-6-phosphate molecules. This formation of unlabeled fructose-6-phopshate affects the calculation if there is isotopic equilibration between hexose phosphates (Schemes 1A, 1B). Neglecting retention of tritium in the cells by pathways other than the OPP cycle, when the retained tritium corresponds to one-half the labeled glucose used, the ratio of glucose-6-phopshate molecules oxidized via the OPP cycle to glucose phosphate molecules formed from glucose is 1/2 if there is no such equilibration (Scheme 1A), and is 3/4 if there is complete equilibration (Scheme 1B). The interconversion of fructose-6-phosphate and glucose-6-phosphate is generally thought to be highly reversible.

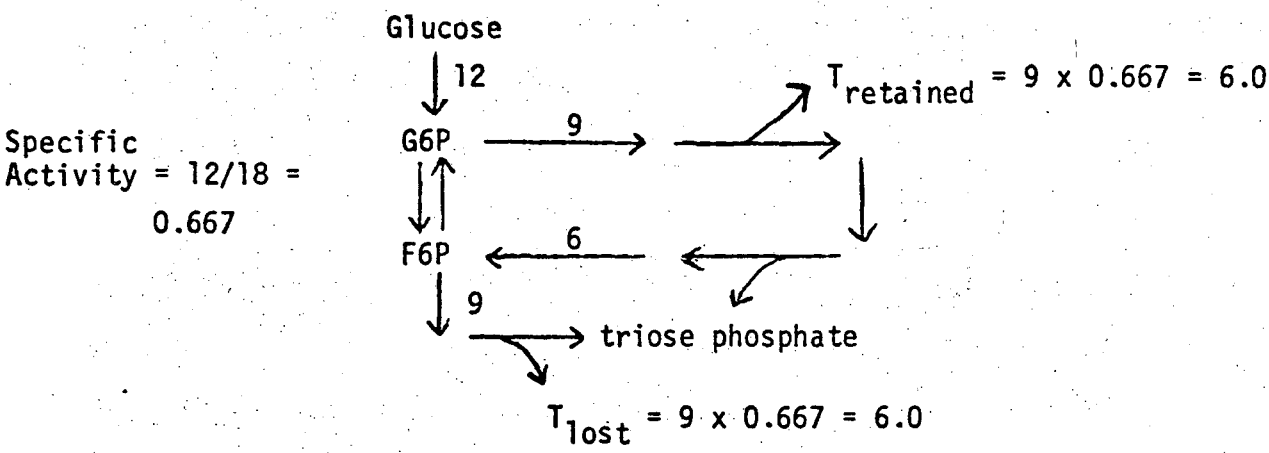
DISCUSSION

In these studies with chick cells in culture, the phenomena related to tritium isotope effects in the metabolism of tritium-labeled substrates generally agree with the effects seen previously with yeasts, algae and bacteria. In addition, some of the results suggest that many

Scheme 1A



Scheme 1B



steps in glucose metabolism are similar in normal and virus-transformed animal cells in tissue culture.

The $T/^{14}C$ values for the intracellular glucose pool indicates that the tritium at the C-6 position is not affected during the transport for either normal or transformed cells (Table IA). Moreover, constancy of this ratio with time indicates that no secondary isotope effects which might diminish the specific tritium radioactivity of glucose occurs during transport.

The small decline in T/^{14}C ratio accompanying the conversion of glucose to dihydroxyacetone and glycerol phosphate is in fact the result of several different effects, and is discussed later.

From a first look at the further reactions of glycolysis, it seems that there should be no possibility for the triosephosphate T-labeled in the 3 position to lose label on its way to pyruvate and lactate. However, we observed that the lactate pool in all our experiments has about 5-10% less tritium than dihydroxyacetone plus glycerol phosphate (Table IA). Rose et al.[16,17] reported the loss of tritium from 3T-phosphoenolpyruvate during the pyruvate kinase reaction. Simon et al.[7] found that during the anaerobic fermentation of 1T and 6T-glucose in yeast there is a loss of tritium into the medium, and they ascribed this loss to the pyruvate kinase reaction. Apparently tritium loss to the medium may occur by this mechanism in the chicken cell cultures also. Since the ratios of pentose phosphate shunt to glycolysis appear to be similar for normal and transformed cells (see below), the 4-10% higher GP-DHAP T/^{14}C ratios may be due to a greater metabolic rate in transformed cells. Also, since the glycolytic pool sizes are different in normal and transformed cells[8], the effects of equilibration of triose phosphate need not be the same for the two cell populations. The T/^{14}C ratios for TCA cycle intermediates and amino acids start at very high values at 5 or 15 min and decrease in the next 60 min to values lower than that of glucose (Table 1B). Pyruvate, which should have the same T/^{14}C ratio as lactate, is decarboxylated to give acetyl-CoA which has one ^{14}C atom less, resulting in a T/^{14}C ratio 1/3 more than that of lactate. In the next step, during condensation with oxalacetate, one

hydrogen atom of these equivalent hydrogen atoms on the methyl group of acetyl CoA is lost and the ratio $T/^{14}C$ should decrease 1/3. However, as Eggerer et al.[18] showed with an in vitro system, instead of 33%, only 18% of the tritium is lost from the tritiated methyl group of AcCoA during the synthase reaction. This isotope effect contributes to the high $T/^{14}C$ ratio in glutamate, citrate, malate and aspartate but does not fully explain them, since the experimental values we find are too high to be explained in this way only.

In the green algae, Chlorella pyrenoidosa, growing in HTO, it was demonstrated[18] that in at least two reactions catalyzed by dehydrogenases of the TCA cycle, tritium is preferentially retained in the pools due to isotope effect. Such isotope effects in the present study would lead to a more rapid increase of tritium than ^{14}C in the pools of TCA cycle intermediates during the time when these pools are far from being fully labeled. The tritium labeling of these intermediate pools thus rises more rapidly at first than the ^{14}C labeling. Since both carbon atoms of acetyl CoA are labeled with ^{14}C, the ^{14}C labeling of TCA cycle intermediate compounds will spread to all carbon positions as the period of metabolism with labeled substrates increases. However, the number of positions in TCA cycle intermediates in which tritium is retained during continued metabolism is less in proportion to carbon positions than in the methyl-T-U-^{14}C acetyl CoA. Thus the ratio $T/^{14}C$ in these TCA cycle intermediates decreases with time.

The experiments with 3-T glucose indicate that about as much glucose is metabolized via the pentose phosphate shunt as via glycolysis (other pathways such as glycogenesis account for less than 1.2% of glucose

metabolism). It should be noted, however, that metabolism of glucose via the shunt produces only one triose phosphate molecule per three glucose molecules used, while the metabolism of three glucose molecules via glycolysis produces six triose phosphate molecules. Thus, the shunt has a relatively small effect on the T/^{14}C ratio of triose phosphates formed from 6T-U-^{14}C-glucose (Table IA). The theoretical T/^{14}C ratios for these triose phosphates formed via the shunt would be 2.0 (compared with the labeled glucose) since they should contain the same amount of tritium for three carbon atoms as the amount of tritium in glucose for six carbon atoms. If the two paths (glycolysis and shunt) are equal, we get [1.0 + 0.167(2.0)]/1.167 = 1.143 as the theoretical T/^{14}C value for glyceraldehyde-3-phosphate and dihydroxyacetone phosphate, provided there were complete equilibration between the two triose phosphates.

We found lower values for T/^{14}C in dihydroxyacetone phosphate and its metabolic derivative, glycerol phosphate. Incomplete equilibration between the two triose phosphates can account for the lower T/^{14}C ratios, since dihydroxyacetone phosphate formed from 6T-U-^{14}C-glucose via glycolysis initially contains no tritium, while glyceraldehyde 3-phosphate would have an initial T/^{14}C ratio of 2.0. If incomplete triose phosphate equilibraiton is responsible for T/^{14}C being 0.9 to 1.0 in dihycroxyacetone phosphate (instead of 1.14), then the glyceraldehyde-3-phosphate converted via 3-phosphoglycerate and pyruvate to lactate must have a T/^{14}C ratio correspondingly higher than 1.14 and must be around 1.3 to 1.4. The drop in T/^{14}C ratio during conversion to lactate would thus be 30-50%.

While the increase in pentose shunt after transformation had been demonstrated for both tumors[20] and chick cells in culture[21], two

important conclusions may be drawn from our results with 3-T glucose. (1) As much as one-half or more of the glucose carbon is metabolized via the shunt in tissue culture cells. (2) The increased shunt after transformation is proportional to the level of glucose uptake. This means that despite the fact that glucose is metabolized twice as rapidly in transformed cells as in normal cells, the ratio of glycolysis to other primary pathways for the conversion of glucose is about the same in normal and transformed cells. This gives further support to the hypothesis[8] that the increased glycolysis and shunt observed after virus transformation is the result, not the cause, of increased glucose transport in these cells. This increased glucose conversion via pentose shunt and glycolysis in transformed cells leads to increased formation of lactate from glucose, not increased conversion of glucose via the TCA cycle[8,21].

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TABLE I

METABOLITE T/^{14}C RATIOS FOLLOWING METABOLISM WITH 6T-U-^{14}C-GLUCOSE*

	Normal cells					Transformed cells				
	1'	5'	15'	30'	60'	1'	5'	15'	30'	60'
					A					
Glucose	0.98	0.97	0.98	1.00	0.97	1.01	1.04	0.96	1.01	0.97
GP-DHAP	-	0.94	0.91	0.91	-	1.04	0.98	0.98	0.95	0.88
Lactate	0.94	0.87	0.89	0.85	0.87	0.93	0.90	0.92	0.95	0.93
UDPG	-	-	0.98	0.94	0.88	-	-	0.96	0.97	0.88
ATP	-	-	0.77	0.79	0.75	-	-	0.72	0.77	0.75
Origin	-	-	1.1	0.95	0.86	-	-	0.91	0.86	0.80
					B					
Citrate	-	-	2-3.3	1.7	1.2	-	-	2-3.3	1.4	1.0
Malate	-	-	2.0	1.4	-	-	-	2.5	1.4	0.95
Aspartate	-	-	2.5	1.4	0.63	-	-	3.3	1.1	0.67
Glutamate	-	1.85	1.15	0.99	0.87	-	1.67	1.05	0.85	0.75

*Average of 5 experiments. Cells were grown and analyzed as described in Methods. The specific radioactivity of 6-T-glucose was 3.6 μC/μM in 2 experiments and 9.0 μC/μmole in 3 experiments.

TABLE II

METABOLITE T/^{14}C RATIOS FOLLOWING METABOLISM OF U-^{14}C-GLUCOSE AND 1T-GLUCOSE OR 6T-GLUCOSE FOR 60 MIN

	1T-U-^{14}C-Glucose*		6T-U-^{14}C-Glucose* ††	
	Normal cells	Transformed cells	Normal cells	Transformed cells
Glucose	0.95	0.99	0.97	0.95
Lactate	0.76	0.78	0.89	0.93
Glutamate	0.58	0.54	0.78	0.72
Citrate	0.60	0.64	1.25	1.11
Aspartate	0.50	0.45	0.63	0.67

*Average of 5 experiments. The specific activity of 1T-glucose was 3.6 μC/μM in 2 experiments and 7.0 μC/μM in 3 other experiments.

††Average of 2 experiments where the specific activity of 6T-glucose was 3.6 μC/μM.

TABLE III

METABOLISM OF 3-T-GLUCOSE*

	1 min	30 min	60 min	120 min
Normal cells				
A. Glucose utilized	4%	12%	17%	24%
B. Tritium in cells after washing	3%	8.5%	10.5%	12%
C. Approximate proportion of glucose metabolized via oxidative pentose phosphate cycle	.75	.71	.62	.50
Transformed cells				
A. Glucose utilized	4%	17%	32%	46%
B. Tritium in cells after washing	3%	10.5%	18.4%	24.4%
C. Approximate proportion of glucose metabolized via oxidative pentose phosphate cycle	.75	.62	.57	.50

*Average of 3 experiments. Specific activity of 3-T-glucose was 3.3 μC/μM.

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