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Radiochromatographic Analysis of Glucose Metabolism in Normal and Virus-Infected Chick Cells in Cultures

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Running Title: Glucose metabolism in culture

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Summary

The concentrations of intermediate compounds and of intracellular lactate in animal cells in tissue culture have been determined by paper chromatography and radioautography, following a period of metabolism in ^{14}C-labeled glucose. The application of these methods appears to be useful for study of metabolic regulation in such cells. The similarity of pattern of virus-transformed cells to that of normal cells in high glucose and normal cells that transport glucose faster after serum addition suggests a similarity in underlying mechanism involved in glucose metabolism.

It is becoming evident that changes in cell surface properties, especially changes in transport of metabolites, accompany malignant transformation in tissue culture [1,2]. An increase of glucose transport can be demonstrated as one of the earliest manifestations of viral transformation [3,4,5,6]. The relation between transport of glucose and the regulatory steps involved in its subsequent metabolism, however, remains to be studied. Such a study of glucose metabolic pathways and their control in tissue culture is complicated by difficulties in maintaining constant environmental conditions during the course of the experiment. Determination of intermediate metabolites and cofactors existing in cells during a kinetic study requires a method for obtaining samples which can be prepared quickly and reproducibly. Each sample should provide, on subsequent analysis, an accurate and detailed picture of the metabolism at the moment of sampling. The steady-state concentration of some metabolites can vary severalfold within a few seconds. Thus, time consuming methods of preparing cell-samples from tissue culture dishes by

scraping and centrifugation or trypsin treatment would result in metabolite pools and patterns significantly changed during the sampling and killing procedure. Furthermore, the pool sizes of some metabolites is very small and a large amount of sample is required for accurate measurements.

Many of these difficulties can be circumvented by using a number of identical small cell cultures, and killing each quickly, after a prescribed period of metabolism with high specific activity ^{14}C-labeled glucose. The killed samples are then analyzed by a combination of two-dimensional paper chromatography and radioautography to provide information about the pool sizes of a large number of intermediary metabolites and cofactors in the cells.

This report describes our early findings using this method, including identification of labeled compounds and amount of labeling under various conditions. Even though strict criteria for steady-state conditions (such as constant pH and temperature) is hard to achieve in standard laboratory incubators, these results indicate the potential value of the method and provide good evidence for the nature of relationships between respiration and glycolysis in normal and virus-transformed animal cells in tissue culture.

MATERIALS AND METHODS

Cell culture and virus infection

Primary cultures were prepared from 10-day old chick embryos as previously described [7,8]. Cells were seeded in medium 199 (Grand Island Biological) supplemented with 2% tryptose phosphate broth (Difco), 1% calf

serum and 1% heated chicken serum (Grand Island Biological; no fungizone was added to these cultures). Secondary cultures were prepared 4 days after the primary seeding by trypsinization of primary cultures and were seeded at 1×10^6 cells per 35 mm dish. For studies with transformed cultures, half the cells of a single embryo were infected 0-4 h after primary seeding with either Bryan or the Schmidt-Ruppin strain of Rous sarcoma virus as previously described [6].

Preparation of samples and chromatography

After 48 h, the secondary cultures were washed 3 times with glucose-free Hank's buffer. Medium containing uniformly labeled ^{14}C-glucose (New England Nuclear), final specific activity 214 mc/mM, and/or $^{32}P_i$, final specific activity 30 μc/μM, was added to each culture. The cells were permitted to take up the radioactive substrates for an hour. The medium was then removed, the cells were washed rapidly with cold unlabeled glucose-containing Hank's buffer, and killed by addition of 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 paper chromatography [9]. The killing is almost instantaneous as no change in metabolite pattern was observed when the cells were left in methanol for various times. The samples were first run with phenol:water:acetic acid (840:160:10) for 24 h. After drying, the paper was turned 90° and run with butanol:water:propionic acid (500:280:220) for another 24 h. After drying, the location of the labeled metabolites was detected by radioautography and the content of ^{14}C and/or ^{32}P was determined as described [10]. The unknown spots were eluted and the procedure was repeated after addition of unlabeled known compounds which later were localized with appropriate chemical reactions.

Rates of DNA synthesis and glucose uptake

The rate of incorporation of [^{3}H] thymidine into DNA was used as a measure of overall rate of DNA synthesis, and the rate of glucose uptake was measured by using [^{14}C] 2-deoxy-D-glucose [5,6].

RESULTS

The radioautographs (Figs. 1,2) show the separation and identification of a large number of metabolites resulting from the metabolism of ^{14}C-glucose in a single sample (a 35 mm dish containing only one million cells). Glycolysis (as measured by the rate of lactate production from glucose) and respiration (as measured by the rate of CO_2 evolution from glucose) becomes linear with time in less than 15 min under our culture conditions [6]. Long before one hour, therefore, most of the metabolites have reached their steady-state concentrations. A comparison of the metabolic pattern of normal and transformed cells (Fig. 1) demonstrates that in transformed cultures the ratio of the intracellular pool of lactate over citrate (as a measure of the TCA cycle) is increased appreciably. Labeled pool sizes of other intermediates of the TCA cycle and those derived from it (malate, glutamate and aspartate shown in the chromatograms) are decreased. The labeled pools of the sugar diphosphate region (DIP), the triose phosphate intermediates (3PGA and DHAP) and the metabolites of the oxidative pentose phosphate pathway (PMP; "hexose monophosphate shunt") are increased measurably. An increased utilization of oxidative pentose phsophate pathway over TCA cycle after transformation was previously observed using CO_2 derived from glucose labeled in 1 or 6 positions [6].

To determine the contribution of the faster growth rate of transformed cells to these patterns, 5% chicken serum was added to normal cells 16 h prior to the onset of the experiment. Serum causes the normal density-inhibited cells to undergo rapid proliferation [11]. While addition of serum increases the glycolytic degradation of glucose two to threefold, the ratio of labeled lactate to citrate is still about 4 times higher in transformed cultures (Table 1). Moreover, the pattern attributable to added serum seems to be due to factors other than growth alone: addition of 5% serum to transformed cultures increases the lactate to citrate ratio further without changing the growth rate appreciably (Table 1). Growth *per se*, therefore, cannot explain the changes observed.

The suppression of respiration by glucose was first described by Crabtree in 1929 [12]. When the normal cells receive decreasing levels of glucose, the ratio of lactate to citrate decreases proportionally to the level of glucose in the medium. A 10-fold change in the level of glucose concentration of the medium causes a 10 to 15-fold change in the ratio of lactate to citrate (Fig. 2, Table 2). Addition of serum to both cultures raises the background level of glycolysis, as indicated by the lactate pool in the cells, yet a substantial difference between cells in high and low glucose remains (Table 2). The labeled pools of the DIP, PMP, and triose phosphate regions are also increased with increased glucose concentration.

DISCUSSION

The techniques employed in this study allow determination of the various pool sizes of metabolites in the cells during a condition of

steady-state metabolism. This information, together with knowledge gained from examination of changes accompanying perturbation of the steady states will permit the elucidation of regulatory steps. Such a procedure has been employed successfully with photosynthetic cells [13]. Changes in glucose metabolism due to pH, serum, hormone and carcinogens addition, cell density and various inhibitors, as well as viral transformation may be effectively examined in this way. Since some metabolites are released into the medium, it will be necessary to make some measurement of metabolites in the medium in order to obtain a complete metabolic picture. However, it is concentration within the cells that provides direct information about regulation of specific reactions, such as the reaction mediated by phosphofructokinase (Tables 1 and 2). Preliminary kinetic experiments after addition of serum to extremely contact inhibited cells or/pH after a increase indicate a rapid rise in the F-1,6-Dip/F6P ratios (as much as a 10-fold increase within one minute), indicating the phosphofructokinase step as one of the controlling points in such transitions as also found in other systems [14].

It is generally accepted that malignant cells are more "glycolytic" than their normal counterparts [15], although the extent and reason for such a difference has been the subject of many controversies [16]. Bissell et al. [6] have demonstrated that the increased lactate production after infection of chick cells with Rous sarcoma virus (RSV) cannot be accounted for by increased growth rate as previously suggested [17]. The results presented in this paper confirm and expand the above findings. We further suggest that the increased glucose (or glucose-6-phosphate) concentration within the transformed cells [18,3,6,5] creates a situation similar to that of normal cells in high glucose medium and causes

the shift to a higher glycolytic metabolism, i.e., the Crabtree effect, and increased glycolysis in transformed cultures may have a similar underlying mechanism. The absolute levels of glycolysis and respiration change from embryo to embryo, with population density [6] and with changes in growth condition. The glucose level within the cell is thus only one of the factors causing the observed balance between glycolysis and respiration. But other factors being equal, whatever increases the glucose entry into the cell seems to cause an inhibition of respiration and an increase in glycolysis and oxidative pentose shunt whether it is addition of serum, an increase in glucose concentration of the medium, or viral transformation.

It is usually reported that transport and phosphorylation of glucose are "coupled processes" in that there is very little or no free glucose inside the cells [4,19]. The presence of a relatively large glucose pool (Gluc) in these chromatograms was therefore surprising. Since the amount of glucose in the medium relative to that of label in the cells is enormous, part or all of the glucose pool could be due to contamination from the medium, despite the extensive washing. However, from this and other experiments with glucose analogues [20; Bissell and Bassham, unpublished], we believe that the possibility that a small free glucose pool does exist within these cells cannot be ruled out. It is possible that the hexokinase step is not necessarily coupled to glucose transport. This problem and the steps involved in the control of glucose metabolism are under investigation. An apparatus is under development to permit rapid sampling, while maintaining more rigorously the necessary steady-state criteria, or during the transient period following a controlled change in a selected external condition.

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Table 1. Relationship of glycolysis and respiration in normal and transformed cultures

	lactate / citrate	F-1,6-DiP / F6P	Growth rate**
T / N *	10.0, 8.1	2.1, 4.3	3.5, 3.5
N + 5% S / N	2.8, 1.9	2.0, 1.7	2.7, 3.2
T / N + 5% S	3.6, 4.2	1.1, 2.5	1.3, 1.1
T + 5% S / N + 5% S	6.0, 7.6	1.7, 3.2	1.4, -

* T, transformed; N, normal; S, chicken serum

** ^{3}H-Thymidine incorporation into DNA; cpm/μg protein

The procedure was as in Fig. 1. The spots were removed from chromatograms and counted. The DiP and HMP regions were eluted with 0.3-0.5 ml water, treated with phosphatase [purified from Polidase S (Schwarz Laboratories) by ammonium sulfate precipitation; 25 μg/0.3 ml, for 3 h at 37°C] and re-chromatographed as described in Methods. The isolated fructose diphosphate (F-1,6-DiP) and fructose-6-phosphate (F6P) were removed and counted. Last column indicates the rate of DNA synthesis as measured by ^{3}H-thymidine incorporation. Glucose concentration was 5.5 mM. Each column includes the values for two experiments. In the second experiment the cells were infected with the Schmidt-Ruppin strain of Rous sarcoma virus, sub-group A.

Table 2. Crabtree effect in normal chick cells

	5% chicken serum	lactate / citrate	F-1,6-DiP / F6P
N cells in 5.5 mM glucose / N cells in 0.55 mM glucose	-	15.5, 11.5	1.8, 2.8
	+	8.4, 7.5	1.7, 2.1
T cells in 5.5 mM glucose / T cells in 0.55 mM glucose	-	9.2, 6.3	-

Legend as in Fig. 2 and Table 1. These are the ratio of ^{14}C counts. The ratio of ^{32}P counts in the last column (taking the 2 phosphates of F-1,6-DiP into consideration) was similar to above values, indicating that the phosphate as well as the carbon pools have approached steady state.

FIGURE LEGENDS

Fig. 1. Pattern of glucose metabolism in normal and Rous transformed cells. The cells were exposed to 0.75 ml of 5.5 mM uniformly labeled ^{14}C-glucose in medium 199 for one hour. After removal of medium and washing, half of the mixture of methanol extracted pools and sonicated cells were applied to paper, as described in Methods. The radioautograms were developed after one week. Abbreviations used: Gluc, glucose; Glut, glutamate; Asp, aspartate; Mal, malate; Lac, lactate; Ala, alanine, DHAP, dihydroxyacetone phsophate; 3PGA, 3-phosphoglyceric acid; DIP, sugar diphosphate region; PMP, pentose monophosphate region; HMP, hexose monophosphate region; UDPG, uridine diphosphate glucose; O, origin.

Fig. 2. Pattern of glucose metabolism in normal chick cells in low and high glucose. Procedure as in Fig. 1. The cultures also received $^{32}P_i$ concomittantly with glucose. During the labeling the phosphate concentration of the medium was 10X less than the experiment reported on Fig. 1. P_i, inorganic phosphate.

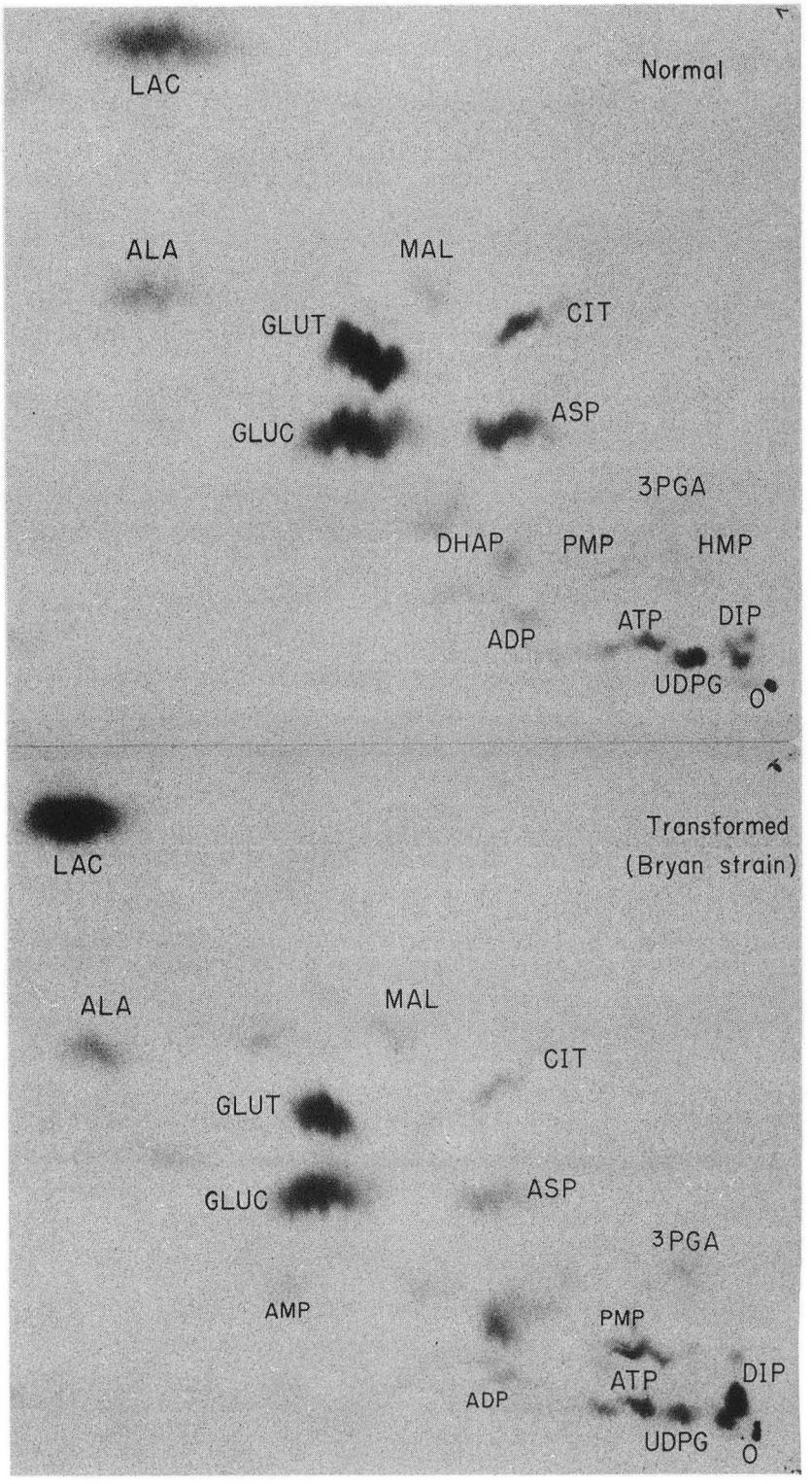

Fig. 1 XBB 7211-5930

0 0 0 0 3 9 0 3 4 1 8

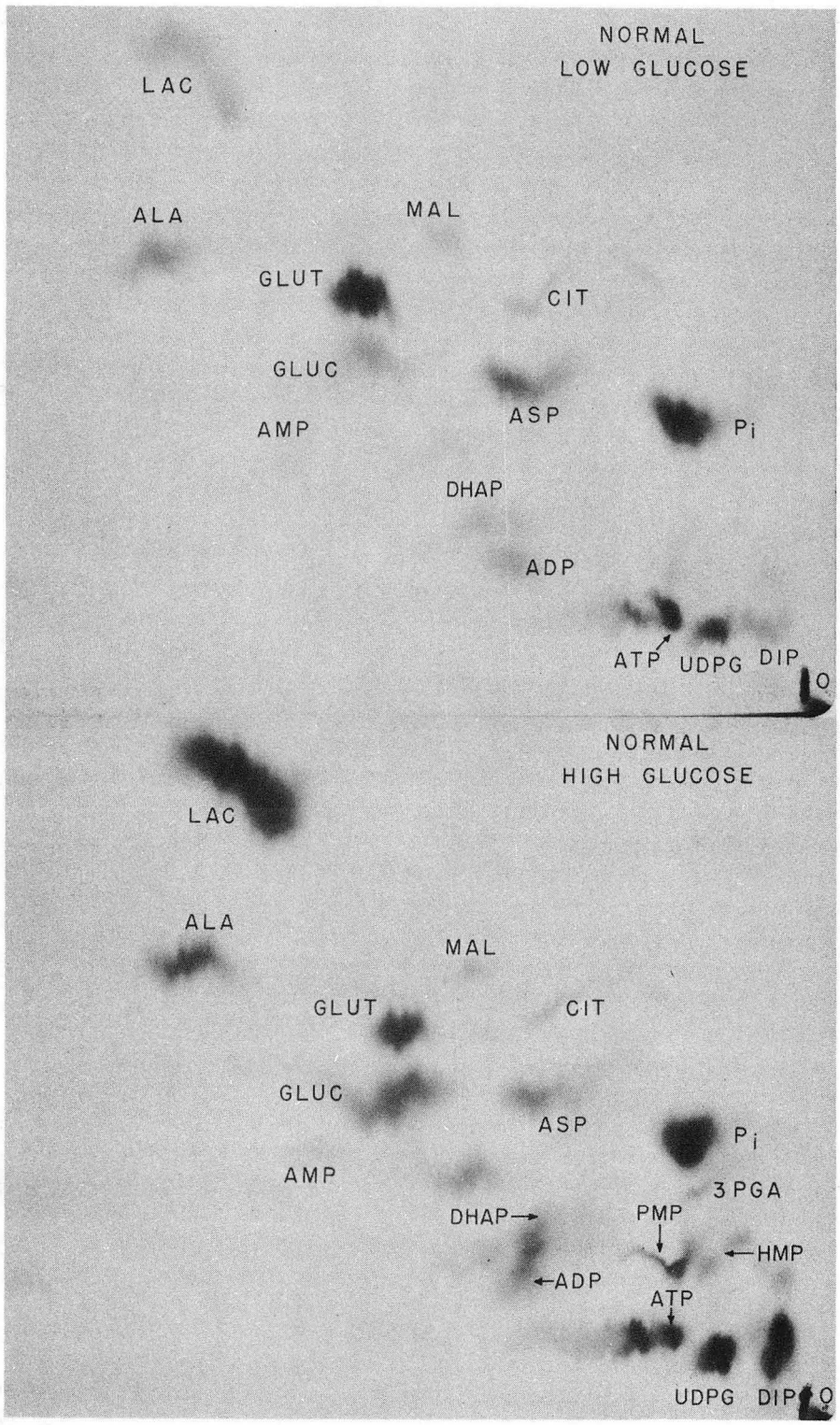

Fig. 2

XBB 7211 -5931

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