Proc. Nat. Acad. Sci. USA
Vol. 71, No. 10, pp. 4129–4132, October 1974

Alterations in Glucose Metabolism in Chick-Embryo Cells Transformed by Rous Sarcoma Virus: Intracellular Levels of Glycolytic Intermediates

(sugar uptake/glycolytic enzymes/glucose phosphorylation)

VISHWA NATH SINGH*, MALATHY SINGH, J. T. AUGUST, AND B. L. HORECKER†

Department of Molecular Biology, Division of Biological Sciences, Albert Einstein College of Medicine, Bronx, New York 10461

Contributed by B. L. Horecker, August 6, 1974

ABSTRACT **Chick-embryo cells, transformed with Rous sarcoma virus, show enhanced rates of sugar transport and glycolysis. Determination of intracellular concentrations of glycolytic intermediates suggests that the enhanced glycolytic flux is due to increased activities of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.-1.1), phosphofructokinase, (ATP:D-fructose-1-phosphate 6-phosphotransferase, EC 2.7.1.56), and pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), and not directly to the increased glucose transport. This conclusion is supported by the finding that the intracellular concentration of free glucose is decreased, rather than increased, in the transformed cells. The present observations suggest that the increased glycolytic flux is related to an increased rate of phosphorylation of glucose, and that hexokinase in the transformed cells is at least partly released from its normal control mechanism involving feedback inhibition by glucose-6-*P*.**

A marked increase in the rate of sugar uptake has been reported for chick-embryo cells transformed by Rous sarcoma virus (1–11). This increased transport of glucose is an early event in transformation (8) and may be related to the increased glycolytic flux that has been reported in transformed cells (3, 12–17). In order to determine the relationship between this increased glycolytic flux and the enhanced sugar transport, and to identify the metabolic step(s) affected by transformation, we have examined the changes in activities of key glycolytic enzymes and also the changes in the levels of glycolytic intermediates. The studies on enzyme levels, reported elsewhere (18), have established that the activities of hexokinase, phosphofructokinase, and pyruvate kinase, the rate-limiting enzymes of glycolysis, are increased several-fold soon after infection of chick embryo cells with the Schmidt-Ruppin strain of Rous sarcoma virus. That these changes are associated with transformation rather than with virus infection was established by experiments with a temperature-sensitive mutant (Ts-68); infection with this mutant at the non-permissive temperatures, where the cells are not transformed, did not affect the levels of the rate-limiting enzymes (18).

In order to confirm that these increases in enzyme activities are indeed related to the increased glycolytic flux *in vivo*, we have now examined the changes in intracellular levels of glycolytic intermediates. The increases and decreases in the levels of these intermediates determine the crossover points, which are the most reliable indicators of the *in vivo* sites of metabolic regulation (19). The studies reported here demonstrate that the increased glycolytic flux in virus-transformed cells is related to the increased intracellular activities of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) phosphofructokinase (ATP:D-fructose-1-phosphate 6-phosphotransferase, EC 2.7.1.56), and pyrivate kinase (ATP: pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40), and perhaps only secondarily to the increased rate of glucose transport.

METHODS

The normal and the Rous sarcoma virus-infected cells cultures were prepared as described elsewhere (18). One half of the primary cell cultures of 10 day-old chick-embryos was infected with the Schmidt-Ruppin strain of Rous sarcoma virus (generously supplied by Dr. H. Hanafusa, The Rockefeller University, New York), while the other half was used as normal control. Secondary cultures were prepared by treating the four or five day-old primary cultures with trypsin and seeding the cells at the desired density in 150-mm Falcon tissue culture dishes unless indicated otherwise. Four day-old growing secondary cultures were used throughout the studies reported here. The culture media were changed daily. Other methods were as described in the tables.

RESULTS

Effect of virus-transformation on sugar uptake and metabolism

Before undertaking the measurements of intracellular concentrations of substrates, it was necessary to confirm that our conditions of infection caused the usual increases in sugar uptake and glycolytic flux. We found that infection with Rous sarcoma virus increased the rate of uptake of 2-deoxyglucose by nearly 6-fold, and that of 3-*O*-methylglucose by more than 2-fold (Table 1). Similarly, the rate of lactic acid production, which was linear for at least 1 hr, was nearly three times greater in the transformed cells (Table 2). The yield of CO_2 was relatively low and was similar in uninfected and transformed cells, but the total yield of anions from glucose, including lactate, pyruvate, phosphorylated intermediates, and intermediates of the citric acid cycle, was more than doubled in the transformed cells.

Effect of transformation on the levels of glycolytic intermediates, adenylates and P_i

Having established that sugar uptake and glycolytic flux were indeed increased in cells transformed under our experi-

* On leave of absence from the Department of Biochemistry, Vallabhbhai Patel Chest Institute, University of Delhi, India.

† Present address: Roche Institute of Molecular Biology, Nutley, N.J.

TABLE 1. *Effect of transformation by Rous sarcoma virus on the uptake of 2-deoxyglucose and 3-O-methylglucose by chick-embryo cells*

Sugar	Sugar uptake* Normal (nmol/mg of protein per min)	Transformed (nmol/mg of protein per min)
2-Deoxyglucose	0.73	4.16
3-*O*-Methylglucose	0.13	0.33

* Measured in three-day-old growing secondary cultures (see *Methods*) in 35-mm Falcon tissue culture dishes, as described by Weber (11). The concentration of the radioactive sugar, the temperature, and the period of incubation were 0.25 mM, 37°, and 10 min in the case of [^{3}H]2-deoxyglucose (New England Nuclear Corp., Boston, Mass.), and 0.5 mM, 22° and 1 min in the case of 3-*O*-methyl-D-[1-^{3}H]glucose (Amersham/Searle Corp., Arlington Heights, Ill.). The specific activities of 2-dGlc and 3-*O*-MeGlc were 2 Ci/mole and 4 Ci/mole, respectively. The cell cultures used for the experiments with 3-*O*-MeGlc were preincubated with phosphate-buffered saline, pH 7.4 (Grand Island Biological Corp., New York) for 1 hr at 37°. After incubation with the radioactive sugars and appropriate washing (11), the CCl_3-COOH extracts were transferred to scintillation vials containing Aquasol (New England Nuclear Corp., Boston, Mass.) for counting. Protein was determined by the method of Lowry *et al.* (20).

mental conditions, we employed the same conditions for the determination of intraceullular levels of glycolytic intermediates (Table 3). Transformation was found to decrease the intracellular steady-state levels of glucose, 2-*P*-glycerate, and *P*-enolpyruvate, with concomitant increases in the levels of hexose monophosphates, fructose-1,6-P_2, triose-*P* and pyruvate (Table 3). The significance of the results is most apparent from the crossover plot, in which the levels of the glycolytic intermeidates in the transformed cells are expressed as the percent of the corresponding values from the normal cells (Fig. 1). The results clearly show forward crossovers

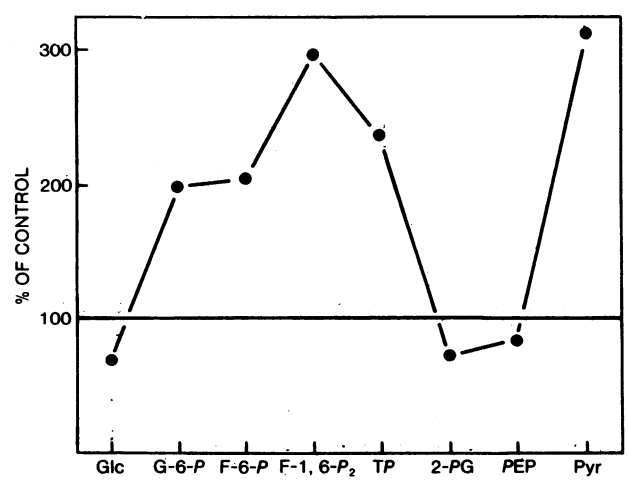

FIG. 1. Crossover plot of the concentrations of glycolytic intermediates in Rous sarcoma virus-transformed chick embryo cells as percentage of the corresponding normal values. Data from Table 3 were used to construct the plot. Glc, glucose; G-6-*P*, glucose-6-*P*; F-6-*P*, fructose-6-*P*; F-1,6-P_2, fructose-1,6-P_2; T*P*, triose-*P*; 2-*P*G, 2-phosphoglycerate; *P*EP, phosphoenolpyruvate; Pyr, pyruvate.

TABLE 2. *Effect of transformation by Rous sarcoma virus on the incorporation of* D-[*U*-^{14}*C*]*glucose into* CO_2, *anions, and lactate by chick-embryo cells*

Determination	Radioactivity incorporated* Normal (cpm/mg of protein × 10^{-3})	Transformed (cpm/mg of protein × 10^{-3})
CO_2	18.3	19.3
Lactate†	143.6	360.2
Anions‡	314.3	740.9

* The medium from four-day-old growing secondary cell cultures (see *Methods*) in tissue culture Falcon plastic flasks (surface area 75 cm^2) was removed by aspiration and the cells were washed twice with prewarmed (37°) phosphate-buffered saline. Ten ml of the buffered saline containing 10 μmol (10 μCi) of D-[*U*-^{14}C]glucose (New England Nuclear Corp.) were added to each flask. The flasks were closed with rubber stoppers, provided with a double-seal septum for air-tight closing and with a polypropylene center well (Kontes Glass Corp., New Jersey) inserted in the center. After incubation at 37° for 30 min, the reaction was stopped by injecting 0.3 ml of 70% $HClO_4$ into the medium followed by injection of 0.2 ml of a strong base (0.5 M Protosol, New England Nuclear Corp.), into the center well. After 60 min the polypropylene wells were transferred directly to the scintillation vials containing Aquasol (New England Nuclear Corp.) for counting. The cells and medium were quantitatively removed and homogenized together. After cooling in crushed ice for 1 hr, the homogenate was centrifuged at 1200 × *g* for 15 min. The acid-insoluble material was dissolved 0.1 N NaOH solution and used for protein estimation (20). The clear supernatant solution was neutralized with 5 N KOH, stored overnight at 4°, and centrifuged to remove the precipitated $KClO_4$. The clear supernatant solution was used for the determination of ^{14}C-labeled anions and lactate. Radioactivity and protein determinations were carried out as described in the footnotes to Table 1.

† For the determination of radioactivity in lactate, the acid-soluble fraction was treated with $CuSO_4$ and $Ca(OH)_2$ (21), and chromatographed on silica gel thin-layer plates (Brinkmann Instruments, Inc., Westbury, N.Y.) (22). The lactate spot was scraped and transferred to scintillation vials for counting.

‡ Determined by the method of Rose and O'Connell (23) as modified by Wu (24).

between glucose and G-6-*P* and between *P*-enolpyruvate and pyruvate. Although there was no crossover between hexose monophosphates and fructose-1,6-P_2, the large increase in the concentration of the latter indicates a marked activation of this phosphorlylation step.

Transformation by Rous sarcoma virus caused a slight increase in the intracellular steady-state concentration of ATP and some decrease in the concentrations of ADP and AMP (Table 4). The "energy charge" (31), calculated from these data was not significantly altered. The concentration of P_i was increased by about 30% in the transformed cells.

DISCUSSION

The results reported here and in the previous communication (18) suggest that the increased glycolytic flux in chick-embryo cells transformed by Rous sarcoma virus is not due to the increased rate of sugar transport, but rather to the enhanced activities of the key enzymes that constitute the control points in the glycolysis. Thus, we observed marked increases in the steady-state levels of glucose-6-*P*, fructose-6-*P*, fruc-

TABLE 3. *Effect of transformation by Rous sarcoma virus on the intracellular concentrations of glycolytic intermediates in chick-embryo cells**

Determination†	Concentration Normal (nmol/mg of protein)	Concentration Transformed (nmol/mg of protein)	% Change
Glucose	21.05	14.73	−30
Glucose-6-*P*	0.348	0.692	+99
Fructose-6-*P*	0.116	0.238	+105
Fructose-1,6-P_2	1.373	4.070	+196
Triose-*P*	1.083	2.566	+137
2-phosphoglycerate	0.524	0.380	−28
P-enolpyruvate	0.134	0.111	−17
Pyruvate	0.827	2.587	+213

* Cells were grown and infected as described under *Methods*. The medium was removed by aspiration and the cells were quickly washed two times with chilled glucose-free Hanks' balanced salt solution, the entire process taking less than 15 sec. Cells were immediately frozen by dipping the plate in ethanol–dry ice and the plates were placed over crushed ice. Two milliliters of prechilled 0.5 N $HClO_4$ was quickly layered over the frozen cells and the cells removed with a rubber policeman. In order to conserve volume, the suspension from one dish was used for removing cells from the next. The cell suspension was homogenized and after keeping in ice for about 30 min, the whole homogenate was centrifuged in the cold. The pellet was dissolved in NaOH solution and used for estimation of protein (20). The supernatant solution was neutralized (pH 7–8) with KOH solution, kept in ice for 1 hr, and centrifuged. The clear supernatant solution was stored frozen at −75° until required for the analyses, which were completed within 12 hr.

† Spectrophotometric procedures were employed for glucose (25), fructose-1,6-P_2 and triose-*P* (26), 2-*P*-glycerate (27), *P*-enolpyruvate (28), and pyruvate (29). Glucose-6-*P* and fructose-6-*P* were estimated fluorometrically (25). Immidazole buffer, pH 7.0, was used for the estimation of fructose-1,6-P_2 and triose-*P* as recommended by Lowry *et al.* (27).

TABLE 4. *Effect of transformation by Rous sarcoma virus on the intracellular concentrations of adenylates and P_i in chick-embryo cells**

Determination†	Concentration Normal (nmol/mg of protein)	Concentration Transformed (nmol/mg of protein)	% Change
ATP	18.63	22.05	+18
ADP	3.43	2.94	−15
AMP	1.11	0.82	−19
P_i	55.26	71.45	+30
Energy charge‡	0.88	0.91	N-S

* Cell cultures were treated as described in Table 3.

† Spectrophotometric procedures were used for ATP (26), ADP, and AMP (29). Precautions suggested by Krebs and Gevers (30) were observed during the estimations of ADP and AMP. The NADH samples were freed of contamination with AMP by treating with bacterial alkaline phosphatase, as suggested by Lowry *et al.* (27).

‡ Energy charge $= \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$ (31). N-S, not significant.

tose-6-P_2, triose-*P* and pyruvate, and concomitant decreases in the intracellular levels of free glucose, 2-*P*-glycerate and *P*-enolpyruvate. The crossover plot, which clearly indicates that the rates of the reactions catalyzed by hexokinase and pyruvate kinase are enhanced in the transformed cells, is consistent with the previously reported increases in the intracellular levels of these enzymes (18). Although we did not observe a clear-cut crossover, in the classical sense, at the step catalyzed by phosphofructokinase, the large increase in concentration of fructose bisphosphate suggests an increased intracellular activity of this enzyme as well (18).

Apparently, the key to the increased glycolytic flux is the enhanced rate of phosphorylation of glucose, which occurs in the face of a 2-fold increase in the intracellular concentrations of glucose-6-*P*. The large increases in concentrations of all these phosphorylated hexose intermediates suggests, that in the transformed cells, hexokinase is not affected by the high concentrations of glucose-6-*P*, which would be expected to inhibit its activity (23, 32, 33). The fact that in the transformed cells hexokinase is present almost entirely as the particulate form of the enzyme (18), which may be less susceptible to allosteric regulation by glucose-6-*P* (34–39), may explain its apparent insensitivity to glucose-6-*P* inhibition in these cells. It will be interesting to examine in detail the nature of the bound hexokinase found in the transformed cells.

Although the rate of sugar uptake is increased in the transformed cells, the steady-state concentration of intracellular free glucose is decreased, presumably due to the increased rate of phosphorylation. Evidence that phosphorylation plays a role in glucose uptake derives from the fact that the rate of uptake of 3-*O*-methylglucose, which is not phosphorylated (40–42), is increased by only 2-fold, compared to an increase of 6-fold in the rate of uptake of 2-deoxyglucose, which accumulates in the cell largely as the phosphate ester (4, 40, 43–45).

Although sugar uptake is significantly enhanced in transformed cells, suggesting that transformation is associated with modification of the plasma membrane (3, 4), this enhanced sugar uptake is evidently not the driving force for the increased glycolytic flux. The increased glycolysis can be interpreted in terms of the same regulatory mechanisms that have been proposed to account for the Pasteur effect in normal cells, with the reactions catalyzed by hexokinase and phosphofructokinase as the key control points (46, 47).

1. Hatanaka, M., Huebner, R. J. & Gilden, R. V. (1969) *J. Nat. Cancer Inst.* **43**, 1091–1096.
2. Hatanaka, M. & Gilden, R. V. (1970) *J. Nat. Cancer Inst.* **45**, 87–89.
3. Hatanaka, M. & Hanafusa, H. (1970) *Virology* **41**, 647–652.
4. Hatanaka, M., Augl, C. & Gilden, R. V. (1970) *J. Biol. Chem.* **245**, 714–717.
5. Hatanaka, M., Todaro, G. J. & Gilden, R. V. (1970) *Int. J. Cancer* **5**, 224–228.
6. Hatanaka, M., Gilden, R. V. & Kelloff, G. (1971) *Virology* **43**, 734–736.
7. Martin, G. S., Venuta, S., Weber, M. & Rubin, H. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2739–2741.
8. Kawai, S. & Hanafusa, H. (1971) *Virology* **46**, 470–479.
9. Isselbacher, K. J. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 585–589.
10. Venuta, S. & Rubin, H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 653–657.
11. Weber, M. J. (1973) *J. Biol. Chem.* **248**, 2978–2983.

12. Levine, A. S., Uhl, R. & Ashmore, J. (1961) *J. Nat. Cancer Inst.* **27,** 597–609.
13. Morgan, H. R. & Ganapathy, S. (1963) *Proc. Soc. Exp. Biol. Med.* **113,** 312–315.
14. Broadfoot, M., Walker, P., Paul, J., Macpherson, I. & Stoker, M. (1964) *Nature* **204,** 79.
15. Paul, J., Broadfoot, M. M. & Walker, P. (1966) *Int. J. Cancer* **1,** 207–218.
16. Steck, T. L., Kaufman, S. & Bader, J. P. (1968) *Cancer Res.* **28,** 1611–1619.
17. Bissell, M. J., Hatie, C. & Rubin, H. (1972) *J. Nat. Cancer Inst.* **49,** 555–565.
18. Singh, M., Singh, V. N., August, J. T. & Horecker, B. L. (1974) *Arch. Biochem. Biophys.*, in press.
19. Rolleston, F. S. (1972) in *Current Topics in Cellular Regulation,* eds. Horecker, B. L. & Stadtman, E. R. (Academic Press, New York), Vol. 5, pp. 47–75.
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
21. Wu, R. (1964) *Anal. Biochem.* **7,** 207–214.
22. Passera, C., Pedrotti, A. & Ferrari, G. (1964) *J. Chromatogr.* **14,** 289–291.
23. Rose, I. A. & O'Connell, E. L. (1964) *J. Biol. Chem.* **239,** 12–17.
24. Wu, R. (1965) *Biochem. Biophys. Res. Commun.* **18,** 402–408.
25. Wu, R. (1964) *Biochem. Biophys. Res. Commun.* **14,** 79–85.
26. Goldberg, E. B., Nitowsky, H. M. & Colowick, S. P. (1965) *J. Biol. Chem.* **240,** 2791–2796.
27. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. & Schulz, D. W. (1964) *J. Biol. Chem.* **239,** 18–30.
28. Maitra, P. K. & Estabrook, R. W. (1964) *Anal. Biochem.* **7,** 472–484.
29. Adam, H. (1963) in *Methods of Enzymatic Analysis,* ed. Bergmeyer, H. U. (Academic Press, New York), pp. 573–577.
30. Gevers, W. & Krebs, H. A. (1966) *Biochem. J.* **98,** 720–735.
31. Atkinson, D. E. (1968) *Biochemistry* **1,** 4030–4034.
32. Crane, R. K. & Sols, A. (1953) *J. Biol. Chem.* **203,** 273–292.
33. Crane, R. K. & Sols, A. (1954) *J. Biol. Chem.* **210,** 597–606.
34. Karpatkin, S. (1967) *J. Biol. Chem.* **242,** 3525–3530.
35. Fromm, H. J. & Zewe, V. (1962) *J. Biol. Chem.* **237,** 1661–1667.
36. Copley, M. & Fromm, H. J. (1967) *Biochemistry* **6,** 3503–3509.
37. Schwartz, G. P. & Basford, R. E. (1967) *Biochemistry* **6,** 1070–1078.
38. Tuttle, J. P. & Wilson, J. E. (1970) *Biochim. Biophys. Acta* **212,** 185–188.
39. Rose, I. A. & Warms, V. B. (1967) *J. Biol. Chem.* **242,** 1635–1645.
40. Csáky, T. Z. & Wilson, J. E. (1956) *Biochim. Biophys. Acta* **22,** 185–186.
41. Narahara, H. T. & Pinar, Ö. (1963) *J. Biol. Chem.* **238,** 40–49.
42. Renner, E. D., Plagemann, P. G. W. & Bernlohr, R. W. (1972) *J. Biol. Chem.* **247,** 5765–5776.
43. Kipnis, D. M. & Cori, C. F. (1959) *J. Biol. Chem.* **234,** 171–177.
44. Smith, D. E. & Gorsky, J. (1968) *J. Biol. Chem.* **243,** 4169–4174.
45. Romano, A. H. & Colby, C. (1973) *Science* **179,** 1238–1240.
46. Krebs, H. A. (1972) in *Essays in Biochemistry,* eds. Campbell, P. N. & Dickens, F. (Academic Press, New York), Vol. 8, pp. 1–34.
47. Ramaiah, A. (1974) in *Current Topics in Cellular Regulation,* eds. Horecker, B. L. & Stadtman, E. R. (Academic Press, New York), Vol. 8, pp. 297–345.