KINETIC AND ELECTROPHORETIC EVIDENCE FOR MULTIPLE FORMS OF GLUCOSE-ATP PHOSPHOTRANSFERASE ACTIVITY FROM HUMAN CELL CULTURES AND RAT LIVER[1]

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Recent studies have shown the presence of multiple forms of glucose-ATP phosphotransferase (hexokinase) activity in mammalian tissues (reviewed by Sols et al., 1964, and Sharma et al., 1964). In the present study, kinetic and electrophoretic evidence has been obtained for 2 forms of this activity from human cell cultures, in comparison to 4 distinct electrophoretic forms from rat liver. Furthermore, in addition to the change in activity of the high Km "glucokinase" in rat liver resulting from nutritional variations, change in activity of at least one of the other molecular forms of hexokinase was noted as well.

METHODS

Cell culture extracts: Established heteroploid clonal cell lines, (Zimmer 2A, 4A, Chang 8A), derived from human liver (Nitowsky and Herz, 1961) were grown as monolayer cultures in Puck's N-16 Medium supplemented with variable concentrations of glucose (cf. Table 1) for periods of up to 4 days following transfer. After harvesting with 0.05% trypsin, the cells were washed with about 100 times the volume of a solution containing 0.15 M KCl, 5 mM EDTA, and 5 mM 2-mercaptoethanol (2-M.E.), adjusted to a final pH of 7.3 with NaOH. The washed cells were then suspended in 0.1 M TRIS buffer, pH 7.3, containing 0.15 M KCl, 5 mM EDTA, 5 mM 2-M.E., and 5 mM $MgCl_2$ (Salas et al., 1963) and sonicated at 2° for 2 minutes.

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The sonicate was centrifuged at 2° and 105,000 x g for 45 minutes. The supernatant is designated cell culture extract.

Rat liver extracts: Male, Sprague-Dawley rats, weighing approximately 250 g, were allowed free access to Purina Laboratory Chow and water, or were fasted but not thirsted for 48 or 72 hours. Enzyme extracts of livers, perfused to remove as much blood as possible, were prepared according to the procedure of Salas et al. (1963).

Starch gel electrophoresis: Glucose-ATP phosphotransferase activities were separated by the vertical starch gel electrophoretic technique of Smithies (1955), using a 0.02 M barbital buffer, pH 8.5, containing 2.7 mM EDTA and 5 mM 2-M.E. Electrophoreses were carried out at 4° for 16 hrs. with a potential gradient of 6 V/cm across the gel. Gels were stained at 25° in the absence of light for 3 hrs. by immersion in 0.1 M TRIS-buffered developer solution at pH 7.4, containing 0.5 mM TPN, 5 mM $MgCl_2$, 5 mM NaATP, 2 mM KCN, 0.4 i.u./ml glucose-6-phosphate dehydrogenase, 40 μg/ml phenazine methosulfate, 400 μg/ml nitro blue tetrazolium, and various concentrations of glucose (cf. Results).

RESULTS

Using a Lineweaver-Burke kinetic analysis, analogous to that employed by Walker (1963) for the determination of two forms of glucose-ATP phosphotransferase activity from rat liver, cell culture extracts showed at least 2 glucose-phosphorylating enzymes which differed in their apparent Michaelis constants (Km). From numerous experiments, such as a typical one depicted in Fig. 1, Km values were calculated from the 2 straight line portions of the curve and designated as Km_1 and Km_2. These averaged 9 (± 1) x 10^{-5} M and 6 (± 1) x 10^{-4} M glucose, respectively. As can be seen in Fig. 1, the reaction velocity fell with increasing glucose concentration (indicating substrate inhibition) prior to the sharp increase at 2.5 mM glucose, which reflects the activity of the Km_2 enzyme. A similar type of inhibition had been found by Walker (1963) with

extracts of rat liver; further evidence for glucose inhibition of enzyme activity in extracts of rat liver and cell cultures is shown in the electrophoretic studies below. Since substrate inhibition of the Km_1 enzyme as well as an expected hyperbolic nature of the Km_2 slope (Dixon and Webb, 1958) might conceivably interfere with measurement of Km_2, the true value of the latter may be higher.

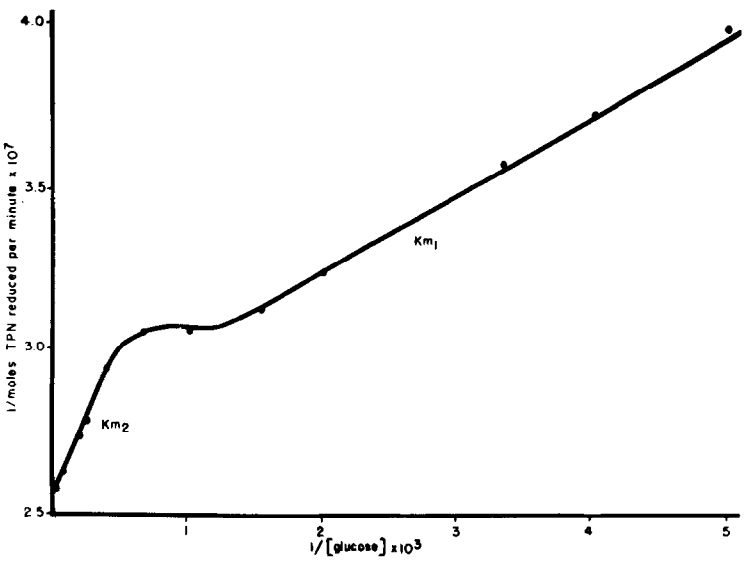

Figure 1. Effect of glucose concentration on velocity of glucose-ATP phosphotransferase activities of extract from Zimmer-2A cells grown on "high" glucose (cf. Table 1, below). 150 µl of extract per incubation.

Although both a 10^{-2} M Km "glucokinase" as well as a 10^{-5} M Km hexokinase have been observed in kinetic analyses using extracts of animal livers, no high Km "glucokinase" could be found in any of the established heteroploid human liver cell lines tested in the present study even when these cells were grown on concentrations of glucose as high as 45 mM. Findings similar to those in Fig. 1 were obtained with extracts of a recently isolated diploid cell strain in the 11th passage derived from the liver of a 3 month old infant.

Although the apparent Km values of the two hexokinases in cell culture extracts in this study are different from those found with liver

extracts by Salas et al. (1963), use of their assay (Table 1) provides a measure of the relative changes in the activities of these enzymes in response to variations in substrate concentrations in the nutrient medium.

TABLE 1

Effect of Glucose Concentration in the Culture Medium on the Activity of Km_1 and Km_2 Hexokinases

Glucose Additions	6×10^{-4} M Km_2 Enzyme	9×10^{-5} M Km_1 Enzyme	No. of Cultures
mMolar	units/mg protein ± S.E.M.		
None	1.3 ± 0.6	48 ± 5	7
10 + 30	13 ± 3	42 ± 4	7

Zimmer-2A cells grown 4 days on 5 mM glucose initially present in culture medium (top row) ("low" glucose culture); supplemented with additional 10 mM glucose on first day plus additional supplement of 30 mM glucose 20 hrs. before harvesting cells on fourth day (bottom row) ("high" glucose culture). Assayed according to Salas et al. (1963).

As shown in Table 1, extracts of cells grown at high glucose concentrations showed an increase of approximately 10 fold in the activity of the Km_2 enzyme but no change in the activity of the Km_1 enzyme. The latter resembles the lack of change of activity of the 10^{-5} M Km hepatic enzyme following feeding reported by others. The time required for appearance of the peak activity of the Km_2 enzyme in cell cultures after glucose supplementation ("induction") with 30 mM glucose was 20 hrs., or approximately one cell generation time.

In order to verify by a different parameter the presence of two forms of hexokinase activity in extracts of cell culture and compare these forms with those from rat liver, extracts were subjected to starch gel electrophoresis, as illustrated in Fig. 2. In support of the kinetic evidence, 2 bands with hexokinase activity were seen after electrophoresis of cell culture extracts. Furthermore, a study of the relative staining intensity of these 2 bands at various concentrations of glucose indicated Km values consistent with those determined from kinetic analyses, i.e. approximately 10^{-5} M for the band that moved 1.5 cm (band 1) and approximately 10^{-4} M for the 3 cm band (band 2). In agree-

ment with the kinetic analysis, there was a distinctly lesser staining intensity of band 2 than of band 1. In addition, there was a somewhat greater intensity of band 2 from extracts of cells grown in the presence of high glucose than from cells grown with low glucose, although this difference was less prominent than the difference between bands 1 and 2.

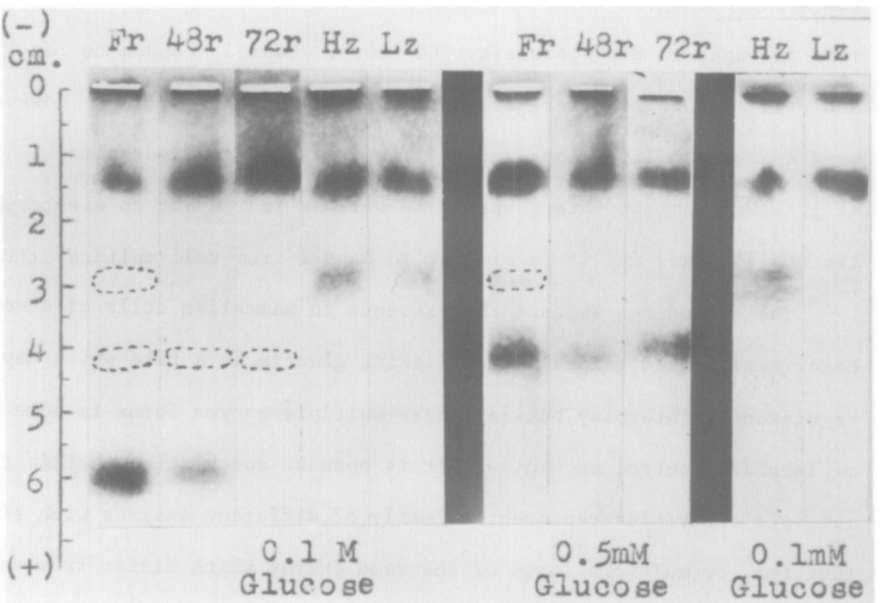

Figure 2. Starch gel electrophoresis of glucose-ATP phosphotransferases in extracts from fed rat (Fr), 48- and 72-hr. fasted rats (48r and 72r) and from cells cultured on high (45mM) glucose (Hz) and on low (5mM) glucose (Lz), but with NaCl added to the latter to maintain equiosmolality. Gels were stained in a developer solution containing 0.1 M, 0.5 mM, or 0.1 mM glucose. Each cell culture extract was applied to origin as 0.3 mg protein; each rat liver extract as 1.5 mg protein. Dotted ovals refer to bands that faded before photography. In gels without ATP in developer solution, no bands appeared.

Of interest is the finding of 4 bands with hexokinase activity in extracts from fed rats (Fig. 2). On the basis of differences in staining intensity with varying concentrations of glucose in the developing solution, approximate Km values of 10^{-5} M, 10^{-4} M, 10^{-6} M and 10^{-2} M glucose could be tentatively assigned to the bands 1, 2, 3, and 4, respectively, in order of increasing mobility. During the course of these studies Gonzalez et al. (1964) reported 4 peaks of rat liver hexokinase

activity which were obtained by DEAE-cellulose chromatography and which had similar Km values. The staining intensity of band 3 was clearly inhibited at 0.1 M glucose. Band 1 exhibited a similar inhibition at 0.3 M glucose, which resembles the substrate inhibition previously alluded to in Fig. 1. Band 4, which corresponds to the high Km "glucokinase", was the most intense in liver extracts from fed rats; in the livers of fasted rats it began to disappear after 48 hours. Band 1, common to rat liver and cell culture, was unaffected by nutritional alterations. However, band 2 from rat liver appeared only in extracts from fed animals. It is of interest that in this respect, as well as in respect to electrophoretic mobility and Km, it is similar to band 2 from cell culture extracts.

These findings suggest the presence in mammalian cells of a complex hexokinase system, capable of utilizing glucose at a rate which may be regulated by interplay between these multiple enzyme forms in some type of feedback control mechanism. It is open to speculation whether these multiple activities represent a family of different enzymes with similar function, or multiple forms of the same enzyme which differ from each other in subunit composition.

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