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Characterization of Isoenzymes of Adenosine Triphosphate:D-Hexose 6-Phosphotransferase from Rat Liver*

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ABSTRACT: Four isoenzymes of adenosine triphosphate (ATP):D-hexose 6-phosphotransferase have been separated from rat liver by DEAE-cellulose. Three of these isoenzymes (A–C) are similar to animal hexokinases inasmuch as they exhibit a low K_m for glucose (10^{-5}–10^{-4} M) and the rate of phosphorylation of fructose is slightly higher than that of glucose. Isoenzyme D has been further purified by fractionation with ammonium sulfate and by chromatography on hydroxylapatite. This isoenzyme would correspond to glucokinase since it presents a high K_m for glucose (1.8×10^{-2} M), and a low activity with fructose as a substrate; it also catalyzes the phosphorylation of mannose and 2-deoxyglucose. The four isoenzymes use only ATP as phosphate donor, with K_m values of about 5×10^{-4} M. The independence of the affinity of glucokinase for glucose or ATP on the concentration of the other substrate is in agreement with the presence of two separate binding sites on the enzyme. The four isoenzymes are competitively inhibited by *N*-acetylglucosamine (K_i about 5×10^{-4} M). High concentrations of glucose inhibit isoenzymes C and D. Mannose also inhibits isoenzyme D. Glucose 6-phosphate inhibits glucokinase ($K_i = 1.5 \times 10^{-2}$ M); the inhibition is competitive with respect to ATP, while it is not clearly defined with respect to glucose.

A great deal of interest has arisen in the last few years on the regulation of glucose phosphorylating activity in rat liver. Changes in the tissue levels of ATP[1]:hexose phosphotransferase have been observed under three main circumstances: (1) variations in the supply of glucose in the diet, including total fasting (Vaughan *et al.*, 1960; DiPietro and Weinhouse, 1960; Niemeyer *et al.*, 1962, 1963; Pérez *et al.*, 1964); (2) availability of insulin (DiPietro and Weinhouse, 1960; Salas *et al.*, 1963; Niemeyer *et al.*, 1967); and (3) initial stages of development after birth (Walker, 1963). Of special significance were the reports by Walker (1963) and Viñuela *et al.* (1963), describing two protein fractions with glucose phosphorylating activity in liver. Only one of these proteins, characterized by its high K_m for glucose and called glucokinase, was amenable to changes under the various above-mentioned conditions. We separated four fractions or isoenzymes[2]

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[1] Abbreviations used: ATP, adenosine triphosphate; $NADPH_2$, reduced nicotinamide–adenine dinucleotide phosphate; $NADP^+$, oxidized nicotinamide–adenine dinucleotide phosphate; NAD^+, oxidized nicotinamide–adenine dinucleotide; $NADH_2$, reduced nicotinamide–adenine dinucleotide; UTP, uridine triphosphate; Glc-6-P, glucose 6-phosphate.

[2] The term isoenzyme is used here in the broad sense accepted by the Standing Committee on Enzymes of the International Union of Biochemistry (Webb, 1964).

of ATP:hexose phosphotransferase by chromatography in DEAE-cellulose (González *et al.*, 1964) and again only one, isoenzyme D, corresponding to glucokinase, varied markedly under the conditions tested.

In the present work the detailed procedures to isolate the four isoenzymes, as well as a study of their kinetic properties, are presented. Special emphasis has been given to the purification and characterization of isoenzyme D or glucokinase because of its potential physiological significance

Experimental Section

Isolation of the Isoenzymes. Adult albino rats were used. They were well fed with a balanced stock diet (Niemeyer *et al.*, 1962). After killing the animals by decapitation without anesthesia the livers were excised and chilled in cracked ice, blotted, and weighed. Homogenates (50%, w/v) were prepared from the pooled livers of 4–16 rats in 10 mM Tris–1 mM EDTA–6–10 mM 2-mercaptoethanol, pH 7.0 (medium A), and centrifuged at 105,000*g* for 60 min in a Spinco preparative ultracentrifuge. All procedures were carried out at 0–4°. The clear supernatant fluids were treated batchwise or in a column with CM-Sephadex (or CM-cellulose) equilibrated with medium A. The material not absorbed by the exchanger was concentrated at low temperature and reduced pressure and chromatographed on DEAE-cellulose ion exchanger. The diameter and height of the columns varied according to the number of animals used in each preparation. Linear gradient elution was carried out with KCl from 0 to 0.5 M in medium A. Fractions were collected and the content of the tubes was examined for glucose phosphorylating activity and for protein.

Further Purification of Glucokinase. The details of the procedure have been reported (Babul and Niemeyer, 1966). In certain experiments the DEAE-cellulose treatment was shortened by washing the column with 0.18 M KCl prepared in medium A, which eluted isoenzymes A, B, and partially C. A linear gradient of concentration from 0.18 to 0.5 M KCl in medium A was then applied. The fractions which were free of isoenzyme C and had the highest specific activities were pooled. Glucose was added to a concentration of 50 mM and then solid ammonium sulfate was added to bring the concentration to 55% saturation. The pH was maintained at 7 with NH_4OH. The precipitate obtained after centrifugation was discarded and glucokinase was precipitated from the supernatant fluid by adding more solid ammonium sulfate to bring the concentration to 80% saturation. The solution was again centrifuged. The precipitated enzyme could be stored as such at 0° for several months without appreciable loss in activity. Several preparations in this stage could be pooled in order to continue the purification procedure. The pooled precipitates were dissolved in a small amount of 5 mM potassium phosphate buffer, pH 6.8, prepared in medium B (50 mM glucose, 6 mM 2-mercaptoethanol, and 1 mM EDTA), and chromatographed on a column of hydroxylapatite (20 ml of Bio-Gel HT, 0.28 g dry/ml of settled bed, mixed with 10 g of cellulose powder) equilibrated with the same buffer. The enzyme was eluted with a linear concentration gradient of potassium phosphate, between 5 and 150 mM at a pH increasing from 6.8 to 7.6, prepared in medium B. The flow rate was adjusted to about 3 ml/min and 5- to 6-ml fractions were collected. The eluates with the highest specific activities were pooled and as soon as possible solid ammonium sulfate was added to bring the concentration to 80% saturation to precipitate the enzyme. The sediment collected by centrifugation was suspended in a small volume of 80% saturation ammonium sulfate and stored at 0°. Aliquots of this suspension were dissolved in 0.1 M KCl prepared in medium A for studies of enzyme properties. Table I presents a typical preparation of glucokinase.

TABLE I: Summary of a Typical Preparation of Highly Purified Isoenzyme D or Glucokinase.

Stage of Purification	Total Act. (units)	Total Protein (mg)	Sp Act. (units/mg of protein)	Yield (%)
Crude extract	45.7	3170	0.014	100
CM-Sephadex	40.4	2260	0.018	88
DEAE-cellulose	24.2	69	0.35	53
$(NH_4)_2SO_4$ (55–80% saturation)	14.5	16.6	0.88	32
Hydroxylapatite	8.85	1.57	5.64	19

Enzyme Assay. ATP:hexose phosphotransferase activity was assayed by either of these two procedures. (a) Measurement of glucose 6-phosphate formation was made by coupling the reaction with an excess of glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. $NADPH_2$ formation was followed in a Beckman DU spectrophotometer, provided with a Photovolt recorder and with a thermospacer to operate at 30°, in 1-ml cells (Pérez *et al.*, 1964). A similar system with ATP omitted acted as a blank. When fructose was used as a substrate, measurement of glucose 6-phosphate was accomplished by the addition of an excess of phosphoglucose isomerase. (b) Measurement of ADP formation was made by coupling the phosphotransferase reaction to pyruvic kinase and lactic dehydrogenase systems. $NADH_2$ oxidation was followed at 340 mμ in a Beckman DU spectrophotometer (Kornberg and Pricer, 1951). The reaction was initiated with the substrate. A system without substrate was used as a blank. This method was reliable only when purified preparations, practically free of adenosine triphos-

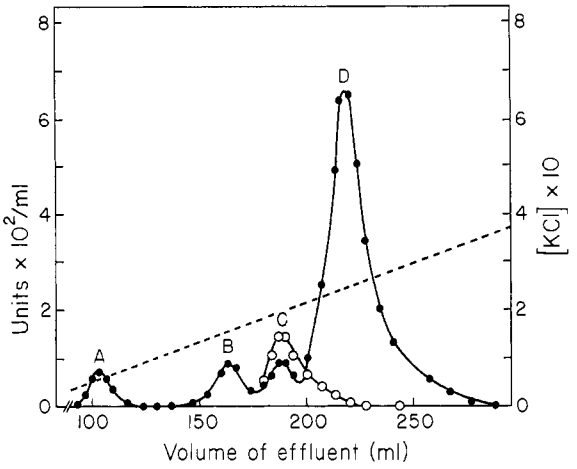

FIGURE 1: Chromatographic fractionation on DEAE-cellulose of ATP:D-hexose 6-phosphotransferase isoenzymes from rat liver. ● and ○ correspond to enzyme activities assayed at glucose concentrations of 100 and 0.5 mM, respectively. - - - - represents the gradient concentration of KCl.

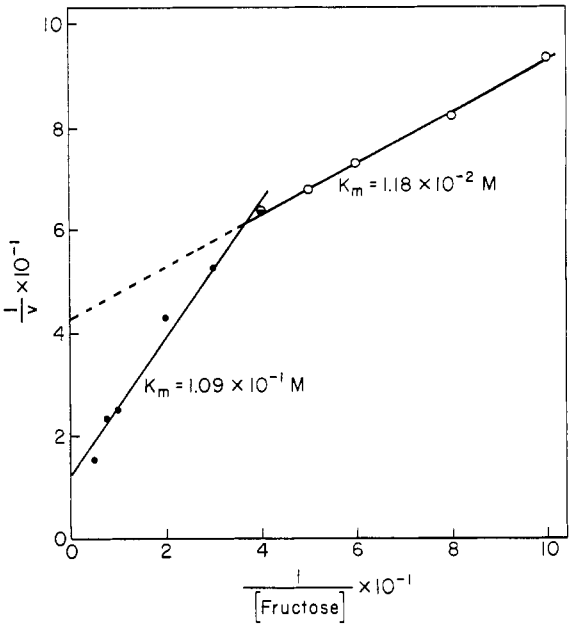

FIGURE 2: Effect of fructose concentration on glucokinase activity. Double-reciprocal plot of initial velocity *vs.* molar concentration of fructose. v = units per milliliter of enzyme preparation. Void points (○) were used to calculate the K_m value of 1.18×10^{-2} M, and points in black (●), to calculate the K_m of 1.09×10^{-1} M. ATP concentration was 5 mM. Further details of the system are given in the Experimental Section.

phatases, were used. One unit of ATP:hexose phosphotransferase corresponds to the amount of enzyme that phosphorylates 1 μmole of substrate in 1 min at 30°. When method a was used, the change in absorbance was divided by 2 since the excess of 6-phosphogluconic dehydrogenase permitted the production of 2 μmoles of $NADPH_2$/μmole of substrate phosphorylated.

Protein Determination. Protein was assayed by the method of Lowry *et al.* (1951) modified by Miller (1959) after the protein was precipitated with 10% trichloroacetic acid and redissolved in 1 N NaOH in order to avoid interference by 2-mercaptoethanol.

Reagents. The following reagents were obtained from Sigma Chemical Co: α-D-(+)-glucose, D-(+)-mannose, D-(−)-ribose, D-(−)-arabinose, D-(+)-glucosamine, *N*-acetylglucosamine, glucose 6-phosphate, purine and pyrimidine triphosphonucleotides, $NADP^+$, $NADH_2$, phosphoglucose isomerase, and lactic dehydrogenase containing pyruvate kinase. L-(+)-Arabinose and 2-deoxyglucose were purchased from Pfanstiehl Laboratories Inc. 2-Mercaptoethanol was purchased from Matheson Coleman and Bell; α-methyl D-glucoside and ammonium sulfate, enzyme grade, from Mann Research Laboratories, Inc.; and CM-cellulose, DEAE-cellulose, and hydroxylapatite from Bio-Rad Laboratories. Phosphoenolpyruvic acid (tricyclohexylammonium salt) and hexokinase-free glucose 6-phosphate dehydrogenase were products from Boehringer u. Soehne; D-(−)-fructose and D-(+)-galactose from E. Merck AG, Darmstadt; sorbitol and mannitol from Hopkin and Williams, Ltd.; CM-Sephadex from Pharmacia, Uppsala. 6-Phosphogluconic dehydrogenase was prepared according to Glock and McLean (1953), and further purified by chromatography on cellulose derivatives (unpublished method).

Results

Chromatographic Fractionation. Figure 1 shows the results of a typical experiment in which four protein fractions with ATP:hexose phosphotransferase activity were eluted from a DEAE-cellulose column. These isoenzymes, named A–D, were detected with 100 mM glucose as a substrate. Fraction C was inhibited by an excess of substrate, as observed when the assay was performed in parallel with 0.5 and 100 mM glucose; this fact was utilized for the recognition of this isoenzyme in former studies (González *et al.*, 1964).

Michaelis Constants. The apparent K_m values for glucose and fructose of the isoenzymes obtained from DEAE-cellulose, without further purification, are shown in Table II. The K_m values were calculated by the method of Lineweaver and Burk (1934). Fraction D has a high K_m (1.8×10^{-2} M) value for glucose and thus would correspond to the high-K_m enzyme or glucokinase (DiPietro *et al.*, 1962; Viñuela *et al.*, 1963; Walker, 1963). The K_m values for mannose and for 2-deoxyglucose are also about 10 mM. Isoenzymes A–C

TABLE II: Michaelis Constants for Several Substrates of ATP:D-Hexose 6-Phosphotransferase Isoenzymes.

Isoenzyme	Glucose (M)	Mannose (M)	Fructose (M)	2-Deoxyglucose (M)
A	4.4×10^{-5}		3.1×10^{-3}	
B	1.3×10^{-4}		3.0×10^{-3}	
C	2.1×10^{-5}		1.2×10^{-3}	
D	1.8×10^{-2}	2.3×10^{-2}	1.2×10^{-2}	4.0×10^{-2}

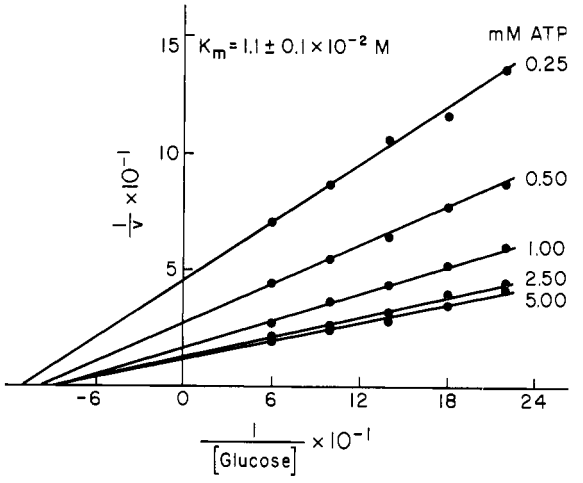

FIGURE 3: Effect of glucose on the velocity of glucokinase reaction at various ATP concentrations. The ATP concentrations are shown in the graph. Glucose concentration was varied in the range from 4.6 to 16.7 mM.

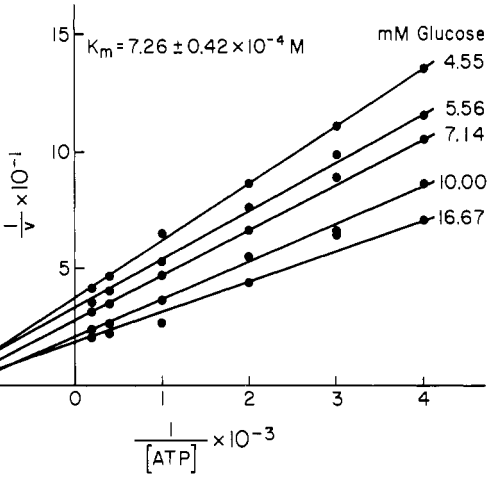

FIGURE 4: Effect of ATP on the velocity of glucokinase reaction at various glucose concentrations. The concentrations of glucose are shown on the graph. ATP concentration was varied in the range from 0.25 to 5 mM.

have low K_m values for glucose and would correspond to the hexokinase that is separated as one protein fraction by ammonium sulfate (Viñuela *et al.*, 1963). The K_m values for fructose of isoenzymes A–C are also lower than the corresponding K_m value of fraction D. Figure 2 is a Lineweaver–Burk plot of fructose concentration against activity of fraction D. The biphasic curve can be interpreted as an indication of the presence of two proteins that can phosphorylate fructose. The component with the lower apparent K_m value (1.1×10^{-2} M) may correspond to glucokinase, since the activity accompanies the isoenzyme during the next purification steps. The high K_m component (1.1×10^{-1} M) could be eluted from hydroxylapatite at a lower ionic strength and thus it may be a different enzyme, with very low affinity for fructose. The problem was not further investigated. All the K_m values for fructose here reported must be lower since fructofuranose, which is the actual substrate (Slein *et al.*, 1950), represents only about 20% of total fructose (Gottschalk, 1944). The ratios between rates of phosphorylation of fructose and glucose, both substrates at a concentration of 100 mM, were 1.09, 1.21, 1.28, and 0.25 for isoenzymes A, B, C, and D, respectively.

The four phosphotransferases use ATP as phosphate donor. Table III shows that the apparent K_m values for the four isoenzymes are very similar. The Mg^{2+}:ATP ratio was kept constant at 2.2 for all determinations. Only isoenzyme D as obtained from the DEAE-cellulose column can use UTP, with a phosphorylating rate of

TABLE III: Michaelis Constants for ATP of ATP:D-Hexose 6-Phosphotransferase Isoenzymes.

Isoenzyme	K_m (M $\times 10^{-4}$)
A	4.2
B	7.0
C	12.9
D	4.9

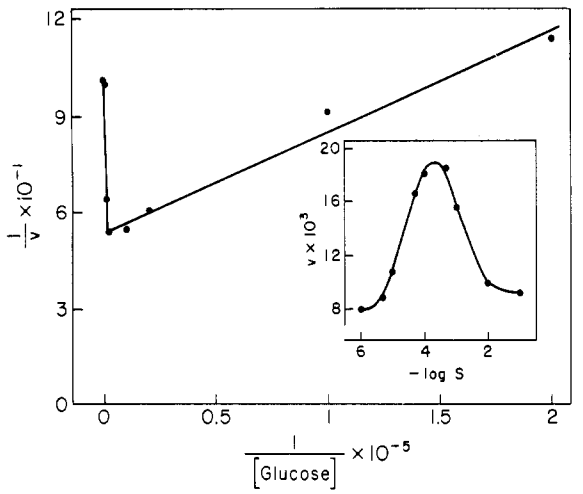

FIGURE 5: Inhibition by substrate of isoenzyme C. Double-reciprocal plot of initial velocity *vs.* molar concentration of glucose. v = units per milliliter of enzyme preparation. The inner figure shows the direct plot of the data.

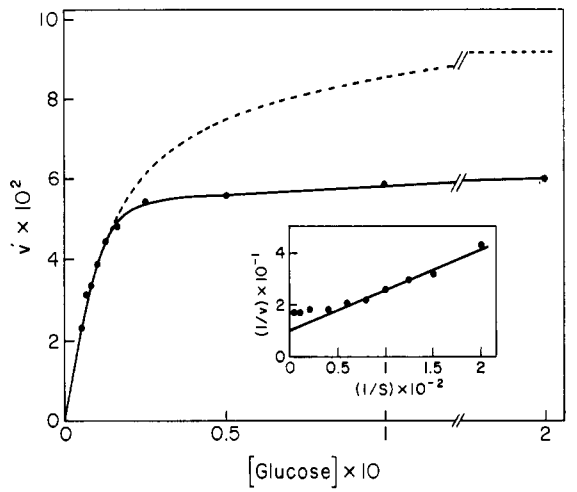

FIGURE 6: Effect of glucose concentration on glucokinase activity. Solid line (—) corresponds to the experimental curve; dashed line (- - -) is the theoretical curve calculated with a K_m value of 1.52×10^{-2} M and a V_{max} of 0.099 unit/ml of enzyme. The regression line of the inner figure was calculated by the method of least squares, using the experimental points from 5 to 16.7 mM glucose.

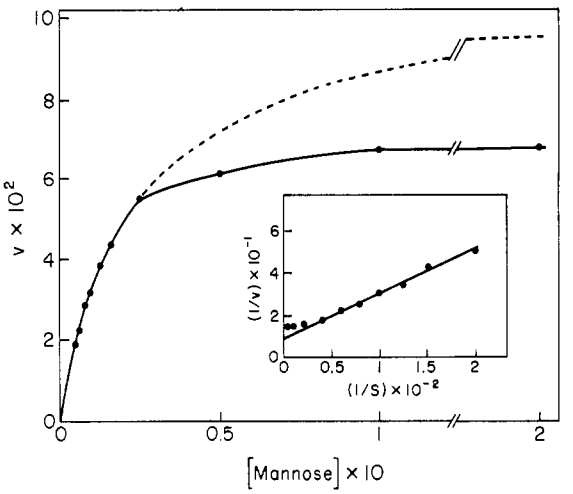

FIGURE 7: Effect of mannose concentration on glucokinase activity. Solid line (—) represents the experimental curve; dashed line (- - -) is the theoretical curve calculated with a K_m value of 2.3×10^{-2} M and a V_{max} of 0.106 unit/ml. The regression line of the inner figure was calculated by the method of the least squares, using the experimental points between 5 and 25 mM mannose.

0.15 relative to ATP. However, the highly purified glucokinase cannot use UTP.

Measurements of enzyme activities were made with purified glucokinase to determine whether the apparent Michaelis constants for glucose and ATP were influenced by ATP and glucose concentrations, respectively. Noninhibitory concentrations of glucose were used. Plots of the reciprocal velocity against reciprocal glucose concentrations at different ATP concentrations and plots of $1/v$ against 1/[ATP] at different glucose concentrations are given in Figures 3 and 4, respectively. All the curves are linear and converge at a common point on the 1/[substrate] axis, indicating that the K_m values for glucose and for ATP, under the assay conditions used, are independent of the concentrations of the other substrate. From data of Figures 3 and 4 the values of the Michaelis constants are: for glucose,

TABLE IV: Relative Phosphorylation Rate of Several Monosaccharides or Derivatives by Glucokinase.[a]

Saccharide	$V_{saccharide}/V_{glucose}$
α-D-(+)-Glucose	1.00
D-(+)-Mannose	0.82
2-Deoxy-D-glucose	0.39
D-(−)-Fructose	0.20
D-(+)-Galactose	0.06
Sorbitol	0.06
Mannitol	0
α-Methyl-D-glucoside	0.03
D-(+)-Glucosamine	0.03
N-Acetyl-D-glucosamine	0
D-(−)-Ribose	0.04
D-(−)-Arabinose	0
L-(+)-Arabinose	0

[a] Concentration of substrates, 100 mM.

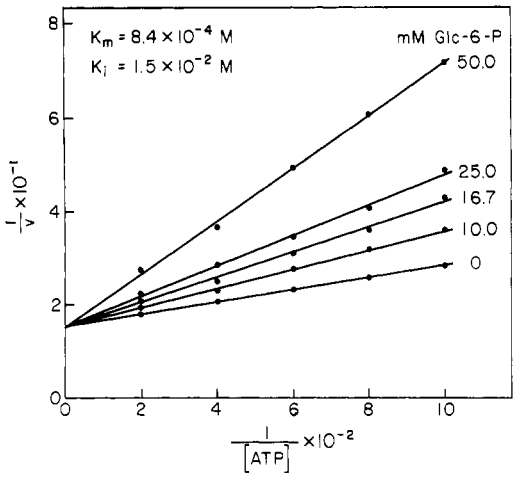

FIGURE 8: Effect of glucose 6-phosphate on the velocity of glucokinase reaction at various ATP concentrations. The concentrations of Glc-6-P are shown in the graph. ATP concentration was varied in the range from 1 to 5 mM. Glucose concentration was 16.7 mM.

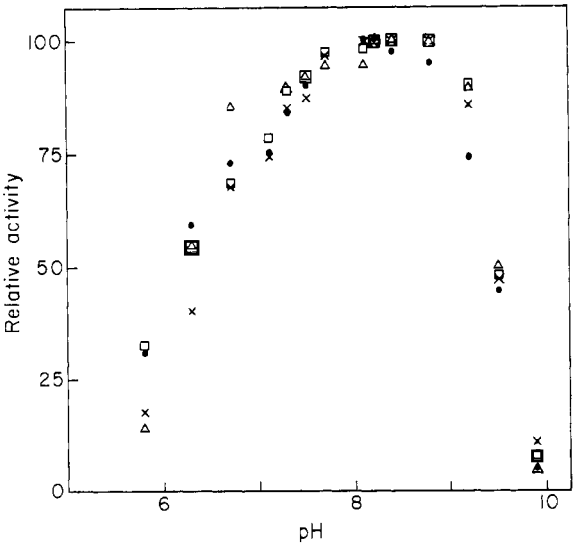

FIGURE 9: Effect of the pH on the activities of isoenzymes A–D. Tris (50 mM)–maleic acid (50 mM) buffer was used between pH 5.5 and 8.5, and 50 mM Tris–50 mM glycine between pH 7.5 and 10.5. Enzyme activity was measured by method a described in the Experimental Section. The amounts of glucose 6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase were increased to compensate for changes in activities of these auxiliary enzymes. ●, isoenzyme A; Δ, isoenzyme B; □, isoenzyme C; ×, isoenzyme D.

$1.1 \pm 0.1 \times 10^{-2}$ M, and for ATP, $7.26 \pm 0.42 \times 10^{-4}$ M.

Specificity of Substrate. Highly purified glucokinase phosphorylates mannose, 2-deoxyglucose, and fructose, although at a lower rate than glucose (Table IV). It does not phosphorylate galactose, pentoses, or pentose derivatives. Most of the hexose derivatives used were not phosphorylated; nevertheless some of them acted as competitive inhibitors with glucose. These results are in general agreement with those of Parry and Walker (1966).

Inhibition by Substrates. As stated above, inhibition of the activity of isoenzyme C is observed with high concentrations of glucose, which is clearly illustrated in Figure 5. Isoenzyme D is also inhibited by excess of glucose (Figure 6) and mannose (Figure 7), although these inhibitions are not so striking as for fraction C.

Inhibition by N-Acetylglucosamine. This compound inhibited competitively the phosphorylation of glucose by the four isoenzymes. The K_i values were 8, 6.5, and 3.8×10^{-4} M for fractions B, C, and D, respectively. The ratios K_m for glucose: K_i for *N*-acetylglucosamine were about 0.1 for B and C, and about 40 for D.

Inhibition by Glucose 6-Phosphate. Glucose 6-phosphate inhibits the phosphorylation of glucose by glucokinase. Figure 8 presents a Lineweaver–Burk graph where ATP was varied in the presence of several concentrations of Glc-6-P. It appears that the hexose phosphate is a competitive inhibitor of ATP. The calculated K_i for Glc-6-P is 1.5×10^{-2} M. When the effect of Glc-6-P was studied in the presence of variable concentrations of glucose in a 4-month aged preparation of purified glucokinase, Glc-6-P acted as a typical noncompetitive inhibitor with respect to glucose. Thus, the inhibition was about 25% with 50 mM Glc-6-P (and 5 mM ATP) at all concentrations of glucose tested. An unexplained effect was observed in two fresh preparations, one of which (2) corresponds to the same enzyme mentioned above as aged preparation. In both preparations the inhibition by Glc-6-P was more pronounced at higher than at lower concentrations of glucose (Table V). In addition, 10–25 mM Glc-6-P slightly activated preparation 2 at low glucose concentration. This activation could not be explained by contamination of the inhibitor with the substrate (less than 0.1%) or by contamination of isoenzyme D with some specific or unspecific phosphatases. Due to the enzyme activities

TABLE V: Inhibition of Fresh Preparations of Glucokinase by Glucose 6-Phosphate in the Presence of Variable Glucose Concentrations.

Glucose Concn (mM)	Inhibition by 50 mM Glc-6-P (%)	
	Prepn 1	Prepn 2
7.14	8	12
16.6	29	26
∞ [a]	58	44

[a] Extrapolated values obtained by the method of Lineweaver and Burk (1934).

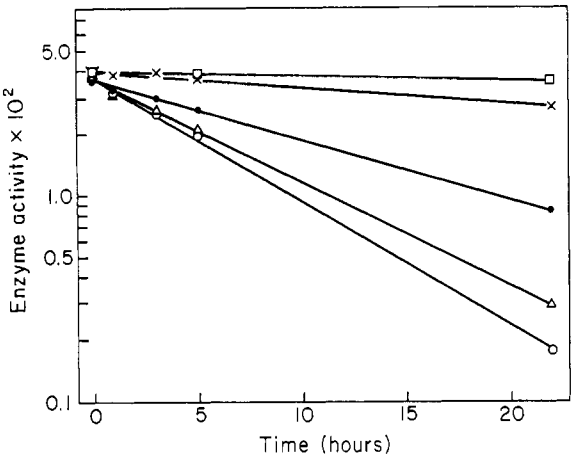

FIGURE 10: Heat stability of glucokinase. The enzyme preparation was dialyzed 1 hr against 10 mM Tris–1 mM EDTA, pH 7.0. Aliquots of the dialyzed enzyme were incubated at 30° in the presence or absence of 0.1 M reagents for the specified time periods: ×, glucose; ●, 2-deoxyglucose; Δ, fructose; ○, no addition. Nondialyzed enzyme was also incubated at 30° as control (□). Activity was measured by method a as described in the Experimental Section.

measured, the concentrations of substrates (glucose and ATP) and inhibitor may be considered constant.

Effect of pH. Isoenzymes A–D show maximum activity between pH 8 and 9. The relative activities of the four fractions at different pH values fall on the same asymmetric curve (Figure 9).

Stability. Isoenzyme D or glucokinase from rat liver is a rather unstable protein when purified. It has been reported that fraction D obtained from DEAE-cellulose is unstable unless the crude extract is previously passed through CM-sephadex or CM-cellulose, in which case this isoenzyme is very stable to storage at 0° and to subsequent treatment (González *et al.*, 1964). Approximately the same stability of isoenzyme D was observed whether the elution from DEAE-cellulose column was performed with NaCl or KCl, with or without addition of 2-mercaptoethanol. After treatment with Sephadex or dialysis against a buffer solution of low ionic strength the enzyme became unstable. As illustrated in Figure 10, glucose and 2-deoxyglucose protected dialyzed glucokinase from thermal inactivation, glucose being more effective. Fructose, on the other hand, did not protect.

The enzyme obtained from the hydroxylapatite column was unstable. For storage, it was precipitated by ammonium sulfate. As a suspension in a 80% saturated ammonium sulfate solution, isoenzyme D was stable, with little loss in activity after several months at 0°. If the enzyme suspension was dissolved, glucokinase was rapidly inactivated, and the protection by glucose could again be demonstrated (Table VI). 2-Mercaptoethanol was ineffective as a stabilizer.

TABLE VI: Relative Activity of Glucokinase after Incubation at 30° for Various Time Intervals.

Addn to the Dilution Medium[a] (mM)	Relative Activities (min)[b]		
	10	22	34
Glucose (50)	0.84	0.72	0.64
2-Mercapto-ethanol (6)	0.54	0.39	0.35
EDTA (1)	0.51	0.47	0.39
None	0.55	0.48	0.38

[a] Aliquots of a suspension of glucokinase in ammonium sulfate were diluted 40-fold in a 10 mM Tris buffer, pH 7.0, with or without the additions indicated. [b] Initial activities were all the same (0.004 unit/0.1 ml of enzyme solution).

Discussion

The presence in rat liver of four isoenzymes of ATP:D-hexose 6-phosphotransferase fractionated by DEAE-cellulose is a reproducible observation. Rechromatography of the separate isoenzymes gives single peaks appearing at about the same ionic strength as in the first chromatography. The finding of these four isoenzymes has been confirmed by separation with ionophoresis in starch gel (Katzen *et al.*, 1965; Katzen and Schimke, 1965); the kinetic properties of the fractions thus obtained generally agree with our previous (González *et al.*, 1964) and present results. The possibility that one or more of the isoenzymes corresponds to blood hexokinase has been ruled out. Contamination in well-bled animals is negligible, since phosphotransferase activity is very low in rat blood and there is little residual blood in the liver. Furthermore, the erythrocyte hexokinase is not retained on DEAE-cellulose under the conditions used for liver extracts (Ureta, 1965).

The relative activities of the four fractions are essentially constant provided that the animals are maintained under the same dietary conditions. Thus, in six experiments performed with liver from rats fed a balanced diet, the percentage of total phosphorylating activity corresponding to isoenzymes A–D was 3.6 ± 0.87, 3.8 ± 0.69, 7.0 ± 0.68, and 85.6 ± 1.95, respectively. After food deprivation or after feeding a high fat, carbohydrate-free diet for several days there is a marked reduction, or even a disappearance of isoenzyme D, while the other isoenzymes remain almost constant (González *et al.*, 1964).

Ureta *et al.* (1965) and Ureta (1965) have shown that the four isoenzymes are present in the liver of other rodents, and very recently Grossbard *et al.* (1966) have demonstrated their existence in several other mammalian species. However, the proportion of the isoenzymes as well as their relative retention by DEAE-cellulose and their electrophoretic mobilities varied in the different species studied. Several other tissues from

the rat differ in the number of ATP:hexose phosphotransferase isoenzymes and in their relative proportion (Moore *et al.*, 1964; Ureta, 1965; Katzen and Schimke, 1965; Grossbard *et al.*, 1966). The properties of the different isoenzymes once identified by certain distinctive kinetic features are uniform from tissue to tissue, and from one species to another.

Isoenzyme D has the properties of glucokinase (Viñuela *et al.*, 1963) with respect to specificity and affinity for substrates, as well as sensitivity to inhibitors. Therefore the name glucokinase, that has been widely accepted for the high-K_m enzyme, has been used alternatively with isoenzyme D. The three low-K_m isoenzymes (A–C) would correspond to the hexokinase fraction described by Sols (Viñuela *et al.*, 1963).

The specificity of isoenzyme D is more restricted than that of hexokinase isolated from other mammalian tissues and microorganisms (Crane, 1962). Similar substrate specificity toward glucose, mannose, 2-deoxyglucose, and fructose, as described here for rat glucokinase, has been shown for the enzyme isolated from rabbit liver (Salas *et al.*, 1965). The purified glucokinase from rat liver can phosphorylate fructose. However, the relative activity with respect to phosphorylation of glucose is low, as compared to that of the other isoenzymes. We are not certain whether glucokinase itself is responsible for fructose phosphorylation, even though fructokinase activity accompanies glucokinase during several steps of purification. Specific ATP:hexose phosphotransferases that can phosphorylate either glucose, fructose, or mannose in position 6 have been described in invertebrates (Bueding and McKinnon, 1955; Agosin and Aravena, 1959). A specific enzyme for glucose has been reported in *Brevibacterium fuscum* (Saito, 1965).

With respect to inhibition by products, different investigators have shown that Glc-6-P inhibits various animal hexokinases (see Crane and Sols, 1955). The group of Sols has emphasized the fact that while rat liver hexokinase shares with the enzyme from other tissues the sensitivity to inhibition by Glc-6-P, the glucokinase from rat and rabbit liver is not appreciably inhibited by physiological concentrations of Glc-6-P (Viñuela *et al.*, 1963; Salas *et al.*, 1965). Our present results show that rat liver glucokinase is in fact inhibited by Glc-6-P, its K_i being of the same order of magnitude as the K_m for glucose. The inhibition was competitive with respect to ATP. The type of inhibition with respect to glucose was not, however, clearly defined inasmuch as we observed with aged preparations a noncompetitive inhibition, while fresh preparations presented more complicated kinetics (Table V). Our results are in general agreement with those obtained by Fromm and Zewe (1962) who showed for brain hexokinase that Glc-6-P acted as a competitive inhibitor of ATP, being of the uncompetitive type with respect to glucose. Parry and Walker (1966) find, with a different rat liver glucokinase preparation, a competitive inhibition by Glc-6-P with respect to glucose. However, the high glucose concentrations used were those that we find to be inhibitory.

The mechanism of different transphosphorylating enzymes seems to be similar (Crane, 1964). Available data would indicate the existence of two separate binding sites on the enzyme permitting both substrates, donor and acceptor, to be in the right position to facilitate the phosphoryl transfer. In this scheme both products could interact with the substrates and inhibit the reaction. The dephosphorylated donor would compete with the donor for its binding site and the phosphorylated acceptor could interact with both the donor, at the overlapping phosphate position, and with the acceptor at its binding site. In the case of glucokinase the independence of the affinity for one substrate on the concentration of the other (Figures 3 and 4) is in agreement with this mechanism. Any mechanism proposed must consider that several monosaccharides or derivatives are not phosphorylated by glucokinase. although they are competitive inhibitors with respect to glucose (Salas *et al.*, 1965; Parry and Walker, 1966). It is notable that whereas glucokinase and the hexokinase-type isoenzymes are inhibited by *N*-acetylglucosamine with the same K_i values. they are readily distinguished by their K_m values for glucose. The conflicting and complex kinetics with respect to product inhibition observed in the present work and that of Parry and Walker (1966) does not permit one to define the mechanism more exactly. One may not exclude, for example, the possibility of changes in allosteric sites in glucokinase. The elucidation of these problems must await a purer enzyme, as well as a better knowledge of its behavior under different experimental conditions.

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Purification and Properties of Yeast Invertase*

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ABSTRACT: Although the enzyme, yeast invertase, has been studied extensively in the past, it has never been chemically characterized and there has been a great deal of uncertainty concerning a number of its physical properties. The present studies describe a rapid method for the isolation of highly purified enzyme in good yield. The enzyme is homogeneous by polyacrylamide gel electrophoresis. Sedimentation velocity studies reveal a major component with an $s_{20,w}$ of 10.4 S. Heavier components which are also present in much smaller amounts are enzymatically active and indicate the presence of an association–dissociation equilibrium. The molecular weight of the enzyme is about 270,000, as determined by sedimentation equilibrium measurements. The chemical properties of the enzyme have been examined and the amino acid composition determined. Invertase is shown to be a glycoprotein which contains about 50% carbohydrate (predominantly mannan with a small percentage of glucosamine).

Recently, Sutton and Lampen (1962) and Islam and Lampen (1962) have described the secretion of invertase by yeast protoplasts. To understand this process, it is essential to characterize the invertase of the intact yeast cell (which is primarily localized in the cell wall) to permit eventual comparison of this material with the secreted enzyme and with the invertase present inside the cell membrane.

Yeast invertase was first isolated by Berthelot (1860) by alcohol precipitation. Since that time, this enzyme has been studied by a number of workers. Much of the early work has been reviewed by Neuberg and Roberts (1946). Myrbäck (1960) has summarized the more recent results. A great deal is known about the enzyme from a kinetic point of view particularly from the inhibition studies by Myrbäck and his coworkers. Nevertheless, relatively little is known about the detailed chemistry of the molecule. Considerable controversy exists concerning physical properties as

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