Multiple Hexokinases of Rat Tissues

PURIFICATION AND COMPARISON OF SOLUBLE FORMS

LIONEL GROSSBARD AND ROBERT T. SCHIMKE
From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service, Bethesda, Maryland 20014

SUMMARY

Rat tissues contain four hexokinases including both the liver-specific high $K_m$ glucokinase and three low $K_m$ hexokinases present in differing proportions in the various tissues. Each of the low $K_m$ hexokinase types designated I, II, and III has been purified approximately 150- to 400-fold from one or more rat tissues (Type I from brain and kidney, Type II from skeletal muscle and epididymal fat pad, and Type III from liver).

Each purified enzyme type, regardless of tissue source, has certain unique properties that distinguish it from the other hexokinase types. The purified types retain their different electrophoretic and chromatographic properties as found in crude extracts. They also differ with respect to apparent $K_m$ values for glucose and probably for adenosine triphosphate, apparent $K_i$ values for adenosine diphosphate and glucose 6-phosphate, and stability to heat and proteolytic inactivation.

The different enzyme types are similar with respect to pH optimum, molecular weight, hexose and nucleotide specificities, $K_m$ for fructose, and the qualitative nature of inhibition by ADP and glucose-6-P.

The available evidence indicates that these hexokinase types represent different molecular forms and are not artifacts of preparation.

Several recent communications have presented evidence for the presence of four hexokinases in supernatant extracts of rat liver. This evidence is based on the separation of the hexokinase types both by DEAE-cellulose chromatography (1) and by starch gel electrophoresis (2, 3). These four hexokinase types include the so-called “high $K_m$ glucokinase” studied extensively by a number of investigators (4-12), as well as three enzymes comprising the “low $K_m$ hexokinase,” previously considered to be a single enzyme activity (6-12). Recent work from this laboratory has indicated that, whereas the glucokinase occurs only in liver, the three low $K_m$ hexokinases constitute a family of three enzymes, uniform in properties from tissue to tissue but present in differing proportions in the various rat tissues (3). In the present paper the properties of the three low $K_m$ hexokinase types of rat tissues have been studied in enzyme preparations purified more extensively than those used in previous studies (1, 3). Each of the three hexokinase types has been purified approximately 150- to 400-fold from one or more tissue sources. Studies have been undertaken to compare their kinetic and physicochemical properties in order to understand the similarities and differences among these multiple forms. The purified hexokinase types retain their unique electrophoretic and chromatographic properties as found in crude extracts. Further, each enzyme type, regardless of the tissue source, has certain properties that distinguish it from the other hexokinase types. Thus, the hexokinases differ in their stability to heat and proteolytic inactivation, as well as apparent $K_m$ values for glucose and probably for ATP, and degree of inhibition by glucose-6-P and ADP. On the other hand, the hexokinases are similar with respect to pH optimum, molecular weight, hexose and nucleotide specificity, and affinity for fructose, as well as the nature of the inhibition by ADP and glucose-6-P. These studies confirm and extend the previous studies on crude enzyme preparations (1, 3).

EXPERIMENTAL PROCEDURE

Animals—Male, Osborne-Mendel albino rats, from the National Institutes of Health colony, weighing 150 to 175 g each, were used for all studies. They were maintained on a regular diet of Purina laboratory chow unless otherwise specified.

Reagents—Purine and pyrimidine nucleotides, glucose-6-P dehydrogenase (Type V from yeast), yeast phosphohexose isomerase, lactate dehydrogenase (Type II from rabbit muscle), D-glucose-6-P, 6-phosphogluconate, nitro blue tetrazolium, TPN+, DPN+, DPNH, p-galactose, and bovine serum albumin were purchased from Sigma. Pyruvate kinase (from rabbit muscle) and 2-phospho-enolpyruvate were obtained from Calbiochem. ADP was obtained from P-L Biochemicals. Yeast alcohol dehydrogenase, trypsin (crystallized two times), chymotrypsin (crystallized three times), and bovine pancreatic protease were obtained from Worthington. 2-Deoxy-D-glucose, D-xyllose, and D-ribose were purchased from Pfannstiel Laboratories. D-Fructose, D-sorbitol, D-mannose, and N-acetyl-D-glucosamine were purchased from Nutritional Biochemical Corporation. 2-Mer-

1 The “low $K_m$ hexokinase” types are designated I, II, and III in order of increasing mobility on starch gel electrophoresis. The high $K_m$ glucokinase, found only in liver, is the most rapidly migrating form and has been designated as Type IV (3).
captoethanol, D-arabinose, and imidazole were obtained from Eastman-Kodak. D-Lyxose, D-glucosamine hydrochloride, phenazine methosulfate, and glycine were purchased from Mann Research Laboratories. DEAE-cellulose was purchased from Schleicher and Schuell as Selectacel and was prepared according to the method of Sober and Peterson (13).

Hydroxylapatite was purchased from Bio-Rad Laboratories as Bio-Gel HT and was equilibrated with 0.001 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na3 EDTA, and 0.01 M glucose prior to use.

The vertical starch gel electrophoresis apparatus was obtained from Buchler Instruments. Hydrolyzed starch was purchased from Connaught Medical Research Industries.

**Assay Systems—Hexokinase activity was assayed spectrophotometrically at 37° by measuring either the glucose-6-P or the ADP formed in the reaction. When glucose-6-P formation was measured, a modification of the method of DiPietro and Weinhouse (4) was used. The routine assay mixture contained in a total volume of 1.0 ml, 74 mM Tris-Cl (final pH 7.4), 25 mM glucose, 0.55 mM TPN+, 3.7 mM ATP, 7.4 mM MgCl2, 5 mM mercaptoethanol, and 0.3 international unit of glucose-6-P dehydrogenase. For Type III hexokinase, assays were carried out at 0.1 mM glucose, as this enzyme type was inhibited by an excess of substrate. When fructose was used as a substrate, 50 μg of crystalline yeast phosphohexose isomerase (specific activity approximately 400 μmoles of substrate converted per min per mg of protein) was included in the incubation mixture. Continuous recordings of the increase in optical density at 340 μm resulting from TPN+ reduction were made in a Beckman DU spectrophotometer equipped with a Gilford model 220 optical density converter. Buffers were kept in a water bath at 37° until used, and the reaction was initiated by the addition of enzyme. The temperature of the spectrophotometer cell housing was maintained at 37° by circulating water from a temperature-controlled bath through thermostatic spacers.

When inhibition by glucose-6-P was studied, the reaction was followed by the rate of ADP formation. ADP formation was measured essentially according to the method of Kornberg and Price (14) in a reaction mixture at 37° containing in a total volume of 1.0 ml, 74 mM Tris-Cl (final pH 7.4), 25 mM glucose, (or 0.1 mM glucose for Type III hexokinase), 0.55 mM DPNH, 3.7 mM ATP, 7.4 mM MgCl2, 5 mM mercaptoethanol, 1 mM P-enolpyruvate, and 0.3 international unit each of pyruvate kinase and lactate dehydrogenase. Continuous recordings of the decrease in optical density at 340 μm resulting from the oxidation of DPNH were made in a Beckman DU spectrophotometer equipped with a Gilford model 220 optical density converter.

6-P-Glucose dehydrogenase activity was determined spectrophotometrically by a modification of the method of Horecker and Smyrniotis (15) in a reaction mixture at 37° containing in a total volume of 1.0 ml, 50 mM Tris-Cl (pH 7.4), 10 mM MgCl2, 0.20 mM TPN, and 0.08 mM 6-P-glucose. Glucose-6-P dehydrogenase was assayed essentially according to the method of Kornberg and Horecker (16). Adenosine triphosphatase activity was assayed spectrophotometrically as described above by measuring the rate of ADP formation from ATP with pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and DPNH, and in the absence of glucose.

Yeast alcohol dehydrogenase was assayed by Martin and Ames’ modification (17) of the method of Racker (18).

One unit of hexokinase activity is defined as the amount of enzyme which transforms 1 μmole of substrate per min at 37° and pH 7.4. Specific activities are expressed as units per mg of protein.

Vertically starch gel electrophoresis was performed according to the technique of Smithies (19, 20) with the use of a 0.02 M sodium barbital buffer, pH 8.4, containing 2.7 mM Na2 EDTA and 5 mM mercaptoethanol. Electrophoresis was carried out at 4° for 16 hours with a potential gradient of 6 volts per cm across the gel. Gel slices were stained for hexokinase activity at 25° in the absence of light for 2 to 3 hours by immersion in 0.1 M Tris-Cl developer solution at pH 7.4 containing 0.5 mM TPN+, 5 mM MgCl2, 5 mM ATP, 2 mM KCN, 0.4 international unit per ml of glucose-6-P dehydrogenase, 40 μg per ml of phenazine methosulfate, 400 μg per ml of nitro blue tetrazolium, and either 0.5 mM or 0.1 mM glucose, as described by Katzen et al. (2). Any slight background staining disappeared after washing the developed gels with a preservative solution of methanol-acetic acid-water (5:1:1).

Sucrose density gradient centrifugation was performed according to the technique of Martin and Ames (17). Yeast alcohol dehydrogenase, having a molecular weight of 150,000 (21), was used as a standard.

Protein was measured by the method of Lowry et al. (22) with crystalline bovine serum albumin as a standard.

**RESULTS**

**Purifications**

The tissue distribution of the three (low K_m) hexokinase types has been described previously (3). Fig. 1, which is derived from that study (3), shows the distributions of these three hexokinases in 31,000 χ g supernatants of homogenates of the various tissues used for the purifications. The hexokinase types are designated Types I to IV in order of increasing mobility on starch gel electrophoresis. Liver contains all three hexokinase types as well as the high K_m glucokinase, here indicated as Type IV (3). Brain and kidney were used for the purification of Type I, and skeletal muscle and epididymal fat pad were used for the preparation of Type II. Type III was isolated from liver alone, since only trace amounts were present in the other tissues.

The purification schemes for hexokinase Types I and II were basically the same, irrespective of tissue source. The general procedure consisted of DEAE-cellulose chromatography followed by ammonium sulfate fractionation, hydroxylapatite chromatography, a second ammonium sulfate fractionation, and finally chromatography on Sephadex G-200. Type III was partially purified by DEAE-cellulose chromatography followed by hydroxylapatite chromatography only. The basic differences in the purification of the various hexokinase types were in the molarities of potassium chloride and potassium phosphate at which the different types were eluted from DEAE-cellulose and hydroxylapatite columns, respectively. Thus, the order of their elution from DEAE-cellulose was in the order of Types I to IV with increasing KCl concentrations (see Figs. 2 to 6). On the other hand, the types were eluted from hydroxylapatite in reverse order from their elution from DEAE-cellulose; thus, Type IV was eluted at the lowest molarity, and Type I was eluted at highest molarity of potassium phosphate.

It is difficult to give an exact fold purification for any of the enzyme types. In all the purification schemes presented the initial...
activity as measured in crude extracts was falsely high for the following reasons. (a) TPNH formation in the initial extract was partially due to the contribution of endogenous 6-P-glucose dehydrogenase. The hexokinase preparation was completely freed of this contaminating enzyme, which is contained in the nonadsorbed protein, during preliminary washing of the DEAE-cellulose column with standard phosphate-glucose buffer. (b) The original extract contains all types of hexokinase present in the original tissue, and these were also separated during DEAE-cellulose chromatography. Hence, the first reliable specific activity is that after DEAE-cellulose chromatography.

Glucose was present during all purification procedures to obtain good yields of enzyme activity. Glucose was particularly necessary during ammonium sulfate fractionations and especially with Type II hexokinase. Glucose has been used previously during purification to stabilize hexokinase from yeast (25) and a "glucokinase" from *Brevisubacterium fuscum* (26). In addition, glucose has been shown to stabilize both yeast (25, 27-29) and mammalian hexokinase (30) against various inactivating agents.

**Type I: Brain**

*Extraction—Rats, which had been fed ad libitum, were killed by decapitation. Thereafter, all steps were performed at 2-4°. Brain (106 g) was homogenized with a Potter-Elvehjem homogenizer equipped with a Teflon pestle in an equal volume (106 ml)* of cold 0.01 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose. This buffer, hereafter denoted as standard phosphate-glucose buffer, was used in all purification procedures. The homogenates were centrifuged in a Servall refrigerated centrifuge at 31,000 × g for 25 min. The resulting supernatant extract (67 ml) was passed through a Sephadex G-25 column (coarse form), 3.9 × 70 cm, that had been equilibrated with the standard phosphate-glucose buffer.

**DEAE-cellulose Chromatography—**The extract (94 ml) was then applied to a DEAE-cellulose column, 3 × 53 cm, that had been packed under 1 pound of pressure and had been equilibrated with standard phosphate-glucose buffer. After application of the extract, the column was washed with 500 ml of the standard phosphate-glucose buffer to remove all nonadsorbed protein. The column was developed with 2000 ml of a linear gradient from 0 to 0.6 M KCl in a standard phosphate-glucose buffer, at a flow rate of about 75 to 90 ml per hour. The Type I hexokinase, regardless of tissue source, was generally eluted at approximately 0.15 M KCl. DEAE-cellulose chromatography of Type I hexokinase from brain is shown in Fig. 2. The peak fractions of en-
zyme activity were combined (98 ml) and solid D-glucose was added to a final concentration of 0.1 M. The eluate was concentrated to a protein concentration of about 5 mg per ml by ultrafiltration through dialysis tubing.

Fig. 2. DEAE-cellulose chromatography of rat brain hexokinase. For details of preparation of the extract and description of the DEAE-cellulose column see text. The column was developed with 2000 ml of a linear gradient of KCl from 0 to 0.6 M in 0.01 M potassium phosphate buffer containing 0.005 M Na2EDTA, 0.005 M mercaptoethanol, and 0.01 M glucose (final pH 7.0); 145 fractions (14 ml each) were collected at a flow rate of 75 to 90 ml per hour. Enzyme activity is expressed as micromoles of product formed at 37°C per min per ml of eluate. Hexokinase assays were performed at 25 mM glucose. The Roman numerals designate hexokinase type. Each peak, designated I, II, III, or IV (on this figure and Figs. 3 to 6), corresponds to the same type on starch gel electrophoresis as shown in Fig. 1.

Fig. 3. DEAE-cellulose chromatography of rat kidney hexokinase. For details see Fig. 2 and the text. The hexokinase assays were performed at two concentrations of glucose: 25 mM glucose, ○—○; and 0.1 mM glucose, ◦—◦. The hexokinase activity at the lower glucose concentration was assayed in Fractions 52 to 69 only. Tubes 35 to 43 were pooled for further purification of Type I.

Fig. 4. DEAE-cellulose chromatography of rat skeletal muscle hexokinase. For details see Fig. 2 and the text. Hexokinase assays were performed at 25 mM glucose. Tubes 49 to 59 were pooled for further purification of Type II.

Fig. 5. DEAE-cellulose chromatography of rat epididymal fat pad hexokinase. For details see Fig. 2 and the text. Hexokinase assays were performed at 25 mM glucose. Tubes 49 to 59 were pooled for further purification of Type II.

Ammonium Sulfate Fractionation—The enzyme solution was fractionated with solid ammonium sulfate according to the formula of Kunitz (31) corrected for the solubility of ammonium sulfate at 0°C. The ammonium sulfate was added slowly with constant stirring. After this, the suspension was permitted to stand for 10 to 15 min and then centrifuged at 18,000 × g for 10 min. The pH of the solution was maintained at 7.0 with addition of concentrated ammonium hydroxide. The hexokinase precipitated in the protein fraction between 40 and 55% of saturation at 0°C. The protein fraction containing enzyme activity was dissolved in 8 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na2EDTA, and
Sulfate was removed by passage through a Sephadex G-25 column containing enzyme activity were pooled (24 ml); solid D-glucose was added to a final concentration of 0.1 M, and the enzyme preparation was concentrated by ultrafiltration through dialysis tubing to a protein concentration of about 4 mg per ml.

Second Ammonium Sulfate Fractionation—The solution was again fractionated with solid ammonium sulfate as described previously, in the presence of 0.1 m glucose. The bulk of the enzyme activity precipitated in the protein fraction between 40 and 55% of saturation at 0 °. The precipitate was dissolved in 0.5 to 1.0 ml of standard phosphate-glucose buffer.

Sephadex G-200 Chromatography—The enzyme fraction (0.5 to 1.0 ml) was applied to a column of Sephadex G-200, 2.0 X 78 cm, which had been equilibrated with standard phosphate-glucose buffer. The column was developed with the same buffer. The fractions containing peak enzyme activity were pooled; solid D-glucose was added to a final concentration of 0.1 M, and the pooled fractions were ultrafiltered to a final volume of about 1.0 ml.

**Type I: Kidney**

Similar steps were used in the purification of hexokinase Type I from rat kidney, which was also prepared as a 1:1 homogenate. Fig. 3 illustrates a typical DEAE-cellulose chromatography of an extract from 125 g of rat kidney.

Table I shows a typical purification protocol of hexokinase Type I from rat kidney, and Table II shows a purification of Type I from kidney. The specific activity of the final preparation of brain Type I was 10.5 to 12.0 units per mg of protein, and the final specific activity of hexokinase Type I from kidney was generally 6.8 to 7.5 units per mg of protein.

**Type II: Muscle**

**Extraction**—Rats, which had been fed ad libitum, were killed by decapitation. All subsequent steps were carried out at 2–4 °. The skeletal muscle of the lower extremities was removed and minced finely. The muscle (97 g) was homogenized with 2 volumes of standard phosphate-glucose buffer in a Waring Blender for 1 min. The homogenates were centrifuged in a refrigerated Servall centrifuge at 31,000 X g for 25 min. After aspiration of the fat layer, the supernatant extract (147 ml) was equilibrated with standard phosphate-glucose buffer by passage through a Sephadex G-25 column (coarse form), 5 X 64 cm, as described above for Type I hexokinase.

**DEAE-cellulose Chromatography**—The equilibrated extract (192 ml) was then placed on a DEAE-cellulose column. Preparation of the column and chromatography of the extract were as described in the text. The assays were performed at two concentrations of glucose: 25 mM glucose, O—O; and 0.1 mM glucose, C—O. The hexokinase activity at the lower glucose concentration was assayed in Fractions 45 to 70 only. Tubes 57 to 66 were pooled for further purification of Type III.

**Table I**

Purification of hexokinase Type I from rat brain

The representative purification scheme is from a 1:1 homogenate of 106 g of rat brain. The activity was assayed by following the rate of glucose-6-P formation as described under "Experimental Procedure." As described in the text, in all purification schemes presented (Tables I to IV), the first reliable specific activity is that after DEAE-cellulose chromatography.

<table>
<thead>
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<th>Step</th>
<th>Volume</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
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<td>Ammonium sulfate (I)</td>
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</table>

* After ultrafiltration.

0.01 M glucose, and was dialyzed overnight (12 to 15 hours) against 4 liters of the same buffer. On occasion, the ammonium sulfate was removed by passage through a Sephadex G-25 column (coarse form), 2.2 X 26 cm, equilibrated with 0.001 M potassium phosphate buffer pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose.

**Hydroxylapatite Chromatography**—The dialyzed enzyme preparation was applied to a hydroxylapatite column, 1.5 X 15 cm, and the column was then washed with 40 ml of 0.001 M potassium phosphate buffer pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose. The enzyme was eluted with 220 ml of a linear gradient of potassium phosphate from 0.001 M to 0.3 M potassium phosphate, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose. The flow rate was about 3.5 ml per hour. The peak fractions of enzyme activity were generally eluted at a potassium phosphate concentration of 0.16 to 0.17 M. The fractions containing enzyme activity were pooled (24 ml); solid D-glucose was added to a final concentration of 0.1 M, and the enzyme preparation was concentrated by ultrafiltration through dialysis tubing to a protein concentration of about 4 mg per ml.

**Table II**

Purification of hexokinase Type I from rat kidney

The representative purification scheme is from a 1:1 homogenate of 125 g of rat kidney.

<table>
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<th>Total activity</th>
<th>Specific activity</th>
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<td>30</td>
<td>6.8</td>
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</table>

* After ultrafiltration.
similar to that described for Type I. The Type II hexokinase, regardless of tissue source, was eluted at approximately 0.22 M KCl. A typical DEAE-cellulose chromatography of rat muscle is shown in Fig. 4. The peak fractions of enzyme activity were combined, and solid n-glucose was added to a final concentration of 0.1 M glucose. The solution was concentrated to a protein concentration of approximately 5 mg per ml by ultrafiltration through dialysis tubing.

Ammonium Sulfate Fractionation—The enzyme solution was fractionated with solid ammonium sulfate, as described for Type I. The enzyme precipitated in the protein fraction between 35 and 50% of saturation at 0°C. The protein fraction containing the enzyme activity was dissolved in 8 ml of 0.001 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose, and was dialyzed overnight (15 hours) against 4 liters of the same buffer. On occasion the solution was passed through a Sephadex G-25 column to free it of ammonium sulfate as described for Type I.

Hydroxyapatite Chromatography—The dialyzed enzyme preparation was then placed on a hydroxyapatite column. Column size, preparation, equilibration, washing, and flow rate were similar to that described for Type I. The enzyme was eluted with 220 ml of a linear gradient of potassium phosphate from 0.01 M to 0.25 M potassium phosphate, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂ EDTA, and 0.01 M glucose. The peak of Type II enzyme activity was eluted at approximately 0.07 to 0.08 M potassium phosphate. The fractions containing enzyme activity were pooled (28 ml); solid n-glucose was added to a final concentration of 0.1 M, and the pooled enzyme fractions were concentrated by ultrafiltration to a protein concentration of about 4 mg per ml.

Second Ammonium Sulfate Fractionation—The solution was again fractionated with solid ammonium sulfate as described for Type I. The bulk of the enzyme activity precipitated in the protein fraction between 40 and 50% of saturation at 0°C. The precipitated fraction was dissolved in 0.5 to 1.0 ml of standard phosphate-glucose buffer.

Sephadex G-200 Chromatography—The dissolved enzyme fraction (0.5 to 1.0 ml) was chromatographed on a Sephadex G-200 column; the size, preparation, equilibration, and development were as described for Type I. The peak fractions of enzyme activity were pooled, and n-glucose was added to a final concentration of 0.1 M. The pooled fractions were ultrafiltered through dialysis tubing to a final volume of about 1.0 ml.

Type II: Epididymal Fat Pad

Steps similar to those described for the purification from muscle were used in the purification of hexokinase Type II from epididymal fat pad. The only difference in procedure in enzyme preparation from fat pad is that the fat was homogenized in 2 volumes of standard phosphate-glucose buffer in a Potter-Elvehjem homogenizer at room temperature. Homogenization was performed at room temperature because, in confirmation of a previous report (32), homogenization at 0-4°C caused the fat to solidify, made homogenization incomplete, and trapped a significant portion of the aqueous phase even after high speed centrifugation. After homogenization, the preparation was centrifuged at 4°C at 31,000 × g for 25 min. The supernatant fat layer was removed, and the resulting supernatant crude extract was carried through a purification procedure similar to that described for Type II for muscle. Fig. 5 illustrates a typical DEAE-cellulose chromatography of an extract from 200 g of rat epididymal fat pad.

Typical purification protocols for hexokinase Type II from skeletal muscle and epididymal fat pad are shown in Tables III and IV, respectively. The specific activity of the final preparations of muscle and epididymal fat pad Type II were 12.5 to 14.5 units per mg of protein.*

**Type III: Liver**

The Type III enzyme from liver was purified essentially as was done by Gonzales et al. (1). This hexokinase type was purified from the livers of rats fasted 60 to 72 hours. The major glucose phosphorylating activity of liver is Type IV (glucokinase) which is eluted from a DEAE-cellulose column almost immediately after and overlapping the Type III enzyme. Fasting markedly diminishes the Type IV enzyme in liver (1, 2, 6-9, 11, 33-35) but causes little change in the level of Type III (1), and thereby makes the purification of Type III much easier. For Type III hexokinase, assays were performed at two concentrations of glucose, 25 mM and 0.1 mM, since the Type III enzyme was identifiable by its inhibition at the higher substrate concentration.

**Extraction**—A typical preparation consisted of homogenizing the livers of 72 fasted animals (liver weight 325 g) in an equal volume of standard phosphate-glucose buffer, with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 25 min in a Servall refrigerated centrifuge at 31,000 × g. Any

*More recent preparations of Type II from skeletal muscle, with the same purification procedures described herein, have specific activities of about 28 units per mg of protein.
supernatant fat was aspirated, and the resulting supernatant extract (223 ml) was equilibrated with standard phosphate-glucose buffer by passage through a Sephadex G-25 column, 7.1 × 52 cm, as for Types I and II.

DEAE-cellulose Chromatography—The equilibrated extract (370 ml) was applied to a DEAE-cellulose column prepared as described for the other types. The peak fractions of Type III hexokinase activity were eluted at approximately 0.27 M KCl. DEAE-cellulose chromatography of rat liver hexokinase is shown in Fig. 6. The peak fractions of enzyme activity, as assayed at 0.1 mM glucose, were combined (126 ml) and immediately passed through a Sephadex G-25 column, 3.6 × 82 cm, equilibrated with 0.001 M potassium phosphate buffer, pH 7.0, containing 0.005 M Na₂EDTA, 0.005 M mercaptoethanol, and 0.01 M glucose.

Hydroxylapatite Chromatography—The equilibrated enzyme fraction was then applied to a hydroxylapatite column, 1.5 × 16 cm, which was then washed with 50 ml of 0.001 M potassium phosphate buffer, pH 7.0, containing 0.005 M Na₂EDTA, 0.005 M mercaptoethanol, and 0.01 M glucose. The enzyme was eluted with 240 ml of a linear gradient of potassium phosphate from 0.001 to 0.2 M potassium phosphate, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂EDTA, and 0.01 M glucose. The peak fractions of enzyme activity were eluted at approximately 0.05 to 0.06 M potassium phosphate. Solid D-glucose was added to a final concentration of 0.1 M, and the pooled enzyme fractions were concentrated by ultrafiltration through dialysis tubing to a final volume of 1.0 ml.

The final specific activity of hexokinase Type III from liver, assayed at 0.1 mM glucose, was 1.2 to 1.4 units per mg of protein.

Table V

<table>
<thead>
<tr>
<th>pH</th>
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</tr>
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<td>8.8</td>
<td>91%</td>
</tr>
<tr>
<td>9.6</td>
<td>52%</td>
</tr>
</tbody>
</table>

No attempt was made to determine the fold purification or yield of Type III from liver, as any assays done on the crude extract would be meaningless in regard to Type III activity.

After purification, starch gel electrophoresis performed with each enzyme preparation showed that: (a) each preparation was a pure hexokinase type and was not contaminated with the other types present in the same tissues; (b) each type retained its original electrophoretic mobility as present in the crude tissue extracts; and (c) the removal of mercaptoethanol from the enzyme preparations, by dialysis for 24 hours against three changes of 4000 volumes of standard phosphate-glucose buffer without mercaptoethanol, resulted in no change in the mobility or patterns of any of the enzyme types on starch gel. Starch gels and the barbitol buffer used for these electrophoreses were prepared without mercaptoethanol.

Rechromatography of the various purified enzyme preparations on DEAE-cellulose, under the original conditions, revealed a single peak of enzyme activity, which was eluted at the expected molarity of potassium chloride, with no other peaks of activity detected.

The purified enzymes were free of measurable adenosine triphosphatase, 6-P-glucose dehydrogenase, and glucose-6-P dehydrogenase activities. In addition, the purified enzymes, in the absence of glucose, caused no reduction of TPN or oxidation of DPNH in the assay systems described under "Experimental Procedure."

After purification, the enzymes were routinely stored at 2°C in 0.01 M potassium phosphate buffer, pH 7.0, containing 0.005 M mercaptoethanol, 0.005 M Na₂EDTA and 0.1 M glucose. The enzymes were stored in the presence of glucose because preliminary studies on the crude enzymes demonstrated increased stability in the presence of this concentration of substrate (3). Prior to all studies, enzyme preparations were initially freed of glucose by passage through small columns of Sephadex G-25 (bead form) equilibrated with 0.01 M potassium phosphate, pH 7.0, with 0.005 M mercaptoethanol and 0.005 M Na₂EDTA. The bed volume was 30 to 40 times that of the applied enzyme solution. This procedure was performed at 4°C. The Sephadex eluate was assayed for hexokinase activity by measuring glucose-6-P formation as described under "Experimental Procedure."

The assay was performed both in the presence and absence of glucose. Only those early fractions of the eluate showing absolutely no activity in the absence of added glucose were considered as being glucose-free and utilized for the studies of the enzyme types.

Properties

Studies on the properties of the purified hexokinases were undertaken to determine what differences existed between the three types, and whether the same hexokinase type obtained from different tissues had similar properties. All studies of a given kinetic or physical parameter, i.e. pH optimum, Michaelis constant, molecular weight, etc., were performed in a similar fashion, with enzyme preparations of similar age and treatment, in order to minimize all differences other than the hexokinase type being studied.

pH Optimum—As shown in Table V, the pH optimum for all three hexokinase types was similar, with a broad optimum between 7.8 and 8.8. Type I from kidney and Type II from fat pad had pH activity curves similar to those shown in Table V.
hexokinase reaction was determined by the rate of 6-P formation as indicated in the figure. The glucose concentration was 20 mm.

For these studies, 2.5 g of brain Type I, 3.9 g of kidney Type I, 2.2 g of muscle Type II, 2.3 g of epididymal fat pad Type II, and 19 g of liver Type III were added to the assay mixture to initiate the reaction.

The specific activities of the enzymes used for these determinations are those final values listed in the purification schemes. For these studies, 2.5 g of brain Type I, 3.9 g of kidney Type I, 2.2 g of muscle Type II, 2.3 g of epididymal fat pad Type II, and 19 g of liver Type III were added to the assay mixture to initiate the reaction.

The different Michaelis constants reported for glucose with hexokinase preparations from various tissues, including brain (39, 41), kidney (42), intestine (43), skeletal muscle (37, 40), heart muscle (43), fat pad (5, 32, 44), and liver are most likely reflections of the activity of the predominant hexokinase type in each tissue and not of the tissue per se. On the other hand, reported values for the Km for fructose have ranged from 1 to 4 X 10^-4 M for crude preparations of hexokinase from all rat tissues studied, including liver (1, 3), fat pad (32, 44), brain (41), and skeletal muscle (37). This is most readily explained by the similarity of the Michaelis constant for fructose to the respective Km for glucose of the three hexokinase types. The apparent Km for fructose and the Vmax ratio of fructose to glucose were quite similar for all three hexokinase types.

Table VII shows the ability of a variety of sugars to inhibit hexokinase activity when glucose is present at a concentration of...
Multiple Forms of Rat Tissue Hexokinase

Fig. 8. Plot of the reciprocal of initial reaction velocity \( v \) versus the reciprocal of molar concentration of D-glucose in the presence and absence of glucose-6-P (G6-P). Initial ATP concentration was 3.7 mM for all hexokinase types. Initial ATP concentration was varied for the three hexokinase types as indicated in the figure. The glucose-6-P concentrations are shown in the figure. The velocity of the hexokinase reaction was determined by the rate of ADP formation as described under "Experimental Procedure." The velocity unit \( v \) is expressed as in Fig. 7. The specific activities of the enzymes used in these experiments are those final values listed in the purification schemes. In these studies, 2.5 µg of hexokinase Type I, 2.1 µg of hexokinase Type II, and 14.2 µg of hexokinase Type III were added to the assay mixture to initiate the reaction.

Fig. 9. Plot of the reciprocal of initial reaction velocity \( v \) versus the reciprocal of molar concentration of ATP in the presence and absence of ADP. Initial D-glucose concentration was 20 mM for Types I and II and 0.25 mM for Type III. ATP concentration was varied from 0.45 mM to 5.0 mM as shown in the figure. The initial MgCl₂ concentration in the assay mixture was 7.4 mM. The ADP concentrations, added with equimolar amounts of MgCl₂, are shown in the figure. The velocity of the hexokinase reaction was approximately 2 times the \( K_m \) value. Those sugars and sugar derivatives which have been reported as either substrates of mammalian hexokinase (41), D-mannose, 2-deoxy-D-glucose, D-glucoamine at pH 7.5, D-fructose, or as competitive inhibitors (41), D-xylene, N-acetyl-D-glucosamine, D-lyxose, inhibited all three hexokinase types to the same extent. Those sugars which have been reported to not be phosphorylated and which do not inhibit hexokinase (D-ribose, D-arabinose, D-sorbitol, and D-galactose) did not inhibit any hexokinase type. Similar results were obtained with Type I from kidney and Type II from fat pad.

The only effective donor of the phosphoryl group in the hexokinase reaction with all three hexokinase types is ATP. The apparent \( K_m \) values for ATP as shown in Table VIII are 4.0 to 4.4 \( \times 10^{-4} \) M for Type I, 7.5 to 7.8 \( \times 10^{-4} \) M for Type II, and 9.8 \( \times 10^{-4} \) M for Type III. Although these differences in apparent \( K_m \) values appear to be slight, we have consistently found them under the assay conditions used. The \( K_m \) values for ATP with Types I and II are similar to those reported for crude preparations of these enzymes from rat liver (1). The \( K_m \) value for the purified calf brain hexokinase (Type I) has been reported as 1.13 to 2.92 \( \times 10^{-4} \) M (39) and that from partially purified rat skeletal muscle (Type II) 4.6 \( \times 10^{-4} \) M (40). Both these values are slightly lower than the values reported for these enzymes in this paper, but the previously reported \( K_m \) values for ATP were obtained at lower glucose concentrations than used here.

As is also indicated in Table VIII, ITP can partially substitute for ATP as a phosphoryl donor for the three hexokinase types. However, other nucleotide triphosphates were ineffective. These results are generally analogous to the nucleotide specificity of crystalline yeast hexokinase (45, 46). Purified glucokinase
Fig. 10. Plot of the reciprocal of initial reaction velocity (\(v\)) versus the reciprocal of the molar concentration of d-glucose in the presence and absence of ADP. Initial ATP concentration was 3.7 mM for all hexokinase types. The MgCl₂ to ATP ratio in all reaction mixtures was constant at 2:1. d-Glucose concentration was varied for the three hexokinase types as indicated in the figure. The ADP concentrations are shown in the figure. The ADP was added with equimolar amounts of MgCl₂. The velocity of the hexokinase reaction was determined by the rate of glucose-6-P formation as described under "Experimental Procedure." The velocity unit (\(v\)) is expressed as in Fig. 7. The specific activities of the enzymes used in these experiments are those final values listed in the purification schemes. In these studies, 2.7 µg of hexokinase Type I, 2.0 µg of hexokinase Type II, and 20.6 µg of hexokinase Type III were added to the assay mixture to initiate the reaction.

Table VII

<table>
<thead>
<tr>
<th>Sugar</th>
<th>I (brain)</th>
<th>II (muscle)</th>
<th>III (liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00 M sugar</td>
<td>0.00 M sugar</td>
<td>0.00 M sugar</td>
</tr>
<tr>
<td>d-Mannose</td>
<td>0</td>
<td>1.3</td>
<td>0</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>4.1</td>
<td>14</td>
<td>10</td>
</tr>
<tr>
<td>d-Fructose</td>
<td>31</td>
<td>71</td>
<td>42</td>
</tr>
<tr>
<td>d-Glucosamine</td>
<td>1.0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>d-Xylose</td>
<td>55</td>
<td>94</td>
<td>63</td>
</tr>
<tr>
<td>N'-Acetyl-d-glucosamine</td>
<td>0</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>d-Lybose</td>
<td>54</td>
<td>89</td>
<td>52</td>
</tr>
<tr>
<td>d-Ribose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>d-Arabinose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>d-Sorbitol</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>d-Galactose</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

(The Type IV) from rabbit liver apparently differs from Types I, II, and III and has an absolute requirement for ATP; the activity with ITP is no different from that with GTP, UTP, and CTP (47). Similarly, crude preparations of rat liver glucokinase show no activity with UTP, ITP, or GTP (5).

Hexokinase activity of all three types is inhibited when the molar ratio of ATP to MgCl₂ is greater than one, as has been reported for rabbit liver glucokinase (47). Increasing the concentration of MgCl₂ up to 20 times greater than that of ATP caused no inhibition of hexokinase activity with any type.

Kinetic Studies of Inhibition of Hexokinase by Glucose-6-P and ATP—Studies of the kinetics of inhibition of hexokinase by its...
products were undertaken to determine if differences existed in the nature or extent of inhibition of the reaction catalyzed by the three types. It is of interest that mammalian hexokinase (30, 39, 45–48) is far more sensitive to inhibition by glucose-6-P than is yeast hexokinase (30, 46, 49) or the glucokinase from rabbit (47) and rat liver (6).

The inhibition of all three hexokinase types by glucose-6-P was competitive with respect to ATP, as shown in Fig. 7. The calculated apparent $K_i$ values (Table IX) were similar for Types I and II, whereas that for Type III was approximately 2 to 3 times greater. Representative studies of the inhibition by glucose-6-P of the three hexokinase types, as a function of the glucose concentration, are shown in Fig. 8. The calculated apparent $K_i$ values for glucose-6-P with respect to glucose (Table IX) were also similar for Types I and II and greater for Type III. The results of product inhibition studies with Type I hexokinase are similar to those reported for calf brain hexokinase (39).

The inhibition of the hexokinase types by ADP, as a function of the ATP concentration, is shown in Fig. 9. The mixed competitive nature of the inhibitions by ADP has also been observed for calf brain hexokinase (39) and rat skeletal muscle hexokinase (40). The calculated apparent $K_i$ values for ADP with respect to ATP are given in Table IX.

**Table IX**

<table>
<thead>
<tr>
<th>Type and source</th>
<th>Glucose-6-P inhibition</th>
<th>ADP inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_i$ glucose-6-P (vs. ATP)</td>
<td>$K_i$ glucose-6-P (vs. glucose)</td>
</tr>
<tr>
<td>I. Brain...</td>
<td>$2.6 \times 10^{-5}$</td>
<td>$2.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>II. Muscle ...</td>
<td>$2.1 \times 10^{-5}$</td>
<td>$1.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>III. Liver ...</td>
<td>$7.4 \times 10^{-5}$</td>
<td>$9.2 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

* Apparent $K_i$ values were calculated from the Lineweaver-Burk plots of Fig. 7. Values were calculated from plots of the reciprocal of $V_{max}$ and are calculated from plots of the reciprocal of $V_{max}$ versus the molar concentration of inhibitor (51). Lineweaver-Burk plots of these product inhibition patterns are shown in Fig. 8.

$K_i$ values were calculated from Dixon plots (52) of the reciprocal of initial velocity versus the molar concentration of inhibitor. Lineweaver-Burk plots of these product inhibition patterns are shown in Fig. 9.

$K_i$ values were calculated from plots of the reciprocal of $V_{max}$ versus the molar concentration of inhibitor (51). Lineweaver-Burk plots of these product inhibition patterns are shown in Fig. 10.

**Fig. 11.** Effect of heat on the stability of the hexokinase types in the presence and absence of glucose. Types I, II, and III hexokinase from the designated tissues were freed of glucose by passage through a Sephadex G-25 column that had been equilibrated with 0.01 M potassium phosphate buffer, containing 0.005 M Na$_2$ EDTA and 0.005 M mercaptoethanol, final pH 7.0. Each preparation was adjusted to a protein concentration of 0.5 mg per ml and heated in the presence or absence of 0.1 M glucose for the specified time periods at 45°. No glucose, — — ; 0.1 M glucose, — — . Activity is expressed as the per cent of initial activity. The specific activities of the enzymes used in these experiments are those final values listed in the purification schemes.
to ATP (Table IX) were similar for Types I and III, but higher for Type II. The inhibition of all hexokinase types by ADP, relative to glucose, is shown in Fig. 10. The apparent $K_i$ values for ADP with respect to glucose (Table IX) were similar for Types I and III, but higher for Type II.

These studies on the inhibition of the three hexokinase types by glucose-6-P and ADP were undertaken for comparative purposes and are not intended to allow for a detailed examination of the mechanism of the hexokinase reaction. The results, however, would suggest that there is no basic difference in the nature of the inhibition of the three hexokinase types. Furthermore, it would appear, on the basis of the Lineweaver-Burk plots in the presence and absence of glucose-6-P or ADP and the calculated apparent $K_i$ values, that there are differences in the degree of inhibition by glucose-6-P and ADP of the different types. Most notable perhaps, is the relatively lesser inhibitory effect of ADP upon Type II as compared with the other types, and the lesser degree of inhibition by glucose-6-P of Type III as compared with the other types.

**Molecular Weights**—Molecular weight estimates of the three hexokinase types were made by the sucrose density gradient method of Martin and Ames (17). As shown in Table X, all three hexokinase types have similar molecular weights of approximately 96,000. It is of interest that this molecular weight is similar to that reported for crystalline yeast hexokinase (53). It is evident, then, that the hexokinase types probably do not represent differing degrees of polymerization of a common subunit.

**Stability Properties**—The three hexokinase types can be distinguished by differences in stability to inactivation by heat and proteolysis. As shown in Fig. 11, Type I is the most stable, Type II is rapidly inactivated, and Type III is intermediate in stability to heat inactivation at 45°. The stability to heat inactivation is similar for Types I and II isolated from different sources. Glucose, at 0.1 M, prevents the inactivation of all three hexokinase types.

The stability of the three hexokinase types against inactivation by trypsin is generally similar to the results obtained with inactivation by heat, as shown in Fig. 12. Again, Type I hexokinase—

![Figure 12](http://www.jbc.org/)

**Figure 12.** Effect of trypsin on the activity of the various types of hexokinase. Types I, II, and III hexokinase from the designated tissues were freed of glucose by passage through a Sephadex G-25 column that had been equilibrated with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.005 M Na$_2$EDTA and 0.005 M mercaptoethanol, final pH 7.0. Each preparation was adjusted to a protein concentration of 0.5 mg per ml and incubated at 22° with trypsin (100 μg per ml) in the presence or absence of 0.1 M glucose. No glucose, ---; 0.1 M glucose, ----. Activity is expressed as the per cent of initial activity. The specific activities of the enzymes used in these experiments are those final values listed in the purification schemes.

<table>
<thead>
<tr>
<th>Type, source, and proteolytic agent</th>
<th>Activity after 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With glucose</td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>97</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>80</td>
</tr>
<tr>
<td>Pancreatic protease</td>
<td>73</td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>100</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>90</td>
</tr>
<tr>
<td>Pancreatic protease</td>
<td>65</td>
</tr>
<tr>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>79</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>60</td>
</tr>
<tr>
<td>Pancreatic protease</td>
<td>58</td>
</tr>
<tr>
<td>Fat pad</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>78</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>59</td>
</tr>
<tr>
<td>Pancreatic protease</td>
<td>57</td>
</tr>
<tr>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>93</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>97</td>
</tr>
<tr>
<td>Pancreatic protease</td>
<td>69</td>
</tr>
</tbody>
</table>
Fig. 13. Effect of trypsin on the starch gel electrophoretic pattern of the various types of hexokinase in the presence and absence of glucose. Types I, II, and III hexokinase (from brain, skeletal muscle, and liver, respectively) were freed of glucose by passage through a Sephadex G-25 column that had been equilibrated with 0.01 m potassium phosphate buffer containing 0.005 m Na₂ EDTA and 0.005 m mercaptoethanol, final pH 7.0. Each preparation was adjusted to a protein concentration of 0.5 mg per ml and incubated at 22°C with trypsin (100 μg per ml) for 30 min in the presence or absence of 0.1 m glucose. After this, the various preparations were subjected to starch gel electrophoresis as described under "Experimental Procedure." Gels with Types I and II hexokinase were stained in the presence of 0.1 m glucose and Type III hexokinase was stained in the presence of 0.5 m glucose. Crude kidney extract is shown here as a marker for the three types. A, electrophoretic pattern of the purified untreated enzyme types. B, electrophoretic pattern of the enzyme types treated with trypsin in the presence of 0.1 m glucose. B', sketch of the results with Type II hexokinase adjacent to actual pattern which stained faintly. C, electrophoretic pattern of the enzyme types treated with trypsin in the absence of glucose (not shown for Type II for which there was no activity detectable by stain). The specific activities of the enzymes used in these experiments are those final values listed in the purification schemes.

DISCUSSION

Mammalian hexokinase can be added to an increasing number of enzymes which have been demonstrated to exist in multiple molecular forms (54, 55). The studies presented in this paper have confirmed and extended previous observations of the presence of three electrophoretically and chromatographically distinct hexokinase isoenzymes present in rat tissues, in addition to the glucokinase that is present only in liver (1, 3). Support for this conclusion includes the following. (a) The hexokinase types differ in electrophoretic and chromatographic properties, most probably representing a difference in net charge. Each hexokinase type retains its unique chromatographic and electrophoretic properties after multiple purification procedures. This would indicate that the various hexokinase types are not artifacts of the preparation. (b) The three hexokinase isoenzymes can be distinguished on the basis of differing stabilities against heat and proteolytic inactivation, differing apparent Kₘ values for glucose and probably for ATP, and differing apparent Kₖ values for ADP and glucose-6-P. On the other hand, the hexokinase isoenzymes are similar in sugar and nucleotide specificity, molecular weight, pH optimum, affinity for fructose, and the qualitative nature of the inhibition by ADP and glucose-6-P.

Although the studies presented in this paper were limited to rat tissues, the presence of multiple forms of hexokinase would appear to be a general phenomenon, at least in mammalian tissues. Thus, a brief survey of the electrophoretic properties of
the hexokinase activity in crude extracts of various tissues of humans, monkeys, and several strains of mice have shown multiple forms of hexokinase with electrophoretic mobilities generally similar, although not identical, to those of rat tissues and with generally the same tissue distribution of the isoenzymes. In addition, several human cell lines in continuous cultures have been shown to have hexokinase activities with the electrophoretic mobilities of Types I and II hexokinase of rat tissues, including HeLa-Sa and Zimmer 2A.

The molecular basis for the multiple forms of mammalian hexokinase is not presently known. They would not appear to result from differing polymeric states of a single active hexokinase subunit, inasmuch as each of the three hexokinase isoenzymes has the same molecular weight as estimated by the sucrose density gradient technique. The multiple forms would also not appear to result from the presence of different cell types in a tissue, each containing a single isoenzyme. Thus, HeLa-Sa, a human cervical cancer cell line that was originally derived from a single clone (56), has hexokinase types corresponding to both I and II.³ The presence of multiple forms of mammalian hexokinase would appear to bear few similarities to the multiple forms of yeast hexokinase. Colowick and his associates (57–59) have recently studied the basis of the multiple forms of hexokinase in Saccharomyces cerevisiae and have concluded that many of their multiple forms result from proteolytic action during purification (59). Attempts to produce interconversion of the mammalian hexokinases by the intrinsic proteolytic activity of homogenates have been unsuccessful (3). On the other hand, the results with trypsin proteolysis presented in this paper indicate that the electrophoretic properties of certain of the mammalian hexokinase types can be readily altered, although the alteration does not result in a conversion of one hexokinase type to another. This effect of trypsin may be analogous to the phenomenon studied by Colowick et al. (57–59). In addition, the multiple forms of yeast hexokinase had similar physical and kinetic properties (58), whereas the multiple forms of mammalian hexokinase can be readily distinguished by kinetic and physical properties.

The presence of the hexokinase isoenzymes with differing charge and kinetic properties may be similar to the classical case of the isoenzymes of lactate dehydrogenase. In the case of lactate dehydrogenase the aggregation of two dissimilar subunits into a tetramer results in the presence of five forms of the enzyme which can be distinguished by differences in kinetic and physical properties (55, 60). It is of interest that Kenkare and Colowick have recently shown that yeast hexokinase with a molecular weight of 96,000 can be disassociated into four subunits of equal molecular weight, and subsequently reaggregated into active enzyme (61). Our results indicate that the mammalian hexokinases also have a molecular weight of 96,000. Studies are now in progress to purify further the hexokinase isoenzymes and to determine whether they result from the aggregation of different subunits or the aggregation of similar subunits into active enzyme having differing conformations.

It should be pointed out that all of the studies in this report have been concerned with hexokinase activity present in the soluble fraction of tissue extracts. Hexokinase of rat tissues is also present in a bound or particulate form, the per cent sedimentable depending on the tissue. For example, brain hexokinase has been reported to be almost completely present in the particulate form (49). Although there may be a difference between the particulate forms and the soluble forms of the enzymes described in this paper, it is unlikely for the following reasons. (a) Kinetic studies of the particulate form (59) of brain hexokinase yielded results similar to those reported here on the soluble form. (b) Recently, in a report on the solubilization and further purification of calf brain hexokinase, the purified enzyme resembled the particulate preparation in its $K_m$ values for glucose and ATP and in its inhibition by low concentrations of glucose-6-P (62). (c) After extraction of the soluble hexokinase, the various sediments, when sonically disrupted and re-extracted, revealed hexokinase activity with electrophoretic mobility indistinguishable from those of the original soluble extracts.⁴ Recently Hernandez and Crane have reported that the binding of hexokinase in pig heart muscle is reversible and is affected by ionic strength, pH, and glucose-6-P (63). Studies on the cellular distribution and binding of hexokinase to subcellular particles of various rat tissues, and their possible relationship to the hexokinase isoenzymes are in progress.

The presence of multiple forms of hexokinase with characteristic tissue distributions in a variety of animals suggests some physiological role for their existence and distribution. Lowry and Pasonneau (64) have shown that in brain, the hexokinase activity along with phosphofructokinase activity, is one of the rate-limiting reactions in glycolysis. Recently, Uyeda and Rackner (30, 65), on the basis of studies with a reconstructed multi-enzyme system, have suggested that glycolysis in animal tissues may be controlled by the interactions of glucose, ATP, ADP, P_i, and glucose-6-P with hexokinase and phosphofructokinase. In view of the differences in $K_m$ for glucose and probably for ATP, and the relatively different degrees of inhibition by ADP and glucose-6-P for the three hexokinase isoenzymes, it is obvious that the control of the hexokinase activity would be potentially very different for each hexokinase type.

Certain studies have also shown variations in the levels of hexokinase activity in specific tissues as a function of the physiological state of the animal. Katzen et al. (2) have shown that growth of the human cell line, Zimmer 2A, in high glucose concentrations resulted in an increase in the activity of a second hexokinase isoenzyme, namely Type II. Chandler and Moore have shown that epididymal fat pad hexokinase is diminished during fasting (66), an effect found limited to Type II hexokinase (3, 67). It is of interest that the reported changes in hexokinase activity would appear to be limited to the Type II hexokinase.

The relationship of the effect of physiological variables on Type II hexokinase to the apparent instability of this isoenzyme may be incidental, or it may indicate that Type II hexokinase is readily subject in vivo to activation and inactivation phenomenon.

The relationship of differences in properties of the hexokinase...
isozymes and their different tissue distributions to physiological requirements must await further studies.

Acknowledgments—The authors would like to express their appreciation to Dr. M. Weksler for his assistance with some of the preliminary studies and to Dr. Howard M. Katzen for discussions during the early phases of these experiments.

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