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The Isolation and Purification of Solubilized Hexokinase from Bovine Brain*

Gerald P. Schwartz† and R. E. Basford

ABSTRACT: Bovine brain hexokinase was purified 1129-fold by a two-phase procedure. The specific activity of the solubilized enzyme was 80 μmoles of glucose 6-phosphate formed/min per mg of protein. The first phase involved purification of particulate hexokinase by successive treatment of the "mitochondrial sediment" with chymotrypsin, deoxycholate, and Triton X-100; the second phase involved solubilization of the particulate hexokinase with Triton X-100 followed by chromatography of the solubilized enzyme on DEAE-cellulose. Analysis by ultracentrifugation and cellulose acetate electrophoresis indicated that the enzyme was homogeneous. The uncorrected sedimentation constant of the purified enzyme was calculated to be 4.44 S.

The absorption spectrum of the enzyme was typical of a protein except that fine structure was observed below 280 mμ. The amino acid analysis indicated that the enzyme had a high content of acidic amino acids, and a low content of tyrosine. Some preliminary kinetic properties of the solubilized enzyme are reported.

Enzyme studies utilizing the technique of differential centrifugation have indicated that brain hexokinase is associated with the mitochondrial fraction of brain homogenates (Utter *et al.*, 1945; Crane and Sols, 1953; Johnson, 1960; Beattie *et al.*, 1963). Only the particulate form of the enzyme had been described (Crane and Sols, 1953, 1955) when this investigation was initiated. Subsequently, Moore and Strecker (1963) and Jagannathan (1963) reported methods for the solubilization and purification of brain hexokinase; however, low yields were obtained and no information regarding purity was reported. This paper describes isolation and purification of solubilized hexokinase from bovine brain and documents some of its physical and chemical properties.

Materials

The following materials were obtained commercially: glucose 6-phosphate dehydrogenase (type 5), ATP,[1] ADP, TPN^+, EDTA, and Tris from Sigma Chemical Co., St. Louis, Mo.; three-times-crystallized α-chymotrypsin (salt free) and purified pancreatic lipase from Worthington Biochemical Corp., Freehold, N. J.; sodium deoxycholate from Matheson Coleman and Bell, Rutherford, N. J.; ammonium sulfate, special enzyme grade, from Mann Research Laboratories, N. Y.; Sephadex gels from Pharmacia, Uppsala, Sweden; DEAE-cellulose (Cellex D) from Bio-Rad Laboratories, Richmond, Calif.; L-histidine monohydrochloride from

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[1] Abbreviations used: ADP and ATP, adenosine di- and triphosphates; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides.

Nutritional Biochemical Corp., Cleveland, Ohio; bovine serum albumin from Pentex Inc., Kankakee, Ill.; Triton X-100 (*p*-(*t*-octyl)phenoxypolyethoxyethanol with an average value for oxyethylene groups of nine to ten) from Ruger Chemical Co., Irvington, N. Y. All other reagents of analytical grade quality were obtained from Fisher Scientific Co., Pittsburgh, Pa. Tris was recrystallized twice from 95% ethanol before use.

Methods

Hexokinase activity was measured spectrophotometrically with a Zeiss Model M4Q spectrophotometer in a system in which glucose 6-phosphate production was coupled to TPNH formation in the presence of glucose 6-phosphate dehydrogenase. A similar assay procedure has been described by Bennett *et al.* (1962). The reaction cuvet at 30° contained 0.2 ml of 0.4 M Tris–0.015 M EDTA–0.015 M $MgCl_2$ (pH 8.0), 0.2 ml of 0.075 M ATP–0.080 M $MgCl_2$ (pH 7.0), 0.1 ml of 0.25 M glucose, 0.1 ml of 0.01 M TPN^+ (pH 7.0), 0.05 ml of glucose 6-phosphate dehydrogenase (0.1 mg of protein/ml) in 0.15 M glycylglycine buffer (pH 8.0), and 0.25 ml of water. The reaction was initiated by addition of 0.1 ml of an enzyme sample diluted with either 0.002 M EDTA (pH 7.0) or 1% bovine serum albumin–0.002 M EDTA (pH 7.0).

The rate of TPNH formation measured at 340 mμ was linear for at least 5 min in the range 0.010–0.100 AU/min. In the same range, the rate of TPNH formation was proportional to hexokinase concentration. The micromoles of glucose 6-phosphate produced per minute were obtained by dividing the observed absorbancy changes by 6.22 $cm^2/\mu mole$. One unit of enzyme activity is defined as the formation of 1 μmole of glucose 6-phosphate/min under the above conditions.

The assay procedure for the kinetic analysis of purified brain hexokinase was similar to the standard assay procedure except that the concentration of glucose and the ATP–$MgCl_2$ mixture (1:2 mole ratio) were varied as desired. In some cases, the EDTA–$MgCl_2$ portion of the buffer mixture was omitted from the reaction mixtures. Rate measurements at 25° were made on a Cary Model 14 spectrophotometer equipped with a 0.0–0.2 AU slide wire.

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Correction for the absolute differences in color development for different proteins was made as indicated below.[2] Aliquots of 0.2, 0.4, 0.6, and 0.8 ml from the standard bovine serum albumin solution (0.250 mg dry weight/ml) or appropriately diluted samples were assayed according to the method of Lowry, and the slope of the curve, absorbancy units at 759 mμ *vs.* volume of protein solution, was determined. All curves obeyed Beer's law in the absorbancy range 0.080–0.500. Comparison of the slopes of sample and standard curves according to the equation $C_2 = DC_1s_2/s_1$ (where D = dilution factor, C_1 = concentration of standard in milligrams of protein per milliliter, C_2 = concentration of sample in milligrams of protein per milliliter, s_1 = slope of standard curve, and s_2 = slope of sample curve) gave the corrected protein concentration of the sample. Particulate enzyme samples were solubilized by the method of Stahl *et al.* (1963) and the protein was determined as above.

Concentration of Enzyme Solutions. The procedure for concentrating enzyme solutions was based on the method of Flodin *et al.* (1960) using G-25 Sephadex (coarse beads). At each concentration step, enough dry Sephadex was added to absorb five-twelves of the volume (G-25 Sephadex absorbs 2.5 ± 0.2 g of water/g of dry gel). The Sephadex beads were allowed to swell in the enzyme solution for 15 min with intermittent stirring. The gel–enzyme suspension was then transferred to the inner chamber of the filter tube shown in Figure 1 and centrifuged at 1000 rpm for 3 min (Servall GSA rotor).

After centrifugation, the concentrated solution was transferred from the outer container to a chilled graduated beaker. The centrifuged gel in the inner chamber was washed twice with 2 or 3 ml of the appropriate buffer, and the wash was combined with the concentrated enzyme solution. The final concentration step was carried out as above except that in order to obtain a final volume of 60 ml, the amount of Sephadex added was usually less than the amount required to absorb five-twelves volume of enzyme solution.

The gel after use was washed with large volumes of water until the conductivity of the wash water was that of deionized water. The gel was regenerated by washing with methanol and drying at 80° as described by Flodin *et al.* (1960).

Preparation of Columns. DEAE-cellulose columns were prepared according to Peterson and Sober (1962) and the Sephadex columns according to directions of the manufacturer.

Electrophoresis. Electrophoretic studies were carried out on cellulose acetate strips (2.5 × 12 cm) by the

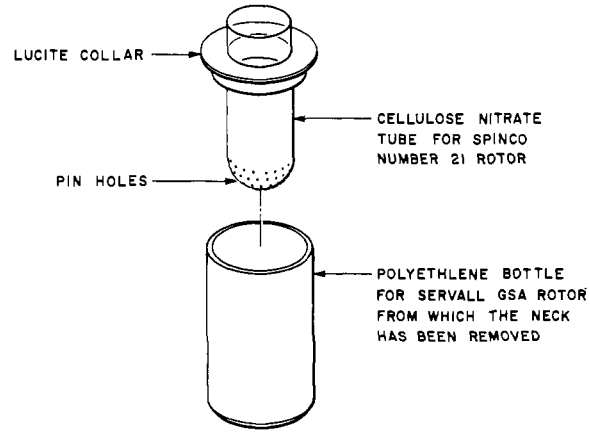

FIGURE 1: Filter tube.

[2] We thank Dr. C. J. Martin for the correction method.

method of Kohn (1960). Electrophoresis was carried out on a Shandon Universal Electrophoresis apparatus at room temperature using a Shandon Vokam No. 2541 power supply (Colab Laboratories, Inc., Chicago Heights, Ill.). Buffers of constant ionic strength were prepared according to the method of Datta and Grzybowski (1961). After the electrophoretic runs, the strips were dried and then stained with napthalene black and overstained with nigrosin as described by Kohn (1960).

The buffer composition of protein solutions was changed by using G-25 Sephadex gel in a special procedure devised for desalting small volumes (1–4 ml). G-25 Sephadex (coarse beads) was allowed to swell for 15 min in the desired buffer. A volume of gel which was 3.3 times the volume of the protein sample to be desalted was transferred to the inner chamber of the filter tube (cellulose nitrate tube for the Servall SS-34 rotor). The gel was washed with 100 ml of buffer, after which the washed gel was centrifuged at 1000 rpm for 3 min in a Servall GSA rotor to remove interstitial buffer. The protein solution was carefully layered on top of the centrifuged gel; after 2 min, the gel–protein suspension was centrifuged at 1000 rpm for 3 min and the desalted protein solution was removed from the outer chamber. The efficiency of the desalting technique was monitored by conductance as measured with a Conductivity Bridge, Model RC 216B2, Industrial Instruments, Cedar Grove, N. J.

Ultracentrifugation. The enzyme suspension in ammonium sulfate was centrifuged at 5000*g* for 10 min (Servall SS-34 rotor). The supernatant was discarded and the precipitate was dissolved in 0.01 M potassium phosphate–0.002 M EDTA (pH 7.2) to give a protein concentration of 8.5 mg of protein/ml. A 0.6-ml aliquot was analyzed in a Spinco Model E analytical ultracentrifuge equipped with the schlierin optical system.

Amino Acid Analysis. A 0.4-ml aliquot of the ammonium sulfate precipitated enzyme dissolved in 0.05 M potassium phosphate–0.001 M EDTA (pH 7.2, 8.0 mg of protein/ml) was hydrolyzed in a sealed tube with 6 N hydrochloric acid for 24 hr at 120°. The hydrolysate was analyzed in a Spinco automatic amino acid analyzer.

Results

Purification of Particulate Hexokinase. All operations were carried out at 0–4° unless stated otherwise. The pH of all solutions was measured at room temperature. The preparation of particulate hexokinase is based on the method of Crane and Sols (1953, 1955) except that the deoxycholate-treated enzyme was further purified by treatment with Triton X-100. Step 2 of the Crane and Sols procedure involves a digestion of the particulate preparation with pancreatic lipase. Great variations in the results of lipase digestion were observed. It was finally determined that the major active ingredient in the lipase preparation was, in fact, α-chymotrypsin. When three-times-crystallized α-chymotrypsin was substituted for pancreatic lipase, reproducible results were obtained. The potassium phosphate buffers were prepared according to the table given by Green and Hughes (1955).

Fresh beef brains from animals killed by exsanguination were obtained from the slaughter house. The brains were placed in a polyethylene bag and stored in ice for transport to the laboratory. After removal of the meninges, 500 g of cortical tissue was carefully scraped from six brains into 1500 ml of 0.1 M potassium phosphate (pH 6.8, buffer 1).

The scrapings were homogenized in a Waring Blendor for 3 min at maximum speed. The average lapse of time between death of animals and homogenization was 90 min. The homogenate was centrifuged at 800*g* for 20 min in the International centrifuge, Model HR 1 (rotor 284).[3] The supernatant material was carefully removed by aspiration and saved. Sediment 1 was reextracted by stirring with 1500 ml of buffer 1 and centrifuged as before. The second supernatant material was carefully removed by aspiration and sediment 2 was discarded. The two supernatant fluids were combined and centrifuged at 5090*g* in the Servall centrifuge (GSA rotor) for 40 min.[4] The supernatant fluid was discarded and sediment 3 was dispersed in 800 ml of 0.05 M potassium phosphate (pH 6.3, buffer 2), using a hand-operated, ground-glass Potter-Elvehjem homogenizer.[5] The homogenate was centrifuged at 6780*g* for 40 min. Sediment 4 was dispersed in buffer 2 (final volume, 600 ml) and centrifuged as before. Sediment 5 was dispersed in buffer 2 (final volume, 500 ml) and stored overnight at 5°.

To the 500 ml of sediment 5 suspension (containing about 4000 mg of protein) were added an additional 480 ml of buffer 2 and 260 mg of three-times-crystallized α-chymotrypsin (65 mg dry weight/g of sediment 5 protein) dissolved in 20 ml of buffer 2. The suspension was incubated for 2 hr in a 30° water bath. At the end of the incubation period, the suspension was centrifuged at 10,800*g* (GSA rotor) for 40 min. The supernatant fluid was discarded and sediment 6 was dispersed in 500 ml of buffer 2 and centrifuged as above. Sediment 7 was dispersed in 360 ml (final volume) of 0.05 M potassium phosphate (pH 7.2, buffer 3), and 40 ml of 3.3% sodium deoxycholate (pH 7.5–8.0) was slowly added with stirring. After stirring for 15 min, the mixture was immediately centrifuged at 13,500*g* (Servall SS-34 rotor) for 30 min.[6] Sediment 8 was dispersed in 300 ml of buffer 3 and centrifuged again at 13,500*g* for 30 min. Sediment 9 was dispersed in 195 ml (final volume) of 0.05 M Tris–0.005 M EDTA (pH 8.0, buffer 4), and 5 ml of 20% Triton X-100 (weight/

[3] All centrifugal forces are given as average values calculated for the center of the tube.

[4] The average radius of the GSA rotor was taken to be 4.25 in.

[5] All homogenizations after the Waring Blendor treatment were performed using hand-operated, ground-glass homogenizers.

[6] For calculation using the SS-34 rotor, a radius of 3 in. was used.

TABLE I: Purification of Particulate Hexokinase.

Step	Vol. (ml)	Protein (mg)	Units	Sp Act. (units/mg of protein)	Recov (%)	Purifcn
Homogenate of 500*g* tissue	3,000	90,112	6,308	0.07	100	—
Sediment 5	500	4,000	2,800	0.70	44	10
Sediment 5, α-chymotrypsin treated	360	2,419	2,670	1.10	42	16
Sediment 9, DOC treated	195	341	1,880	5.50	30	80
Sediment 12, Triton treated	70	205	1,500	7.30	24	104

volume) was added with stirring. After stirring for 20 min, the material was centrifuged at 20,000*g* (SS-34 rotor) for 30 min. Sediment 10 was dispersed in 200 ml of 0.01 M histidine–0.01 M Tris–0.002 M EDTA (pH 7.0, buffer 5) and centrifuged at 20,000*g* for 30 min. Sediment 11 was dispersed in 175 ml of buffer 5 and centrifuged as above. Sediment 12 was transferred from the centrifuge tubes to the homogenizer with three 10–12-ml portions of buffer 5. After dispersing the sediment, an equal volume of cold glycerol was added with gentle stirring and the glycerol suspension was stored at 2°. The final particulate preparations regardless of purity were stable for several months when stored in 50% glycerol as stated by Crane and Sols (1955). Typical results are shown in Table I.

Solubilization of Brain Hexokinase. The procedure for obtaining reproducible solubilization of hexokinase depends on a preliminary centrifugation of the particulate enzyme in 25% glycerol to remove lipid contaminants which reduce the solubilizing action of Triton X-100. The method given is for the solubilization of four particulate preparations. The solubilization procedure may be used for any quantity of particulate enzyme; however, optimum purification of the soluble enzyme is obtained when no more than four particulate preparations are used as a source of soluble enzyme.

Four particulate brain preparations (sediment 12) in 50% glycerol were diluted to a final glycerol concentration of 25% with buffer 5. The suspension was centrifuged at 20,000*g* for 30 min (Servall SS-34 rotor) and the lipid-containing supernatant was discarded. The sediment was dispersed with buffer 5 using one-third the volume of the diluted glycerol suspension and centrifuged as before. The sediment, freed from as much supernatant as possible, was dispersed in enough 0.08 M Tris–0.005 M EDTA containing 1% Triton X-100 (pH 9.1) to provide a concentration of 4–7 mg/ml.[7] After 20 min with intermittent stirring, the pH was adjusted to 8.0 (measured at 2°) with 2.0 M potassium phosphate–0.002 M EDTA (pH 7.7). The mixture was centrifuged at 78,000*g* for 30 min. The supernatant fluid (first soluble fraction) was carefully collected to exclude any sedimented material. Table II shows the large dependence of the solubilization process on pH, and the absolute requirement of Triton X-100 for solubilization.

TABLE II: Solubilization of Brain Hexokinase as a Function of pH and Triton X-100 Concentration.

pH	Triton X-100 Concn (%)	Units of Triton-Treated Mixture[a]	Units of First Soluble Fraction	Solubilizn (%)
9.00	1.0	5310	4900	92
8.85	1.0	5100	3100	60
8.70	1.2	5400	1620	30
9.00	0.0	1000	0	0
9.00	0.5	1000	680	68

[a] There was no loss of hexokinase activity during the Triton incubation.

Purification of Solubilized Enzyme. The solubilized enzyme obtained from four particulate preparations after centrifugation at 78,000*g* was concentrated to a volume of 60 ml. The dark red solution was desalted on a 2 × 70 cm G-25 Sephadex column (coarse beads) which had been washed with 500 ml of 0.05 M potassium phosphate–0.002 M EDTA (pH 7.7).[8] The desalted material was placed on a DEAE-cellulose column (2 × 70 cm DEAE-cellulose, 0.73 mequiv/g) which had been washed with 600 ml of the phosphate–EDTA buffer as above, and then with 500 ml of the same buffer containing 0.3% Triton X-100 (eluting buffer). Fractions of 5 ml were collected at a flow rate of 0.5 ml/min. A dark brownish green material[9] slowly moved down the column, but usually did not interfere

[7] Protein was determined before centrifugation of the washed sediment; the volume of solubilizing buffer used for four particulate brain preparations was never more than 180 ml.

[8] The flow rate was 2 ml/min; the column was monitored by following the movement of the dark red solution through the column.

[9] The brownish green contaminants could be eluted from the column with 0.4 M potassium phosphate (pH 7.8) containing 1% Triton X-100 and the major contaminant was shown to be cytochrome oxidase (unpublished observation).

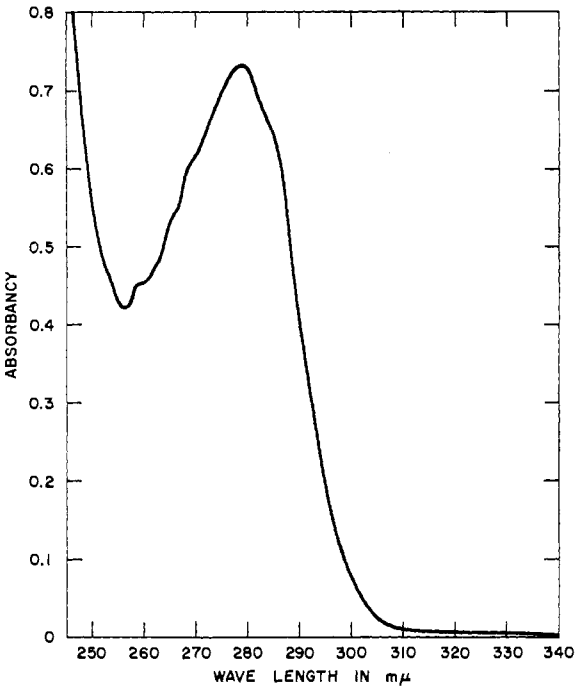

FIGURE 2: Absorption spectrum of purified brain hexokinase in 0.05 M potassium phosphate–0.002 M EDTA (pH 7.8, 1.32 mg of protein/ml). Measurements were made in a Cary Model 14 spectrophotometer using cells of 1-cm light path. The reference cell contained the buffer mixture.

with the much faster elution of hexokinase as detected by enzyme assay.[10] The material obtained gave erratic activity measurements when the enzyme was diluted with 0.002 M EDTA (pH 7.0). Reproducible activity measurements which were twofold higher were obtained when 1% bovine serum albumin–0.002 M EDTA (pH 7.0) was used as the enzyme diluent.

The clear hexokinase fractions were collected and concentrated to 60 ml with G-25 Sephadex. The concentrated solution was desalted by passage through a 2 × 70 cm G-25 Sephadex column[11] which had been washed with 500 ml of 0.005 M potassium phosphate–0.002 M EDTA (pH 7.7). The conductance of the desalted material was determined, and if identical with the phosphate–EDTA buffer, the material was placed on a DEAE-cellulose column (1 × 20 cm, 0.73 mequiv/g) which had been washed with 200 ml of phosphate–EDTA buffer as above. The column was washed with the same buffer until the detergent was removed (as detected by absorption at 280 mμ). The enzyme was eluted from the column with 0.05 M potassium phosphate–0.02 M EDTA (pH 7.7). Fractions of 1.5 ml at a flow rate of 0.5 ml/min were collected and those containing enzyme were detected by absorption at 280 mμ.

The purified enzyme was concentrated to a protein concentration of 2–2.5 mg/ml and solid glucose was added to a final concentration of 0.02 M. Saturated ammonium sulfate (adjusted with ammonium hydroxide to pH 7.2–7.3 after a 1:5 dilution with distilled water) containing 0.02 M glucose and 0.005 M EDTA was slowly added with gentle stirring to a saturation of 0.64. The suspension which formed was stable at 2° for at least 1 week. Purification and recoveries of enzymic activity at the various stages of purification are shown in Table III.

Remarks

It was necessary to free the gray matter as much as possible from the white matter in order to obtain efficient detergent action at the deoxycholate and Triton X-100 steps. Inefficient detergent action due to white matter (lipid) contamination was evidenced by a fairly large amount of tan, creamy material overlayering the centrifuged sediment after the deoxycholate step. Such preparations had only a slightly lower specific activity but were unsuitable as a source of soluble enzyme because of their large cytochrome oxidase content. When these particulate preparations were used as the starting material for preparation of the solubilized enzyme, the contaminants occupied 80–100% of the first DEAE-cellulose column. In such cases, adequate separation of the hexokinase from these contaminants was sometimes difficult to obtain. The same results were observed when more than four particulate preparations were used as a source of soluble enzyme.

Both the enzyme and Triton X-100 were excluded from G-25 Sephadex. The detergent concentration after concentrating the first soluble fraction to 60 ml was 3%. At this detergent concentration, the cytochrome contaminants usually did not occupy more than 50% of the DEAE-cellulose column. Under these conditions, hexokinase was easily separated from the contaminants. When the amount of solubilizing buffer used was greater than 180 ml, the Triton concentration after concentrating was greater than 3% and the cytochrome contaminants occupied a greater percentage of the column space. Recovery of enzymic activity from the first DEAE-cellulose column in many cases was less than 80% (40–55%). The reason for the low recoveries has not been determined, but the extent of recovery may also be a function of the manner in which particulate enzyme was prepared. Because of the low recoveries, the amount of purified enzyme obtained after the second DEAE-cellulose chromatography varied from 8 to 28.5 mg.

The ammonium sulfate fractionation was used only to concentrate the enzyme for the physical measurements. As shown in Table III, the amount of enzyme recovered after the fractionation was only 65% with

[10] The high absorbancy of the detergent at 280 mμ prevented the detection of protein by spectrophotometric techniques. Enzyme activity usually appeared after the first 100 ml of buffer was collected.

[11] Fractions of 5 ml were collected and the column was monitored by enzyme assay.

TABLE III: Purification of Solubilized Enzyme.

Step	Vol. (ml)	Protein (mg)	Units[a]	Sp Act. (units/mg)	Recov (%)	Purifcn
First soluble fraction	230	700	5290	7.50	100	107
Concentrating and desalting	70	—	4232	—	80	—
First DEAE-column eluate	200	—	3385	—	64	—
Concentrating and desalting	70	—	2538	—	48	—
Second DEAE-column eluate	10	28.5	2284	80.0	43	1129
$(NH_4)_2SO_4$ fractionation[b]	2.5	18.7	1496	80.0	28	1129
Mother liquor from $(NH_4)_2SO_4$ fractionation	30	—	304	—	5.7	—

[a] The enyzme after the first DEAE column was diluted with 1% bovine serum albumin–0.002 M EDTA, pH 7.0, for activity measurements. [b] The ammonium sulfate suspension stored for either 1 day or 1 week was centrifuged at 5000*g* for 10 min (Servall SS34 rotor). The supernatant was decanted, and the precipitate was dissolved in 0.05 M potassium phosphate–0.002 M EDTA, pH 7.2, and any insoluble material remaining was removed by centrifugation.

TABLE IV: Kinetic Constants of Purified Brain Hexokinase.[a]

ATP (M)	App K_m of Glucose (M)	V_m of Glucose (μmoles of glucose-6-P/min)	Glucose (M)	App K_m of ATP (M)	V_m of ATP (μmoles of glucose-6-P/min)
2.0×10^{-3}	5.0×10^{-5}	2.5×10^{-3}	1.2×10^{-3}	4.89×10^{-3}	8.3×10^{-3}
7.5×10^{-4}	3.0×10^{-5}	1.5×10^{-3}	5.0×10^{-5}	2.83×10^{-3}	4.3×10^{-3}
2.5×10^{-4}	1.3×10^{-5}	3.2×10^{-4}			

[a] Rate measurements were made as described in text; the enzyme concentration in the reaction mixtures was 7.0×10^{-2} μg/ml.

respect to the second DEAE column eluate. Glucose was present during the salt fractionations in the hope that it would protect against the denaturation. Mild techniques for the addition of saturated ammonium sulfate such as dialysis against ammonium sulfate solutions or addition of the salt solution by use of micropipets gave the same results. However, it was observed that the active enzyme in the suspension remaining after addition of ammonium sulfate to 0.65 saturation was stable for at least 1 week. In all cases, the soluble portion of the ammonium sulfate precipitates (active enzyme) had the same specific activity as the second DEAE column eluate (80 units/mg of protein).[12] The ammonium sulfate precipitates dissolved in 0.05 M potassium phosphate were stable for at least 1 month at −20°.

Ultraviolet Absorption Spectrum. A spectrum of purified brain hexokinase from the second DEAE column is shown in Figure 2. The spectrum is typical of protein except for the low absorbancy at 280 mμ ($A_{1\,cm}^{1\%}$ 5.53) and the fine structure observed below 280 mμ. The ratio of absorbancy at 280 and 260 mμ is 1.6.

Ultracentrifuge Analysis. The ammonium sulfate precipitated enzyme dissolved in 0.1 M potassium phosphate–0.002 M EDTA (pH 7.2, 8.5 mg of protein/ml) was analyzed in a Spinco Model E ultracentrifuge for evidence of inhomogeneity. The schlieren patterns (Figure 3) indicated that the material was homogeneous in the ultracentrifuge. The uncorrected sedimentation constant calculated from the data is 4.44 S at 21°.

Cellulose Acetate Electrophoresis. The ammonium sulfate precipitated enzyme was examined for inhomogeneity by electrophoresis on cellulose acetate paper. Short-term electrophoresis at four different pH values in phosphate and Tris buffers indicated that the enzyme was negatively charged between pH 6.4 and 8.0. It was observed that there was an abnormally large amount of trailing in the Tris buffers. Long-term electrophoresis of the enzyme sample in phosphate buffers in the pH range 6.4–8.0 indicated that the enzyme was electrophoretically homogeneous.

Kinetic Properties. Kinetic data for the ammonium sulfate precipitated enzyme dissolved in 0.1 M potassium

[12] The insoluble material was found to have only trace amounts of hexokinase activity.

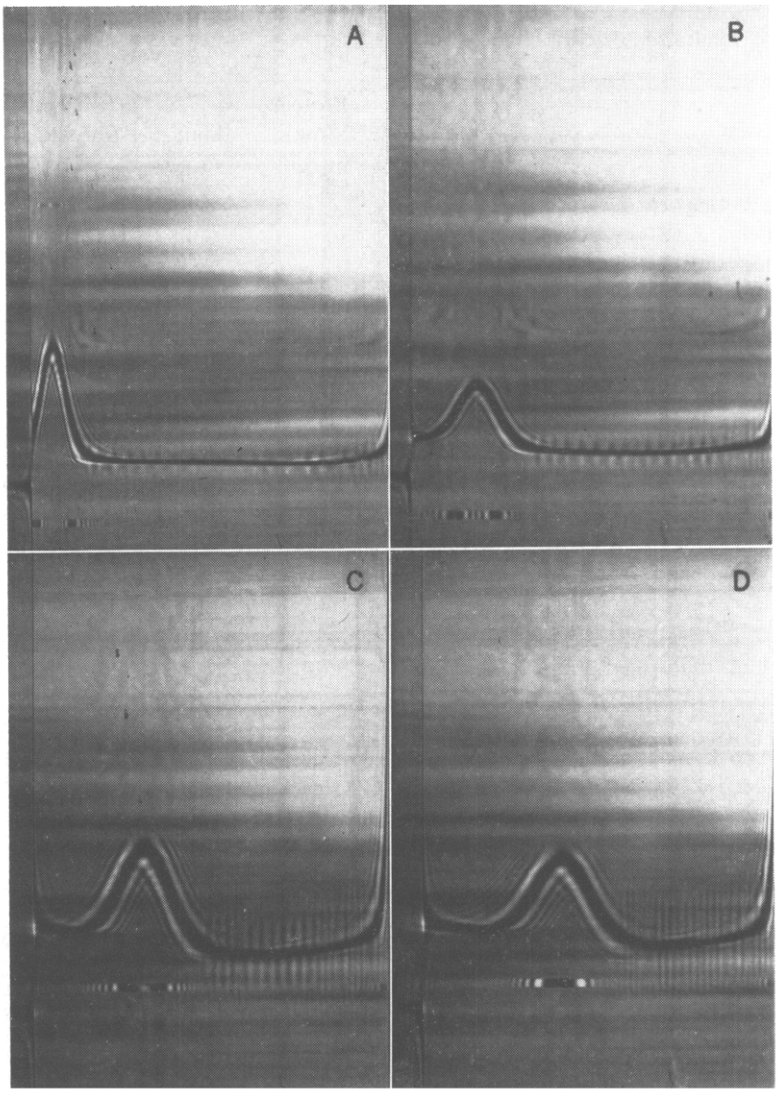

FIGURE 3: Sedimentation of purified hexokinase in the Spinco Model E analytical ultracentrifuge. Velocity, 42,040 rpm; temperature, 21°. Photographs were taken at (A) 15, (B) 60, (C) 105, and (D) 135 min after maximal velocity was obtained. The bar angle was 70° for photographs A and B and 50° for photographs C and D.

phosphate–0.002 M EDTA (pH 7.0) were plotted according to the equation of Dixon and Webb (1957).

$$\frac{S}{V} = \frac{K_m}{V_m} + \frac{1}{V_m}S$$

The plots for glucose as the variable substrate at various fixed ATP concentrations are shown in Figure 4. These results indicated that the apparent K_m and V_m for glucose decreased as the concentration of ATP was lowered, but the ratio of K_m to V_m remained constant. Omission of the EDTA–$MgCl_2$ portion of the Tris buffer did not alter the kinetic properties. The K_m and V_m values calculated from the plots are shown in Table IV.

Similar plots for ATP as the variable substrate are shown in Figure 5. In this case, the apparent K_m and V_m for ATP decreased as the fixed glucose concentration decreased, but the ratio of K_m to V_m was not constant. Omission of the EDTA–$MgCl_2$ portion of the Tris buffer did not alter the kinetic properties. The K_m and V_m values calculated from the plots are shown in Table IV.

Amino Acid Analysis. A partial amino acid analysis of brain hexokinase is shown in Table V. The results indicated that the enzyme has a low content of tyrosine, proline, and histidine and a high content of glycine, leucine, aspartic acid, and glutamic acid. The other amino acids detected varied between the two extremes. Only a trace amount of half-cystine was detected in the hydrolysate; however, analysis for the major transformation product of half-cystine, cysteic acid, was not carried out.

TABLE V: Partial Amino Acid Analysis of Pure Brain Hexokinase.

Amino Acid	μmole[a]	Molar Ratio
Histidine	0.06	1.00
Lysine	0.19	3.17
Arginine	0.15	2.50
Ammonia	0.35	5.83
Glutamic acid	0.32	5.33
Aspartic acid	0.30	5.00
Half-cystine	trace	—
Glycine	0.26	4.33
Alanine	0.16	2.66
Valine	0.19	3.17
Leucine	0.29	4.83
Isoleucine	0.14	2.33
Serine	0.17	2.83
Threonine	0.17	2.83
Methionine	0.10	1.67
Phenylalanine	0.13	2.17
Tyrosine	0.06	1.00
Proline	0.07	1.17

[a] Analysis of a hydrochloric acid hydrolysate of 3.2 mg of purified hexokinase.

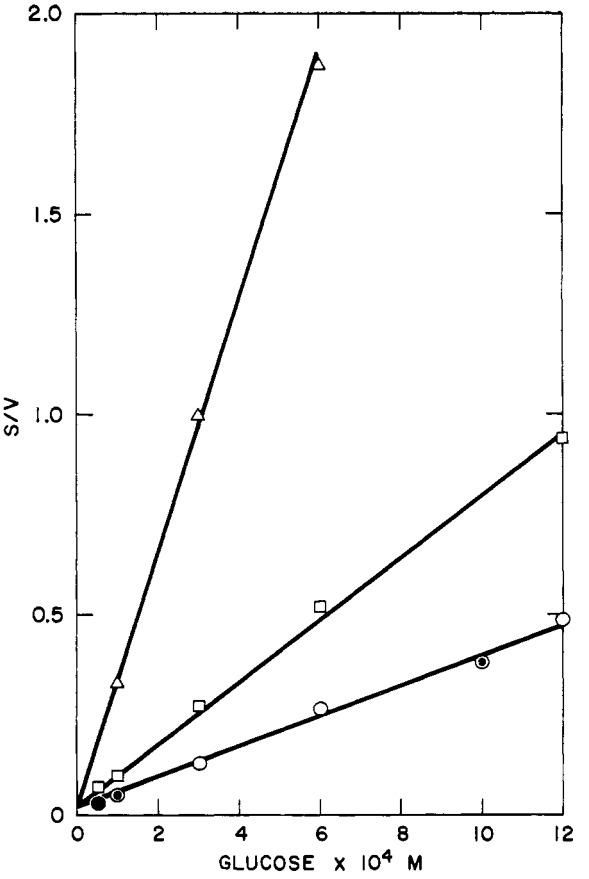

FIGURE 4: Plot of the molar concentration of glucose (S)/initial reaction velocity (V) *vs.* the molar concentration of glucose. Velocities (V) are expressed as micromoles of glucose 6-phosphate produced per minute. ATP concentrations were held constant at 2×10^{-3} (open circles), 7.5×10^{-4} (squares), and 2.5×10^{-4} M (triangles). In one case, the measurements for an ATP concentration of 2×10^{-3} M were repeated with $MgCl_2$–EDTA portion of the Tris buffer omitted (closed circles). The enzyme concentration in the reaction cuvet was 7.0×10^{-2} μg/ml. Other experimental details are described in the text.

Although alkaline hydrolysis was not carried out to determine tryptophan content, the low absorbancy at 280 mμ (Figure 2) indicates an absence or low content of this amino acid. The amino acid analysis can only be considered approximate because the completeness of hydrolysis and the degree of hydrolytic destruction for each amino acid have not been determined.

Discussion

The method for the isolation of highly purified brain hexokinase involved the following major items: (1) an initial removal of contaminants from the particulate material by successive treatments of the sediment with chymotrypsin, deoxycholate, and Triton X-100; (2) solubilization of the treated sediment with Triton X-100 at a high pH; and (3) purification of the solubilized enzyme by chromatography on DEAE-cellulose. This procedure has resulted in a 1129-fold purification of brain hexokinase.

The only step in the procedure which gave unreproducible recoveries was the chromatography of hexokinase on the first DEAE-cellulose column. The requirement of a small amount of Triton for the elution of enzyme indicated that the chromatography was not based solely on ion-exchange principles. One explanation is that hexokinase was bound to certain contaminants adsorbed to the column. A small amount of Triton would then break the contaminant–hexokinase complex without desorption of the contaminants from the column. The frequent low recoveries from the DEAE column can also be explained by the contaminant–hexokinase hypothesis. In some cases, the purified particulate enzyme preparations contained hexokinase-binding contaminants to such a degree that the amount of Triton used was not sufficient for efficient elution of enzyme. Although increasing the detergent concentration probably would increase the yield of hexokinase, higher concentrations of detergent were not used because the binding of contaminants to DEAE-cellulose would be greatly reduced.

Regardless of the yield of enzyme, this procedure has resulted in the isolation of brain hexokinase with a specific activity greater than previous solubilized brain hexokinase preparations (80 units/mg of protein compared to 40 units/mg of protein for the preparation

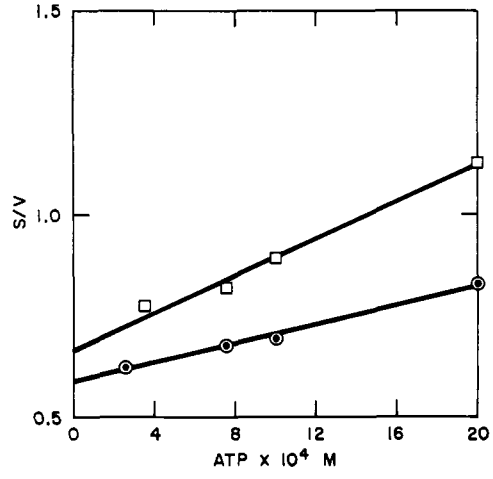

FIGURE 5: Plot of the molar concentration of ATP (S)/initial reaction velocity (V) *vs.* the molar concentration of ATP. V is expressed as in Figure 4. Glucose concentrations were held constant at 1.2×10^{-3} (open circles) and 5×10^{-5} M (squares). The measurements at a glucose concentration of 1.2×10^{-3} M were repeated with the $MgCl_2$–EDTA portion of the Tris buffer omitted (closed circles). The enzyme concentration in the reaction cuvet was 7.0×10^{-2} μg/ml. Other experimental details are described in the text.

of Jagannathan (1963) and 23 units/mg of protein for the preparation of Moore and Strecker (1963)).

Ultracentrifugation and electrophoresis on cellulose acetate paper indicated that the purified enzyme was homogeneous with respect to these two criteria of purity. Immunochemical evidence[13] of purity will be presented in a subsequent publication.

The low absorbancy of hexokinase at 280 mμ indicates the protein has a low content of tryptophan and tyrosine. This observation is not unusual for kinases since crystalline 3-phosphoglycerate kinase from yeast (Malmström and Larson-Raznikiewicz, 1962) and purified pyruvate kinase from rabbit and human muscle (Boyer, 1962) have absorbancies at 280 mμ almost identical with that of brain hexokinase. In addition, the ratio of absorbancies at 280 and 260 mμ are very similar for all four enzymes.

The appearance of fine structure in the spectrum of brain hexokinase is an unusual observation; however, Kawahara *et al.* (1962) have observed detailed fine structure in crystalline Δ^5-3-keto steroid isomerase. The fine structure of the isomerase was attributed to the spectral contributions of phenylalanine and tyrosine. The authors reasoned that the fine structure was observable because of the absence of tryptophan which, if present would obscure the absorption bands of tyrosine and phenylalanine because of its much higher molar absorbancy index.

The kinetic properties of purified brain hexokinase with respect to glucose (Table IV, Figures 4 and 5) are the same as reported by Fromm and Zewe (1962) for the particulate enzyme. The properties of the enzyme with respect to ATP do not appear to be the same as the particulate enzyme. The K_m values for ATP (Table IV) are much higher than the values reported for the particulate enzyme under the same conditions ($2.92–1.13 \times 10^{-4}$ M) and K_m/V_m term for the solubilized enzyme is not independent of the fixed glucose concentration. Whether these differences can be reconciled with the known properties of the particulate enzyme must await further studies.

Acknowledgments

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Properties of Cyclic 3′,5′-Nucleotide Phosphodiesterase from Rat Brain*

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ABSTRACT: A cyclic 3′,5′-nucleotide phosphodiesterase from a 30,000*g* supernatant of rat brain cortex showed maximal activity around pH 8 with a K_m of 0.1–0.3 mM. The energy of activation was 7.5 kcal/mole between 25 and 35° and 3.5 kcal/mole between 38 and 45°. Optimal activity was observed at 45°. The enzyme required Mg^{2+} or Mn^{2+} for full activity. In the absence of added divalent cations, it exhibited about one-third of maximal activity. When brains were homogenized in water or isotonic sucrose solution, one-half of the potential enzymic activity was latent, which could be unmasked by the addition of Triton X-100 The enzyme was strongly inhibited by inorganic polyphosphates and nucleoside triphosphates. Citrate and methylxanthines were also inhibitory, but less potent than the nucleoside triphosphates. The inhibition by adenosine triphosphate was pH dependent, but that by inorganic pyrophosphate was not. It is believed that the active form of phosphodiesterase is a metal–enzyme complex and that the mechanism of inhibition by inorganic polyphosphate, nucleoside triphosphates, and citrate is *via* chelating the metal ion in the enzyme.

Adenosine 3′,5′-phosphate (cyclic AMP)[1] exerts its action in a variety of ways at the level of cellular metabolism. As a mediator of catecholamines, it triggers glycogenolysis by activating phosphorylase *b* kinase, which in turn converts phosphorylase *b* to *a* (Posner *et al.*, 1962). It stimulates phosphofructokinase (Mansour and Mansour, 1962), usually the rate-limiting step of glycolysis, thus allowing increased glycolytic flux. On the other hand, glycogen synthesis is probably kept low as cyclic AMP increases the conversion of glycogen synthetase I to D (Rosell-Perez and Larner, 1964). Cyclic AMP also affects pigmentation of the frog skin (Bitensky and Burnstein, 1965), hydroxylation of steroid hormones (Roberts *et al.*, 1964), permeability of the toad bladder (Orloff and Handler, 1962), and the oscillations of NADH in a cell-free extract of *Saccharomyces carlsbergensis* (Cheung, 1966a). Although tissue levels of cyclic AMP are generally low, marked increases have been noted in *Escherichia coli* changing from a glucose medium to one containing no glucose (Makman and Sutherland, 1965), in perfused rat heart following administration of epinephrine (Cheung and Williamson, 1965; Robison *et al.*, 1965), and in the brain cortex after decapitation (Rall and Kakiuchi, 1965). The physiological significance of the cyclic AMP change observed in the brain is not known.

Sutherland and Rall (1958) first described a cyclic 3′,5′-nucleotide phosphodiesterase from beef heart. This enzyme hydrolyzes cyclic AMP into 5′-adenosine monophosphate. Studies on tissue distribution show that it is widely distributed and brain cortex is the richest source (Butcher and Sutherland, 1962). Using a subcellular fractionation technique according to De Robertis *et al.* (1962, 1963), we have established that phosphodiesterase of rat brain is mostly microsomal and that considerable soluble activity is concentrated inside the nerve endings (Cheung and Salganicoff, 1966). We have also found that both adenosine 5′-triphosphate and inorganic pyrophosphate are potent inhibitors of phosphodiesterase, suggesting to us that the enzyme might exist in a greatly inhibited state *in vivo* (Cheung, 1966b). This communication describes further some of the properties of phosphodiesterase from the rat brain.

Materials and Methods

Cyclic 3′,5′-Nucleotide Phosphodiesterase. Brain cortices from young male rats of Wistar strain were homogenized in five volumes of chilled glass-distilled water. The homogenate with about pH 6.8 was centrifuged for 30 min at 30,000*g* and the supernatant fluid

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[1] Abbreviations used: cyclic AMP, adenosine 3′,5′-phosphate; TCA, trichloroacetic acid; GMP, guanosine monophosphate; NADH, reduced nicotinamide–adenine dinucleotide; ATP, CTP, UTP, GTP, TTP, and ITP, adenosine, cytidine, uridine, guanosine, thymidine, and inosine triphosphates, respectively.