THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 247, No. 23, Issue of December 10, pp. 7576–7584, 1972
Printed in U.S.A.

Bovine Brain Mitochondrial Hexokinase

SOLUBILIZATION, PURIFICATION, AND ROLE OF SULFHYDRYL RESIDUES

(Received for publication, March 6, 1972)

VILAS D. REDKAR AND UMAKANT W. KENKARE
From The Molecular Biology Unit, Tata Institute of Fundamental Research, Bombay 5, India

SUMMARY

Bovine brain mitochondrial hexokinase, type I, has been solubilized by extraction of the mitochondria in 0.2 M acetate buffer, pH 5.0, containing 0.9 M NaCl. The solubilized enzyme has been purified to apparent homogeneity as shown by ultracentrifugal and electrophoretic criteria. The purification procedure included fractionation of the solubilized enzyme with ammonium sulfate and two successive diethylaminoethyl cellulose chromatographic steps. The sedimentation coefficient, $s_{20,w}$, was found to be 5.9 S at a protein concentration of 1.7 mg per ml. The approximate molecular weight as determined by gel filtration on Sephadex G-200 is 107,000.

The enzyme has 11 to 13 sulfhydryl residues per mole as determined by reaction of the denatured enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Almost all of these residues react with DTNB in the native enzyme though with differing degrees of reactivity. Reaction of the enzyme with excess DTNB caused its rapid inactivation. A comparison of the progress of this inactivation with the progress of the reaction of the sulfhydryl residues of the enzyme with DTNB showed that a maximum of only 2 residues could be involved in the inactivation process. If 2-mercaptoethanol is added to the enzyme immediately after complete inactivation, a rapid and total recovery of enzyme activity ensues. These results have been analyzed in terms of involvement of sulfhydryl residues, in the active conformation of the enzyme.

Substrate glucose partially protects the enzyme against inactivation by DTNB and also modifies the reactivity of the sulfhydryl residues of the enzyme toward this reagent. MgATP, MgADP, and inorganic phosphate even at 10 mM concentration do not protect the enzyme against inactivation by DTNB. Product inhibitor glucose 6-phosphate affords a complete protection to the enzyme against inactivation by DTNB and drastically changes the reactivity of its sulfhydryl residues. Fructose 6-phosphate is without a comparable effect.

Hexokinase activity of brain homogenates is located predominantly in the mitochondrial fraction (1, 2). The work of Katzen, Schimke, and their co-workers (3–5) has shown that the hexokinase in the brain is almost exclusively present as the type I isoenzyme with type II isoenzyme occurring only in very small amounts. The enzyme from bovine brain was partially purified in its particulate form by Crane and Sols (1). Jagannathan (6), Moore and Strecker (7), and Schwartz and Basford (8) described methods for the solubilization and purification of the particulate enzyme. Using pancreatic elastase to release the enzyme from the mitochondrial particles, Joshi and Jagannathan (9) obtained a preparation which was about 90% pure. Schwartz and Basford (8) treated the mitochondria with α-chymotrypsin, deoxycholate, and Triton X-100 and purified the enzyme thus solubilized to a state of homogeneity. A few physical, chemical, and kinetic properties of the solubilized enzyme have been presented in the papers of Schwartz and Basford (8) and of Joshi and Jagannathan (9). Grossbard and Schimke (4) have also partially purified type I hexokinase from the soluble fraction of rat brain homogenates and described some of its kinetic properties.

However, for a study of the physiological and control properties of the enzyme, it seemed desirable to prepare pure brain hexokinase by a procedure which avoids the use of a proteolytic or detergent treatment of the mitochondria. In this paper we describe such a procedure for the solubilization and purification of this enzyme which also gives substantially higher yields than the previously described methods. On the basis of the starch gel electrophoretic criteria of Katzen and Schimke (3), this purified brain hexokinase appears to be exclusively the type I isoenzyme.

Inactivation of the purified enzyme in the absence of EDTA or 2-mercaptoethanol and the reports by Joshi and Jagannathan (9) and Moore (10) on the inhibition of their hexokinase preparations by low concentrations of *p*-CMB[1] led us to an investigation of the role of sulfhydryl residues in the function of this enzyme. The relevant results which have been obtained with the Ellman reagent, DTNB (11), also form the subject matter of the present paper.

MATERIALS AND METHODS

Chemicals—Glucose 6-phosphate dehydrogenase, aldolase, pyruvate kinase, NADP, NADPH, NAD, NADH, ATP, ADP, glucose 6-phosphate, and fructose 6-phosphate were obtained

[1] The abbreviations used are: *P*-CMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.

from Boehringer Mannheim, West Germany. Alkaline phosphatase was prepared as described by Krishnaswamy and Kenkare (12). Myoglobin, ovalbumin, crystalline bovine albumin, and apoferritin were obtained from Mann Research Laboratories. Sephadex G-200 was a product of Pharmacia, Uppsala, Sweden. 2-Mercaptoethanol was a product of Koch-Light Laboratories, Colnbrook, Bucks, England. DTNB and SDS were supplied by Pierce Chemical Company. Analar grade EDTA was a product of BDH division of Glaxo Laboratories, Bombay, India. Guanidine hydrochloride was obtained from Fluka, Switzerland. DEAE-cellulose, sodium pyruvate, phenazine methosulfate, cytochrome *c*, nitro blue tetrazolium, and 2,6-dichlorophenolindophenol were products of Sigma Chemical Company. Crystalline bovine albumin used as a standard for measurement of protein concentrations was obtained from Nutritional Biochemicals Corporation. Acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′,*N*′-tetramethylenediamine, and ammonium persulfate were supplied by Eastman Organic Chemicals. Hydrolyzed starch was supplied by Connaught Medical Research Laboratories, Toronto, Canada.

Enzyme Assays—Hexokinase was assayed spectrophotometrically in a Carl Zeiss spectrophotometer equipped with a Honeywell recording device and a temperature-controlled cell compartment using 1-cm quartz cuvettes. The activity was measured by coupling the formation of glucose 6-phosphate to the reduction of TPN in the presence of glucose 6-phosphate dehydrogenase. The assay system was essentially that developed by Sharma, Manjeshwar, and Weinhouse (13). The reaction mixture maintained at 30° contained 80 mM Tris-chloride buffer, pH 7.4; 0.16 mM TPN; 6 mM ATP; 27 mM glucose; 8 mM $MgCl_2$; 5 mM 2-mercaptoethanol; and 0.4 international unit of glucose 6-phosphate dehydrogenase in a total volume of 3 ml. The reaction was normally started by the addition of 1 to 5 μl of the enzyme sample. The amount of the enzyme was so adjusted that the rate of TPN reduction followed at 340 nm was linear for at least 8 min. Under these conditions, the rate of enzyme activity was first order with respect to enzyme concentration. The optical density change occurring between the 2nd and 5th min after starting the reaction was used for the estimation of enzyme activity.

A unit of enzyme activity is defined as the amount of enzyme which brings about the conversion of 1 μmole of glucose to glucose 6-phosphate under the above conditions.

Alkaline phosphatase was assayed as described by Krishnaswamy and Kenkare (12). Pyruvate kinase and aldolase were assayed on an Eppendorf fluorimeter as described by Maitra and Lobo (14). Succinate dehydrogenase activity was assayed by the spectrophotometric procedure of King (15). Lactate dehydrogenase activity was assayed according to the method of Stolzenbach (16). NADPH-cytochrome *c* reductase activity was determined by the method described by Sottocasa *et al.* (17).

Estimation of Sulfhydryl Residues—Studies on sulfhydryl residues in the enzyme were carried out by the method of Ellman (11) with the use of DTNB. The uumber of sulfhydryl residues reacted was calculated by using an ϵ of 13,600 cm^{-1} M^{-1} for the product, 5-thio-2-nitrobenzoate (11). The concentration of the DTNB stock solution was tested by allowing a suitably diluted aliquot to react with a 100-fold excess of a freshly prepared solution of L-cysteine. Stock DTNB solutions were made in 0.7 M Tris-chloride buffer, pH 7.0, and were used only for the experiments performed on the same day. The reaction with DTNB was carried out in 104 mM Tris-chloride buffer, pH 8.0, containing 0.5 mM EDTA.

Protein Concentration—For experiments with the purified enzyme, protein concentration was determined by the method of Lowry *et al.* (18), with bovine plasma albumin as the standard. Protein concentrations required to monitor enzyme purification were measured by the spectrophotometric procedure of Warburg and Christian (19).

Absorption Spectrum—The ultraviolet spectrum of the enzyme was taken on a Carl Zeiss spectrophotometer.

Sephadex G-200 Chromatography—Chromatography on Sephadex G-200 to determine the approximate molecular weight of purified brain hexokinase was carried out by the method of Andrews (20). Sephadex G-200, Lot To 3379, particle size 40 to 120 μ, was prepared for chromatography according to instructions contained in the Sephadex manual. The column (2.1 × 70 cm) was equilibrated either with a 100 mM phosphate buffer, pH 7.0, containing 100 mM glucose or with a 50 mM Tris-chloride buffer, pH 7.5, containing 150 mM KCl and 100 mM glucose. Myoglobin, ovalbumin, bovine serum albumin, alkaline phosphatase, aldolase, pyruvate kinase, and apoferritin were used as marker proteins. Myoglobin, ovalbumin, bovine serum albumin, and apoferritin were estimated as described by Andrews (20). The enzymes were assayed as described in the section on enzyme assays.

Acrylamide Gel Electrophoresis—Polyacrylamide disc gel electrophoresis of the purified enzyme was carried out at pH values 8.9 and 7.0. For electrophoresis at pH 8.9, the method of Davis (21) was followed with the slight modification that the enzyme sample was dissolved in 20% sucrose and directly loaded on the separating gel. The gels employed at this pH were 7.5 and 5%. For electrophoresis at pH 7.0, the buffer system used was that of Weber and Osborn (22) but without SDS, since the object was only to study the homogeneity of the protein and not to dissociate it into its polypeptide chains. Ten and 5% gels were used at this pH. In either case electrophoresis buffer contained, in addition to the buffer components, 0.5 mM 2-mercaptoethanol, 0.5 mM EDTA, and 10 mM glucose. Electrophoresis was performed at 3–5° at a constant current strength which varied depending on the experiment, between 4 and 8 ma per gel. The positive electrode was located in the lower vessel. Bromphenol blue was used as the tracking dye. Electrophoresis was carried out for about 4 hours. About 50 to 100 μg of the enzyme protein in 150 μl of 50 mM Tris-chloride buffer, pH 7.0, containing 5 mM mercaptoethanol, 5 mM EDTA, and 10 mM glucose were applied to the gels. A 1% solution of Amido black in 7% acetic acid was used to identify the protein bands. Destaining was carried out electrophoretically. Hexokinase activity was detected with an assay solution containing glucose, Mg^{++}, ATP, glucose 6-phosphate dehydrogenase, NADP, phenazine methosulfate, and nitro blue tetrazolium in concentrations employed by Grossbard and Schimke (4) for hexokinase staining in starch gel electrophoresis.

The diameter of the acrylamide gels used in staining for protein bands was 8 mm. For hexokinase activity staining, narrower gels of about 5 mm diameter were employed, as gels of this thickness appeared to give better results. All gels were prerun in appropriate buffers before use. Prerunning of gels was found to be essential for activity staining.

Starch Gel Electrophoresis—Vertical starch gel electrophoresis was carried out according to the procedure of Smithies (23, 24). The conditions of the experiments were the same as those em-

ployed by Grossbard and Schimke (4). The starch gels were stained by the procedure described by these authors (4).

Ultracentrifugal Studies—Sedimentation velocity studies were carried out in a Beckman model E analytical ultracentrifuge equipped with a rotor temperature indicator and a control unit, using AN-D rotor. Schlieren optics was employed for studies on the homogeneity of the protein, and ultraviolet optics was used for measurement of the sedimentation coefficient. In the latter case, the images obtained on the photographic film strips were converted into plots of concentration *versus* distance from the axis of rotation, by using a Beckman Analytrol film densitometer. All sedimentation experiments were carried out at speeds of 59,780 rpm. A 12-mm, 4° sector cell with an aluminum centerpiece was employed for the ultracentrifuge runs. The sedimentation coefficient was corrected to a value corresponding to a solvent with the viscosity and density of water at 20° ($s_{20,w}$).

Sucrose density gradient centrifugation studies were performed by the method of Martin and Ames (25). The gradient was linear between 5 and 20% sucrose dissolved in 50 mM Tris-chloride buffer, pH 8.0, containing 0.5 mM EDTA. Each tube contained the marker proteins, pyruvate kinase, aldolase, *Escherichia coli* alkaline phosphatase, and myoglobin. Their $s_{20,w}$ values are 10.0 (26), 7.35 (27), 6.3 (28), and 2.0 (29), respectively. The enzyme solution together with marker enzymes, in 0.2 ml of the buffer in which the gradient was made, was layered on top of the gradient. The centrifugation was carried out at 36,000 rpm for 20 hours at 0–2° in the Beckman model L_2-65B ultracentrifuge with the use of an SW 39 rotor. Seventy-six 1-drop fractions were collected, and these were assayed for various proteins as described earlier.

RESULTS

Purification of Enzyme

Beef brain mitochondrial hexokinase was solubilized and purified to homogeneity by the procedure described below. Unless otherwise indicated, the purification steps were carried out at 0–4°. Table I summarizes the yields and specific activities through all purification steps. All centrifugations were carried out on a Sorvall RC_2B centrifuge.

Step 1: Homogenate and Combined Supernatants—Beef brains from freshly killed animals were obtained from the slaughterhouse and immediately chilled in ice. In the early stages of this work they were processed within 2 hours of the death of the animal. Experience, however, showed that the yield of the enzyme was not affected if the brains were processed after overnight storage in ice. Since the latter practice was more convenient, it was followed during the course of the work described here.

TABLE I

Summary of typical purification procedure for bovine brain mitochondrial hexokinase

Purification step	Volume	Total protein	Total activity	Specific activity	Yield
	ml	*mg*	*units*	*units/mg*	%
1. Homogenate	7,300	153,300	10,220	0.07	100
2. Combined supernatants	13,500	71,550	7,425	0.10	72
3. Mitochondrial fraction	2,000	16,850	5,900	0.35	58
4. Acetate extract	2,420	4,160	5,445	1.30	53
5. $(NH_4)_2SO_4$ fraction	160	880	4,000	4.50	39
6. 1st DEAE-cellulose column chromatography	32	54	2,880	53.0	27
7. 2nd DEAE-cellulose column chromatography	4.5	22	1,820	83.0	18

Cortical tissue, 1300 g, was carefully scraped from several brains and was suspended into 6500 ml of 250 mM sucrose solution containing 5 mM EDTA. A few drops of caprylic alcohol were added to this suspension. The suspension was then homogenized in a Braun homogenizer at top speed for a total of 3 min made up of three 1-min periods separated by an interval of about 3 min. The homogenate (A) was centrifuged at 800 × *g* for 10 min. The supernatant (B1) was saved, and the sediment was resuspended in 6500 ml of 250 mM sucrose-5 mM EDTA medium. This suspension was homogenized for 1 min and centrifuged at 800 × *g* for 10 min. The sediment obtained at this stage was discarded, and the supernatant (B2) was added to the supernatant (B1).

Step 2: Mitochondrial Fraction—The combined supernatant fraction ($B_1 + B_2$) amounting to about 13,000 ml was centrifuged in a Sorvall RC_2B centrifuge at 13,000 × *g* for 15 min. The mitochondrial sediment was collected and dispersed in 100 mM NaCl solution made up in 10 mM potassium phosphate buffer, pH 7.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 10 mM glucose, henceforth referred to as standard glucose phosphate or buffer A after Grossbard and Schimke (4), who used it first for studies on soluble brain hexokinase type I. The suspension was stirred for some time, then centrifuged at 13,000 × *g* for 10 min. The supernatant was discarded. The mitochondrial sediment at this stage was completely free of lactate dehydrogenase activity and possessed only a trace of NADPH-cytochrome *c* reductase activity. On the other hand, it had more than 80% of the succinate dehydrogenase activity present in the starting homogenate. This mitochondrial preparation was thus reasonably free of cytoplasmic and microsomal contamination (30, 31) and was considered suitable for our purpose.

The mitochondrial sediment thus obtained was washed with glycerol as described by Schwartz and Basford (8). The sediment was collected in about 1000 ml of Buffer A, and to the resulting solution an equal volume of 50% glycerol made up in Buffer A was added. The suspension was stirred vigorously for some time and centrifuged at 13,000 × *g* for 30 min. The supernatant was rejected, and the sediment was taken up in about 1,000 ml of 25% glycerol made up in Buffer A. After vigorous stirring for a few minutes, the suspension was centrifuged at 13,000 × *g* for 30 min. The supernatant was discarded, and the sediment was taken up and dispersed in 5 volumes of 0.2 M sodium acetate buffer, pH 5.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, 10 mM glucose, and 900 mM NaCl. This mitochondrial suspension generally amounted to about 2,000 to 2,500 ml.

Step 3: Acetate Extract—The mitochondrial suspension in acetate buffer was stirred for about 2 hours and then dialyzed overnight in 20 volumes of the same buffer. This dialysis was followed by another dialysis for a few hours in the same buffer but now without NaCl. The dialyzed suspension was again stirred for about 1½ hours and then centrifuged at 20,000 × *g* for 30 min. The sediment was discarded, and the supernatant was adjusted to pH 7.0 with liquor ammonia. The neutralized supernatant amounted to about 2,500 ml and was turbid in appearance.

Step 4: $(NH_4)_2SO_4$ Fraction—The neutralized acetate extract obtained in the above step was subjected to ammonium sulfate

fractionation. Ammonium sulfate was added in small quantities with constant stirring. During addition of ammonium sulfate the pH of the solution was maintained around neutrality with ammonium hydroxide. Ammonium sulfate was first added to 45% saturation. The precipitate formed after 30 min was removed and discarded. The supernatant was then taken to 70% saturation with respect to ammonium sulfate. The solution was centrifuged after 30 min, and the precipitate was taken up in minimum volume of Buffer A and dialyzed overnight against 15 liters of Buffer A in 5-liter portions. The dialyzed 45 to 70% ammonium sulfate fraction amounted to about 150 to 200 ml.

Step 5: First DEAE-cellulose Chromatography—The dialyzed $(NH_4)_2SO_4$ fraction was applied to a DEAE-cellulose column (2 × 56 cm) which was equilibrated with Buffer A. Elution was carried out with the same buffer containing 100 mM potassium chloride. The flow rate was maintained at 10 to 12 ml per hour with a Sigmamotor pump. Generally 1.5- to 2-ml fractions were collected. Bulk of inactive protein emerged from the column in about 1½ column volumes of the eluting buffer, and the enzyme emerged immediately thereafter. The fractions were assayed for enzyme activity, and their absorbance at 280 nm was measured. All of those fractions which exhibited a ratio of units to absorbance of at least 30 were collected, and the pool was subjected to $(NH_4)_2SO_4$ fractionation. The precipitate obtained between 45 and 70% saturation with $(NH_4)_2SO_4$ was taken up in minimum volume of Buffer A and dialyzed overnight against 6 liters of Buffer A in 2-liter portions. The dialyzed material amounted to about 30 to 40 ml.

Step 6: Second DEAE-cellulose Chromatography—The dialyzed material obtained as above was applied to a DEAE-cellulose column (1.5 × 23 cm) equilibrated with Buffer A. The elution was carried out with a linear salt gradient of 0 to 120 mM KCl in 250 ml of Buffer A. The flow rate was maintained at about 6 ml per hour, and 1.8- to 2.0-ml fractions were collected. The fractions were assayed for enzyme activity, and their absorbance at 280-nm was measured. Under these conditions the enzyme was eluted from the column between 1.4 and 1.7 column volumes. The peak of enzyme activity emerged at 30 mM KCl concentration. All those fractions which had a ratio of units to absorbance of at least 60 were collected (Fig. 1), and the pool was fractionated with $(NH_4)_2SO_4$ as described in Step 5. The 45 to 70% $(NH_4)_2SO_4$ precipitate was taken up in about 2 to 3 ml of 0.05 M Tris-chloride buffer, pH 7.0, containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 10 mM glucose or in Buffer A and dialyzed overnight in 2 liters of the same buffer in 500-ml portions. The inactive precipitate formed was centrifuged off and discarded, and the supernatant enzyme solution (about 4 ml) was stored at 4°.

Stability of Enzyme

The enzyme as prepared above was stable for several months at 4°. Simultaneous omission of glucose, 2-mercaptoethanol, and EDTA from the dialysis buffer resulted in a rapid loss of enzyme activity. A slow loss of activity occurred if glucose and 2-mercaptoethanol but not EDTA were omitted from the dialysis medium.

Purity of Enzyme

Sedimentation in Ultracentrifuge—Sedimentation velocity patterns of the purified enzyme at various time intervals showed a single symmetrical peak at a protein concentration of 6 mg per ml at an ionic strength of 0.6 (Fig. 2). The preparation was thus apparently homogeneous.

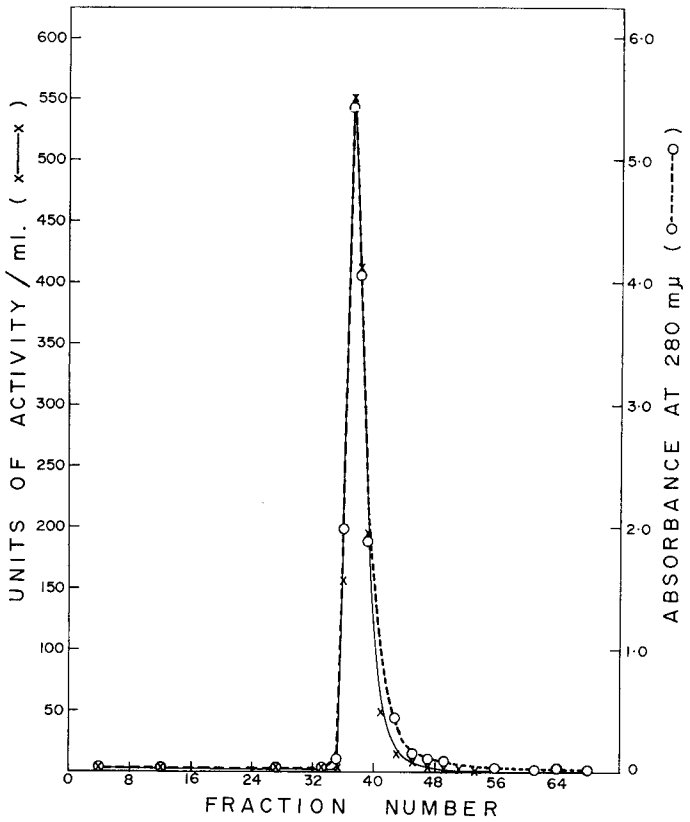

FIG. 1. Second DEAE-cellulose chromatographic step in the purification of bovine brain mitochondrial hexokinase. Thirty milliliters of the dialyzed material from the first DEAE-cellulose chromatographic step containing 39 mg of protein were loaded on the column. Details are described in the text. Tubes 36 to 39 which had a units to absorbance ratio of about 70 and above were pooled. The volume of the pool including washings was 10 ml and contained 24 mg of protein.

Disc Electrophoresis—Polyacrylamide gel electrophoresis of the purified fraction at pH values 7.0 and 8.9 and at gel concentrations of 5, 7.5, and 10% showed only one protein band (Fig. 3*A*); 5 and 10% gels run at pH 7.0 were also stained for hexokinase activity. These gave only one band at positions corresponding to those occupied by the protein bands (Fig. 3*B*). These results indicated that the purified hexokinase fraction consisted of only one isoenzyme, presumably type I, which is the predominant form present in brain tissue (3–5).

Starch Gel Electrophoresis—In order to assure ourselves that the purified hexokinase was the type I isoenzyme, we subjected the purified preparation to starch gel electrophoresis, using the conditions employed by Katzen and Schimke (3) to characterize hexokinase isoenzymes by this method. The results are shown in Fig. 3*C*. The mitochondrial extract and the purified fraction show only one band each. This corresponds to the slower moving band shown by the beef skeletal muscle preparation. Our purified preparation is thus exclusively the type I hexokinase which has the slowest movement toward the anode of all hexokinase isoenzymes.

Some Properties of Enzyme

Sedimentation Coefficient—The sedimentation velocity method yielded an $s_{20,w}$ value of 5.9 S at a protein concentration of 1.7 mg per ml and at an ionic strength of 0.1 (experiment not shown).

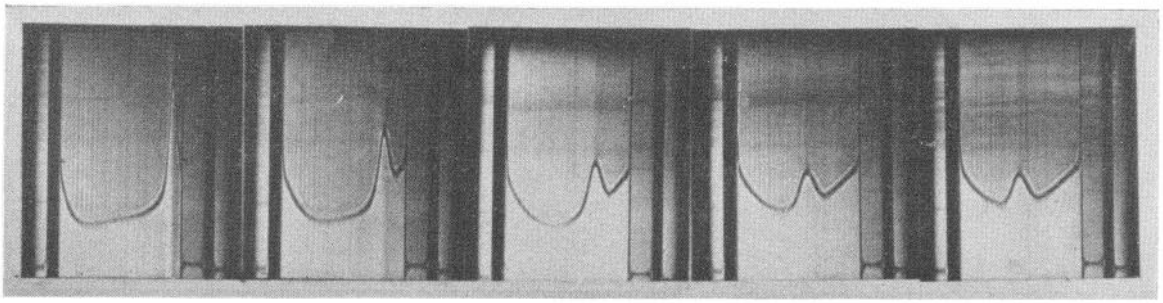

FIG. 2. Sedimentation velocity patterns of purified bovine brain mitochondrial hexokinase at 20°. The pictures, from *left to right*, were taken at 10, 34, 58, 90, and 106 min, respectively, after the rotor reached the speed of 59,780 rpm. The direction of sedimentation shown in the pictures is from *right to left*. The pictures were taken at a bar angle of 50°. The enzyme at a concentration of 6 mg per ml was dissolved in Buffer A to which KCl was added to give a total ionic strength of 0.6.

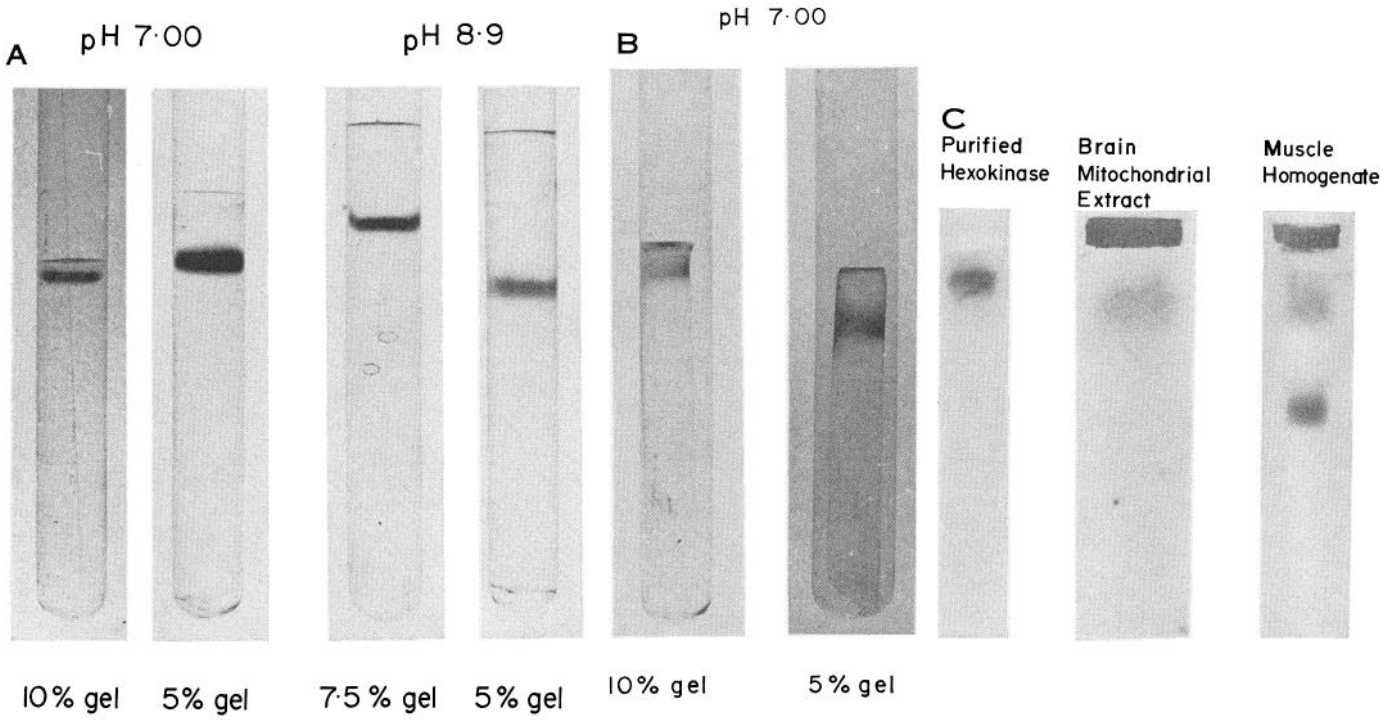

FIG. 3. *A*, polyacrylamide disc gel electrophoresis of purified bovine brain mitochondrial hexokinase. Bands were obtained by staining for protein. For details, see section on "Materials and Methods." One hundred micrograms of the enzyme were loaded for the pH 7.0 experiment and 50 μg were loaded for the pH 8.9 experiment. In all cases, the marker dye was allowed to reach the bottom of the tube before the electrophoresis was stopped. *B*, polyacrylamide disc gel electrophoresis of purified bovine brain mitochondrial hexokinase. Bands were obtained by staining for enzyme activity. Other conditions of the experiment were the same as for the pH 7.0 experiment described in *A*. *C*, vertical starch gel electrophoresis of different hexokinase preparations. Bands were obtained by staining for enzyme activity. The following quantities of various enzyme fractions were loaded. Purified fraction, 120 μg; brain mitochondrial extract, 500 μl of acetate extract of mitochondria; muscle homogenate, 500 μl of a 50% (w/v) muscle homogenate; for details, see section on "Materials and Methods."

The $s_{20,w}$ value for the enzyme using the sucrose gradient method was found to be 6.3 S (Fig. 4). The sedimentation coefficient determined by the latter method can be considered as only approximate as the marker enzymes employed in the experiment may differ from each other in shape and partial specific volume. The value obtained by the sedimentation velocity method is identical to the value reported by Joshi and Jagannathan (9) for their preparation. However, it differs markedly from the value of 4.4 S (at 21°) reported by Schwartz and Basford (8). The reason for this discrepancy is not clear. Recently, a value of 5.3 S has been reported for type I hexokinase from porcine heart (32).

Molecular Weight—An approximate value for the molecular weight of the enzyme was obtained by gel filtration on Sephadex G-200 by the method of Andrews (20). An average value of 107,000 has been determined for the molecular weight of the enzyme on the basis of the two experiments presented in Fig. 5. The two experiments are represented by *Lines A* and *B*. The variation in the slope of the lines relating logarithm of molecular weight to elution volume (V_e) can be ascribed to differences in the swelling of the gels employed in the two experiments (20). In spite of differences in the slopes of the two lines, the values for the molecular weight of hexokinase obtained in the two experiments are quite close. Easterby (32) has reported a value of 97,000 for the molecular weight of type I hexokinase from porcine heart. This value is the same as the one found by Grossbard and Schimke (4) for hexokinase isoenzymes, types I, II, and III. Paranjpe and Jagannathan (33) have reported a value of 90,000 for a purified particulate ox heart hexokinase.

Ultraviolet Absorption Spectrum—The absorption spectrum of

the purified fraction is shown in Fig. 6. The spectrum is typical of a protein. The absorption maximum is around 278 to 279 nm. The ratio of absorbance at 280 and 260 nm is 1.64. The protein has a relatively low absorption at 280 nm, since a protein concentration of 1 mg per ml (as measured by Lowry procedure) gave an optical density of only 0.55. These results are in complete agreement with those reported by Schwartz and Basford (8) for their hexokinase preparation obtained by detergent and proteolytic treatment. However, these values for the absorbance at 280 nm can only be considered as provisional since insufficiency of the enzyme precluded the determination of an absolute value for the absorbance of the protein at 280 nm.

Role of Sulfhydryl Residues

Free Sulfhydryl Residues of Enzyme—The total number of free sulfhydryl residues per mole of enzyme was determined by Ellman's procedure with DTNB (see "Materials and Methods"). The enzyme was treated with 4 M guanidine hydrochloride or 0.30% SDS prior to the addition of excess DTNB. Fig. 7 represents results which are typical of several experiments per-

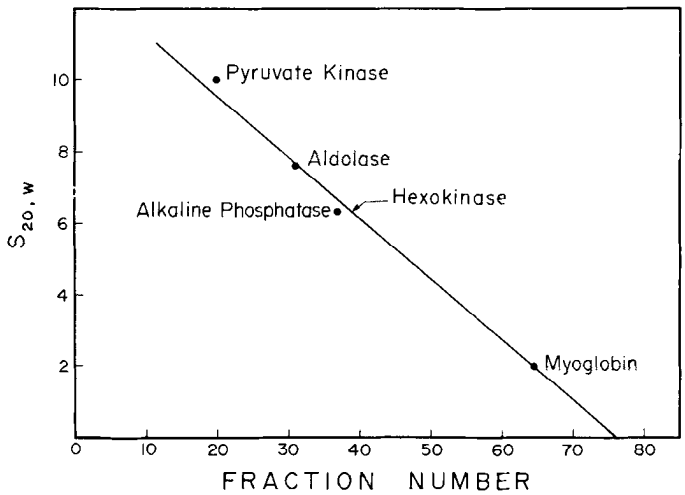

FIG. 4. Determination of sedimentation coefficient on sucrose gradient. For details see section on "Materials and Methods." The fractions are numbered from *bottom to top*.

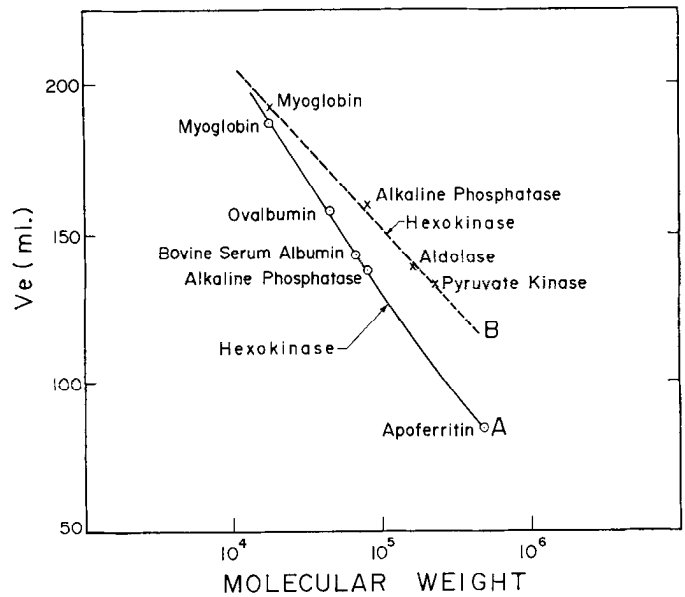

FIG. 5. Determination of molecular weight of purified bovine brain mitochondrial hexokinase by gel filtration on Sephadex G-200. For details see section on "Materials and Methods." For *Curve A*, the column was equilibrated with 100 mM phosphate buffer containing 100 mM glucose. For *Curve B*, the column was equilibrated with 50 mM Tris-chloride buffer, pH 7.5, containing 150 mM KCl and 100 mM glucose.

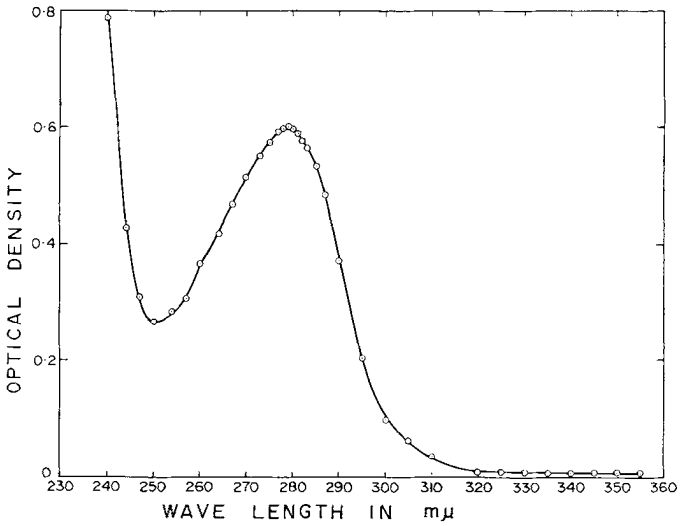

FIG. 6. Ultraviolet absorption spectrum of purified bovine brain mitochondrial hexokinase. The sample cuvette contained 1.1 mg per ml of the purified enzyme in 50 mM Tris-chloride buffer, pH 8.0, containing 0.5 mM EDTA. The reference cuvette contained only the buffer EDTA mixture.

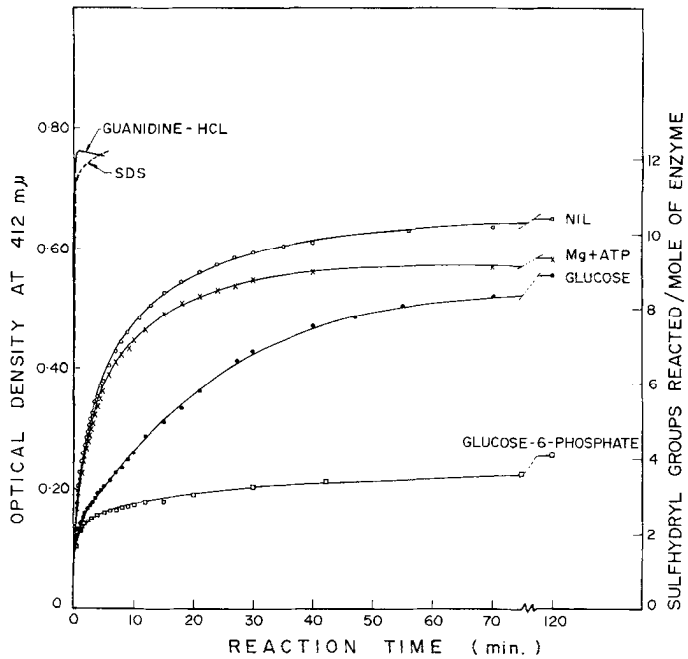

FIG. 7. Progress of reaction of DTNB with the sulfhydryl groups of the purified bovine brain mitochondrial hexokinase in the presence of denaturing agents and metabolites. See section on "Materials and Methods" for details. Both the reaction and reference cuvettes (1-cm path length) contained 104 mM Tris-chloride buffer, pH 8.0, 0.5 mM EDTA, and the various additions as indicated on the *curves*. The concentrations of the added substances were: guanidine HCl, 4 M; SDS, 0.30%; Mg and ATP, 10 mM each; glucose, 10 mM; glucose 6-phosphate, 10 mM. The reaction cuvette contained in addition 4.66 μM enzyme. The reaction was started by the addition of 0.833 mM DTNB to both cuvettes. The solutions of the enzyme, DTNB, and of the various added substances were made in 50 mM Tris-chloride buffer, pH 8.0, containing 0.5 mM EDTA. The reaction was carried out at 25°.

formed with different enzyme preparations. It can be seen from the figure that in the presence of denaturing agents like guanidine hydrochloride or SDS about 12 sulfhydryl residues per mole of enzyme reacted almost instantaneously. Reaction in the presence of guanidine hydrochloride was somewhat faster than in SDS. In the absence of any denaturing agent only 10 groups reacted at 25° in 2 hours. At 30° (experiment not shown), all the 12 groups reacted in the same time interval. The results thus show that the enzyme possesses 12 free sulfhydryl residues per mole. When the progress of reaction between DTNB and sulfhydryl residues of the enzyme was plotted in a semilogarithmic fashion (plot not shown), it became evident that the sulfhydryl residues consisted of two or more types of subclasses having differing degrees of reactivity toward DTNB.

Effect of DTNB on Activity of Enzyme—Reaction of sulfhydryl residues with excess DTNB resulted in a rapid loss of enzyme activity. The kinetics of the inactivation process is clearly first order. The rate constant is 1.54 min^{-1} (Fig. 8).

Reversibility of Enzyme Inactivation—Hexokinase inactivated under the conditions of the experiment described in Fig. 8 can be reactivated by the addition of 10 mM 2-mercaptoethanol (experiment not shown). The extent of reactivation, however, depends on the time that elapses between addition of DTNB and addition of 2-mercaptoethanol. If mercaptoethanol is added immediately after complete inactivation, the enzyme recovers most of the activity almost immediately. If, however, some time is allowed to elapse before the addition of 2-mercaptoethanol, recovery is incomplete. Recovery is also total if the enzyme is treated with just enough DTNB to cause about 95% inactivation, and excess 2-mercaptoethanol is added thereafter.

Number of Sulfhydryl Residues Involved in Inactivation of Enzyme—Kinetics of enzyme inactivation showed that the enzyme lost about 90% of its activity when only 4 out of 12 free sulfhydryl residues of the enzyme had reacted with DTNB (Figs. 7 and 8). This made it clear that reaction of some of these 4 residues with DTNB caused the inactivation of the enzyme. Of these 4 residues which react, 2 undergo reaction within the first 10 s before any significant inactivation of the enzyme has occurred (Figs. 7 and 8). These 2 residues obviously cannot be implicated in the inactivation process. The choice then narrows down to the remaining 2 residues, the reaction of which with DTNB accompanies the inactivation of the enzyme. It is thus reasonable to suppose that reaction of any one or both of these residues with DTNB causes loss of enzyme activity.

Effect of Some Metabolites on Reaction of Enzyme with DTNB

This section describes the effect of various metabolites related to hexokinase on the reaction of its sulfhydryl residues with DTNB. Fig. 7 shows the effect of glucose, MgATP, and glucose 6-phosphate on this reaction. Fig. 9 describes the kinetics of activity loss which occurred when the sulfhydryl residues of the enzyme reacted with DTNB in presence of various metabo

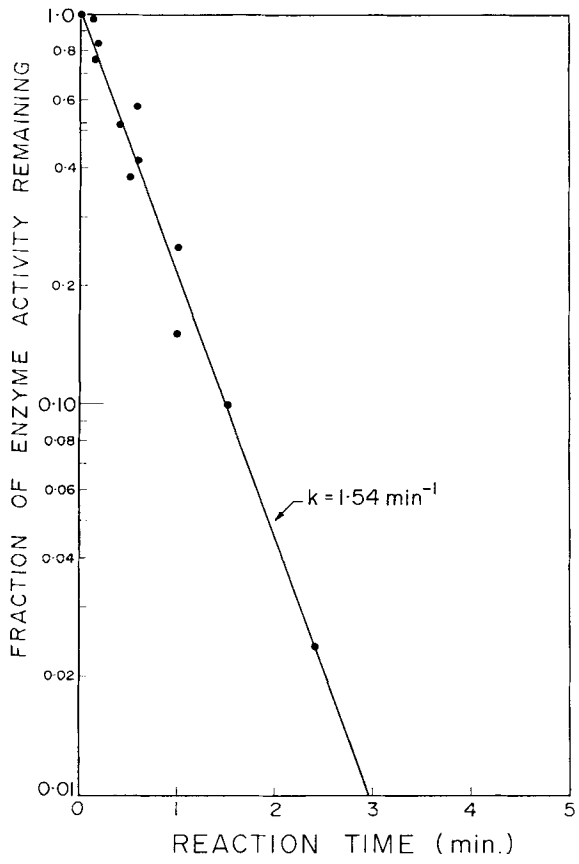

FIG. 8. Kinetics of enzyme activity loss by DTNB treatment. Details for the DTNB reaction and other particulars are the same as described for the *curve* marked *NIL* in Fig. 7. From this reaction mixture, 1-μl aliquots were taken at the time intervals indicated above and assayed for hexokinase activity as described in the section on "Materials and Methods." The points shown here were obtained in two different experiments.

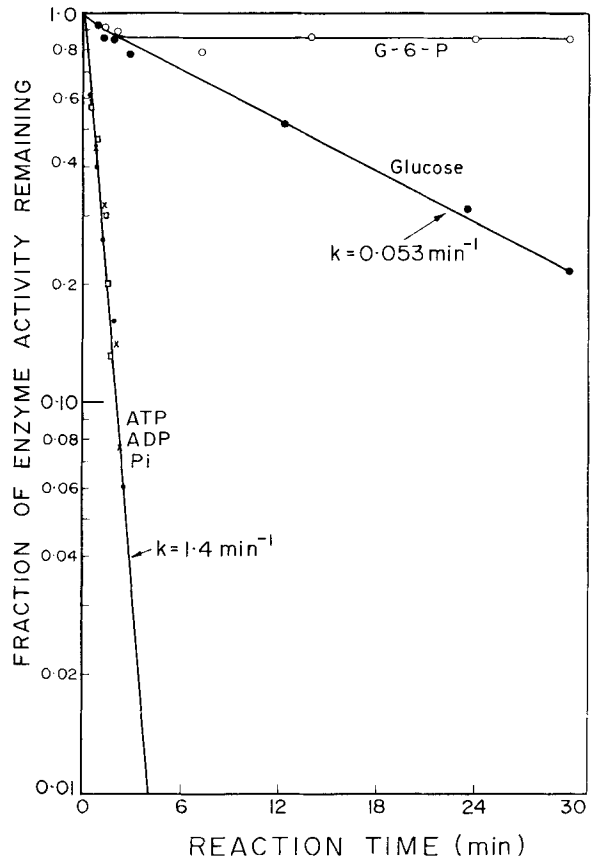

FIG. 9. Kinetics of enzyme activity loss by DTNB treatment in presence of various metabolites. Details for the DTNB reaction and other particulars are the same as described for Fig. 7. All the metabolites were added at 10 mM concentration. □——□, ATP supplemented by 10 mM Mg; ×——×, ADP supplemented by 10 mM Mg; ●——●, P_i. The rate constant for inactivation in the presence of glucose 6-phosphate (*curve* labeled as *G-6-P*) is not shown, as it was too low to be measured accurately.

lites. The results presented in Figs. 7 and 9 are summarized below.

Glucose—The progress of reaction of the sulfhydryl residues of the enzyme with DTNB in presence of 10 mM glucose is seen in Fig. 7. Although 1 mM glucose had the same effect (experiment not shown), a concentration of 10 mM was chosen for the present experiment for better comparison with other substances. This figure shows that in presence of glucose, 1 sulfhydryl residue is prevented from reacting with DTNB. A qualitative comparison of the curves representing reaction of the enzyme with DTNB in the presence and absence of glucose also indicates that glucose changes the reactivity of some sulfhydryl residues of the enzyme toward this reagent. The rate constant of activity loss in the presence of glucose is about 30-fold lower than that in its absence (Figs. 8 and 9).

MgATP and MgADP—Fig. 7 shows that 1 sulfhydryl residue of the enzyme is protected from reaction in the presence of 10 mM MgATP. Identical results were obtained in the presence of 10 mM MgADP (experiment not shown). However, these substances offered no protection to the enzyme against inactivation by DTNB, as the rate constant of enzyme inactivation in the presence of these substances is close to that in the case of the unprotected enzyme (see figs. 8 and 9).

Inorganic Phosphate—One sulfhydryl residue of the enzyme is protected from reaction with DTNB in the presence of inorganic phosphate (experiment not shown). Inorganic phosphate, however, provides no protection to the enzyme against inactivation by DTNB, as the rate constant of enzyme inactivation in the presence of phosphate is almost the same as that for the unprotected enzyme (see Figs. 8 and 9).

Glucose 6-Phosphate and Fructose 6-Phosphate—Only 4 residues of the enzyme react with DTNB in the presence of 10 mM glucose 6-phosphate and 8 do not (Fig. 7). Enzyme inactivation, too, is negligible in the presence of glucose 6-phosphate (Fig. 9). On the other hand, the corresponding fructose ester shields only 1 sulfhydryl residue from reaction with DTNB and provides only a partial protection to the enzyme against inactivation by DTNB. (Experiments with fructose 6-phosphate are not shown.)

DISCUSSION

The procedure for the solubilization and purification of bovine brain mitochondrial hexokinase type I described in this paper gives about 3 times the yield of the enzyme as obtained by the procedure of Schwartz and Basford (8). The avoidance of any proteolytic treatment in solubilizing the enzyme should make this preparation much more acceptable in studies seeking to correlate the structure of the enzyme with its function and regulation.

All determinations with DTNB have given a value of about 11 to 13 free sulfhydryl residues per mole of enzyme, 12 residues being the mean value.[2] The ease with which these residues react with DTNB at 30° shows either that none of them is deeply buried in the molecule or that gradual unfolding of the molecule as each sulfhydryl is titrated by DTNB exposes the buried sulfhydryl residues for attack by this reagent.

Results presented in this paper indicate that inactivation of the enzyme by DTNB is due to its reaction with a maximum of 2 sulfhydryl residues of the enzyme. The evidence is as follows. (*a*) Excess DTNB (about 200-fold) inactivates the enzyme almost completely within 1½ min of addition. Within this time only 4 out of 12 sulfhydryl residues of the enzyme undergo reaction. Of these 4, 2 residues react before any significant inactivation of the enzyme has occurred. This leaves 2 residues, any 1 or both of which may be involved in the inactivation process. (*b*) Addition of 2-mercaptoethanol immediately after complete inactivation of the enzyme leads to a speedy and total recovery of enzyme activity. Furthermore, we have recently determined the reaction order with respect to DTNB for the inactivation of the enzyme.[3] This was done by the method of Levy *et al.* (35). The reaction order was found to be one, which means that on an average 1 molecule of DTNB reacts with 1 molecule of enzyme to cause inactivation. This finding is consistent with the suggestion that not more than 2 sulfhydryl residues of the enzyme are involved in inactivation by DTNB.

These data are, however, insufficient to decide whether these particular residues are located at the active site of the enzyme or are merely important for its active conformation. The considerable protection afforded by substrate glucose and the almost complete protection by product inhibitor glucose 6-phosphate against inactivation by DTNB (Fig. 9) appear to indicate at first glance that these residues are part of the active site region of the enzyme. But protection by these metabolites against inactivation can also be explained by a possible conformational change in the enzyme brought about by these two substrates. Such a conformational change can make the sulfhydryl residues less accessible to DTNB.

The changed reactivity of the sulfhydryl residues toward DTNB in the presence of glucose and the complete absence of reactivity of some residues in the presence of glucose 6-phosphate (but not in the presence of fructose 6-phosphate) is worthy of note. Small molecules like glucose and glucose 6-phosphate could not have changed the reactivities of so many sulfhydryl residues by steric hindrance alone. One of the possibilities to be considered is that changes in the reactivities of sulfhydryl residues might reflect changes in the conformation of the enzyme molecule as a result of its interaction with its substrates. Such a possibility is strengthened by our recent observations that in the presence of 1 mM glucose 6-phosphate the sedimentation constant of the enzyme changes from 5.9 to about 8.0 S.[3] It is perhaps possible that the inhibitory (1, 36) and regulatory (37–39) roles of glucose 6-phosphate in the hexokinase reaction are related to this change.

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[2] The number of sulfhydryl residues was estimated using a molar extinction coefficient of 13,600 per cm for the 5-thio-2-nitrobenzoate anion (11). In a recent paper, however, Robyt *et al.* (34) have pointed out that this extinction coefficient is really of the order of 11,400 per cm. Using the latter value, the number of sulfhydryl residues in the protein would come to around 14 to 15.

[3] U. Chakrabarti, V. D. Redkar, and U. W. Kenkare, unpublished experiments.

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