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STUDIES ON HOG SPLEEN *N*-ACETYLGLUCOSAMINE KINASE

I. PURIFICATION AND PROPERTIES OF *N*-ACETYLGLUCOSAMINE KINASE

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SUMMARY

A highly specific kinase (ATP:2-acetylamino-2-deoxy-D-glucose 6-phosphotransferase) which phosphorylates 2-acetylamino-2-deoxy-D-glucose (GlcNAc) has been isolated from the extracts of hog spleen. The enzyme has been purified more than 3500-fold from the crude extract. Isolation and analysis procedures are described.

The kinase reaction is optimal within a pH range of 8.6–9.4. The enzyme is strictly specific for GlcNAc as phosphate acceptor; ATP is the phosphoryl group donor for the kinase reaction and to a lesser extent GTP. K_m values for GlcNAc and ATP are 1.1 and 1.8 mM, respectively. The enzyme requires Mg^{2+}, which may be replaced by other bivalent metal ions such as Mn^{2+} and Co^{2+} for lesser degrees of effectiveness.

The enzyme is inhibited by P_i and PP_i. The enzyme is also inactivated by *p*-chloromercuribenzoate (PCMB) and the inactivation can be reversed by –SH compounds, *e.g.* cysteine and 2-mercaptoethanol.

The enzyme is inhibited by the reaction products ADP and GlcNAc-6-*P*; ADP competes with ATP whereas the inhibition by GlcNAc-6-*P* is non-competitive. Other inhibitors studied are UTP, CTP and uridine diphosphate GlcNAc (UDP-GlcNAc), the end product of the pathway of GlcNAc metabolism.

INTRODUCTION

In mammals, aminosugars play an important role in connective tissue metabolism, but little information could be found in literature about the enzymes catalyzing the phosphorylation of aminosugars. LELOIR *et al.*[1] first reported that the extracts of rat kidney and other tissues had the ability of phosphorylating GlcNAc and 2-acetylamino-2-deoxy-D-galactose with the agency of specific kinases. The only other report

Abbreviations: GlcNAc, 2-acetylamino-2-deoxy-D-glucose; ManNAc, 2-acetylamino-2-deoxy-D-mannose; UDP-GlcNAc, uridine diphosphate GlcNAc; PCMB, *p*-chloromercuribenzoate.

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about the mammalian *N*-acetylglucosamine kinase (ATP:2-acetylamino-2-deoxy-D-glucose 6-phosphotransferase) came from PATTABIRAMAN AND BACHHAWAT[2] who purified the enzyme about 25-fold from sheep brain extracts and reported that the enzyme is inactivated by dialysis. This kinase also has been found in different bacteria, *Staphylococcus aureus*[3], *Escherichia coli*[4] and *Staphylococcus pyogenes*[5]. The kinase which had been purified from various sources had different properties.

In this report, the purification and properties of *N*-acetylglucosamine kinase from hog spleen is described.

MATERIALS AND METHODS

The following compounds were purchased from Sigma Chemical Co., U.S.A.: ATP, UTP, CTP, GTP, ADP, GlcNAc, Glc-6-*P*, NADH, protamine sulphate, DEAE-cellulose, wheat germ acid phosphatase, and bovine serum albumin. GlcNAc-6-*P* was prepared by phosphorylation of GlcNAc by the procedure of GHOSH AND BANERJI[3]. 2-Acetylamino-2-deoxy-D-mannose (ManNAc) was prepared by the method of CARROLL AND CORNFORTH[6] by epimerization of GlcNAc; calcium phosphate gel was made by the procedure of KEILIN AND HARTREE[7]; pyruvate kinase was isolated from goat muscle[8]. Other compounds were obtained from commercial sources in the highest purity available.

Enzyme assays

N-Acetylglucosamine kinase catalyzes an irreversible phosphorylation according to Eqn. 1,

$$N\text{-Acetylglucosamine} + \text{ATP} \xrightarrow{Mg^{2+}} N\text{-acetylglucosamine-6-}P + \text{ADP} \qquad (1)$$

The *N*-acetylglucosamine kinase activity could be measured either by the rate of disappearance of GlcNAc or formation of ADP.

Assay 1. This method is based on the specific removal of phosphorylating sugar by the addition of $ZnSO_4$ and $Ba(OH)_2$ solutions after enzyme incubation according to the procedure of SOMOGYI[9] and the estimation of free sugar disappeared from the assay mixture during enzyme reaction. This procedure was used throughout the enzyme purification. Routine assay mixture contained (in μmoles) in a total volume of 0.20 ml: GlcNAc, 1.0; ATP, 1.0; $MgCl_2$, 2.5; glycine–NaOH (pH 9.0), 12.5 and enzyme fraction. Incubation was carried out at 37° for 10 min unless otherwise stated. After incubation, the reaction was stopped by adding 1.0 ml of $ZnSO_4$, followed by the addition of an equivalent amount of saturated $Ba(OH)_2$ solution. After centrifugation, 0.2 ml of the supernatant was taken for the estimation of free sugar left in the reaction tube by the Morgan–Elson color reaction[10]. Standards, boiled enzyme controls, and complete but unincubated mixtures were also assayed in the same way.

Assay 2. This procedure was used with the purified enzyme and involved estimation of the amount of ADP formed in the kinase reaction by the procedure of KORNBERG AND PRICER[11]. The composition of the incubation mixture was the same as for Assay 1. After incubation, the reaction was stopped by heating in a boiling-water bath for 1 min, and the reaction mixture was diluted with water to 1.0 ml.

An aliquot of this diluted mixture (0.1 ml) was used for ADP assay. Assay mixture without sugar or without enzyme served as control.

Protein was estimated by the method of LOWRY *et al.*[12] with bovine serum albumin as the standard. Phosphorus was determined by the method of CHEN *et al.*[13].

RESULTS

Distribution of N-acetylglucosamine kinase in different tissues

A survey work was undertaken with the intention of determining the levels of *N*-acetylglucosamine kinase in different tissues of animal origin and selecting a good source of the enzyme. As shown in Table I, the spleen has the highest concentration of the kinase when compared to the other tissues of hog and goat.

TABLE I

DISTRIBUTION OF *N*-ACETYL-D-GLUCOSAMINE KINASE IN DIFFERENT ANIMAL TISSUES

Tissue extracts, prepared as described in the text, were assayed for the kinase activity by Assay 1. Specific activity is expressed as μmoles of GlcNAc esterified per mg of protein in 10 min at 37° under the assay conditions described in the text.

Tissue	*Specific activity of crude extracts*
Hog	
Spleen	0.25
Kidney	0.12
Liver	0.08
Muscle	—
Brain	0.12
Goat	
Spleen	0.09
Kidney	0.08
Liver	0.036
Muscle	0.02
Brain	0.016

Purification of N-acetylglucosamine kinase from hog spleen

Unless otherwise indicated, all operations were conducted between 0 to 4°, and all phosphate buffers contained 1 μmole of EDTA and 10 μmoles of 2-mercaptoethanol per ml.

Step 1. Homogenization. Hog spleen, collected at the abattoir were immediately chilled in ice. The organs can be stored in ice for as long as 5 days or in the deepfreeze at −18° for several months, before kinase extraction, without much loss of activity. The spleen (50 g) was homogenized in a Waring Blender with 100 ml of 0.03 M potassium phosphate buffer (pH 7.6) for two periods of 30 sec each. The mixture was centrifuged at 18 000 × *g* for 30 min and the supernatant was collected and the residue discarded.

Step 2. Protamine sulphate fractionation. The kinase was precipitated from 100 ml of the collected supernatant by the addition of 15 ml of a 2% protamine sulphate solution (dissolved at 37°) over 10 min with continuous but gentle stirring. The protamine precipitate was collected by centrifugation at 8000 × *g* for 10 min and

washed once with 100 ml and again with 50 ml of a solution containing 0.1 M Tris. The enzyme was then extracted from the residue by stirring with increasing concentrations of potassium phosphate buffer (pH 7.6). The first few extractions, each consisting of 50 ml of 0.02, 0.04, and 0.05 M potassium phosphate buffer (pH 7.6) were rejected. Active enzyme was obtained by four further extractions with 50 ml each of 0.075, 0.075, and 0.1 M potassium phosphate buffer (pH 7.6).

Step 3. $(NH_4)_2SO_4$ fractionation. The protamine extract obtained from the previous step was brought up to 25% saturation with solid $(NH_4)_2SO_4$. After standing for 10 min, the insoluble proteins were removed by centrifugation for 10 min at 16 000 × *g*. The $(NH_4)_2SO_4$ concentration of the supernatant was then increased to 50% saturation by the addition of solid $(NH_4)_2SO_4$. The precipitate, collected by centrifugation at 16 000 × *g* for 10 min, was then suspended in 5 ml of 25% saturated $(NH_4)_2SO_4$ solution (pH 7.0). The suspension was stirred for 10 min and insoluble proteins were removed by centrifugation of the suspension for 10 min at 16 000 × *g*. Enough $(NH_4)_2SO_4$ was then added with constant stirring to the resulting supernatant to give 50% saturation. After 10 min of standing, the resulting precipitate was collected by centrifugation and dissolved in 30 ml of 0.02 M potassium phosphate buffer (pH 7.0).

Step 4. Charcoal adsorption. The solution obtained above (30 ml) was treated with 65 mg of acid-washed charcoal (protein to charcoal ratio 1:3). After 10 min, the supernatant was collected by centrifugation and dialyzed against 5 mM potassium phosphate buffer (pH 7.6) for 24 h.

Step 5. Calcium phosphate gel adsorption. Calcium phosphate gel (9 ml; dry wt. 16.2 mg/ml) was sedimented by centrifugation. The sediment was resuspended in 30 ml of dialyzed charcoal fraction (protein to gel ratio 1:8) by gentle stirring with a glass rod. After 10 min, the residue was collected by centrifugation. After preliminary washing with water (30 ml), the active enzyme was eluted from the residue first with 30 ml of 0.05 M, then with 15 ml of 0.05 M and finally with 15 ml of 0.075 M potassium phosphate buffer (pH 7.6). These extracts were then combined and brought to 0.5 saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved and dialyzed against 5 mM potassium phosphate buffer (pH 7.6).

Step 6. DEAE-cellulose column chromatography. The enzyme solution obtained n Step 5 was applied to a DEAE-cellulose column (1.5 cm × 7.5 cm) which had been

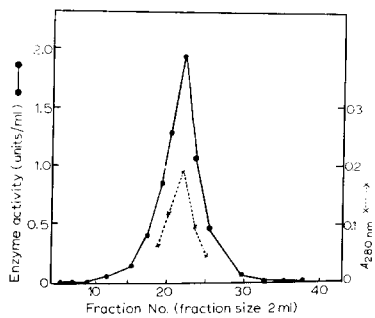


Fig. 1. Chromatography of *N*-acetylglucosamine kinase on DEAE-cellulose. The ordinate on the left and filled circles represent *N*-acetylglucosamine kinase activity. The ordinate on the right and cross refer to protein concentration.

equilibrated with 0.02 M KCl–0.01 M potassium phosphate buffer (pH 7.6). After a preliminary washing of the column with 25 ml of 0.1 M potassium phosphate buffer (pH 7.6) the active enzyme was eluted with 15 ml of 0.2 M potassium phosphate buffer (pH 7.6). The enzyme was then concentrated by $(NH_4)_2SO_4$ as in the previous step and dialyzed against 5 mM potassium phosphate buffer (pH 7.6).

The dialyzed enzyme solution (2 mg protein) was applied again to a DEAE-cellulose column (2 cm × 10 cm) as described above, and eluted with a 200-ml linear gradient of 0.125–0.25 M potassium phosphate buffer (pH 7.6) in 0.02 M KCl–0.001 M EDTA. The *N*-acetylglucosamine kinase activity and protein content of the eluted fractions are illustrated in Fig. 1.

The procedure resulted in a purification of approx. 3600-fold with an overall recovery of about 21%. A summary of the purification procedure is presented in Table II.

TABLE II

PURIFICATION OF *N*-ACETYL-D-GLUCOSAMINE KINASE FROM HOG SPLEEN

Enzyme activity was measured by Assay 1. A unit of enzyme activity was defined as the amount of enzyme producing 1 μmole of GlcNAc-6-*P* per 10 min under the standard assay conditions and specific activity was expressed as the number of units per mg of protein.

Fractions	*Total units*	*Activity yield (%)*	*Specific activity*	*Purification factor*
Homogenate	638	100	0.25	1
Protamine sulphate	572	90	6.7	27
$(NH_4)_2SO_4$	488	76	22.9	91
Charcoal	480	75	27.3	109
Calcium phosphate gel	452	71	156.0	624
DEAE-cellulose	134	21	900.0	3600

Properties of N-acetylglucosamine kinase

Stability. Enzyme stored at 4° in 0.02 M potassium phosphate buffer (pH 7.6)–0.001 M EDTA–0.01 M 2-mercaptoethanol, was stable for at least a week. The enzyme was unstable to freezing and thawing, and labile to heat and low pH. Approx. 50%

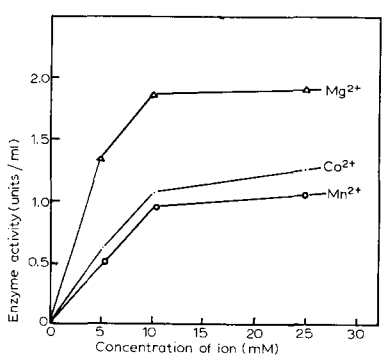


Fig. 2. Dependence of reaction rate on Mg^{2+}, Mn^{2+} and Co^{2+} concentrations. Activity measurements were made by Assay 1 with 0.4 μg of Step 6 protein.

TABLE III

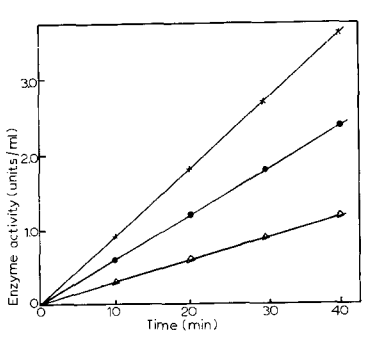
EFFECT OF RATIO OF Mg^{2+} TO ATP ON REACTION RATE OF *N*-ACETYL-D-GLUCOSAMINE KINASE

Concentration of ATP (mM)	*Concentration of Mg^{2+} (mM)*	*Relative activity (%)*
5	1	25
	2.5	48.5
	5	67.8
	10	100
	20	100
10	2.5	38.8
	5	59.6
	10	75
	15	86.2
	20	100
	25	100

of the total activity was lost on heating at 70° for 2 min. The loss of activity during storage could be reversed by 2-mercaptoethanol. As indicated in the purification procedure, the enzyme was stable to dialysis.

Substrate specificity. The enzyme displayed a high degree of specificity for GlcNAc, and it did not act on a variety of other sugars, including ManNAc, *N*-acetylgalactosamine, glucosamine, glucose, mannose, galactose and fructose. The phosphoryl donor, ATP, could not be replaced by UTP, CTP, ADP or phosphoenol pyruvate. Only GTP had some donating capacity (30% of ATP).

The kinase required Mg^{2+} for activity. Other bivalent metal ions, Mn^{2+} and Co^{2+} for instance, could replace the Mg^{2+} requirement partly, while monovalent cations, *e.g.* Na^+ and K^+ and bivalent cations such as Ca^{2+} and Ba^{2+} were completely ineffective. The comparative activities of Mg^{2+}, Mn^{2+}, and Co^{2+} with respect to the stimulation of the reaction rate is presented in Fig. 2. As shown in Table III, the Mg^{2+}



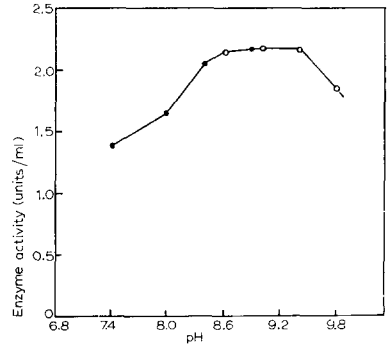


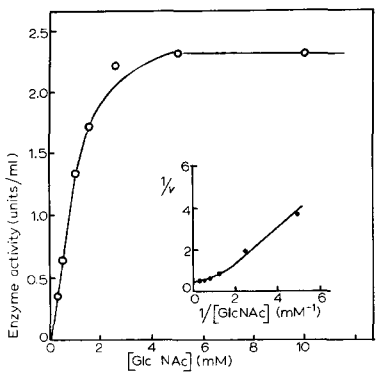
Fig. 3. Effect of incubation time and protein concentration on reaction rate. Enzyme activity was measured by Assay 2 with different amounts of Step 5 protein. △—△, 5 μg; ●—●, 10 μg; ×—×, 15 μg.

Fig. 4. *N*-acetylglucosamine kinase activity as the function of pH. Activity measurements were made by Assay 2 with 0.4 μg of Step 6 protein. Tris–HCl buffer (●) between pH 7.4 and 8.9 and glycine–NaOH buffer (○) between pH 8.6 and 9.7 were used.

to ATP ratio for optimal activity of the kinase was approx. 2:1 which is unusual, since most hexokinases show a ratio of about 1:2 (ref. 14).

Effect of pH, enzyme concentration and incubation time. Assay 2 was used to study these variables, and the results are shown in Figs. 3 and 4. The reaction rate was proportional to enzyme concentration and was linear with respect to time up to 40 min of incubation. The optimum pH was 8.6–9.4.

Effect of substrate concentration. The rate of reaction varied with substrate concentration as shown in Figs. 5 and 6. The apparent K_m values, obtained by Line-



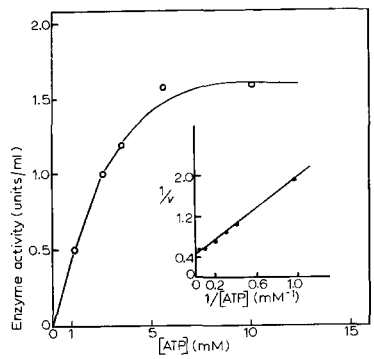


Fig. 5. Effect of GlcNAc concentration on the rate of kinase reaction. The inset shows the data plotted in the form of a Lineweaver–Burk plot. Conditions were the same as described in Assay 2, except that indicated concentrations of GlcNAc were used in 0.2-ml incubation mixtures containing 1.0 μmole of ATP.

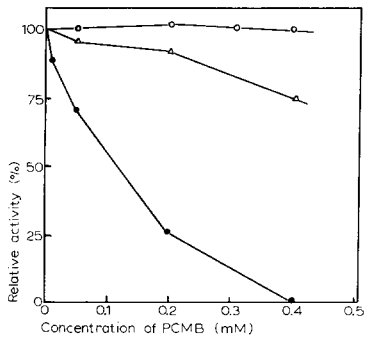
Fig. 6. Effect of ATP concentration on the rate of kinase reaction. The inset shows the data plotted in the form of a Lineweaver–Burk plot. Conditions were the same as described in Assay 2 except that indicated concentrations of ATP were used in 0.2-ml incubation mixtures containing 1.0 μmole of GlcNAc.

weaver–Burk plots, were 1.1 mM for GlcNAc measured with fixed concentration of ATP (5 mM) and 1.8 mM for ATP when GlcNAc concentration (5 mM) in the incubation mixture was fixed. But in the case of GlcNAc, the Lineweaver–Burk plot yielded a slightly curved line instead of a straight line.

Effect of various substances on the activity of N-acetylglucosamine kinase. Sulphydryl reagents, such as PCMB, markedly inhibited the enzyme activity. The kinase was preincubated at 27° for 3 min with PCMB, and the treated fractions were immediately diluted with the assay medium. For a protein concentration of 20 μg/ml, PCMB at a concentration of 0.4 mM inactivated the kinase completely. This inactivation could be reversed almost completely by cysteine and 2-mercaptoethanol (Fig. 7).

As shown in Table IV, the activity of *N*-acetylglucosamine kinase was inhibited by PP_i and P_i. Moreover, ADP, UTP, CTP, UDP-GlcNAc and GlcNAc-6-*P* also inhibited the activity of *N*-acetylglucosamine kinase. As shown in Fig. 8, inhibition due to ADP was competitive with ATP, K_i being 0.9 mM.

Products of reaction. A typical incubation mixture which contained 10 μmoles each of GlcNAc, ATP and Mg^{2+} was incubated with *N*-acetylglucosamine kinase



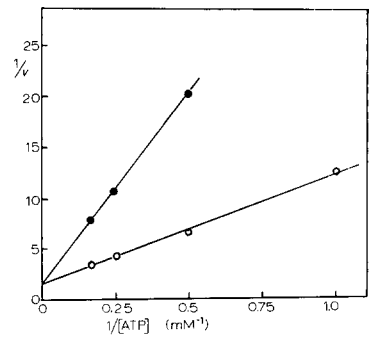


Fig. 7. Inhibitory effect of PCMB on *N*-acetylglucosamine kinase. A, inhibition by PCMB (●—●); B, inhibition by PCMB in presence of cysteine (△—△); C, inhibition by PCMB in presence of 2-mercaptoethanol (○—○). DEAE-cellulose fraction (20 μg protein/ml and dialyzed) was pre-incubated with PCMB at 27° for 3 min and then diluted with the assay medium to assay the kinase activity. Conditions were the same as described in the text except that 2-mercaptoethanol or freshly neutralized cysteine·HCl was added (final concentration 20 mM) where indicated.

Fig. 8. Effect of ATP concentration on the rate of reaction: A, in absence of ADP (○—○); B, in the presence of ADP (●—●). The composition of incubation mixtures and the other assay conditions were the same as described in Assay 1 except that ADP was added (final concentration 8 mM) in each of the duplicate tubes.

TABLE IV

EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF *N*-ACETYL-D-GLUCOSAMINE KINASE

Inhibitor	*Concentration (mM)*	*Inhibition (%)*
ADP	1	14.2
	2	30.4
	4	59.3
	6	85.6
UTP	2	30.2
	4	50
	6	60.3
CTP	2	18.5
	4	40.1
	6	43.5
UDP-GlcNAc	0.4	16.2
	0.8	35.4
	1.2	60.2
	2.0	67.5
GlcNAc-6-*P*	1	19.2
	2	40
	3	60.5
	4	62.5
PP_i	7.5	32.8
	15	65.6
	30	100
P_i	15	17
	30	31.5
	45	40.4
	60	50.6

(Step 6) in 1 ml of 0.03 M glycine–NaOH (pH 9.0). The reaction was terminated by the addition of 1 ml of 0.1 N acetic acid and the products put on a column (1.5 cm × 5 cm) of Dowex 1 (acetate form). Free sugar (GlcNAc) was eluted with water (10–15 ml) and estimated by the Morgan–Elson color reaction[10]. The adsorbed sugar ester was then eluted quantitatively with 0.1 N H_2SO_4 (15–20 ml). The effluent was evaporated to dryness under reduced pressure and the residue dissolved in water. Electrophoresis[15] carried out on a suitable aliquot revealed that the product migrated at the same rate as an authentic sample of GlcNAc-6-*P*. The product was incubated with acid phosphatase[16] and the amounts of P_i and free GlcNAc formed were estimated. The ratio of P_i to GlcNAc was found to be 1.00:0.99. The position of the phosphate group of the product was established by periodate oxidation in acetate buffer (pH 4.5) by the

TABLE V

STOICHIOMETRY AND REQUIREMENTS FOR *N*-ACETYL-D-GLUCOSAMINE KINASE REACTION

Experimental conditions are described in the text.

System	*GlcNAc (μmoles)*	*GlcNAc-6-P (μmoles)*	*ADP (μmoles)*
Complete	−5.72	+5.81	+5.8
−enzyme			0
−GlcNAc			0
−ATP			0
$-Mg^{2+}$			+0.001

method of JEANLOZ AND FORCHIELLI[17]. The identification of glycolaldehyde phosphate[18] as a periodate oxidation product confirmed the position of phosphate ester at C-6 of GlcNAc. Table V shows the stoichiometry and requirements for the reaction performed under these conditions.

DISCUSSION

Though a specific kinase for GlcNAc was detected in mammalian tissues[1,2], no extensive purification or detailed properties of the enzyme have been described previously. LELOIR *et al.*[1] first reported that rat kidney contains the maximal activity of *N*-acetylglucosamine kinase followed by heart, spleen, and liver; the specific activity of *N*-acetylglucosamine kinase from rat kidney extracts being 0.1 μmole of *N*-acetylglucosamine esterified per h per mg protein. However, a specific activity (defined as above) of 1.5 has been found in the hog spleen (Table I). The presence of a highly active enzyme in hog spleen is probably due to the fact that blood cell-degradative activities of the spleen make free aminosugars available for rephosphorylation in that organ.

The kinetic properties of the kinase from hog spleen differ greatly from those of other kinases isolated from different sources[2–5]. As mentioned in the purification procedure, unlike the sheep brain enzyme[2], the purified spleen enzyme is not inactivated by dialysis.

Strong inhibition of the kinase by PCMB (100% inhibition by 0.4 mM) and its

complete reversal by cysteine and 2-mercaptoethanol suggests a –SH group is involved in the activity of the enzyme.

Of various inhibitors (Table IV), ADP, GlcNAc-6-*P* and UDP-GlcNAc could intensively inhibit the activity of the purified enzyme. However, the inhibitory effect of ADP could be competitively prevented by ATP whereas the other two inhibitors are noncompetitive. This effect of ADP could be due to a competitive displacement of ATP by the former from a common receptor site (catalytic site) on the enzyme. The above possibility is also suggested by another observation (A. DATTA, manuscript in preparation) that, during heat treatment of the enzyme, the catalytic activity and the inhibitory activity of ADP were abolished at the same rate even when the heating period was extended to a point of complete inactivation of the enzyme.

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