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PURIFICATION AND SOME PROPERTIES OF HUMAN ERYTHROCYTE HEXOKINASE

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Summary

1. Human erythrocyte hexokinase (ADP:D-hexose 6-phosphotransferase, EC 2.7.1.1) was purified 50 000—100 000-fold with a final specific activity of about 25—50 units/mg protein using gel-filtration, ion-exchange chromatography and affinity chromatography.

2. After isoelectrofocusing of the preparation one major protein band could be detected besides a minor band. The isoelectric point of the major protein band was found to be 4.7.

3. After purification the enzyme could be stabilized in a medium containing inorganic phosphate, glucose, glycerol and mercaptoethanol.

4. The molecular weight was determined by gel-filtration and was found to be 132 000 ± 8000.

5. The enzyme shows a broad pH optimum ranging from 7.0 to 8.4.

6. The kinetic behaviour of the purified enzyme at 37°C was somewhat different from the normal Michaelis-Menten kinetics due to its instability. The affinity constants were 0.048—0.080 mM for glucose and 0.57—1.0 mM for Mg · ATP.

7. The enzyme was specific for Mg · ATP as the nucleotide substrate. Mg · UTP, Mg · ITP, Mg · GTP and Mg · CTP were not converted to corresponding diphosphates. Several hexoses could be phosphorylated by the enzyme. Mannose could be phosphorylated at the same rate as glucose, although the affinity for the enzyme was lower (K_m = 0.60 mM). Much lower rates and lower affinities were found with 2-deoxy-D-glucose (K_m = 1.0 mM), D(+)-glucosamine (K_m = 4.5 mM) and fructose (K_m = 10 mM). *N*-acetyl-D-glucosamine, galactose and sorbose were not phosphorylated at all.

Introduction

Hexokinase (ADP:D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes the conversion of glucose to glucose 6-phosphate with Mg · ATP as the phosphate

donor and is a key enzyme in glycolysis. Although there is a voluminous literature about mammalian hexokinase from many species, comparatively little is known about human erythrocyte hexokinase, mainly because it has never been extensively purified. This is probably due to the low activity of the starting material and the problems in separating haemoglobin from hexokinase. Apart from a more extended purification procedure of Gerber et al. [1], who purified human erythrocyte hexokinase 680-fold with a final specific activity of 0.29 I.U./mg protein, only crude preparations have been reported [2,3].

In order to allow additional research on the kinetic, physical and regulatory properties of human erythrocyte hexokinase, in particular in order to explain the decreased activity of hexokinase in erythrocytes of some patients with haemolytic anaemia, we tried to prepare a highly purified hexokinase from human erythrocytes.

The present paper describes a method of purification by chromatography on a matrix-bound *N*-acetyl glucosamine, which in free form is a potent competitive inhibitor with respect to the sugar substrate of bovine brain hexokinase [4]. A similar procedure has been reported for the purification of rat liver glucokinase by Chester et al. [5], who did not observe any interaction with the liver hexokinase under their conditions.

Materials and Methods

Chemicals. All nucleotide phosphates, glycolytic intermediates and enzymes used for the measurement of hexokinase activity were obtained from Boehringer Mannheim. DEAE-Sephadex A-50 and activated CH-Sepharose 4B were obtained from Pharmacia (Uppsala, Sweden). DEAE-cellulose (DE 32) was purchased from Whatman (Maidstone, England). Ultrogel AcA 44 and the Ampholine PAG-plates, used for the electric focusing experiments, were obtained from LKB (Stockholm, Sweden). 2-Merceptoethanol was obtained from Fluka A.G. (Switzerland) and D(+)-glucosamine from Sigma (St. Louis, Mo., U.S.A.). The other sugars used for the substrate specificity studies were from Merck (Darmstadt, Germany).

All other reagents were of analytical grade of purity. Human erythrocytes were supplied by the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Enzyme assay. Hexokinase activity was routinely measured at 37° C spectrophotometrically in a system coupled with glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. The assay mixture contained, in a total volume of 3 ml, 0.033 M Tris · HCl (pH 7.25), 10.0 mM glucose, 5.0 mM ATP, 10 mM $MgCl_2$, 0.33 mM $NADP^+$, 0.15 I.U. glucose-6-phosphate dehydrogenase and 0.15 I.U. 6-phosphogluconate dehydrogenase.

Initial rate measurements were performed by following the reduction of $NADP^+$ at 340 nm with a Perkin-Elmer spectrophotometer Model 124. For each molecule of glucose utilized two molecules of NADPH are formed. For hexokinase samples free of 6-phosphogluconate dehydrogenase the 6-phosphogluconate dehydrogenase in the assay mixture can be omitted; in this case one molecule of NADPH is formed per molecule glucose. For testing samples con-

taining an unknown amount of endogenous 6-phosphogluconate dehydrogenase, an excess of exogenous 6-phosphogluconate dehydrogenase has to be used to make sure its influence can be calculated.

One unit of hexokinase activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose-6-*P* per min at 37° C.

In the sugar specificity studies the initial rates of ADP production were measured in a coupled enzyme system with pyruvate kinase and lactate dehydrogenase.

In addition to the sugar substrate the assay mixture contained, in a total volume of 3.0 ml. 0.033 M Tris · HCl (pH 7.25), 5.0 mM ATP, 10.0 mM $MgCl_2$, 3.0 mM phospho*enol*pyruvate, 1.0 mM NADH, 3 I.U. pyruvate kinase and 3 I.U. lactate dehydrogenase.

Hence one unit of hexokinase activity is defined as the amount of enzyme which catalyzes the production of 1 μmol of Mg · ADP per min at 37° C.

Protein determination. Protein content was determined by the method of Lowry et al. [6] using bovine serum albumin as a standard.

Coupling of D(+)-glucosamine to activated CH-Sepharose 4B. Activated CH-Sepharose 4B provides a six-carbon space group and an active ester group for covalent coupling of ligands containing primary amino groups, with the capacity to couple 15—20 μmol of ligand per g powder. The activated CH-Sepharose 4B was swollen in 10^{-3} M HCl. The gel was washed with distilled water on a sintered glass filter using approx. 200 ml per g dry powder. 4 mg D(+)-glucosamine hydrochloride per mg dry powder was dissolved in the coupling solution (0.1 M $NaHCO_3$ containing 0.5 M NaCl), of which there were 5 ml per g dry powder, and was mixed with the gel. Excess of ligand was removed by washing with coupling solution. The product was washed with three cycles of alternating pH, consisting of a wash at pH 4 (0.1 M acetate buffer, 1 M NaCl) followed by a wash at pH 8 (0.1 M Tris · HCl, 1 M NaCl), which at the same time blocks and remaining active groups. The product was stored at 4° C in the presence of 3 mM KF.

Isoelectrofocusing. Isoelectrofocusing experiments were performed on Ampholine polyacrylamide gel plates, (having a pH gradient of 3.5—9.5) according to the instructions of the manufacturer.

20-μl samples containing 40—100 μg protein were applied using sample application pieces which were removed after 0.5 h focusing. The inital voltage setting was 280 V increasing to 1200 V within 40 min. The focusing was completed after 2 h.

Detection of the protein bands was performed according to the instructions of the manufacturer with Coomassie Brilliant Blue R 250 (Merck).

The pH-gradient was determined by cutting a strip of the gel into pieces after the run was finished. These pieces were extracted in 2 ml of distilled water, followed by pH determination at the same temperature as maintained during the run (4° C).

Electrophoresis. Electrophoresis experiments were performed on starch gel according to the method of Holmes et al. [7], on polyacrylamide gel according to the method of Altay et al. [8] and on cellulose acetate gel according to the method of Sato et al. [9].

Molecular weight determination. Molecular weight was determined according

to the method of Andrews [10]. Gel filtration was performed using both Sephadex G-200 and Ultrogel AcA 44 as molecular sieve.

Results

Purification of hexokinase

In addition to the buffers described in the text, all the buffers contained 3 mM 2-mercaptoethanol to prevent oxidation of sulphydryl groups of the enzyme and 3 mM KF to prevent bacterial growth. The whole purification procedure was carried out at 4° C.

1. Preparation of haemolysate. Approx. 800 ml of packed red cells were washed twice with isotonic sodium chloride. The buffy coat was removed by suction. After adding an equal amount of 0.4% saponin solution the washed cells were haemolysed for 1 h.

2. Batch-by-batch treatment with DEAE-Sephadex A-50. The haemolysate was mixed with approx. 3 l of DEAE-Sephadex A-50 suspension, equilibrated in 0.01 M sodium potassium phosphate buffer (pH 7.3), and stirred for 1 h. The suspension was rinsed on a filter with the same buffer until the eluate was colourless. This procedure removed the bulk of the haemoglobin, while the hexokinase remained bound. The enzyme was eluted with approx. 1.5 l 0.5 M phosphate buffer (pH 7.3) containing 10% $(NH_4)_2SO_4$.

3. Ammonium sulphate fractionation. The enzyme solution was brought to 75% of saturation with $(NH_4)_2SO_4$. After 15 h of storage at 4° C the precipitate was collected by centrifugation (16 000 × *g*, 15 min) and was redissolved in approx. 100 ml 0.01 M phosphate buffer (pH 7.0).

4. Chromatography on DEAE-cellulose (DE 32). The enzyme solution was dialysed for 20 h against 0.01 M phosphate (pH 7.0). After this procedure the precipitated material was removed by centrifugation (16 000 × *g*, 10 min). The dialysed solution was then applied to a DE-32 column (5.0 × 25 cm) equilibrated in 0.01 M phosphate buffer (pH 7.0).

The column was washed with 500 ml of 0.01 M phosphate buffer and afterwards with 300 ml of the same buffer containing an additional 10 mM KCN. During the first wash most of the haemoglobin was removed, whereas during the second wash the remaining haemoglobin was more firmly bound due to its charge having been altered by the presence of the KCN. The enzyme was not inhibited by KCN in this concentration.

A linear 500 ml gradient from 0 to 0.5 M KCl in phosphate buffer containing 10 mM KCN was used to elute the hexokinase. Fractions of 4 ml were collected at a flow rate of approx. 100 ml/h. The hexokinase was eluted at a KCl concentration of approx. 0.3 M. The elution profile is shown in Fig. 1.

5. Gel-filtration on Ultrogel AcA 44. The fractions containing hexokinase activity were pooled and concentrated by ultrafiltration on an Amicon UM 20 E filter up to 10 ml. This concentrate was applied to an Ultrogel AcA 44-column (5.0 × 35 cm) equilibrated in 0.05 M Tris · HCl (pH 8.0) containing 0.5 M NaCl. The enzyme was eluted with 500 ml of the same buffer. Fractions of 5 ml were collected at a flow rate of 40 ml/h. The hexokinase activity peak appeared between the two main peaks of protein (Fig. 2).

6. Chromatography on immobilized N-acetylglucosamine. The hexokinase

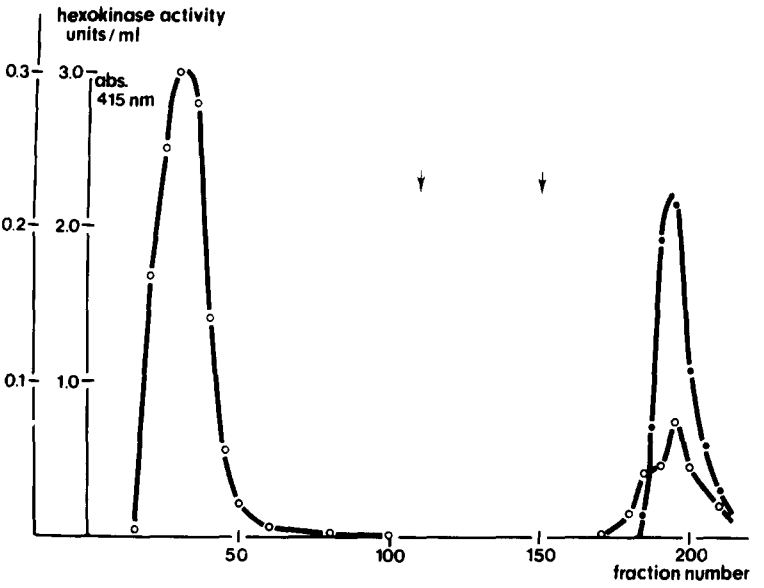

Fig. 1. Elution pattern from DEAE-cellulose (DE-32) column. The arrows indicate successively the addition of 10 mM KCN and the starting point of the 0.5 M KCl gradient (for further conditions see text). ○———○, haemoglobin (Absorbance at 415 nm); ●———●, hexokinase activity (units/ml).

fractions from stage 5 were collected and concentrated by ultrafiltration (as mentioned under 5) up to approx. 6 ml. The sample was then applied to a column (1.0 × 35 cm) of glucosamine coupled to activated CH-Sepharose 4B, which was equilibrated in 0.05 M Tris · HCl (pH 8.0) containing 0.5 M NaCl. The enzyme was eluted with 150 ml of the same buffer. The hexokinase activ-

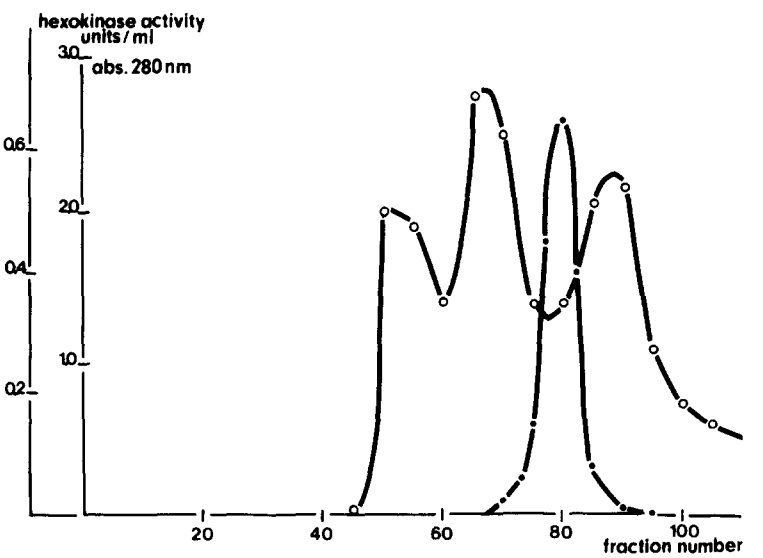

Fig. 2. Gel-filtration on Ultrogel AcA 44. ○———○, protein (Absorbance at 280 nm); ●———●, hexokinase activity (units/ml).

ity was retarded with respect to the main protein fraction. A typical elution profile is shown in Fig. 3. The column can be used several times and can be regenerated with 6 M urea followed by equilibration with the elution buffer.

A typical purification procedure representing six similar experiments is given in Table I.

Only the most active hexokinase fractions were pooled and used for isoelectrofocusing experiments and kinetic measurements. The specific activity of these fractions ranged from 25 to 50 units/mg protein in the final preparations, representing a purification of 50 000—100 000-fold.

Stability

Purified hexokinase was very unstable, showing a half-life of 10 min at 37°C, but the enzyme could be stabilized by dialysis against 0.05 M phosphate (pH 7.5) containing 0.1 M glucose, 3 mM mercaptoethanol, 3 mM KF and 25% glycerol (v/v). In this medium the enzyme was stable for several months when stored at −10°C.

Other procedures

Electrophoresis. Electrophoresis was carried out on starch, polyacrylamide and cellulose acetate (see Methods) both with haemolysate and with purified preparations. In all these cases only one band of hexokinase activity could be detected if the leucocytes were carefully removed.

Isoelectrofocusing. After isoelectrofocusing of the purified preparation one major protein band could be detected, besides a minor band (see Fig. 4). It was

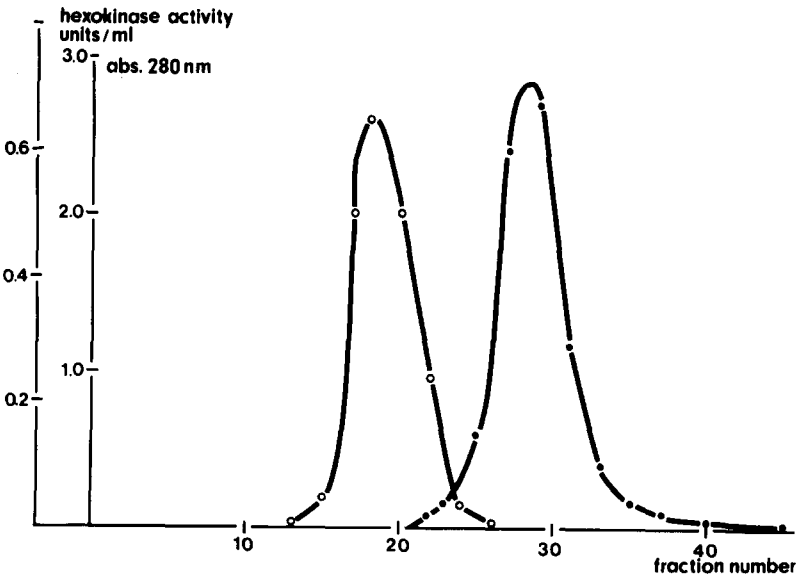

Fig. 3. Elution profile of chromatography on immobilized *N*-acetylglucosamine. ○———○, protein (Absorbance at 280 nm); ●———●, hexokinase activity (units/ml). For further conditions see text. The protein content of the fractions containing the top activities of hexokinase was very low and could not be detected by measuring absorbance at 280 nm.

TABLE I

PURIFICATION OF HUMAN ERYTHROCYTE HEXOKINASE

Fraction	Volume (ml)	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Yield (%)	Purification (-fold)
1. Haemolysate	1690	39	83 000	0.00047	100	—
2. DEAE-Sephadex A-50-eluate	1600	38.5	2 400	0.016	99	34
3. $(NH_4)_2SO_4$ precipitate	104	34.5	2 000	0.017	89	36
4. DEAE-cellulose (DE-32) eluate	165	24	770	0.031	62	66
5. Ultrogel AcA 44 eluate	58	17	116	0.146	44	310
6. Immobilized *N*-acetylglucosamine eluate	22	5.3	0.37	14.3	14	30 500

not possible to stain for hexokinase activity because of the presence of ampholines, which inhibited the enzyme activity. The isoelectric point of the major protein band was found to be 4.7.

Molecular weight. The molecular weight was determined by gel-filtration with the reference proteins cytochrome *C* (M_r 13 000), Peroxidase (horse radish, M_r 40 000), albumin (bovine serum, M_r 68 000), glutathione reductase (yeast, M_r 122 000) and aldolase (rabbit muscle, 160 000).

The molecular weight of hexokinase was found to be 132 000 (S.D. = 4000, $n = 4$). No difference was obtained using a polydextran gel (Sephadex G-200) or a polyacrylamide/agarose gel (Ultrogel AcA 44).

pH optimum. Hexokinase activity was tested in the range pH 6.0—9.0 (0.033 M Tris · HCl buffers). Above pH 8.0 the amount of glucose-6-phosphate dehydrogenase in the assay mixture was doubled. A broad pH optimum ranging from pH 7.0 to 8.4 was observed.

Kinetics

Aging of the enzyme preparations appeared to produce a decrease in the affinity for glucose. Therefore all kinetic measurements were performed on the

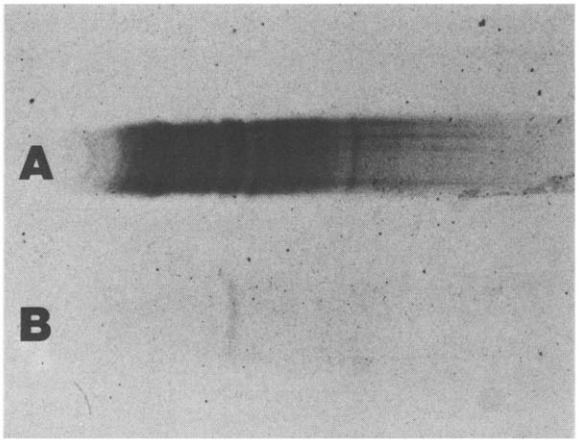

Fig. 4. Isoelectrofocusing of hexokinase before (A) and after (B) the immobilized *N*-acetylglucosamine column.

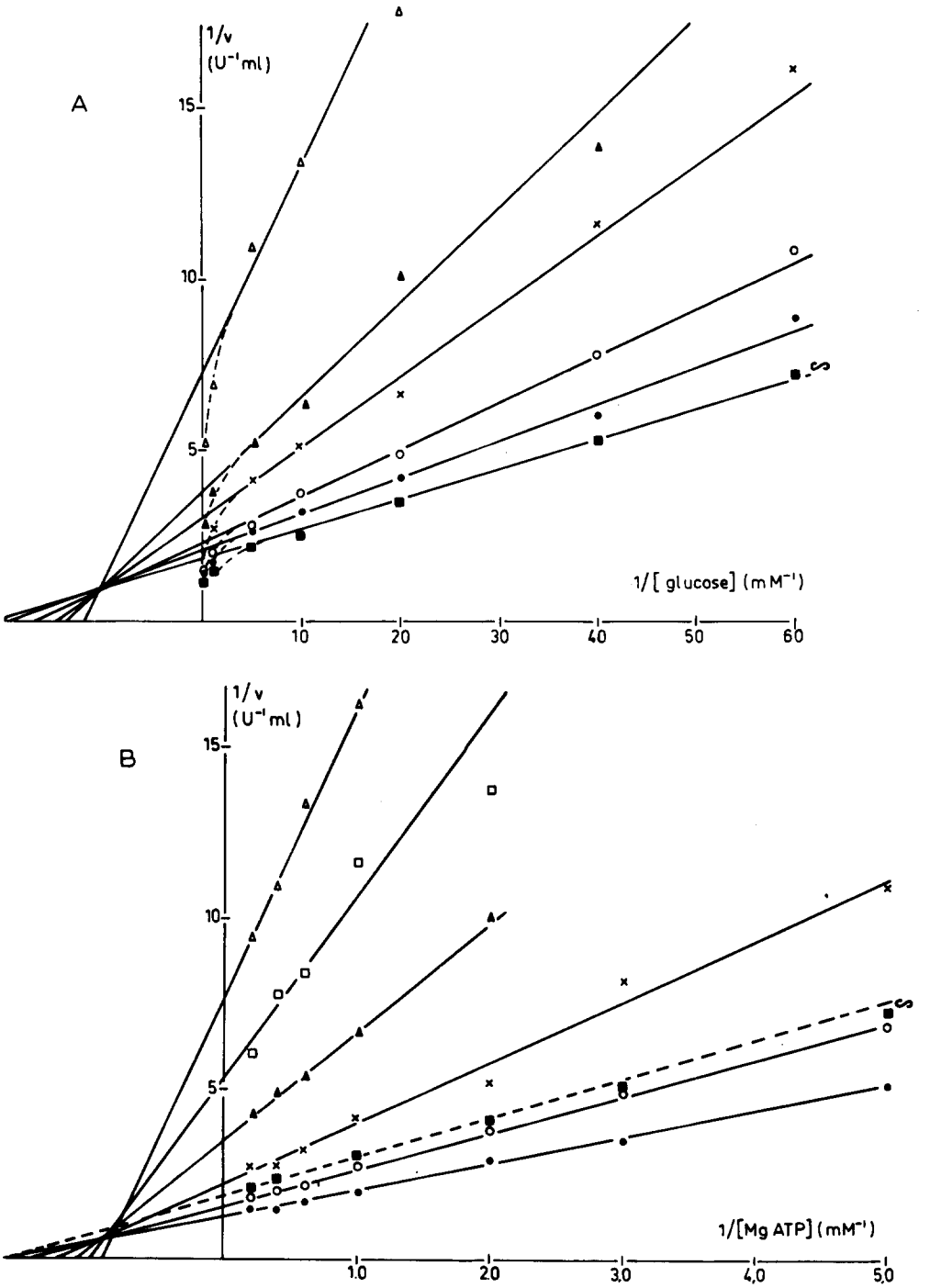

Fig. 5. The $1/v$ vs. 1/[glucose] plot at Mg · ATP concentrations of 5 mM (●), 2.5 mM (○), 1.0 mM (+), 0.5 mM (*) and 0.2 mM (△), The values for 1/[Mg · ATP] = 0 (■) are extrapolated from Fig. 5B. Conditions are as described in the Materials and Methods section. B. $1/v$ vs. 1/[Mg · ATP] plot at glucose concentrations of 10 mM (●), 1.0 mM (○), 0.2 mM (+), 0.05 mM (*), 0.025 mM (□) and 0.0166 mM (△). The values for 1/[glucose] = 0 (■) are extrapolated from the linear part of the curves of Fig. 5A. Conditions are as described in the Materials and Methods section.

same day as and immediately after stage 6 of the purification procedure.

The kinetic behaviour of the enzyme at 37° C is somewhat different from the normal Michaelis-Menten kinetics.

Fig. 5A shows $1/v$ vs. 1/[glucose]. The curves deviate from linearity at glucose concentrations over 1 mM. This results in Fig. 5B, plotting $1/v$ vs. 1/[Mg · ATP], in a greater slope of the extrapolated curve for 1/[glucose] = 0 than the slopes of the curves for [glucose] = 10 mM and 1.0 mM. Furthermore the curves in Fig. 5B do not intersect at one point.

Extension of the high-concentration part of Fig. 5A (1.0—100 mM glucose) gave no evidence for a different affinity for glucose in this concentration range, as might be expected from Fig. 5A. Moreover the Lineweaver-Burk plots at 25° C were completely linear (Results not shown).

The abnormal kinetics observed in Fig. 5 are due to the instability of the purified enzyme and the stabilisation effect of glucose, as can be seen in Fig. 6. The enzyme is unstable at 37° C without glucose or in the presence of low concentrations of glucose, but is stabilized at higher concentrations of glucose (over approx. 5 mM). The affinity constant of the enzyme for glucose was 0.048—0.080 mM and for Mg · ATP 0.57—1.0 mM (ranges of four different experiments). The same values were found at 25° C.

Substrate specificity

Human erythrocyte hexokinase is specific for Mg · ATP as the nucleotide substrate. Mg · ITP, Mg · GTP, Mg · UTP and Mg · CTP are not converted to their diphosphates at concentrations of 10 mM.

Several hexoses however can be phosphorylated by the enzyme (Table II). Mannose is phosphorylated at the same rate as glucose, although the affinity and therefore the phosphorylation coefficient is less. Much lower rates and affinities are found for 2-deoxy-D-glucose, D(+)-glucosamine and D(—)-fructose. *N*-acetyl-D-glucosamine, D(+)-galactose, D(+)-xylose and L(—)-sorbose are not phosphorylated at all.

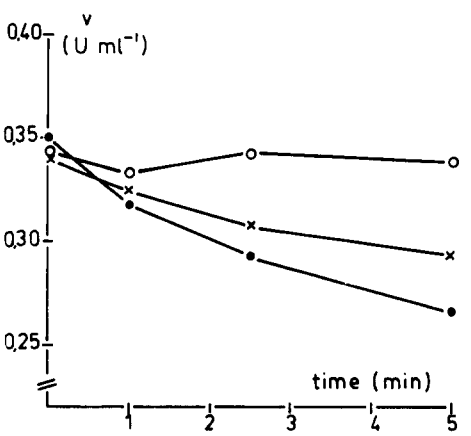

Fig. 6. Hexokinase activity after preincubation at 37°C in the presence of 10 mM glucose (○), 1.0 mM glucose (+) and 0 mM glucose (●). The activities are measured as described in the Materials and Methods section.

TABLE II

SUGAR SPECIFICITY OF PURIFIED HUMAN ERYTHROCYTE HEXOKINASE

The Michaelis-Menten constants at 5 mM Mg · ATP (K_m app.) and the maximum velocities were determined from initial rate measurements of ADP formation, as described in Materials and Methods, using the Lineweaver-Burk plot. Maximum velocities are expressed relative to the V for glucose (100%). The phosphorylation coefficient is defined as:

$$\frac{V\text{ (substrate)}}{K_m\text{ (substrate)}} \cdot \frac{K_m\text{ (glucose)}}{V\text{ (glucose)}}.$$

Sugar substrate	K_m app. (mM)	Relative V (%)	Phosphorylation coefficient
D(+)-Glucose	0.07	100	1
D(+)-Mannose	0.6	111	$1.3 \cdot 10^{-1}$
2-Desoxy-D-glucose	1.0	33	$2.3 \cdot 10^{-2}$
D(+)-Glucosamine	4.4	33	$5.3 \cdot 10^{-3}$
D(—)-Fructose	10	38	$2.7 \cdot 10^{-3}$
N-acetyl-D-glucosamine	N.P. *	—	—
D(+)-galactose	N.P.	—	—
D(+)-xylose	N.P.	—	—
L(—)-sorbose	N.P.	—	—

* N.P.: not phosphorylated at concentrations of 100 mM of sugar substrate.

Discussion

The purification procedure described here provides a rather simple method for obtaining highly purified red blood cell hexokinase by chromatography on glucosamine coupled to the six-carbon spacer group of activated CH-Sepharose 4B, so providing an immobilized *N*-acetyl-glucosamine.

However, there is some doubt about the true nature of the affinity of the interaction of hexokinase with this material. Hexokinase was only retarded, although *N*-acetylglucosamine is a potent inhibitor of hexokinase. Our purified hexokinase preparation was inhibited by *N*-acetylglucosamine competitively to glucose with a K_i of 0.3—0.5 mM (results not shown). Furthermore the sample applied to the column had to be rather pure; if in stage 4 of the purification procedure KCN was omitted, the specific activity of the product of stage 5 was much lower and no interaction at all was observed. So the interaction witht he immobilized ligand is rather weak and is easily masked by other proteins. There are a few possible explanations:

1. The conformation of the immobilized *N*-acetylglucosamine is changed thus prohibiting the inhibitory action.

2. The interaction is not a biospecific but rather an aspecific interaction e.g. a hydrophobic interaction with the six-carbon spacer group as in what Shaltiel [11] designates "hydrophobic chromatography".

In favour of the first assumption is the observation of Chesher et al. [5] that the immobilized *N*-acetylglucosamine did not inhibit yeast or rat-liver hexokinase whereas the free ligand did. Up to now we did not try to verify this assumption. Hydrophobic interaction between the enzyme and the column could be ruled out by using an activated CH-Sepharose 4B column to which no ligand was bound. No retardation of the enzyme fraction with respect to the main protein fraction was observed. However, the phenomenon of the hydro-

phobic chromatography may indeed be responsible for the minor contaminating protein fraction of the preparation.

Several authors have described the hexokinase patterns of erythrocytes but there has been no general agreement about the results [7,8, 12—15]. In general two bands of activity are found corresponding in mobility to hexokinases Type I and III in the nomenclature of Katzen and Schimke [16]. The predominant form, which accounts for approx. 90% of the hexokinase activity [1], corresponds to Type I.

In our electrophoresis experiments we failed to detect hexokinase Type III in haemolysates from which the leucocytes had been carefully removed. This is in agreement with the results of Povey et al. [15]. Since leucocytes contain a relatively high hexokinase activity with the predominant form corresponding to Type III [15], the hexokinase Type III activity normally detected in erythrocytes might be of leucocyte origin.

The isoelectric point for the pure Type I hexokinase from rat brain has been reported to be 6.0 [17]. The value of 4.7 found by us is not in agreement with this finding.

Purified Type I hexokinases are reported to have molecular weights close to 100 000, whether obtained from rat brain [17], beef brain [18] or porcine heart [19]. Our results for the human erythrocyte hexokinase do not agree with these findings.

Preliminary results however indicate the presence of approx. 15% of lipid * in our purified hexokinase preparation, which may be responsible for the lower isoelectric point and the higher molecular weight (of 132 000) found by us. The latter result is in agreement with the observations of Kumura and Gourly [10] who demonstrated in rat brain the presence of a lower-molecular-weight hexokinase and a higher-molecular-weight hexokinase. The latter appeared to be an aggregate of the low-molecular-weight hexokinase and phospholipid moieties.

The kinetics of the purified enzyme show, apart from the abnormalities due to the instability of the preparation, a rapid equilibrium reaction mechanism as is now generally accepted for most hexokinases. The affinity constants are in the same range as is reported for hexokinase Type I from erythrocytes [1—3] and many other mammalian tissues [21].

The substrate specificity studies show that glucose is the most effective hexose substrate, although mannose is a reasonably good substrate too. This confirms the conclusion of Beutler and Teeple [22] that in the red cell mannose and glucose are phosphorylated by the same enzyme.

Additional research is in progress to study more closely the properties of purified human erythrocyte hexokinase and the possible lipid effects on the enzyme activity.

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