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FRUCTOKINASE FROM RAT LIVER

I. PURIFICATION AND PROPERTIES

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SUMMARY

Fructokinase (ATP:D-fructose-1-phosphate transferase, EC 2.7.1.3) from rat liver has been purified 400-fold. The purification procedure involves an acid treatment, a heat step at 65°, $(NH_4)_2SO_4$ fractionation, chromatography on Sephadex G-100 and finally $(NH_4)_2SO_4$ extraction.

The enzyme appears nearly homogeneous by density gradient centrifugation but gives a single peak in sedimentation velocity analysis. Purified liver fructokinase has a K_m of 0.46–0.80 mM for fructose and 1.56–1.33 mM for MgATP at a K^+ concentration of 0.4 and 0.1 M, respectively. The enzyme also phosphorylates L-sorbose and D-tagatose. No difference could be found in the phosphorylation of the pyranose and furanose forms of fructose. The enzyme is inhibited by *p*-chloromercuribenzoate and is stable up to 50–55°.

INTRODUCTION

Numerous biochemical and physiological investigations[1–24] clearly indicate that fructose 1-phosphate is the sole initial product of the hepatic utilization of fructose. The phosphorylation of fructose is catalyzed by fructokinase (ATP:D-fructose 1-phosphate transferase, EC 2.7.1.3). This enzyme presents an interesting behaviour that distinguishes it from other hexokinases as it shows an absolute requirement for K^+ (ref. 7). It is also strongly and noncompetitively inhibited by one of the reaction products, ADP[7]. The present study was undertaken to obtain information concerning these problems. Furthermore, although fructokinase has been purified by various methods[5,7,10,18], little is known about the chemical properties of the enzyme. This paper reports a method for the purification of fructokinase from rat liver to near homogeneity as well as some chemical properties of the protein.

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MATERIALS AND METHODS

Chemicals

Fructose, ATP (disodium salt), and creatine kinase were purchased from Sigma Chemical Company; lysozyme 3 times crystallized from Mann Research Laboratories; and Sephadex G-100 from Pharmacia Fine Chemicals. $(NH_4)_2SO_4$ was recrystallized from EDTA according to the method of SUTHERLAND AND WOLISAT[25].

Analytical techniques

Fructose was determined by the thiobarbituric acid method according to the method of PERCHERON[26] in a total volume of 1.2 ml. Protein was estimated according to the procedures LOWRY *et al.*[27] and WARBURG AND CHRISTIAN[28] (in connection with gel filtration). Measurement of protein in fractions of the sucrose gradient was determined also by the method of MURPHY AND KIES[29].

Enzyme assays

Assay a. A colorimetric assay was based on the measurement of the fructose disappearance with a similar test as the one described by HERS[5]. The standard incubation mixture was as follows: 0.25 μmole of fructose, 0.3 μmole of ATP, 5 μmoles of Tris buffer (pH 7.4), 25 μmoles of KCl, 0.3 μmole of $MgCl_2$, 0.375 μmole of creatine phosphate, 1 μg of creatine kinase, and enzyme in a total volume of 0.05 ml. Incubation was carried out for 15 min at 37° and the reaction was stopped by adding 50 μl of 0.15 M $ZnSO_4$ and 50 μl of 0.15 M $Ba(OH)_2$. After centrifugation, aliquots of the supernatant were used for the thiobarbituric acid reaction. An incubation mixture to which ATP was added after stopping the reaction by adding $ZnSO_4$, was used as a blank.

Fructokinase activity was also measured by coupling the formation of ADP with phosphoenolpyruvate and pyruvate kinase. The pyruvate formed was estimated either colorimetrically with 2,4-dinitrophenylhydrazine or spectrophotometrically with lactate dehydrogenase.

Assay b. A standard incubation mixture was as follows: 0.25 μmole of fructose, 0.3 μmole of ATP, 5 μmoles of Tris buffer (pH 7.4), 25 μmoles of KCl, 0.3 μmole of $MgCl_2$, 0.25 μmole of phosphoenolpyruvate, 3 μl of pyruvate kinase, and enzyme in a total volume of 0.05 ml. Incubation was carried out for 15 min at 37° and the reaction was stopped by heating the tubes for 1 min at 100°. After cooling, 10 μl of a mixture of NADH (0.1 μmole) and lactate dehydrogenase (1 μg) were added, followed by a second incubation for 5 min at 37°. Water was added to 0.5 ml and the change in absorbance at 340 nm was followed in Beckman DU spectrophotometer. A blank without fructose was used as reference.

This assay is not suitable to measure enzyme activity in the first fractions of the purification procedure, as the addition of ATP alone causes a rapid ADP formation (presumably due either to ATPase or adenylate kinase which might be inactivated during the heat treatment).

Assay c. The standard incubation mixture was as for Assay b, but the reaction was stopped by adding 80 μl of 10% trichloroacetic acid, followed by 0.15 ml of 2,4-dinitrophenylhydrazine (0.1% in 2 M HCl). After 5 min at 37°, 0.4 ml of 2.5 M NaOH

was added, and the tubes were mixed and centrifuged. The absorbance of the supernatant at 520 nm was measured.

Unit

One unit of fructokinase is defined as the amount of enzyme that will catalyze the disappearance of 1 μmole of fructose or the formation of 1 μmole of ADP or fructose 1-phosphate per min at 37°.

Gel filtration

Sephadex G-100 was prepared by swelling in distilled water on a boiling-water bath for 1 h. The gel suspension was allowed to cool down and settle. The supernatant was poured out, removing small particles. The cleaning cycle was repeated until the supernatant was cleared. Usually it was sufficient to perform the operation twice.

Sucrose gradient centrifugation

Sucrose gradients were 5–20% (w/v) in 0.05 M Tris buffer (pH 7.4) prepared by the method of MARTIN AND AMES[30]. The gradients had a volume of 4.6 ml. A 30-μl sample (200 μg protein) was layered on top. After running for 5 h at 40 000 rev./min in a SW-50 rotor in a Spinco Model L, the rotor was allowed to coast to a stop. Fractions of 150 μl were collected. As sedimentation control lysozyme was used. Lysozyme activity was measured in a mixture containing 3 mg of *Bacillus stearothermophilus* 1503-4R (ref. 31), 5 μl of 0.1 M EDTA (pH 7), 60 μl of 0.01 M Tris buffer (pH 7.5), and 15 μl of each gradient fraction. Incubation was carried out for 15 min at 20°, and the absorbance at 520 nm was measured.

Disc gel electrophoresis

Electrophoresis of the enzyme was carried out in polyacrylamide gel (pH 8.9) by the method of ORNSTEIN AND DAVIS[32,39]. The degree of polymerization of the gel was 7.5%; it was stained for proteins with 1% Amido Schwarz in 7% acetic acid for 30 min and washed continously with 7% acetic acid for 24–36 h or run through electrophoresis. Runs were performed at 4° for 2 h with 5 mA per tube. Fructokinase activity in the gel was measured after electrophoresis by cutting the gel cylinder longitudinally. One-half was used for staining. The other half was divided in 2-mm thick sections. Enzyme activity was tested with each section according to Assay a.

Analytical ultracentrifugation

Ultracentrifuge studies were done in a Spinco Model E analytical ultracentrifuge. Analyses were performed with the enzyme dissolved in 0.01 M Tris buffer (pH 7.5). Runs were for 214 min at 42000 rev./min and at 7°.

The sedimentation constant of the protein was calculated according to the procedure of SCHACHMAN[36].

Conformation studies

Differently prepared solutions of fructose were used in these studies. Solution a was a 50 mM fructose solution prepared 24 h before use; this solution corresponds to an equilibrium mixture of the pyranose and furanose forms. Solution b was prepared by dissolving, just before incubation started, the amount of crystalline fructose

(pyranose) necessary to make a 50 mM solution. The time lapse between the addition of water to dissolve the fructose and the start of incubation was approx. 15 sec.

Activity determinations with both fructose solutions were carried out under the condition of Assay b except that the incubation times were as follows: 0.5, 1, 1.5, 2, 4, and 6 min (Fig. 7). The ADP formed in the reaction was estimated as described in Assay b after stopping the reaction by heating the tubes for 1 min at 100°.

RESULTS

Purification of fructokinase

Wistar rats weighing 150–200 g were decapitated and allowed to bleed. Livers were removed, sliced and chilled in ice. The slices were freed of fat tissue and repeatedly washed in ice–water to remove blood. Liver slices were then weighed and homogenized with 3 volumes of ice-cold 0.15 M KCl in a Waring blender for 3 min. All the following steps in the purification procedure were carried out at 0–4°. The homogenate was centrifuged for 15 min at 20 000 × *g* in a Sorvall refrigerated centrifuge. The supernatant (crude extract) was adjusted to pH 5 by addition of 1 M acetic acid with constant stirring. The acid extract was then centrifuged for 15 min at 20000 × *g*.

The acid supernatant was heated in a water bath (85°) under constant stirring. When the temperature had reached 65 ± 1°, the enzyme solution became cloudy and was heated for a further period of 2 min at this temperature. The solution was then cooled immediately in an ice-bath until its temperature reached 4°. This took approx. 2–3 min. Attempts to perform the acid purification and heat treatment in one step resulted in loss of enzyme activity.

The cold enzyme solution was clarified by passing it through a layer of cheesecloth to remove the denatured proteins or by centrifuging it for 15 min at 20 000 × *g*. The filtrate was mixed with one-twentieth its volume of 1 M acetate buffer (pH 5.0). Solid $(NH_4)_2SO_4$ was added very slowly to bring the solution to 45% saturation.

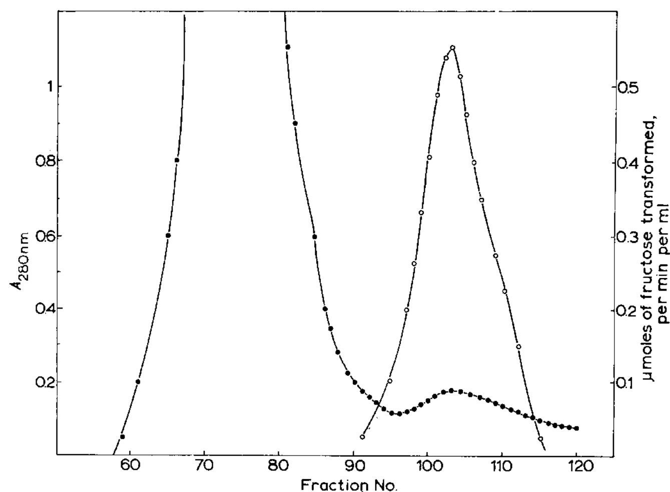


Fig. 1. Sephadex G-100 chromatography. For details see text. ●—●, absorbance at 80 nm (protein); ○—○, enzyme activity determined by Assay a in aliquots of 25 µl.

After 30 min stirring, the precipitate was collected by centrifugation for 15 min at 20000 × *g* and dissolved in ice-cold water. The solution was dialyzed for 3 h against 500 vol. of 0.01 M Tris buffer (pH 7.5). The dialysis step could be omitted without modifying the purification obtained by gel filtration.

1 ml of enzyme solution (50 mg protein) was applied to a 2 cm × 80 cm column of Sephadex G-100 previously equilibrated overnight with 0.01 M Tris buffer (pH 7.5). The column was eluted with the same buffer, collecting 1-ml fractions at a flow rate of 0.2 ml/min. The fractions containing fructokinase activity (Fig. 1) were pooled and freeze-dried to about one-hundredth volume of the homogenate. The concentrated preparation was then dialyzed overnight against 0.01 M Tris buffer (pH 7.5). $(NH_4)_2SO_4$ solution (saturated at room temperature, pH 6.8) was added to the dialysate to bring the enzyme solution to 70% saturation. After standing for 30 min, the precipitate was collected by centrifugation for 20 min at 20 000 × *g*. The precipitate was extracted twice with 0.4 ml of 70, 50, and 30% saturated $(NH_4)_2SO_4$, successively. In each case the remaining precipitate was centrifuged for 20 min at 20 000 × *g*. The last two extractions with 30% $(NH_4)_2SO_4$ contained the fructokinase activity.

The enzyme was purified 250–400-fold over the initial homogenate with an overall yield of 30%. Typical data of a purification are shown in Table I.

TABLE I

PURIFICATION OF FRUCTOKINASE

Step	*Vol. (ml)*	*Units*	*Protein (mg/ml)*	*Specific activity (units/mg)*	*Purification*	*Yield (%)*
Homogenate	134	0.868	56	0.015	1	100
Crude extract	100	1.17	40	0.029	1.9	100
pH 5 supernatant	78	1.20	22	0.055	3.7	80
Heat supernatant	69	1.02	7	0.146	9.7	61
0–45% $(NH_4)_2SO_4$	1.5	35.7	105	0.340	22.6	47
Sephadex G-100 eluate	75	0.57	0.20	2.85	190	37
30% $(NH_4)_2SO_4$ extraction	1	34.4	6.14	5.6	370	30

Criteria of purity

Sucrose gradient centrifugation. The profile of the enzyme is depicted in Fig. 2. A single absorption peak calculated by the difference between the absorbances at 215 and 225 nm (ref. 29), closely fitting the fructokinase activity, was observed. The profile or the position of the peak was not affected by 0.25 M KCl and 0.04 M mercaptoethanol. The sedimentation coefficient with lysozyme as a standard was found to be 2.85 (ref. 30).

Ultracentrifugal pattern. The sedimentation pattern of the enzyme preparation is shown in Fig. 3. The boundary of the purified enzyme moved as a single symmetrical peak with a sedimentation constant of $s_{20,w} = 2.95$ S.

Disc gel electrophoresis. When the purified enzyme preparation was subjected to electrophoresis in polyacrylamide gel at pH 8.9, two bands of protein migrating towards the cathode were observed. Both bands, as can be seen in Fig. 4, overlap with the enzyme activity detected in gel slices as described under MATERIALS AND METHODS.

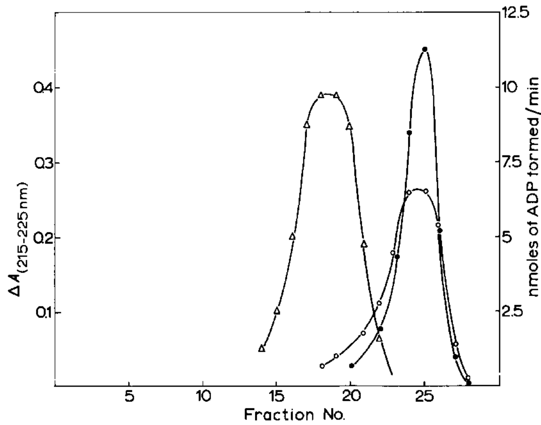


Fig. 2. Sucrose density gradient centrifugation. For details see text. ○—○, difference between absorbances at 215 and 225 nm (protein); △—△, lysozyme activity; ●—●, fructokinase activity (Assay c).

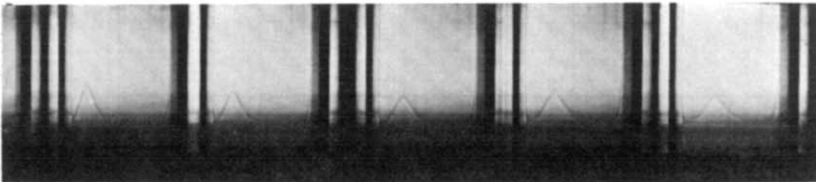


Fig. 3. Sedimentation velocity pattern of fructokinase (12 mg/ml) in 0.01 M Tris buffer (pH 7.5). The photographs were taken at 99, 129, 186, and 214 min after speed of 42 040 rev./min was reached. Direction of sedimentation is from left to right.

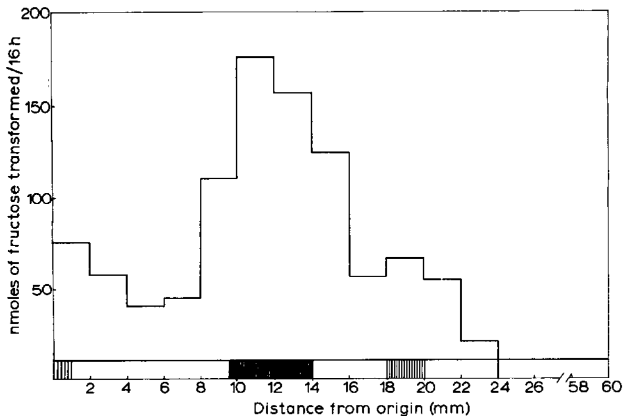
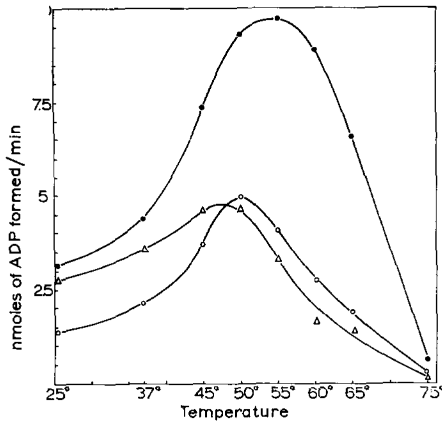


Fig. 4. Polyacrylamide gel electrophoresis of fructokinase. For details see text. The intensity of the hatched areas corresponds to the colour developed with Amido Schwarz.



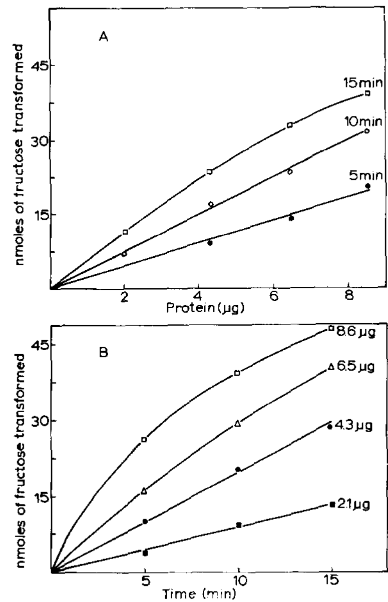


Fig. 5. Effect of temperature on fructokinase. Activity (Assay a) with different substrates: ●—●, fructose; ○—○, sorbose; △—△, tagatose.

Fig. 6. Protein concentration (A) and tissue curves (B) of fructokinase reaction. Assay a. Similar curves can be obtained with Assays b and c. In all cases incubations were carried out in the absence of ATP-regenerating system.

Properties of enzyme

Stability. No loss of activity occurs upon storage of the purified enzyme in 30% saturated $(NH_4)_2SO_4$ or in 0.01 M Tris buffer (pH 7.4) at −15° for at least 7 months.

When fructokinase activity was tested as a function of temperature, the enzyme was stable up to 50–55° and then decreased. A similar behaviour was found out with other ketoses also phosphorylated by fructokinase, as can be seen in Fig. 5. From these data the energy of activation for the phosphorylation of fructose was calculated as 11 kcal per mole and a Q_{10} of about 2. A similar value was obtained for sorbose.

Kinetics. The broad pH optimum already reported[5] was also found for the

TABLE II

MICHAELIS CONSTANTS FOR FRUCTOSE AND MgATP AT DIFFERENT K^+ CONCENTRATIONS

The Michaelis constants were determined by Assay a and by Assay d. See subsequent paper[37].

Substrate	*Concn. (mM)*	K^+ *(M)*	K_m *(mM)*
Fructose	0.5–10	0.4	0.46
	0.5–10	0.1	0.8
MgATP (Ratio ATP/Mg^{2+} = 1)	0.5–10	0.4	1.56
	0.5–10	0.1	1.33

TABLE III

SUBSTRATE SPECIFICITY OF FRUCTOKINASE AT DIFFERENT K^+ CONCENTRATIONS

Fructokinase activity (Assay a) towards the various substrates referred to activity with fructose at 0.15 M K^+ as unity.

Ketose (4 mM)	*0.15 M K^+*	*0.5 M K^+*
D-Fructose	1	1.35
L-Sorbose	0.45	1.03
D-Tagatose	0.69	0.86

purified enzyme. A higher activity was found with Tris buffer as compared with phosphate or acetate-borate-cacodilate buffers. With Tris buffer, enzyme activity presented a plateau at pH 7.2-8.5.

As shown in Fig. 6, proportionality between fructose transformed and enzyme concentration or time only held when the reaction was allowed to proceed to a small extent in the absence of an ATP-regenerating system. This behaviour has been shown to be caused by inhibition of the ADP formed in the reaction[7].

The Michaelis constants determined for fructose and MgATP are presented in Table II for different K^+ concentrations. It should be noticed that an increase in K^+ concentration brings a diminution of the K_m for fructose.

Substrate specificity: structure. The purified enzyme phosphorylates also L-sorbose and D-tagatose. Moreover, K^+ stimulation of enzymatic activity towards these sugars can be seen in Table III. However, at concentrations of 4 mM, D-tagatose and L-sorbose inhibit the phosphorylation of fructose 36 and 24%, respectively, at 0.5 M K^+. It should be mentioned here that metabolites like sorbitol, fructose 6-

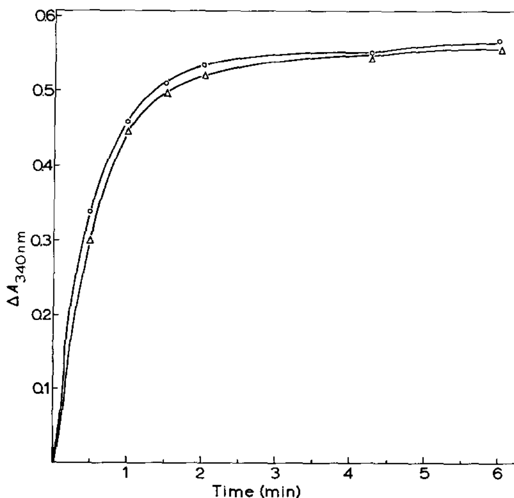


Fig. 7. Fructokinase activity towards the pyranose and furanose forms of fructose. For details see text. △—△, fructopyranose; ○—○, equilibrium mixture of fructopyranose and fructofuranose.

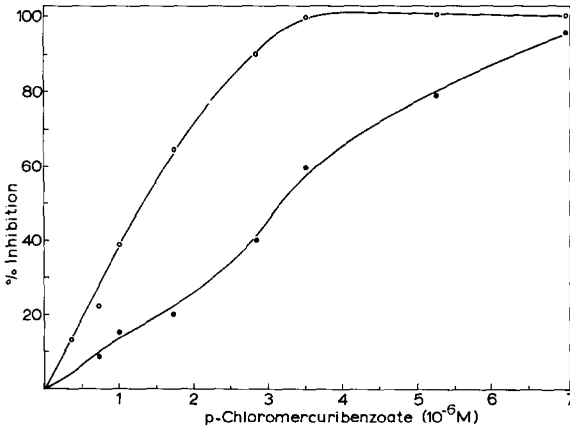


Fig. 8. Effect of *p*-chloromercuribenzoate on fructokinase. Activity (Assay a) at different temperatures. For details see text. ○—○, 55°; ●—●, 37°.

phosphate, fructose 1,6-diphosphate, glucose 1-phosphate, dihydroxyacetone phosphate, and glyceraldehyde have no effect on the activity of fructokinase.

Substrate specificity: conformation. It can be seen in Fig. 7 that fructokinase phosphorylates at the same rate a newly prepared solution of fructose (pyranose form) and a solution of fructose where the pyranose and furanose forms were equilibrated.

Effects of thiols. It has been shown that fructokinase can be inhibited by *p*-chloromercuribenzoate[9,10,18], suggesting the presence of essential sulfhydryl groups for enzyme activity. When the purified enzyme was treated with *p*-chloromercuribenzoate, it was found that 50% inhibition was attained at a concentration of $3.2 \cdot 10^{-6}$ M at 37° (see Fig. 8). However, the same *p*-chloromercuribenzoate concentration caused 100% inhibition at 55°. The concentration of *p*-chloromercuribenzoate which causes 100% inhibition, was found for the phosphorylation of sorbose to be $2.5 \cdot 10^{-6}$ M and for tagatose $1.75 \cdot 10^{-6}$ M.

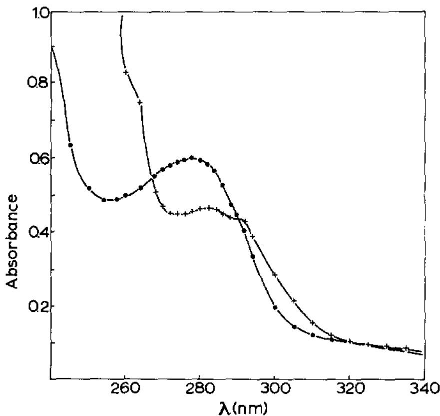


Fig. 9. Absorption spectra of fructokinase. The protein (0.3 mg/ml)was dissolved in 0.12 M phosphate buffer (pH 7) (●—●) and was brought to pH 12 with 1 M NaOH (+—+).

Molecular weight. The molecular weight determined by density gradient centrifugation using lysozyme as standard, according to the method of MARTIN AND AMES[30], averaged 28 000.

Absorption spectra. In Fig. 9 are shown the ultraviolet spectra of the purified protein at both neutral and alkaline pH. The $A_{280\,nm}/A_{260\,nm}$ ratio was 1.2. The characteristic absorption maximum at 278 nm contributed by aromatic amino acid residues was seen at neutral pH.

DISCUSSION

The purification procedure reported here for fructokinase from rat liver permits the isolation of the enzyme with a yield of about 35% by a rather small number of purification steps.

The enzyme preparation appears to be homogeneous by sedimentation velocity, but only near-homogeneous by density gradient centrifugation. By disc gel electrophoresis two protein bands were obtained. However, both proteins showed enzyme activity. The significance of this result is not clear, but it could indicate the existence of two enzyme forms. These results are approximate and require further study, but nevertheless they might have some significance.

The fructokinase is very stable under a variety of conditions, such as aging, heat inactivation and acid denaturation. The enzyme possesses sulfhydryl groups which are related to the activity, but contradictory results regarding its number are obtained with the *p*-chloromercuribenzoate experiments. Effectively, assuming a molecular weight of 30 000 for the protein, the number of sulfhydryl groups calculated according to the inhibition data would be about 1 at 55°, which is not in agreement with the number obtained at 37° which is about 2. A possible explanation for the difference observed could be a change in conformation of the enzyme with increased temperature.

The K_m values reported here for fructose (0.46–0.8 mM) and for MgATP (1.56–1.33 mM) at K^+ concentrations of 0.4 and 0.14 M, respectively, are in good agreement with those previously found by ADELMAN *et al.*[18] and HERS[5]. The value for MgATP obtained by PARKS *et al.*[7] is about 10 times lower than any other reported.

We have confirmed that fructokinase catalyzes the phosphorylation of L-sorbose[10,18]. Also we have shown that the enzyme is active towards D-tagatose. The purity of both sugars was ascertained by paper chromatography and shown to be free of fructose. Moreover, the change of enzyme activity with temperature is very similar for D-fructose, L-sorbose, and D-tagatose. Measurement of the equilibrium of aqueous fructose solution indicates 80% pyranose, 20% furanose and considerably less than 0.1% of the open form[33–35]. Which of those forms is the substrate for fructokinase? The experiments reported here seem to indicate that the enzyme is unable to distinguish between the cyclic forms. Similar experiments carried out with yeast hexokinase[38] show that this enzyme phosphorylates β-D-fructofuranose. Our method of measure does not permit to detect if the open form would have been phosphorylated more specifically. In other words, the question remains open if the open-chain form is preferred.

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