*Biochimica et Biophysica Acta*, 657 (1981) 468—481


BBA 69192

# TWO HEXOKINASES OF *HOMARUS AMERICANUS* (LOBSTER), ONE HAVING GREAT AFFINITY FOR MANNOSE AND FRUCTOSE AND LOW AFFINITY FOR GLUCOSE

MARJORIE R. STETTEN and PAUL K. GOLDSMITH

*National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, MD 20205 and The Marine Biological Laboratory, Woods Hole, MA 02543 (U.S.A.)*

(Received August 4th, 1980)

*Key words Hexokinase isozyme; Glucose-6-phosphate dehydrogenase; Substrate affinity; (Lobster)*

## Summary

Two major hexokinases (ATP: D-hexose 6-phosphotransferases, EC 2.7.1.1) have been identified in tissues of *Homarus americanus* (lobster) and separated from each other by DEAE-cellulose ion-exchange chromatography and by polyacrylamide gel electrophoresis. The molecular weight of each, determined by gel filtration, is about 50 000.

Hexokinase II, named for its column elution order, resembles hexokinase isozymes I and II of vertebrates. $K_m$ values for glucose, mannose and fructose are 0.08, 0.13 and 6.7 mM, respectively. It is strongly inhibited by the reaction products, ADP and glucose-6-*P* ($K_i$ = 0.8 mM).

Hexokinase I appears to be different from any animal hexokinase previously described. It has a high affinity for mannose and fructose and low affinity for glucose. $K_m$ values are 6, 0.07 and 1.2 mM and relative maximum rates 100, 520 and 1070 for glucose, mannose and fructose, respectively. Hexokinase I is not inhibited by physiological concentrations of ATP nor by glucose-6-*P*, mannose-6-*P* or fructose-6-*P* even at high concentrations. Both enzymes occur in muscle at about 10% of the concentration found in the hepatopancreas.

The use of *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49), with NAD as cofactor, is recommended for measuring hexokinases in crude tissue preparations to avoid the variable further reduction of nucleotide caused by the action

---

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-$N^1$-2-ethanesulfonic acid; Tricine, *N*-Tris(hydroxymethyl)methylglycine.

of 6-phosphogluconate dehydrogenase when NADP is used with yeast glucose-6-phosphate dehydrogenase.

## Introduction

A particulate glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9) with properties like those of the enzyme of vertebrate liver and kidney has been found to occur in the hepatopancreas of *Limulus polyphemus* and Crustaceans [1]. This specific enzyme was absent in many other invertebrate animals studied. We have proposed the hypothesis that a specific glucose-6-phosphatase evolved in those animals which utilized free glucose as an important circulating form of energy [1]. In the hepatocytes of higher animals hexokinase type IV (glucokinase)(ATP: D-glucose 6-phosphotransferase, EC 2.7.1.2), with a high $K_m$ for glucose, acts in conjunction with glucose-6-phosphatase in the hepatic 'futile' cycling of glucose and glucose 6-phosphate coincident with the hormonal and substrate regulation of the level of blood glucose [2,3]. We sought an answer to the question of whether such a glucokinase might also be found in those invertebrates which have a vertebrate type of glucose-6-phosphatase.

Whereas the hexokinases of yeast and other microorganisms, as well as those of a variety of vertebrates, have been extensively studied [4—7], relatively little is known in detail about the corresponding enzymes of invertebrate animals. In this study we have used lobster for the isolation of hexokinase isozymes because its hepatopancreas is discrete and accessible and lacks many of the interfering enzymes encountered in *L. polyphemus*.

## Materials and Methods

*Animals.* Male lobsters, weighing between 1 and 3 pounds, were obtained from a local sea food supplier and were used at once. No attempt was made to control their state of nutrition.

*Materials.* Enzymes used in the analytical procedures, glucose-6-phosphate dehydrogenases from *Leuconostoc mesenteroides* (Type XXII), from yeast (Types XI, XII and XV) and from Bakers yeast (Type VII), as well as phosphoglucose isomerase (Type III), phosphomannose isomerase, lactic dehydrogenase (Type II) and pyruvate kinase (Type II) were obtained from Sigma Chemical Co. Phenazine methosulfate was from Eastman, nitro blue tetrazolium chloride from the Aldrich Chemical Co., and DEAE-Sephacel and Sephacryl S-200 from Pharmacia Fine Chemicals.

*Lobster enzyme preparations.* Freshly excised hepatopancreas was homogenized in a Servall stainless steel Omni-mixer for 1 min at 0°C with enough 0.05 M Tricine buffer/5 mM dithiothreitol (pH 8.1) to give a 25 or 33.33% homogenate (w/v). The homogenate was centrifuged twice for 10 min at 10 000 × *g*, filtering the supernatant portion through gauze each time to remove the appreciable accumulation of a floating yellow fatty layer. The 10 000 × *g* supernatant was centrifuged for 1 h at about 150 000 × *g* and the supernatant again filtered to remove fat before a final recentrifugation for 30

min. Supernatants of tail muscle were obtained by a similar procedure, omitting the filtrations through gauze, since the fatty layer does not occur with muscle. The resulting clear, high-speed supernatants were used for enzyme analysis, DEAE-cellulose ion-exchange chromatography and polyacrylamide gel electrophoresis.

*Hexokinase assays.* The rate of glucose, mannose or fructose phosphorylation was usually determined by coupling the glucose-6-*P* formed with reduction of NAD catalyzed by *L. mesenteroides* glucose-6-*P* dehydrogenase. Unless otherwise specified, the reaction mixture consisted of 5 mM ATP/6 mM $MgCl_2$ /0.5 mM NAD/0.5 or 100 mM sugar/1 unit of glucose-6-phosphate dehydrogenase/a suitable aliquot of the lobster enzyme solution/0.1 M Hepes buffer, pH 7.5, in a final volume of 1 ml. In addition, for phosphorylation of fructose and mannose, 1 unit phosphoglucose isomerase, and for mannose, 1 unit of phosphomannose isomerase were added. Alternatively, with other sugars and for sugar phosphate inhibition studies, the rate of formation of ADP was measured by coupling the reaction to the oxidation of NADH by pyruvate kinase and lactic dehydrogenase in the presence of an excess of phospho*enol*pyruvate [8].

*DEAE-cellulose ion-exchange chromatography.* Separation of the enzymes by DEAE-cellulose ion-exchange chromatography was performed as described in the captions to Figs. 1 and 2.

*Polyacrylamide gel electrophoresis.* This was done in a Pharmacia electrophoresis apparatus GE-4 with a slab containing 7.5% polyacrylamide (19 : 1 acrylamide: bisacrylamide) in 0.89 M Tris-HCl, pH 8.37/0.08 M borate/2.5 mM EDTA into which 1 unit/ml glucose-6-phosphate dehydrogenase had been incorporated as suggested by Harrison [9]. After electrophoresis, the hexokinases were located in the gels by incubation with a developing solution at room temperature, in the absence of light for an appropriate length of time. The solution contained 100 mM Hepes buffer, pH 7.5/10 mM $MgCl_2$/0.5 mM NADP/40 μg phenazine methosulfate/400 μg nitro blue tetrazolium chloride ml/0.5 or 100 mM glucose/5 mM ATP where appropriate. For visualization of fructokinase 1 U phosphoglucose isomerase was added to each ml of the incubation mixture along with the appropriate concentration of fructose. Reaction was stopped by fixation in 10% (w/v) acetic acid.

*Molecular weight determinations.* Molecular weights were estimated by the gel chromatography method of Andrews [10], using a 2.2 × 90 cm column of Sephacryl S-200 equilibrated with 0.05 M Tricine/5 mM dithiothreitol, pH 8.1. Reference proteins were obtained in a kit from Pharmacia Fine Chemicals. Elution volumes of hexokinases were determined by enzyme assays of column fractions.

Protein was determined by a modification [11] of the method of Lowry et al. [36].

## Results

### *Use of NAD with glucose-6-phosphate dehydrogenase from L. mesenteroides to measure hexokinases in impure enzyme preparations*

Several methods have been used to correct for the contribution of endogenous 6-phosphogluconate dehydrogenase to the reduction of NADP

when determining hexokinase activities in crude preparations from animal tissues, using the coupled reaction with yeast glucose-6-phosphate dehydrogenase. The early method of Di Pietro and Weinhouse [12], used by most others, was to assume that crude tissue preparations had a sufficiently high concentration of 6-phosphogluconate dehydrogenase to catalyze the overall reduction of 2 mol NADP. Some have checked this assumption by showing that the occasional addition of purified 6-phosphogluconate dehydrogenase to their enzyme assays did not increase the rate of NADPH production [13,14]. Ureta et al. [15] have, when measuring total hexokinases in liver extracts of a variety of species, routinely added an excess of both dehydrogenases to their assay mixtures. Shonk and Boxer [16] noted that high concentrations of purified 6-phosphogluconate were required for the activity of 6-phosphogluconate dehydrogenase. At the levels of hexokinase activity that they were measuring in human tissues, even if purified 6-phosphogluconate dehydrogenase were added to the assay, the rate of NADPH production was unaffected in short-term experiments. When reactions were run for 20 min, a maximum of about 1.5 mol NADP were reduced/mol glucose phosphorylated. Sheer et al. [17] have found it necessary to make individual corrections for 6-phosphogluconate dehydrogenase in each sample isolated.

In our preliminary measurements of hexokinases in various invertebrate tissues, we also found that 2 mol NADP were not reduced even when an apparent excess of 6-phosphogluconate dehydrogenase was added to the assay mixture. We have therefore measured glucose-6-*P* oxidation using NAD as cofactor with a purified preparation of glucose-6-phosphate dehydrogenase from *L. mesenteroides* (Sigma). Under these conditions, since most 6-phosphogluconate dehydrogenases have a specific requirement for NADP, the presence of variable quantities of 6-phosphogluconate dehydrogenases in tissues in which hexokinases are being measured does not interfere with measurement of the rate of glucose-6-*P* production. Only 1 mol NADH is formed in the coupled reaction with *L. mesenteroides* glucose-6-phosphate dehydrogenase/mol glucose-6-*P* formed by hexokinase action in impure preparations. This stoichiometry has been confirmed by measuring the ADP formed in the hexokinase reaction. Since this bacterial glucose-6-phosphate dehydrogenase uses either NAD or NADP [18], its use with the two cofactors in measuring hexokinases in tissue homogenates gives an approximate measure of the extent of the contribution of endogenous 6-phosphogluconate dehydrogenase to NADPH production. For lobster hepatopancreas homogenates and supernatants about 1.7-times as much NADP was reduced as NAD (Table I). Although the quantity of hexokinase measured varied from animal to animal and differed for high and low glucose concentrations, the relative contribution of the secondary reaction due to endogenous 6-phosphogluconate dehydrogenase in the tissue was independent of the glucose concentration and was relatively constant for crude lobster hepatopancreas preparations. We have made appropriate corrections in some of our early assays in which yeast glucose-6-phosphate dehydrogenase and NADP were used, but have usually avoided this complication by using NAD with the *L. mesenteroides* enzyme. Muscle preparations had much less hexokinase activity and little or no 6-phosphogluconate dehydrogenase activity. The inclusion of an excess of 6-phosphogluconate dehydrogenase in the assay of

TABLE I

USE OF *LEUCONOSTOC MESENTEROIDES* GLUCOSE-6-PHOSPHATE DEHYDROGENASE WITH NAD AND NADP IN THE ASSAY OF HEXOKINASES IN CRUDE ENZYME PREPARATIONS

Assay mixtures contained 5 mM ATP/6 mM $MgCl_2$/0.5 mM NAD or NADP/0.5 or 100 mM glucose/1 unit of *L. mesenteroides* glucose-6-phosphate dehydrogenase, appropriate amounts of crude lobster enzyme preparation and 0.1 M Hepes buffer, pH 7.5, in a volume of 1 ml. The rate of reduction of nucleotide was measured at 340 nm and 30° C. The reaction was started by the addition of sugar after preincubation for about 10 min to remove preexisting glucose-6-*P*.

| Enzyme preparation | Glucose in assay (mM) | Nucleotide in assay (μmol reduced/min per g tissue) | | NADP/NAD (ratio) |
|---|---|---|---|---|
| | | NADP | NAD | |
| 1. Hepatopancreas — 150 000 × *g* supernatant | 0.5 | 1.58 | 0.94 | 1.68 |
| | 100 | 1.98 | 1.18 | 1.68 |
| 2. Hepatopancreas — 10 000 × *g* supernatant | 0.5 | 1.55 | 0.93 | 1.67 |
| | 100 | 1.77 | 1.07 | 1.65 |
| 3. Hepatopancreas — 150 000 × *g* supernatant | 100 | 2.24 | 1.33 | 1.68 |
| 4. Supernatants after removal of small molecules by Sephadex G-25 filtration | | | | |
| Hepatopancreas | 100 | 1.35 | 0.86 | 1.57 |
| Muscle | 0.5 | 0.125 | 0.120 | 1.04 |
| Muscle + excess 6-phosphogluconate dehydrogenase | 0.5 | 0.238 | 0.119 | 2.00 |

muscle hexokinase doubled the rate of NADPH formation (Table I).

Another disadvantage in the use of yeast glucose-6-phosphate dehydrogenases for hexokinase assays is the occurrence in many of the commercially available purified enzymes of significant amounts of glucose dehydrogenase activity which may contribute appreciably to NADPH formation with high glucose concentrations [19]. In a survey of a few of the many different preparations

TABLE II

GLUCOSE DEHYDROGENASE ACTIVITY OF SOME COMMERCIALLY AVAILABLE PREPARATIONS OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Assay mixtures consisted of 100 mM glucose/6 mM $MgCl_2$/0.5 mM NAD or NADP/approx. 1 U glucose-6-phosphate dehydrogenase/0.1 M Hepes buffer, pH 7.5, with or without 5 mM ATP in a volume of 1 ml. When the concentration of glucose-6-phosphate dehydrogenase was increased 2-fold the reaction rate was nearly doubled.

| Source of glucose-6-phosphate dehydrogenase | Nucleotide | $\Delta A$ (340 nm) × $10^3$/min per ml | |
|---|---|---|---|
| | | —ATP | +ATP |
| *Leuconostoc mesenteroides* | NADP | <0.1 | <0.1 |
| | NAD | 0.1 | <0.1 |
| Bakers yeast — Type VII | NADP | 0.9 | 0.2 |
| | NAD | 0 | 0 |
| Torula yeast — Type XII | NADP | 1.2 | 0.5 |
| | NAD | 0 | 0 |
| Torula yeast — Type XV | NADP | 0.1 | <0.1 |
| | NAD | 0 | 0 |

available we have found, in assays without ATP or lobster enzyme, (Table II) that the extent of the contribution of glucose dehydrogenase to the reduction of the nucleotides varies greatly with the type of yeast enzyme as well with the amount and age of the solution used. *L. mesenteroides* glucose-6-phosphate dehydrogenase shows only a small amount of glucose dehydrogenase activity with both cofactors. The usual correction of hexokinase assays by subtraction of values obtained with the same assay mixture in the absence of ATP was found to be inadequate to correct for the contribution made by glucose dehydrogenases, since these enzymes are partially and variably inhibited by ATP (Table II). The application of the conventional 'no ATP blank' correction will, in the presence of glucose dehydrogenase activity, give false low values for hexokinase.

The use of *L. mesenteroides* glucose-6-phosphate dehydrogenase with NAD as cofactor, instead of yeast glucose-6-phosphate dehydrogenase with NADPH, is recommended for routine hexokinase assays in crude tissue preparations for three reasons. 1. Further reduction of nucleotide by tissue 6-phosphogluconate dehydrogenase is avoided. 2. Contribution to reduction of nucleotides by glucose dehydrogenase is minimized. 3. It is more economical since NAD is much less costly than NADP.

*Separation of hexokinases in lobster hepatopancreas by DEAE-cellulose ion-exchange chromatography*

Two major hexokinases could be completely separated from each other by application of a high-speed supernatant solution of lobster hepatopancreas homogenate to a DEAE-Sephacel column followed by elution with a linear gradient of 0—0.5 M KCl. An example of such a separation is shown in Fig. 1. The enzymes have been named I and II in order of their elution from the column. Both enzymes were found to have a low $K_m$ value for mannose, so assay of elution fractions with 0.5 mM mannose was most convenient for locating the peaks of hexokinase activity. The enzymes differed greatly in their reactivity with glucose. With either 0.5 or 100 mM glucose, hexokinase II gave about the same level of activity as with mannose, while hexokinase I showed much less reactivity with glucose, even at high concentration, and very little activity at low glucose concentration. The peak of activity was about twice as high for both enzymes with 100 mM fructose (data not shown) as with mannose.

Muscle of lobster was found to contain what appeared to be the same two hexokinase isozymes. A comparison of the separation of the enzymes from the muscle and hepatopancreas of a single lobster is shown in Fig. 2. There was usually about 10% as high a concentration of each enzyme in muscle as in hepatopancreas.

In a preliminary study of the quantity of total hexokinase activity in homogenates of lobster hepatopancreas measured with both 0.5 and 100 mM glucose, it was apparent that a considerable portion of the hexokinase was labile and that, as the total decreased, the relative concentration of a portion having a high Michaelis constant for glucose increased. Subsequent study showed that, whereas hexokinase I is relatively stable in purified elution fractions, hexokinase II loses activity rapidly both in homogenates and in elution

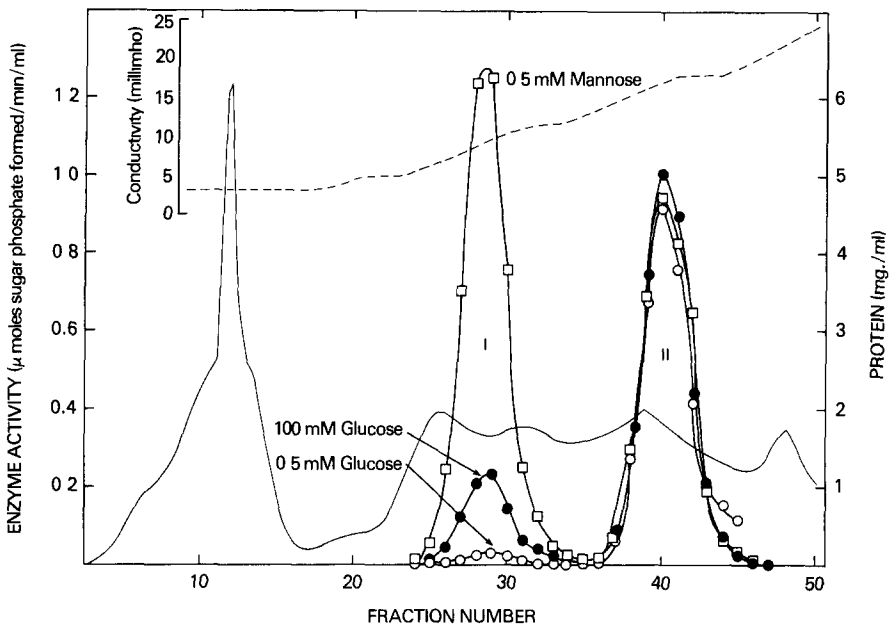


Fig. 1. DEAE-cellulose ion-exchange chromatography of lobster hepatopancreas hexokinases. 80 ml of a high speed supernatant of a 33.3% homogenate of lobster hepatopancreas in 0.05 M Tricine/5 mM dithiothreitol (pH 8.1) was applied to a 4.4 × 6.3 cm column (96 ml bed vol) of DEAE-Sephacel (Pharmacia) equilibrated with the same buffer mixture at 4°C. After a wash with 80 ml buffer, the column was eluted with 400 ml of a linear gradient of 0—0.5 M KCl in the buffer mixture. Flow rate was about 100 ml/h and fractions of about 8.5 ml were collected. Conductivity of the fractions is indicated by the broken line, and protein concentrations by the thin solid line. Aliquots of each fraction were analyzed for hexokinase activity with mannose and with two concentrations of glucose: 0.5 mM mannose (□——□); 0.5 mM glucose (○——○); 100 mM glucose (●——●).

fractions and does not withstand freezing. Therefore, the relative heights of the peaks of activity seen in Figs. 1 and 2 do not well represent the relative normal abundance of the two hexokinases in the animal. By extrapolation we have estimated that there was approx. 4—5-times as much enzyme II as enzyme I in the animals studied.

Fractions having the peak activity for each enzyme appeared to contain only one hexokinase isozyme. Assays were carried out under standard conditions with 0.5 and 100 mM glucose, fructose and mannose and enzyme fractions which had been progressively aged or heat treated. While absolute values for enzyme activities decreased up to 10-fold, ratios of activities of the three sugars remained approx. constant as did the ratios of activities of each sugar at the two concentration levels. For enzyme I the ratio for 100 mM/0.5 mM sugar was about 9 for glucose, 3.3 for fructose and 1.1 for mannose. The corresponding ratios for enzyme II were 1.0, 9 and 1.0.

No attempt has been made to isolate the enzymes as pure proteins but some further degree of purification and removal of contaminating proteins, which interfered with assay methods, was achieved by the gel filtration chromatography procedure used to estimate molecular weights of the enzymes. Phospho-

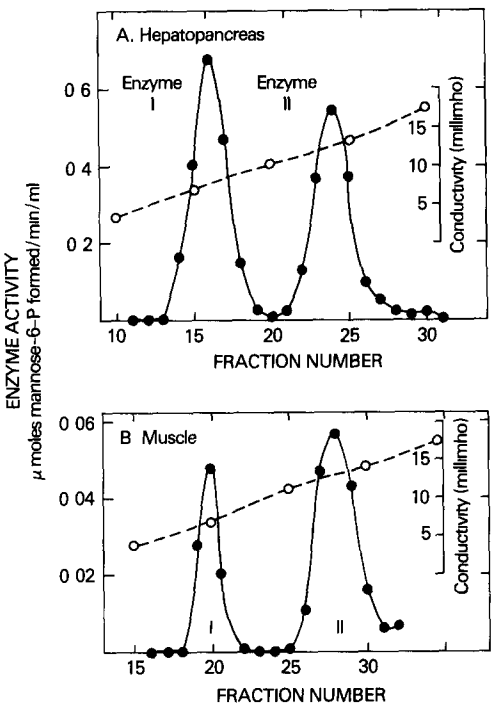


Fig. 2. Separation of the two lobster hexokinases. Comparison of hepatopancreas with muscle. Supernatant fractions, prepared by centrifugation of 25% homogenates of lobster hepatopancreas or tail muscle in 0.05 M Tricine/5 mM dithiothreitol buffer (pH 8.1) for 1 h at 150 000 × *g*, were used for separation of the hexokinases by DEAE-cellulose ion-exchange chromatography. Phosphorylation of mannose was used to locate the hexokinase activity in the fractions collected. A. 54 ml of hepatopancreas supernatant was applied to a 2.6 × 12 cm column (64 ml bed vol.) of DEAE-Sephacel equilibrated with the Tricine/DTT buffer at 4° C. The column was washed with 75 ml buffer and then eluted with 400 ml of a 0—0.8 M linear gradient of KCl in the same buffer. B. 88 ml muscle supernatant was applied to a 3.2 × 11.5 cm column (93 ml bed vol.) and the column washed with 90 ml buffer and eluted with 500 ml of a 0—0.8 M linear gradient of KCl.

glucose isomerase in peak fractions of hepatopancreas enzyme I and adenosine triphosphatase in fractions of muscle enzyme I were removed by this method. By comparison with proteins of known molecular weights, both hexokinases I and II had molecular weights of approx. 50 000.

*Polyacrylamide gel electrophoresis*

The enzymes could also be separated by polyacrylamide gel electrophoresis and visualized as purple bands which precipitated when incubated with ATP, NADP, $Mg^{2+}$, sugar and a dye. Fig. 3 shows the results of an experiment in which aliquots of unfractionated supernatants of hepatopancreas and muscle homogenates were run along with aliquots of peak fractions from the ion-exchange chromatography separation of Fig. 2. Controls (data not shown) were carried out with identically prepared slabs but incubated after electrophoresis with each sugar, NADP, $Mg^{2+}$ and dye solution in the absence of ATP. Those bands which were seen only in the presence of ATP are indicated by arrows as hexokinases. All other bands, which were located nearer to the origin, were

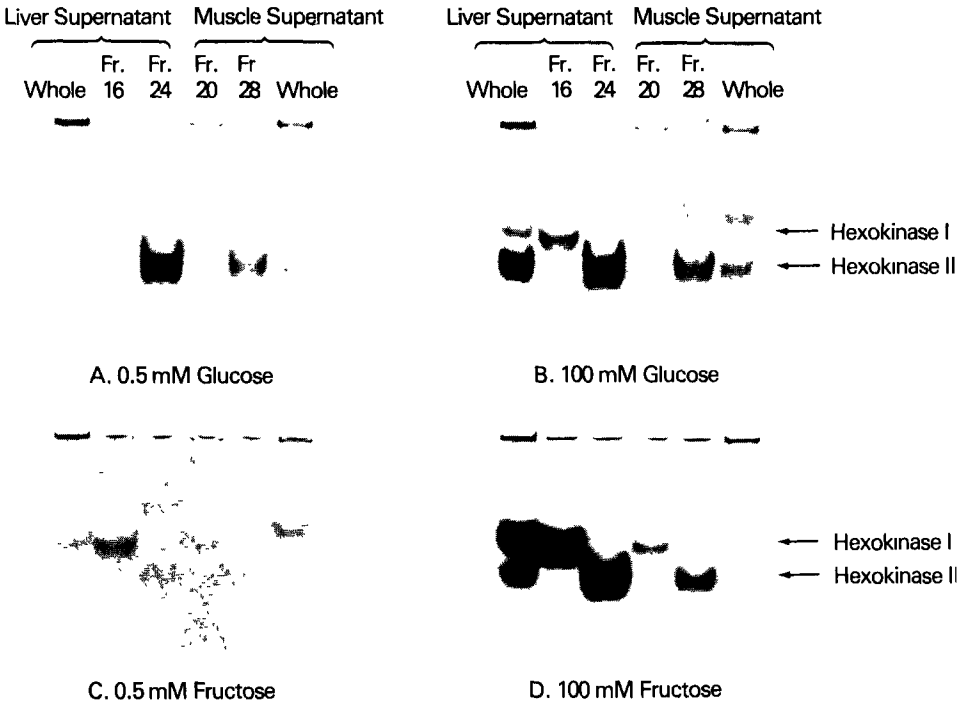


Fig. 3. Polyacrylamide gel electrophoresis of lobster hexokinases. Aliquots of unfractionated high-speed supernatants of the lobster hepatopancreas and muscle homogenates used in the ion-exchange column chromatography experiment described in Fig. 2 and of the fractions having peak levels of enzyme activity were used for slab polyacrylamide gel electrophoresis. Details are given in the experimental section. 50 λ aliquots were applied to gel slabs into which glucose-6-phosphate dehydrogenase had been polymerized [9]. After electrophoresis, hexokinase activity was visualized as purple bands which precipitated when slabs were incubated in a buffered solution at pH 7.5 with nitro blue tetrazolium chloride and phenazine methosulfate and ATP, NADP, $Mg^{2+}$ and sugar. A. 0.05 mM glucose. B. 100 mM glucose. C. 0.5 mM fructose. D. 100 mM fructose.

constant in the presence or absence of ATP, NADP or sugar.

With 0.5 mM glucose, only one hexokinase band appeared and only in the fraction from the second peak, corresponding to hexokinase II. At high concentrations of glucose both hexokinases appeared and were located only in one or other of the fractions. With 0.5 mM fructose, hexokinase I, which has a $K_m$ value of 1.2 mM for fructose, was visible, but not hexokinase II, with its high $K_m$ value for fructose, With 100 mM fructose, both enzymes gave deeply stained bands, in accord with their high $V$ values for fructose relative to that of glucose. Since identical aliquots of supernatants of 25% homogenates of hepatopancreas and muscle were used, a comparison of the density of staining of the two tissues is in accord with the observation that hexokinases are about 10-times more concentrated in hepatopancreas than in muscle.

*Effect of pH*

The two enzymes differ appreciably in their pH-related activities (Fig. 4). Hexokinase I has a narrow optimum at about pH 7, while hexokinase II, like all

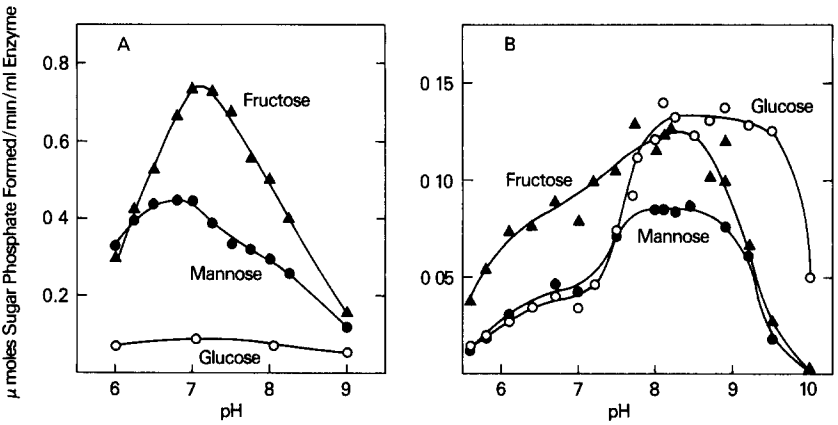


Fig. 4. Effect of pH on lobster hexokinase activities. A. Hepatopancreas enzyme I. B. Muscle enzyme II. Buffers used were cacodylate (A) or MES (2(*N*-morpholino)ethanesulfonic acid) (B) in the region pH 5.6—6.7, Hepes between pH 6.8 and 7.8, Tricine between pH 8.0 and 9.0, glycylglycine-NaOH between pH 8.1 and 8.9 and glycine-NaOH between pH 8.9 and 10.0. All reaction mixtures contained 200 mM buffer/5 mM ATP/6 mM $MgCl_2$/0.5 mM NAD/sugar/lobster enzyme and 1 U *L. mesenteroides* glucose-6-phosphate dehydrogenase and, where required, 1 U phosphoglucose isomerase and 1 U mannose phosphate isomerase/ml of reaction mixture. Sugar concentrations for enzyme I (A) were 50 mM glucose, 5 mM fructose or 0.5 mM mannose, and for enzyme II (B) 20 mM glucose, fructose or mannose. Reactions were started by the addition of enzyme. Enzyme fractions used were those which showed peak activities after successive absorption and elution on DEAE-Sephacel and on Sephacryl S-200. ○———○, glucose; ▲———▲, fructose; ●———●, mannose.

TABLE III

SUGAR SPECIFICITY OF LOBSTER HEXOKINASES

Enzyme activities were determined by measuring the rate of formation of ADP or of sugar phosphate as described under Materials and Methods, using either Hepes buffer, pH 7.5, or Tricine buffer, pH 8.2 and aliquots of peak fractions from ion-exchange chromatography separation of the two enzymes of hepatopancreas and muscle. Approximate relative maximum rates were determined using 100 mM sugars. Relative max rate is relative to glucose = 100. Compounds negative with both enzymes: D-galactose, L-sorbose, 2-deoxygalactose, D-ribose, 2-deoxyribose, D-arabinose, L-arabinose, D-xylose, D-lyxose, arabitol, sorbitol, mannitol, D-glucoheptose, mannoheptulose, glucoheptulose, α-$CH_3$ glucoside, α-$CH_3$ mannoside, maltose, trehalsoe, *N*-acetylglucosamine.

| Sugar | Hexokinase I | | | | Hexokinase II | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Hepatopancreas | | Muscle | | Hepatopancreas | | | Muscle | |
| | Rel. max rate | $K_m$ pH 7.5 (mM) | Rel. max rate | $K_m$ pH 7.5 (mM) | Rel. max rate | $K_m$ pH 7.5 (mM) | $K_m$ pH 8.2 (mM) | Rel. max rate | $K_m$ pH 7.5 (mM) |
| D-Glucose | 100 | 6 | 100 | 6 | 100 | 0.08 | 0.15 | 100 | 0.08 |
| 2-Deoxyglucose | 160 | 0.56 | 170 | — | 110 | | 0.13 | 120 | 0.25 |
| D-Mannose | 520 | 0.07 | 490 | 0.09 | 110 | 0.13 | 0.22 | 100 | 0.13 |
| D-Fructose | 1070 | 1.2 | 1010 | 1.3 | 260 | 6.7 | 5.0 | 240 | 6.7 |
| D-Glucosamine | 150 | 5.0 | 130 | — | 90 | | 0.36 | 70 | — |
| D-Mannosamine | 20 | — | — | — | 10 | | — | 0 | — |
| D-Allose | 20 | 60 | — | — | 10 | | — | 30 | — |
| D-Altrose | 100 | 2.7 | 100 | — | 0 | | — | 0 | — |
| Sedoheptulose | 20 | — | — | — | 8 | | — | 0 | — |
| 1,5-Anhydroglucitol | 0 | — | — | — | 30 | | — | 30 | — |

four isozymes occurring in vertebrates [20,21], shows a broad optimum between about pH 8 and 9. With enzyme II, the pH profiles differed with the different sugar acceptors, a phenomenon previously noted for hexokinases of *L. mesenteroides* [22] and *E. coli* [23].

*Sugar specificity*

About 30 different sugars and related compounds were tested as phosphoryl acceptors with the two hexokinases, and Michaelis constants were determined for some of the sugars which gave positive results (Table III). The properties of each enzyme were similar in hepatopancreas and in muscle. Enzyme II, like the common vertebrate hexokinase isozymes I and II of brain and muscle [24,5] has a low $K_m$ value for glucose and mannose, a high $K_m$ value for fructose, and efficiently catalyzes the phosphorylation of 2-deoxyglucose and glucosamine. For enzyme I, mannose is the preferred acceptor at low sugar concentration, while fructose is phosphorylated well at low concentrations and to a greater extent than mannose at high sugar concentrations. Glucose is a relatively poor substrate, with a Michaelis constant of about 6 mM. 2-Deoxyglucose, which can also be called 2-deoxymannose, has an affinity, as indicated by its $K_m$ value, between those of mannose and glucose. Altrose reacts, with a relatively low $K_m$ value, with enzyme I but not at all with enzyme II, while allose is not a good substrate for either enzyme.

In spite of the accumulation of much data on the specificity of various hexokinase, it has not been possible to explain satisfactorily hexokinase activities on the basis of conformation and functional groups of sugar substrates, as discussed by Crane [24]. Nevertheless, it may be of interest to note that altrose differs from allose in the same way that mannose differs from glucose, i.e., only in the configuration about C-2. Lobster hexokinase I has a much greater affinity for the sugar isomers with the -OH of C-2 on the left hand side of the molecule in the Fisher notation, i.e., mannose and altrose.

*Inhibition studies*

The two enzymes differ greatly in product inhibition. Enzyme II, like most vertebrate hexokinases, is strongly inhibited by low concentrations of both products, glucose-6-*P* and ADP. ADP is a competitive inhibitor of ATP and glucose-6-*P* a non-competitive inhibitor of glucose phosphorylation. Inhibition constants were determined from Dixon plots [25] of enzyme activities determined by varying the glucose-6-*P* concentration between 0 and 2 mM at constant levels of 0.067, 0.1 and 0.2 mM glucose. $K_i$ = 0.8 mM glucose-6-*P* for both hepatopancreas and muscle hexokinase II. On the other hand, hexokinase I was not greatly inhibited by ADP and was not at all inhibited by physiological concentrations of glucose-6-*P*. Neither glucose-6-*P*, fructose-6-*P* nor mannose-6-*P* at up to 10 mM levels inhibited phosphorylation of their respective sugars, while concentrations as high as 50 mM sugar phosphates had only a small inhibiting effect. In this property, lobster hexokinase I resembles the hexokinases of yeast and vertebrate type IV hexokinase [4].

Since mannose and fructose appear to be the preferred substrates for hexokinase I, we have studied the mannose inhibition of the phosphorylation of fructose by this enzyme. A Dixon plot of the data obtained with hepa-

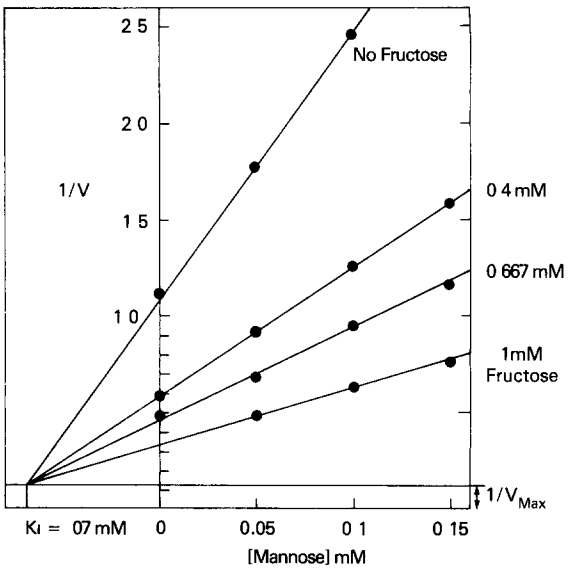


Fig. 5. Mannose inhibition of the fructokinase activity of lobster hexokinase I. The rate of phosphorylation of fructose in the presence of various concentrations of the inhibitor, mannose, was determined with the substrate concentration held constant at several levels. The data are plotted by the method of Dixon [25].

topancreas enzyme I (Fig. 5) shows a typical competitive inhibition type of kinetics. The inhibition constant for mannose was 0.07 mM, identical with the $K_m$ value for mannose. Thus, mannose and fructose may be assumed to act on the same site on hexokinase I.

## Discussion

Hexokinases have been found in all species of microorganisms, plants and animals in which they have been sought. Their often widely differing properties have been the subject of extensive studies [4—7]. Whereas a few enzymes with absolute specificity for a single sugar have been described in certain bacteria [26,27,29,30], slime mold [28] and parasitic worms [31], most hexokinases have a broad substrate specificity, while showing a strong preference for one or another sugar. In almost all species there is one or more hexokinase having a great affinity for glucose and mannose at low concentrations and a relatively low affinity for fructose. The much studied isozymes of yeast hexokinase [4] and isozymes I, II and III of higher vertebrates [5,6] are of this type, as is lobster hexokinase II. Although not much is known about the hexokinases of most invertebrates, Mochizuki and Hori [32] have isolated and studied the properties of a hexokinase from pyloric caeca of the star fish, *Asterias amurensis*. This enzyme closely resembles lobster hexokinase II in its substrate specificity, kinetic properties, inhibition by glucose-6-*P* and molecular weight. The presumed function of such hexokinases is to catalyze the initial step in the utilization of glucose when the free sugar occurs at low concentrations.

The unique vertebrate liver hexokinase type IV (glucokinase), with its high $K_m$ value for glucose, has been much studied because its adaptive and kinetic

properties give it an important role in the regulation of glucose metabolism in the liver [33]. Enzymes with some kinetic properties similar to this hexokinase have been reported in a few species of plants and microorganisms, for example, hexokinases with a high $K_m$ value for glucose in germinating maize seeds [34] and in *E. coli* [23].

Lobster hexokinase I has some properties similar to those of the vertebrate type IV enzyme but also differs from it in many ways. Both have molecular weights of about 50 000, a $K_m$ value for ATP of about 0.5 mM, a high $K_m$ value for glucose, and are not inhibited by physiological concentrations of glucose-6-*P*. Their affinities for sugars other than glucose are very different. The vertebrate type IV enzyme is nearly specific for glucose, with $K_m$ values of about 33, 800 and 55 mM for mannose, fructose and 2-deoxyglucose, respectively [35], while the corresponding values for lobster type I hexokinase are 0.07, 1.2 and 0.6 mM. The lobster enzyme can catalyze the phosphorylation of glucosamine while the vertebrate enzyme can not.

Lobster hexokinase I has the property, not previously noted for an animal enzyme, of a greater affinity for mannose and fructose than for glucose. The differences in relative affinities of hexokinase I for fructose, mannose and glucose are not as well shown by their respective Michaelis constants and relative maximum rates as by their relative rates at 0.5 mM sugar. At these more nearly physiological concentrations, the rates are 50 for mannose and 32 for fructose, relative to glucose at 1. The most comparable known enzyme of which we are aware is the 'mannokinase' of *E. coli* reported by Sebastian and Asensio [23] as having $K_m$ values of 0.2 mM for mannose, 0.3 mM for fructose, 10 mM for glucose, 10 mM for 2-deoxyglucose and 5 mM for glucosamine. Like the lobster enzyme, it is not inhibited by sugar phosphates. A mannofructokinase with similar properties but unable to react with glucose or other sugars has been isolated from *L. mesenteroides* by Sapico and Anderson [22].

What the normal physiological substrates for lobster hexokinase I are and whether it participates in a substrate-controlled adjustment of the level of circulating blood sugars is not at present known. Perhaps the most important feature of enzyme I to the animal is its lack of product inhibition. Hexokinase II should be able to phosphorylate glucose and mannose efficiently at the usual low levels of free sugars found in marine invertebrates, but at high substrate concentrations might be significantly inhibited by the glucose-6-*P* formed, if sugar phosphate were not rapidly removed. These animals are adapted to use food which is obtained intermittently. Perhaps hexokinase I is tailored to function well when absorbed food is plentiful. When the sugar concentration is high, hexokinase I might act in lobster the way glucokinase does in hepatocytes, in utilizing sugars at high concentrations and without being subject to product inhibition.

## References

1 Stetten, M.R. and Goldsmith, P.K. (1976) Biochim. Biophys. Acta 444, 835–852
2 Hue, L. and Hers, H.-G. (1974) Biochem. Biophys. Res. Commun. 58, 540–548
3 Clark, D., Lee, D., Rognstad, R. and Katz, J. (1975) Biochem. Biophys. Res. Commun. 67, 212–219
4 Colowick, S.P. (1973) in The Enzymes (Boyer, P.D., ed.), Vol IX, pp. 1–48, Academic Press, New York

5 Ureta, T. (1975) in Isozymes — Developmental Biology (Markert, C.L., ed.), Vol. III, pp. 575—601, Academic Press, New York
6 Walker, D.G. (1966) in Essays in Biochemistry (Campbell, P.N. and Greville, G.D., eds.), Vol. 2, pp. 33—67, Academic Press, New York
7 Purich, D.L., Fromm, H.J. and Rudolf, F.B. (1973) in Advances in Enzymology (Meister, A., ed.), Vol. 39, pp. 249—326, John Wiley and Sons, Inc., New York
8 Balinsky, D. and Fromm, H.J. (1978) Comp. Biochem. Physiol. 60B, 71—75
9 Harrison, R.A.P. (1974) Anal. Biochem. 61, 500—507
10 Andrews, P. (1964) Biochem. J. 91, 222—233
11 Peterson, G.L. (1977) Anal. Biochem. 83, 346—356
12 Di Pietro, D.L. and Weinhouse, S. (1960) J. Biol. Chem. 235, 2542—2545
13 Walker, D.G. (1963) Biochim. Biophys. Acta 77, 209—226
14 Bontemps, F., Hue, L. and Hers, H.-G. (1978) Biochem. J. 174, 603—611
15 Ureta, T., Radojkovic, J., Diaz, N., Slebe, J.C. and Longano, C. (1978) Arch. Biochem. Biophys. 186, 235—247
16 Shonk, C.E. and Boxer, G.E. (1964) Cancer Res. 24, 709—721
17 Scheer, W.D., Lehmann, H.P. and Beeber, M.F. (1978) Anal. Biochem. 91, 451—463
18 De Moss, R.D., Gunsalus, I.C. and Bard, R.C. (1953) J. Bacteriol. 66, 10—16
19 Anderson, W.B. and Nordlie, R.C. (1968) Biochemistry 7, 1479—1485
20 Grossbard, L. and Schimke, R.T. (1966) J. Biol. Chem. 241, 3546—3560
21 González, C., Ureta, T., Babul, J., Rabajille, E. and Niemeyer, H. (1967) Biochemistry 6, 460—468
22 Sapico, V. and Anderson, R.L. (1967) J. Biol. Chem. 242, 5086—5092
23 Sebastian, J. and Asensio, C. (1972) Arch. Biochem. Biophys. 151, 227—233
24 Crane, R.K. (1962) in The Enzymes (Boyer, P.D., ed.), 2nd edn., Vol. VI, pp. 47—66, Academic Press, New York
25 Dixon, M. (1953) Biochem. J. 55, 170—171
26 Kamel, M.Y., Allison, D.P. and Anderson, R.L. (1966) J. Biol. Chem. 241, 690—694
27 Saito, N. (1965) J. Biochem. (Tokyo) 57, 363—371
28 Baumann, P. (1969) Biochemistry 8, 5011—5015
29 Sabater, B., Sebastián, J. and Asensio, C. (1972) Biochim. Biophys. Acta 284, 406—413
30 Sabater, B., Sebastián, J. and Asensio, C. (1972) Biochim. Biophys. Acta 284, 414—420
31 Bueding, E. and MacKinnon, J.A. (1955) J. Biol. Chem. 215, 495—505
32 Mochizuki, Y. and Hori, S.H. (1977) J. Biochem. (Tokyo) 81, 1849—1855
33 Weinhouse, S. (1976) in Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R., eds.), Vol. 11, pp. 1—50, Academic Press, New York
34 Cox, E.L. and Dickinson, D.B. (1973) Plant Physiol. 51, 960—966
35 Salas, , J., Salas, M., Viñuela, E. and Sols, A. (1965) J. Biol. Chem. 240, 1014—1018.
36 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol Chem. 193, 265—275