*Biochimica et Biophysica Acta*, 334 (1974) 328–342


BBA 67126

# PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF THE HUMAN HEXOKINASE ISOENZYMES I AND III (ATP-D-HEXOSE 6-PHOSPHOTRANSFERASE EC 2.7.1.1)*

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(Received August 14th, 1973)

## SUMMARY

In extracts of human tissues three different hexokinases were demonstrated by starch gel electrophoresis. Hexokinase I was purified from heart tissue, and hexokinase III was isolated from spleen tissue. The enzymes have a similar molecular size, but differ in their electrophoretic and chromatographic properties and in their Michaelis constants for glucose, hexokinase III being a substrate-inhibited form. In electroimmunodiffusion experiments hexokinase I did not crossreact with antihexokinase III and vice versa.

## INTRODUCTION

In mammalian tissues four major fractions with hexokinase activity have been demonstrated, i.e. the three low $K_m$ hexokinase isoenzymes I, II and III*** and a high $K_m$ glucokinase [1]. In a given species the multiple molecular forms of hexokinase occur in varying concentrations dependent on tissue, age and nutritional conditions, as was first shown in rat tissues [2, 3]. The activities of hexokinase II and glucokinase are thought to be regulated by insulin [4–8].

In man the occurence of multiple molecular forms of hexokinase has been demonstrated in extracts from tissue cultures [9], various tissues [10–13] and in blood cells [12, 14–17]. The classification of hexokinases in blood cells as to number and types has been controversial [18, 19]. The molecular basis for the multiple forms of mammalian hexokinase is not known at present. There is immunological evidence for the different nature of the hexokinase proteins: antiserum prepared against glucokinase of rat did not precipitate the low $K_m$-hexokinases [20], and anti-hexokinase II did not inhibit the enzymatic activity of hexokinase I of that species [21]. We have

* Some of the results presented here are from a thesis submitted in partial fulfilment of the requirements for a doctoral degree at the University of Bochum by Siegfried Neumann.

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*** The nomenclature of Katzen and Schimke [3] is adopted which assigns the numerals to the enzymes in the order of their increasing mobility towards the anode in starch gel electrophoresis.

purified hexokinase I from human heart and partially purified hexokinase III from human spleen and prepared antisera against these enzymes. In electroimmunodiffusion experiments it was found that hexokinase I does not crossreact with anti-hexokinase III and vice versa. This is the first report of the existence of at least two immunologically unrelated hexokinases in human tissues.

## MATERIALS AND METHODS

### *Chemicals*

Glucose in analytical grade and bovine hemoglobin were obtained from Serva Entwicklungslabor, Heidelberg. ATP (disodium salt), $NADP^+$, aldolase from rabbit muscle (EC 4.1.2.12), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), lactate dehydrogenase Band 1 from pig heart (EC 1.1.1.27) and malate dehydrogenase from pig heart mitochondrial (EC 1.1.1.37) were commercial preparations of Boehringer Biochemicals, Tutzing.

2-Mercaptoethanol was a product of analytical grade from Schuchardt, München. All other common chemicals were products of analytical grade from Merck, Darmstadt. 2,5-Diphenyl-3-(4,5-dimethylthiazolyl-2)tetrazolium bromide was obtained from Serva Entwicklungslabor, Heidelberg, methylphenazonium methosulphate from EGA-Chemie, Steinheim, Albuch and lightgreen yellow from Merck, Darmstadt. Agarose from Behringwerke Marburg, starch hydrolysed from Connaught Inc. (Toronto), ion exchangers from Whatman, W & R Balston and Dextran blue and Sephadex resins from Pharmacia (Uppsala) were used.

### *Homogenization of tissue*

Human tissue was obtained from autopsy 12–14 h after death and was stored at −20 °C until use. Before homogenization the tissue was thawed out, and adjacent fat and connective tissue was removed. Homogenization was done in 0.01 M phosphate buffer (pH 8.5) with 5 mM EDTA and 5 mM 2-mercaptoethanol in a Sorvall Omnimix Homogenizer for 1 min at full speed. This buffer was used throughout most of the purification procedures and is referred to as standard buffer. One part of tissue (wet wt) was homogenized in two vol. of buffer unless otherwise stated. After stirring in an ice bath for 30 min the homogenate was centrifuged in a Sorvall refrigerated RC 2-B centrifuge at 48 200 × *g* for 30 min at 0 °C. The supernatant was filtered through glass wool and dialyzed against approx. 100 vol. of standard buffer for 3–5 h unless otherwise stated.

### *Hexokinase assay*

Hexokinase activity was assayed by the method of Salas et al. [22] with some minor modifications. The reaction mixture contained in a final volume of 2.0 ml 0.074 M Tris–HCl buffer (pH 7.4) 25 or 0.5 mM glucose as indicated, 10 mM ATP, 20 mM $MgCl_2$, 0.55 mM $NADP^+$, 5 mM 2-mercaptoethanol, 0.5 I.U. glucose-6-phosphate dehydrogenase per ml and 0.1 ml of appropriately diluted enzyme preparation. The increase of absorbance at 366 nm was measured in a thermocuvette at 25 °C with an Eppendorf filter photometer with automatic recorder. Cuvettes from which ATP was omitted were used as blanks. Modifications of assay conditions are given in the text.

*Protein assay*

Protein content was estimated by the modified biuret method [23]. The extinction due to heme in coloured proteins at 546 nm was measured in blanks, in which copper sulphate was omitted, and subtracted.

*Definition of units*

1 unit of hexokinase activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 $\mu$mole of glucose per min at 25 °C. Specific activity is expressed as units per mg of protein.

*Horizontal starch gel electrophoresis*

The enzyme samples were inserted on filter paper 4 mm × 6 mm into 11.5% starch gel. 0.02 M barbital–HCl buffer (pH 8.6) with 2.7 mM Na-EDTA and 5 mM 2-mercaptoethanol was used as gel and electrophoresis buffer. Electrophoresis was run at 250 V for 4–5 h on a high-voltage pherograph type Original Frankfurt (Hormuth and Vetter, Wiesloch, German Federal Republic) with surface cooling by methanol at −5 °C. Then the gel was sliced with a wire into three horizontal layers which were stained for hexokinase activity at different glucose concentrations as described by Katzen and Schimke [3]. Staining was performed at 37 °C in the dark overnight. Samples without ATP were used for identification of unspecific staining.

*Agarose electrophoresis*

The method of Altay et al. [17] was used. Samples were inserted on filter paper and run at 18 V/cm of gel for 2 h.

*Calcium phosphate gel adsorption*

Calcium phosphate gel was prepared by the method of Keilin and Hartree [24].

*$(NH_4)_2SO_4$ fractionation*

Prior to $(NH_4)_2SO_4$ precipitation the enzyme solution was brought to 100 mM glucose for stabilization of the enzyme [25]. Then solid salt was added to give the degree of saturation as calculated by the formula given by Beisenherz et al. [23].

*Gel filtration*

Molecular weights were determined by gel filtration on a Sephadex G-200 column (23 mm × 1165 mm) in 0.02 M phosphate buffer (pH 8.0) with 5 mM EDTA and 5 mM 2-mercaptoethanol. Dextran blue (mol. wt $2 \cdot 10^6$), aldolase ($1.6 \cdot 10^5$), lactate dehydrogenase Band 1 from pig heart ($1.47 \cdot 10^5$ [27]), malate dehydrogenase mitochondrial ($6.8 \cdot 10^4$ [28]) and hemoglobin ($6.45 \cdot 10^4$) were used as markers. The elution was done with a flow rate of 33 ml/h and 3.3-ml fractions were collected. The apparent molecular weight of hexokinases was calculated from a plot of log mol. wt vs $v_e/v_0$.

*Preparation of antisera*

Hexokinase I from an intermediary stage of purification (preparation after first step chromatography on DEAE-cellulose as described below) was used as immunogen. 9 mg of protein in emulsion with complete Freunds adjuvant were given

to 5 rabbits in approximately equal doses. 3 injections were given: on Day 1 in the hind foot pads, on Day 20 intramuscularly and on Day 34 subcutaneously. 7 and 10 days after the third injection the rabbits were bled from the ear vene. Those three animals with suitable anti-hexokinase I titre as judged by immunoelectrophoresis received an additional subcutaneous injection on Day 52 and were bled again on Days 59 and 62. A combined serum was obtained by mixing equal volumes of the sera of these three rabbits. Anti-hexokinase III serum was produced by immunization of rabbits with partially purified hexokinase III protein from spleen as isolated in two separate purification experiments described below. 24.5 mg of protein in emulsion with complete Freunds adjuvant were given into the hind foot pads of two rabbits. The enzyme preparation had a specific activity of 0.75 hexokinase units (assayed at 0.5 mM glucose, 10 mM ATP, 20 mM $MgCl_2$, pH 7.4, 25 °C) per mg of protein. A second dose was injected intramuscularly on Day 16. The booster injection was given on Day 39 subcutaneously. This time the emulsion was prepared from a hexokinase III preparation with a specific activity of 3.2 units per mg of protein, and 2.8 mg of the protein were administered to each animal. On Days 46 and 48 the rabbits were bled. The sera obtained were stored at −20 °C.

*Immunoelectrophoresis*

The method of Scheidegger [29] was used.

*Single radial immunodiffusion [30]*

Immunodiffusion was allowed for 96 h at 4 °C in a moist chamber. Then the gels were washed in saline for 48 h and stained for hexokinase activity as described in the following section.

*Electroimmunodiffusion [31]*

In the two-dimensional version of this method the enzyme sample was filled into a well on the cathodic side of 0.8% Agarose gel in 0.15 M barbital–HCl buffer (pH 8.6). Electrophoresis was carried out for 22 min with approx. 38 V/cm of gel. 0.06 M barbital–HCl buffer (pH 8.6) was used as bridge buffer. Then 0.8% Agarose in gel buffer containing antiserum was added on the plate to one side of first dimension gel, and the enzyme was electrophorized into the antibody-containing gel at 16 V/cm of gel for 12 or 24 h (as indicated in the text). The plates were placed on a metal block which was cooled by tap water. In one-dimensional electroimmunodiffusion the enzyme samples were filled into wells in a strip of 0.8% Agarose and electrophorized directly into the antibody-containing gel.

After electrophoresis the gels were washed in 0.9% NaCl (saline) for 48 h at 4 °C. For detection of hexokinase activity the immunogels were overlayed with staining gel and incubated in a moist chamber in the dark at 37 °C for 60–120 min. The staining gel was prepared by mixing at 46–48 °C 3.0 ml of 1.6% Agarose in 0.15 M barbital–HCl buffer (pH 8.6) with 7.0 ml of the same buffer in which the following reagents were dissolved to give the concentrations in the final mixture as indicated in brackets: glucose (0.5 mM), ATP (4 mM), $MgCl_2 \cdot 6\,H_2O$ (10 mM), glucose-6-phosphate dehydrogenase (0.5 I.U./ml), KCN (2 mM), 2,5-diphenyl-3-(4,5-dimethylthiazolyl-2)tetrazolium (0.4 mg/ml) and traces of methylphenazonium methosulphate. Glucose-6-phosphate dehydrogenase was added prior to use. In precipitates with

hexokinase activity a dark blue colour was developed. Then the staining gel was poured off and residual reagents were washed out of the immunogels with water. The gels were dried at 70 °C. For staining of protein the dried gels were immersed in 0.5% lightgreen yellow in 5% trichloroacetic acid for 30–60 min.

## RESULTS

*Number and types of hexokinases in human tissues*

The hexokinase activity found in extracts of human tissues from autopsy could be separated into three different fractions by starch gel electrophoresis (Fig. 1). They were classified as hexokinases I, II or III according to their electrophoretic mobilities. Hexokinase II was observed in only one extract from muscle, but not in

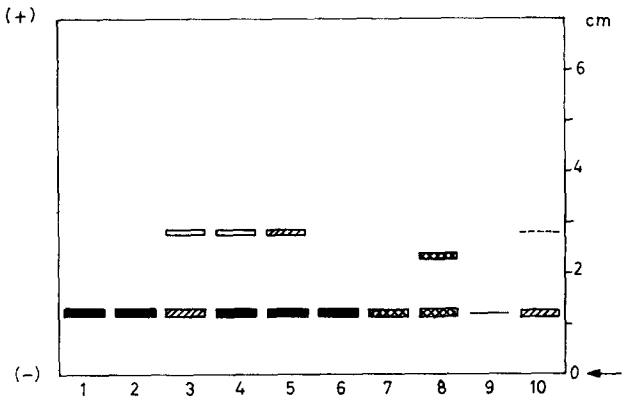


Fig. 1. Tissue distribution of hexokinase in man. Diagramatic representation of hexokinases after starch gel electrophoresis of tissue extracts and hemolysates and subsequent enzyme-specific staining at 100 mM glucose. The tissues studied are (number of samples are given in parentheses): 1, heart (3); 2, brain (3); 3, liver (3); 5, lung (3); 5, spleen (3); 6, kidney (2); 7, skeletal muscle (3); 8, skeletal muscle with an additional band corresponding to hexokinase II (1); 9, fat tissue (1); 10, hemolysates (5). The position of insertion is indicated by an arrow. Intensity of staining is indicated by the thickness of the band.

any other tissue. No glucokinase activity could be demonstrated. Hexokinase I was present in all tissues studied. Its mobility was slightly slower than that of hemoglobin $A_1$. Variations of the hexokinase I band were seen in staining intensity and electrophoretic "tailing". This was found to correspond to the activity of the sample.

Hexokinase III was found in extracts from liver, spleen, lung and in traces in hemolysates. The hexokinases I and II stained optimal at 100 mM glucose and hexokinase III at 10–100 mM glucose. Under these conditions hexokinase III was not significantly inhibited at 100 mM glucose. In the optical hexokinase assay, however, tissue extracts from livers, spleens and lungs showed higher activities at 0.5 mM than at 100 mM glucose. When the activity at 0.5 mM is divided by the activity at 100 mM, quotients which exceed unity were found for livers (values about 1.5), spleens (1.23–1.58) and lungs (1.04–1.34). The highest hexokinase activities per mg of protein were found in extracts from brain or heart. On the basis of specific hexokinase

activity in tissue extracts and the availability of autopsy material, heart tissue was selected for preparation of Type I isoenzyme and spleen for the isolation of Type III isoenzyme. No effort was made to purify hexokinase II isoenzyme because of lack of tissue with sufficient activity of this enzyme.

*Preparation of hexokinase I*

2 kg of heart muscle tissue were minced first and then homogenized in 6 l of standard buffer. The homogenate was centrifuged for 15 min at 3290 × *g*, and the resultant supernatant was centrifuged again at 27 300 × *g* for 30 min in a Sorvall refrigerated centrifuge RC 2-B (0 °C). The sediments from both centrifugations were extracted once again with 6 l of standard buffer. The supernatants from both extractions were pooled (10.2 l). Calcium phosphate gel was added to give a final concentration of 3 mg of calcium phosphate per ml of extract. The gel was centrifuged, and desorption was done in 0.3 M phosphate buffer (pH 7.2) with 5 mM EDTA and 5 mM 2-mercaptoethanol twice. The pool of the two eluates (1.66 l) was submitted to $(NH_4)_2SO_4$ fractionation. The main part of hexokinase was found in the precipitate from 40 to 60% saturation.

The precipitates from four parallel preparations were pooled (Table I). Further purification was achieved by chromatography on DEAE-cellulose fibrous form (DE-

TABLE I

PURIFICATION OF HEXOKINASE ISOENZYME I FROM HUMAN HEART

Hexokinase activity was assayed at 100 mM glucose, 3.7 mM ATP and 7.4 mM $MgCl_2$ in 0.074 M Tris–HCl buffer (pH 8.5) The data presented for the first three steps of the preparation, i.e extracts, calcium phosphate gel adsorption and $(NH_4)_2SO_4$ fractionation, are summarized from four parallel experiments starting with 2000 (2×), 1500 and 2400 g heart muscle tissue. Further explanations are given in the text.

| Step | Volume (ml) | Total protein (mg) | Total activity (units) | Spec. act. (units/mg) | Fold purification | Yield (%) |
|---|---|---|---|---|---|---|
| Extracts | 38 570 | 156 200 | 29 190 | 0.187 | | 100 |
| Calcium phosphate gel | 7 370 | 38 370 | 22 740 | 0 593 | 3.2 | 78 |
| $(NH_4)_2SO_4$ I | 435 | 13 000 | 18 180 | 1.400 | 7.5 | 62 |
| DEAE-cellulose I* | 965 | 821 | 12 230 | 14.9 | 80 | 42 |
| DEAE-cellulose II | 62 | 174 | 5 570 | 32 0 | 171 | 19 |
| Sephadex G-150 | 84 | 90 3 | 4 570 | 50.6 | 270 | 16 |
| $(NH_4)_2SO_4$ II and III | 7.5 | 10.2 | 727 | 71.3 | 381 | 2.5 |

* Results after two successive chromatographies on DEAE-cellulose DE-22.

22, Fig. 2a). The fractions with the highest specific activity (No. 135–150) were combined. The enzyme in fractions with intermediary hexokinase activity (No. 120–134 and 151–190) was rechromatographed on a second column of DE-22. It appeared in a single peak with maximal activity at 0.15 M NaCl, as had the peak enzyme in the first column. The peak enzymes from the two columns were both concentrated by $(NH_4)_2SO_4$ precipitation (80% satn), and the two suspensions were pooled. Then an aliquot of 51 mg of protein was removed for immunization. The rest of the enzyme was chromatographed on DEAE-cellulose microgranular form (DE-52, Fig.

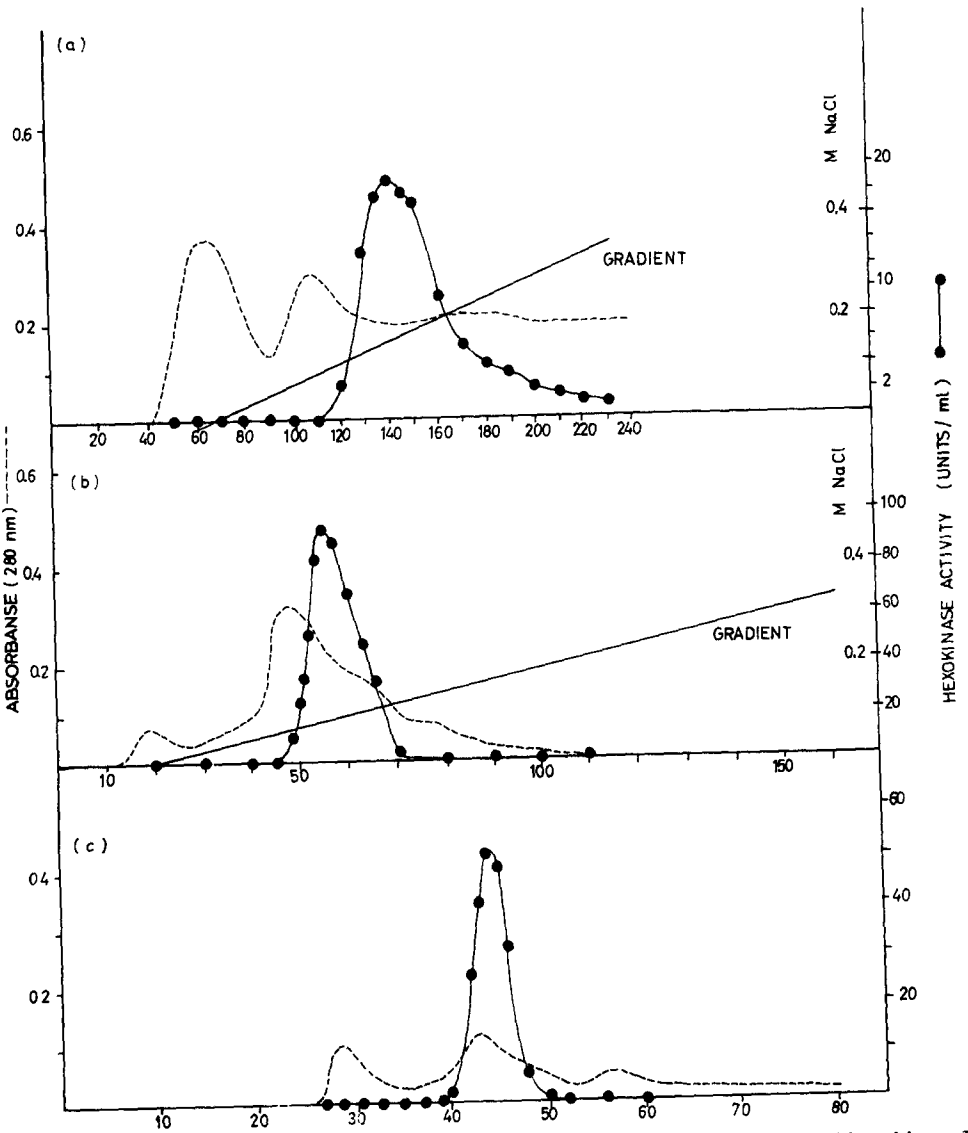


Fig. 2. Chromatography of hexokinase I from heart. a. First step chromatography of hexokinase I on DEAE-cellulose (DE-22). The enzyme solution (540 ml) was applied to a column (75 mm × 310 mm) previously equilibrated with standard buffer. Hexokinase was eluted by a linear gradient from 0 to 0.4 M NaCl in 4 l of standard buffer. 18-ml fractions were collected. b. Second step chromatography of hexokinase I on DEAE-cellulose (DE-52). The enzyme solution (32.5 ml) was applied to a column (50 mm × 130 mm) in standard buffer. Elution was carried out with a linear gradient from 0 to 0.3 M NaCl in 1 l of standard buffer. 7-ml fractoins were collected. c. Gel filtration chromatography on a Sephadex G-150 column (23 mm × 1130 mm) in standard buffer. The flow rate was 18.3 ml/h, and the eluate was collected in 5-ml fractions.

2b). The enzyme peak was then concentrated by $(NH_4)_2SO_4$ precipitation as described above and applied to a column of Sephadex G-150 (Fig. 2c) in three portions. The enzyme peak from gel filtration was concentrated by $(NH_4)_2SO_4$ precipita-

tion once again and submitted to two successive $(NH_4)_2SO_4$ fractionations. The final enzyme preparation was taken from the precipitate from 60 to 64% salt satn. It had a specific activity of 71.3 units/mg of protein. It was stored at 4 °C in suspension with 70% $(NH_4)_2SO_4$ in the presence of 100 mM glucose and kept its full activity over months.

*Preparation of hexokinase III*

On chromatography of extract from spleen on DEAE-cellulose at pH 8.5 hexokinase activity was repeatedly found in three peaks, i.e. in the wash (Peak 1), at 0.1–0.15 M NaCl (Peak 2) and at 0.18–0.25 M NaCl (Peak 3) as shown in Fig. 3. Substrate inhibition was seen with the fractions of Peak 3 only. Peak 1 enzyme was found in the wash again when it was rechromatographed on DEAE-cellulose. Thus it appears not to be the result of overloading the ion exchanger.

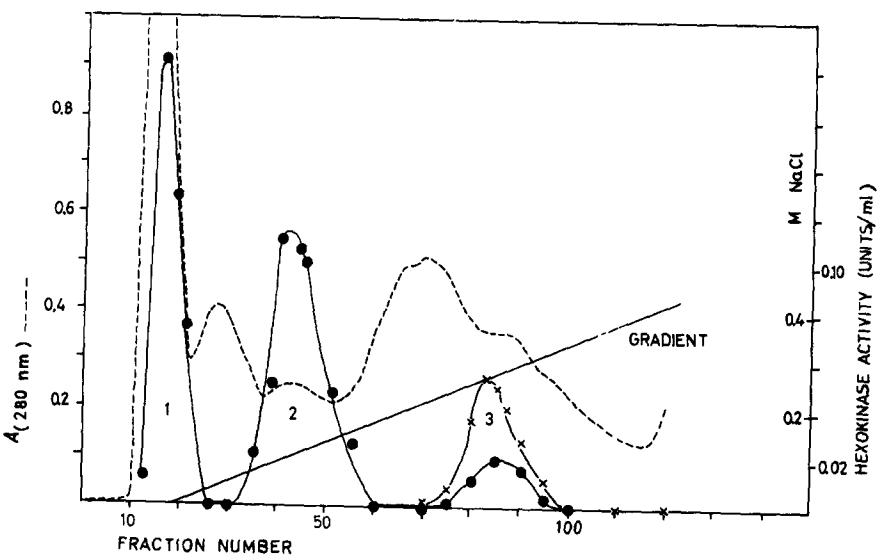


Fig. 3. Chromatography of extract from spleen on DEAE-cellulose. The 48 200 × *g* supernatant of a homogenate from 50 g spleen tissue (homogenized in standard buffer (1:1, w/v)) was applied to a column of DEAE-cellulose DE-32 (50 mm × 165 mm) previously equilibrated with standard buffer. Then a linear gradient from 0 to 0.4 M NaCl in 2 l of standard buffer was applied to the column, and the eluate was collected in 15-ml fractions. Hexokinase activity was assayed at 25 mM (●—●) and 0 5 mM glucose (×—×, Peak 3 fractions only).

The Peak 1 and 2 enzymes differed only slightly in electrophoretic mobility (Fig. 4). The Peak 3 enzyme moved faster. It was identical with the band classified as Form III in tissue extracts. 25–29% of the total hexokinase activity in the fractions after DEAE-cellulose chromatography were found in Peak 3. This enzyme was labile.

For the isolation of Form III on a preparative scale 660 g spleen tissue (wet wt) were homogenized in 660 ml of standard buffer. The crude homogenate was centrifuged at 27 300 × *g* for 60 min (0 °C). The supernatant was centrifuged for further 30 min to remove particles. The sediments from the two centrifugations were


Fig. 4. Starch gel electrophoresis of extract from spleen before chromatography on DEAE-cellulose and of the hexokinase fractions obtained by chromatography. 1, spleen extract, 2, Peak 1 enzyme; 3, Peak 2 enzyme; 4, Peak 3 enzyme, which is hexokinase III still contaminated by a hexokinase with lower electrophoretic mobility. Staining was performed at 100 mM glucose. The start is indicated by the arrow

extracted once again with buffer (660 ml). The final supernatants were combined, and the pH of the solution was adjusted to 8.5 with 1 M NaOH. The extract was applied directly to a column of microgranular DEAE-cellulose (DE-32, 100 mm × 287 mm) previously equilibrated with standard buffer. Elution was carried out with buffer (700 ml) first and was continued with a linear gradient from 0 to 0.4 M NaCl in buffer (4 l). The peak fractions of the three hexokinase peaks were combined separately. Peak 3 enzyme (544 ml) was dialyzed against standard buffer and rechromatographed on a DEAE-cellulose DE-32-column in a similar procedure as described for the first column. A small trace of Peak 2 enzyme was separated from the Peak 3 enzyme. The latter enzyme peak (512 ml) was dialyzed against buffer and concentrated by ultradialysis through dialysis tubing to a final volume of 38.5 ml. The enzyme was subsequently chromatographed on a column of Sephadex G-100 (50 mm × 1065 mm). Hexokinase activity was found in a single peak. The peak enzyme (67 ml) was concentrated by ultradialysis to a final volume of 4 ml. The final enzyme preparation had a specific activity of 3.2 units/mg of protein (Table II). It was stored at −20 °C. A parallel procedure led to an enzyme preparation with a specific activity of 0.75 units/mg of protein. Both preparations were used in the course of the immunization.

*Biochemical properties*

The molecular weights were determined by gel filtration on Sephadex G-200 as described in Materials and Methods. Hexokinase I from heart and the Peak 1 and 2 enzymes from spleen all were found to have an apparent molecular weight of approx. 111 000. Hexokinase III from spleen had an apparent molecular weight of 116 000.

TABLE II

PURIFICATION OF HEXOKINASE ISOENZYME III FROM HUMAN SPLEEN

Hexokinase activity was assayed at 25 mM and 0.5 mM glucose, 20 mM ATP, 20 mM $MgCl_2$ in 0 074 M Tris–HCl buffer (pH 7 4) as described in Materials and Methods.

| Step | Volume (ml) | Total protein (mg) | Total activity (units) | | Spec. act. (units/mg) | | Q* |
|---|---|---|---|---|---|---|---|
| | | | 25 mM glucose | 0.5 mM glucose | 25 mM glucose | 0.5 mM glucose | |
| Extracts | 1080 | 24 600 | 793 | 877 | 0.0323 | 0.0356 | 1.11 |
| DEAE-cellulose I | | | | | | | |
| Peak 1 | 230 | 207 | 54.7 | 50.6 | 0.254 | 0.244 | 0.96 |
| Peak 2 | 575 | 1 090 | 338.0 | 338.0 | 0.307 | 0.307 | 1.00 |
| Peak 3 | 544 | 975 | 50 6 | 131.0 | 0.052 | 0.134 | 2.58 |
| DEAE-cellulose II | 512 | 191 | 29.3 | 75.3 | 0.153 | 0.394 | 2.60 |
| Sephadex G-100 | 67 | 14.6 | 16.1 | 42.4 | 1.103 | 2.905 | 2.63 |
| Ultrafiltration | 4.0 | 13.5 | 17.5 | 43.2 | 1.295 | 3 205 | 2.47 |

* Quotient of hexokinase activities at 0.5 and 25 mM glucose.

The Michaelis constants for glucose did not differ significantly for hexokinase I from heart ($9.8 \cdot 10^{-5}$ M) and the Peak 1 and 2 enzymes from spleen ($1.03 \cdot 10^{-4}$ M and $9.7 \cdot 10^{-5}$ M, respectively). A different $K_m$ for glucose was obtained with hexokinase III from spleen ($3.0 \cdot 10^{-5}$–$4.2 \cdot 10^{-5}$ M). Hexokinase III was inhibited at glucose concentrations exceeding 1 mM. For all $K_m$ determinations the initial ATP concentration was 10 mM (20 mM $MgCl_2$), the buffer was 0.074 M Tris–HCl buffer (pH 7.4), and the temperature was maintained at 25 °C. The Michaelis constants for ATP were all in the range of 1.4–1.6 mM. In these experiments the molar ratio of ATP to $MgCl_2$ was 1 to 2, the initial glucose concentration was 20 mM for hexokinase I from heart and the Peak 1 and 2 enzymes and 0.5 mM for hexokinase III.

Hexokinase I from heart had a low absorbance at 280 nm ($A^{1\%}_{1cm} = 7.62$), as has been reported for bovine brain hexokinase ($A^{1\%}_{1cm} = 5.53$ [32]).

*Immunological properties*

In electroimmunodiffusion experiments the anti-hexokinase I serum has been used to demonstrate the removal of individual contaminant proteins in the course of the enzyme preparation (Figs 5a and 5b). Hexokinase activity was found in only one of the precipitates, which is seen as the dark stained area in Fig. 5b. In the final preparation only traces of two immunologically active contaminants were seen. Nearly identical precipitation areas with hexokinase activity were obtained with samples having the same total hexokinase activity from different stages of the enzyme preparation. This would indicate the absence of components interfering with the reaction of the enzyme with the anti-hexokinase I antibodies.

Precipitates other than the hexokinase precipitate were also seen in electroimmunodiffusion of hexokinase III with its homologous antiserum.

The binding titres of the antisera were calculated from the areas under the hexokinase precipitates in electroimmunodiffusion experiments. 56.2 units of hexokinase I from heart (assayed at 25 mM glucose) were bound by 1 ml of anti-

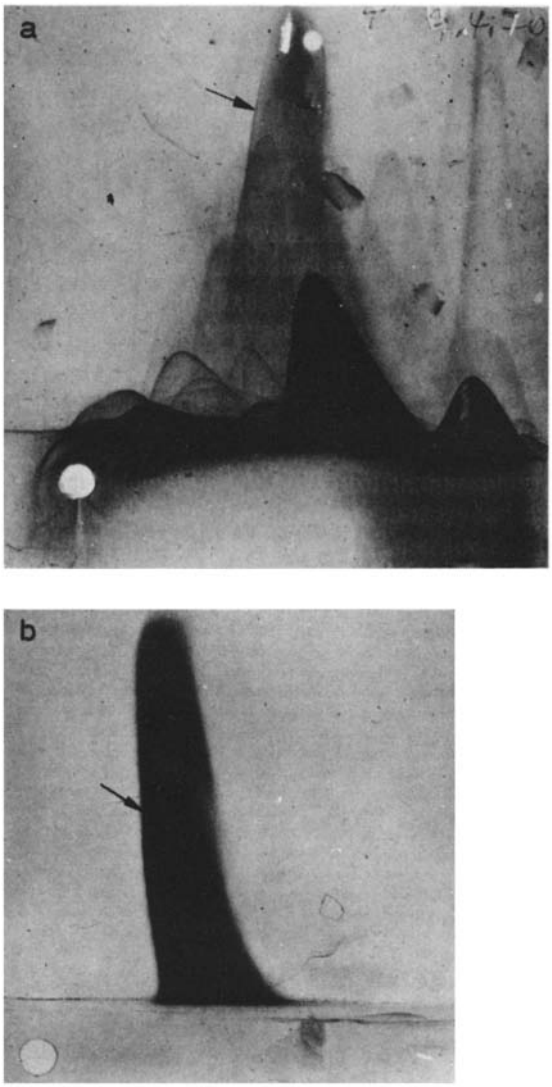


Fig. 5. Antigenic analysis of hexokinase I from heart at different stages of purification. Samples with 2.18 units hexokinase activity from the enzyme preparation following DEAE-cellulose chromatography I (a) and from the final enzyme (b) were analysed by two dimensional electroimmunodiffusion in immunogel containing 20% (v/v) anti-hexokinse I. The anode was first to the right of the agarose (22 min) and then at the top of the immunogel (24 h). The precipitates with hexokinase activity were identified first by incubation with the staining gel (as described in Methods and Materials) and are marked here by arrows. Then the precipitates were stained for protein.

hexokinase I, and 13.5 units hexokinase III of spleen (assayed at 0.5 mM glucose) were bound by 1 ml of anti-hexokinase III.

The crossreactivity of hexokinases I and III was tested by electroimmunodiffusion. Hexokinase I did not form insoluble immune complexes with anti-hexokinase III, nor did it influence the precipitation of type III enzyme (Fig. 6).

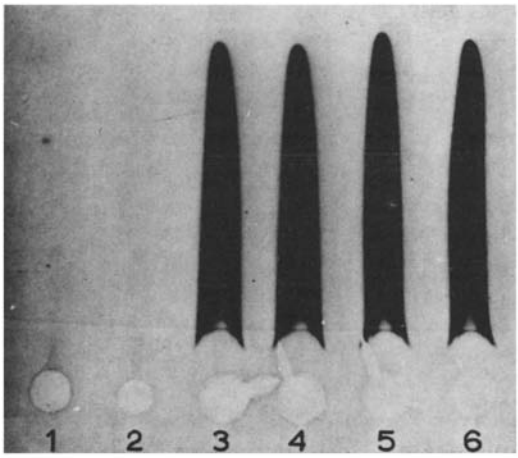


Fig. 6. One-dimensional electroimmunodiffusion of the hexokinases I and III in agarose containing anti-hexokinase III. Samples containing 180 munits hexokinase I from heart (Wells 1 and 2), 90 munits hexokinase III from spleen (Wells 5 and 6) or mixtures with both 180 munits hexokinase I and 90 munits hexokinase III (Wells 3 and 4) were placed into the wells of the sample gel. Electrophoresis was carried out for 12 h with the anode at the top of the plate. The agarose was made 5% (v/v) with anti-hexokinase III. After electrophoresis the gels were washed in saline for 48 h and then stained for hexokinase activity.

In corresponding experiments hexokinase III was not precipitated by anti-hexokinase I.

The immunological relationship of the Peak 1 and 2 enzymes from spleen and hexokinase I from heart was studied in single radial immunodiffusion. When samples with the same total hexokinase activity were allowed to diffuse into gel containing anti-hexokinase I, nearly identical areas of gel staining positive for hexokinase activity were obtained. There was evidence for the immunological identity of these three enzymes, i.e. the absence of spur formation with the fusion of the boundaries of the hexokinase precipitates and the competition for precipitating antibodies in the areas between the depots.

## DISCUSSION

Three different hexokinase fractions can be demonstrated in extracts of human tissues by starch gel electrophoresis and subsequent enzyme staining (Fig. 1). They were classified as hexokinase I, II and III on the basis of their relative electrophoretic mobilities. The nomenclature of Katzen and Schimke [3] defined initially for rat hexokinases was adopted since homology of number and types of hexokinase isoenzymes has been reported for all mammalian species studied [1], and four hexokinase isoenzymes with similar electrophoretic mobilities and glucose affinities as reported for the hexokinases of rat liver have been demonstrated in human liver [10, 13].

The tissue distribution in our material is, however, different from that reported for rat tissues [33] due to the lack of glucokinase in liver and the rare occurrence of hexokinase II. In the rat both glucokinase and hexokinase II are adaptive enzymes [4–8], as is glucokinase in man [10, 34]. In addition, both enzymes are

rather labile in the rat [25]. Therefore they may only be present in low concentrations in autopsy material and/or undergo inactivation during autolysis in tissue or starch gel electrophoresis. This makes quantitative estimates of the relative concentrations of the different hexokinase isoenzymes difficult.

The effect of varying glucose concentration on the apparent activity of hexokinase III was different in starch gel electrophoresis and in the optical enzyme assay. There was no evidence for substrate inhibition of this enzyme in the zymograms. It is assumed that during the enzyme staining the enzymatic inhibition at 100 mM glucose is counteracted by the stabilizing effect of glucose [25] in the course of the incubation. This effect may be inefficient at lower concentrations. The relative high tissue concentrations of Type III activity in extracts from liver, spleen and lung, as shown by the staining intensity in electrophoresis, are in accordance with the distribution pattern observed in the rat [33].

It can be demonstrated that hexokinase I from human heart and hexokinase III from spleen differ in electrophoretic mobility, chromatographic behaviour on DEAE-cellulose and in their Michaelis constants for glucose. The $K_m$ values for glucose differ by a factor of two to three, with hexokinase III being a substrate-inhibited form. These differences are very similar with those observed with the low $K_m$ hexokinases I and III from rat [25]. Unlike the observations in rat, in human spleen extract there are two hexokinases with Type I reactivity. The Peak 1 and 2 enzymes from DEAE-chromatography do not differ in their affinities for glucose, but Peak 1, enzyme may have a lower net charge as judged from its chromatographic behaviour. The Type I hexokinases do not differ in immunological reactivity, regardless of tissue source and chromatographic properties. At present the mechanism leading to two Type I hexokinase isoenzymes in extracts from spleen is not known.

For the purpose of evaluing the immunological characterization of the hexokinases I and III it seems important to demonstrate (a) the presence of only the required hexokinase isoenzyme in the immunogenic material, and (b) the absence of contaminant hexokinases when the immunological reactivity of a particular hexokinase are studied. The presence of Type I activity in the immunogen is demonstrated by rechromatography (Fig. 2b). Type I activity only is present in the final enzyme preparation (Fig. 7). Similarly, the absence of hexokinases other than hexokinase III

Fig. 7. Agarose electrophoresis of the hexokinases I and III. Samples from the final preparations of Type I enzyme from heart (1) and of hexokinase III from spleen ((2) spec. act. 0.75 unit/mg and (3) spec. act. 3.20 units/mg) were electrophorized with the anode at the top of the gel. The origin is indicated by the arrow. Hexokinase staining was carried out as described for electroimmunodiffusion.

in the preparations from spleen was assured by rechromatography. This is verified by agarose electrophoresis (Fig. 7).

In the comparison of the hexokinases I and III the most striking result was the complete lack of immunological crossreactivity as demonstrated by electroimmunodiffusion. Additional evidence from immunoinhibition and precipitin tests will be given in a subsequent paper [35]. The immunological relationship of the hexokinases I and III with hexokinase II is not clear at present. It would be of interest to study the reaction of antisera against human hexokinases I or III with Type II enzyme from man or, for convenience, from other mammalian tissues, from which preparation may be more successful.

ACKNOWLEDGEMENTS

S.N. received a fellowship of the Stiftung Volkswagenwerk (Hannover). F.F. was supported by grant No. Fa 71/1 of the Deutsche Forschungsgemeinschaft. The authors wish to acknowledge the assistance of Dr D. A. Fitzpatrick in the preparation of the manuscript.

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