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IMMUNOLOGICAL SPECIFICITY OF THE ISOENZYMES I AND III OF HUMAN HEXOKINASE (ATP:D-HEXOSE 6-PHOSPHOTRANSFERASE EC 2.7.1.1)

ESTIMATION OF ISOENZYME PATTERN BY QUANTITATIVE IMMUNOTECHNIQUES*

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SUMMARY

The immunological relationship of hexokinase isoenzymes I and III from human tissues was tested with antisera made against purified forms of these enzymes. There was no evidence for crossreactivity as tested by immunoinhibition and precipitin experiments. In mixtures of the two hexokinases made from stock solutions the relative concentration of each form could be determined by specific precipitation with the homologous antiserum. When the method is applied to extracts from human tissues high concentrations of hexokinase form III were found in liver (more than 50% of total hexokinase activity), spleen (up to 50%) and lung (below 20%).

From the immunological data it is concluded that the low K_m hexokinases from mammalian tissues constitute a family of distinct proteins with similar catalytic properties. The general applicability of immunoinhibition methods for the evaluation of the tissue concentrations of the hexokinase isoenzymes is discussed.

INTRODUCTION

In mammalian tissues four major hexokinases with different electrophoretical, chromatographic and kinetical properties have been demonstrated [1], i.e. the three low K_m hexokinases I, II and III and a high K_m glucokinase termed according to electrophoretic mobility and affinity to glucose [2]. The concentrations of these enzymes were found to vary as a function of tissue, age and nutritional conditions [2, 3], and evidence for the regulation of hexokinase II and glucokinase by insulin was given [4–8]. In these studies the estimation of the hexokinase concentrations in tissue ex-

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tracts was done by staining intensity of the hexokinase bands in starch gel electrophoresis [2, 4–8], evaluation of peak activity after DEAE-cellulose chromatography [5] or by evaluation of activity in the hexokinase assay at different glucose concentrations [6].

Information has accumulated now on the different immunological properties of the low K_m hexokinases on the one side and glucokinase on the other [9] and on the different immunological reactivities of the low K_m hexokinases [10]. We have seen complete lack of immunological crossreactivity of human hexokinases I and III in electroimmunodiffusion [11]. This report presents additional data from neutralization and precipitation studies with these enzymes and the antisera made against them. Since it was observed, that the antisera neutralized or precipitated only the hexokinase isoenzyme against which they were produced, immunoinhibition studies were also done with tissue extracts for the estimation of the tissue concentrations of these forms. The general applicability of immunoinhibition methods for the determination of the concentrations of hexokinase isoenzymes in tissues and pathological sera is discussed.

MATERIALS AND METHODS

Chemicals

Glucose was obtained in analytical grade from Serva Entwicklungslabor, Heidelberg. ATP (disodium salt), $NADP^+$, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) 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.

Homogenization of tissue

Human tissue (autopsy material) was obtained 12–14 h after death and stored at −20 °C. Before homogenization the tissue was thawed and cut with removal of fat tissue. 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 1 min at full speed. The crude homogenate was stirred in an ice bath for 30 min and then centrifuged in a Sorvall refrigenerated RC 2-B centrifuge at maximal 48 200 × *g* for 30 min at 0 °C. The supernatant was filtered on glass wool and dialyzed against approx. 100 vol. of the homogenization buffer for 3–5 h at 4 °C.

Hexokinase assay

Hexokinase was assayed as described previously [11]. 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 in the text, 10 mM ATP, 20 mM $MgCl_2$, 0.55 mM $NADP^+$, 5 mM 2-mercaptoethanol, 0.5 I.U./ml glucose-6-phosphate dehydrogenase and 0.1 ml of appropiately diluted enzyme preparation. The increase of absorbance at 366 nm was measured in a thermocuvette at 25 °C with an Eppendorf filter photometer with automatical recorder. Cuvettes from which ATP was omitted were used as blanks.

Protein assay

Protein content was estimated by the modified biuret method [13] with corrections for extinction of coloured proteins [11].

Definition of units

One unit of hexokinase activity is defined as the amount of enzyme catalyzing the phosphorylation of one micromole of glucose per minute at 25 °C. Specific activity is expressed as units/mg of protein.

Preparation of antisera

Anti-hexokinase I and anti-hexokinase III sera were prepared by immunization of rabbits with partially purified hexokinase I from human heart or partially purified hexokinase III from human spleen respectively as described elsewhere [11]. The antisera had a binding titre of 56.2 units hexokinase I (assayed at 25 mM glucose) in anti-hexokinase I and 13.5 units hexokinase III (assayed at 0.5 mM glucose) in anti-hexokinase III serum, as determined in electroimmunodiffusion [11].

Isoenzyme neutralization by antiserum

Stock solutions with 3 I.U. hexokinase activity per ml were prepared from hexokinase I from human heart (spec. act. 47.3 I.U./mg of protein) and hexokinase III from human spleen (spec. act. 0.48 I.U./mg of protein). Various volumes of these solutions were mixed for the preparation of hexokinase mixtures as indicated below. Samples with 15 munits total hexokinase activity (assayed at 0.5 mM glucose) in a volume of 5 μl were incubated with 0.1 ml serum in 1.795 ml of the reaction mixture of the hexokinase assay from which ATP had been omitted. The serum additions were: 0.1 ml preimmunization serum (control), 0.05 ml anti-hexokinase I serum, 0.05 ml anti-hexokinase III serum or 0.05 ml of both antisera. If necessary the serum addition was brought to 0.1 ml final volume by addition of preimmunization serum. After incubation at 25 °C in the thermocuvette for 10 min the enzymatic reaction was started with 0.1 ml solution of ATP. The activity was assayed at 0.5 mM glucose, 10 mM ATP, 20 mM $MgCl_2$ (pH 7.4), 25 °C and was recorded automatically. Each experiment was run in duplicate. Means of calculated activity are expressed as percentage of control.

Immunoprecipitation

Mixtures of hexokinases I and III were prepared from stock solutions as described for the neutralization experiments. 4 aliquots with a total hexokinase activity of 45 munits each in a final volume of 0.2 ml were mixed with 0.3 ml preimmunization serum (control), 0.15 ml anti-hexokinase I serum (Assay a), 0.15 ml anti-hexokinase III serum (Assay b) or 0.15 ml of both antisera (Assay c). To Assay a and b 0.15 ml preimmunization serum was also added. The mixtures were incubated at 4 °C for 30 min. The precipitate was centrifuged off in a Christ centrifuge at 4500 rev./min for 10 min. The hexokinase activity in the supernatant was assayed with 0.1 ml aliquots. The reaction was started with ATP. The assay was run in duplicate. Supernatant activity is expressed as percentage of control. The activity of the control did not differ significantly from the activity of samples incubated with assay buffer instead of serum for 30 min at 4 °C or tested after dilution without delay. All sera were dialyzed overnight.

RESULTS

Isoenzyme neutralization

Whereas hexokinases I and III were not affected by the addition of preimmunization serum in the optical enzyme assay, a rapid decay of enzymatic activity was observed after the addition of appropiate antiserum to the cuvette (Figs 1a and 1b). Hexokinase I was inhibited by anti-hexokinase I only and hexokinase III by anti-hexokinase III only. The hexokinase activity of serum was either found to be negligible or was otherwise taken into account.

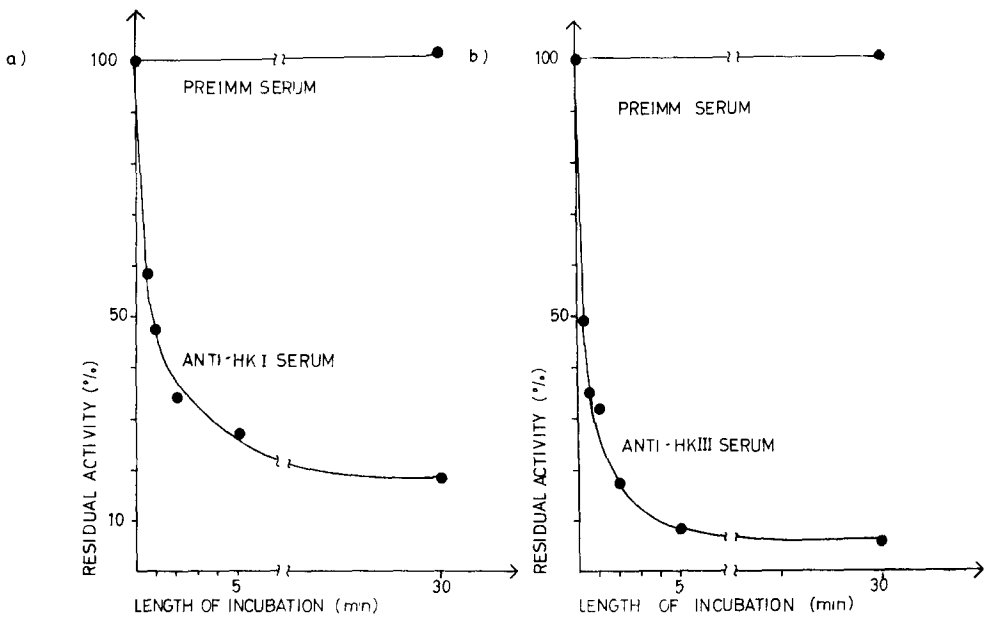

Fig. 1. Hexokinase isoenzyme neutralization by the homologous antiserum. The hexokinase activity was assayed after different periods of incubation of enzyme with antiserum in the cuvette. (a) Inhibition of hexokinase I from heart (30 munits) by anti-hexokinase I serum (80 μl). (b) Inhibition of hexokinase III from spleen (30 munits) by anti-hexokinase III serum (50 μl). The incubation of hexokinase with serum was done at 25 °C in the presence of 0 5 mM glucose. The hexokinase assay was started by addition of ATP.

The immunological neutralization of hexokinases I and III alone or in mixtures of both isoenzymes is shown in Fig. 2. The diagram presents the residual activity after incubation with one corresponding antiserum or both antisera. Activity is expressed as percentage of control, i.e. activity found when preimmunization serum was added to the cuvette. The activity of the control was essentially the same as when assayed without serum addition. As demonstrated by curve a the degree of inhibition by anti-hexokinase I increases linearly with the relative concentration of Form I in the isoenzyme mixture. A residual activity of 29% is found when Form I makes up all hexokinase activity. Similarly the degree of inhibition by anti-hexokinase III shown in Curve b increases linearly with the relative concentration of Form III (read

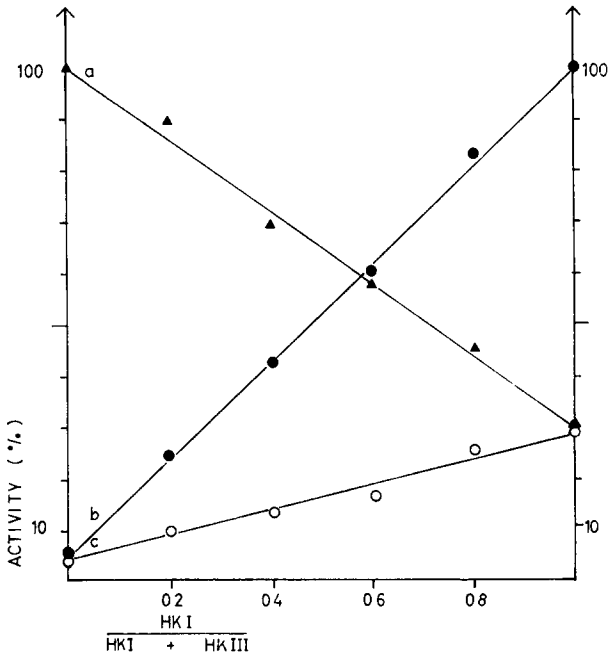

Fig. 2. Analysis of isoenzyme composition of mixtures of hexokinase I and III by specific antisera. Mixtures of hexokinases I and III with a total hexokinase activity of 15 munits were incubated with 0.1 ml preimmunization serum (control), 0.05 ml anti-hexokinase I serum and 0.05 ml preimmunization serum (Curve a, ▲—▲), 0.05 ml anti-hexokinase III serum and 0.05 ml preimmunization serum (Curve b, ●—●) or 0 05 ml of both antisera (Curve c, ○—○) as described in Methods and Materials in detail. Residual activity is plotted as a function of the isoenzyme composition, which is given as relative concentration of Form I in the abscissa. By regression analysis of the experimental data the following equations were calculated for the plots: Curve a: residual activity = 100.7 − 71.1 (rel. conc. of Form I), $r = -0.9968, P < 0.001$. Curve b: residual activity = 4.8 + 95.3 (rel. conc. of Form I), $r = 0.9994, P < 0.001$. Curve c: residual activity = 4.8 + 23.9 (rel. conc. of Form I) $r = 0.9830, P < 0.001$.

on the horizontal axis from right to left). In the sample with Form III only a residual activity of 5% is found. Prolonged incubation of hexokinase I or III with the homologous antisera did not result in lower residual activities under the experimental conditions chosen. In samples with hexokinase I or III as the single component the residual activity after incubation with anti-hexokinase I or anti-hexokinase III, respectively, was nearly identical to the activity which was obtained after incubation with both antisera (Curve c; cf. left and right intersections of the Curves a, b and c with the vertical axes). In samples with mixtures of both hexokinases, however, the residual activity after incubation with both antisera was lower than in the assays with one serum only. This is interpreted as additive inhibition of the two hexokinase components by their homologous antisera. As calculated by linear regression analysis, the correlation between isoenzyme composition and activity was high for all three curves in the diagram.

Precipitation experiments

Quantitation of hexokinases in mixtures was also tried by specific precipitation

with the homologous antiserum. The residual activity of the supernatant was found to be directly proportional to the concentration of the heterologous enzyme. In Fig. 3 the supernatant activity of mixtures of hexokinases I and III is plotted versus the isoenzyme composition of the sample. It is seen from the curves of the diagram that hexokinase I is precipitated completely by the chosen amount of anti-hexokinase I serum

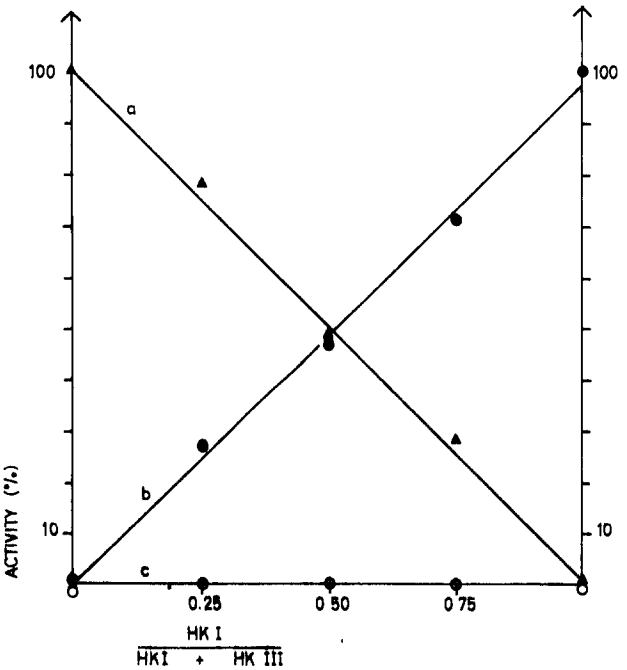

Fig. 3. Precipitation of hexokinases I or III from mixtures by the homologous antiserum. Hexokinase I and hexokinase III were mixed from stock solutions with tested activity to give the relative composition indicated in the abscissa as relative percentage of hexokinase I. Precipitation and assay of the supernatant activity was done as described in Methods and Materials. The supernatant activity is expressed as percentage of control, with Curve a (▲—▲) activity after precipitation with antihexokinase I, Curve b (●—●) after precipitation with antihexokinase III and Curve c (○—○) after precipitation with both antisera. From the data given in the diagram equations were calculated for the curves of the diagram by regression analysis as follows: a, sup. act. $= 100.3 - 99.4\,x$, $r = -0.9954$, $P < 0.001$; b, sup. act. $= 0.5 + 97.0\,x$, $r = 0.9986$, $P < 0.001$; with x, relative concentration of Form I.

in every sample tested (Curve a), hexokinase III is completely precipitated by antihexokinase III (Curve b) and both forms are precipitated in mixtures in the presence of both antisera (Curve c). In a given mixture the concentration of a component enzyme could be calculated either from the activity lost by precipitation with the homologous antiserum or from the supernatant activity found after precipitation with the heterologous antiserum. The two calculations gave nearly identical results.

Hexokinase activity in extracts from human tissues

Some results on hexokinase activity in extracts from human tissues are represented in Table I. High specific hexokinase activity was found in extracts from

TABLE I

HEXOKINASE ACTIVITY IN EXTRACTS OF HUMAN TISSUES FROM AUTOPSY

Tissue samples were taken from five individuals. Homogenization and hexokinase and protein assay was done as described in Materials and Methods. Hexokinase activity was assayed at 25 and 0.5 mM glucose.

Case No.	Tissue	Hexokinase activity		Protein concn (mg/ml)	Q*	Spec. act.	
		25 mM glucose (I.U./ml)	0.5 mM glucose (I.U./ml)			25 mM glucose (munits/mg)	0.5 mM glucose (munits/mg)
I	Kidney	0.247	0.240	17.9	0.97	13.8	13.4
	Liver	0.205	0.289	23.0	1.41	8.9	12.6
II	Heart	0.872	0.812	8.9	0.93	98.0	91.3
	Spleen	0.496	0.603	22.6	1.22	21.9	26.7
	Lung	0.460	0.449	26.9	0.98	17.1	16.7
III	Spleen	0.445	0.467	35.7	1.05	12.5	13.1
IV	Heart	0.710	0.629	9.8	0.89	72.5	64.2
	Spleen	0.280	0.287	27.4	1.03	10.2	10.5
	Lung	0.223	0.234	25.6	1.05	8.7	9.1
V	Spleen	0.689	0.850	22.7	1.23	30.3	37.4

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

heart and brain. In some extracts of liver, lung and spleen higher hexokinase activity was found at 0.5 mM glucose than at 25 mM glucose. This was taken as evidence for the presence of hexokinase III which is a substrate-inhibited form [11].

Quantitation of hexokinase isoenzyme concentration in tissue extracts

Neutralization experiments with tissue extracts gave unsatisfactory results because both antisera produced a bulky turbidity with the extract in the cuvette, and interference with the enzyme assay was noticed. For precipitation experiments aliquots with 45 munits total hexokinase activity were treated as described in the preceding chapter for artificial mixtures of hexokinase I and III. Results from experiments with different tissues are given in Table II. In some extracts a supernatant activity is found after incubation with both anti-hexokinase I and anti-hexokinase III serum (Column C). It cannot be demonstrated when ATP is omitted from the cuvette. This activity may represent an additional hexokinase (or even more than one type) which is not or only partially precipitated by either of the two antisera. The first hypothesis is favored because the totals of the activities presented in Columns A and B, following correction for the residual supernatant activity (Column C), are nearly identical to the control (Column D). The tissue concentration of the hexokinase components can also be calculated from the activity which is precipitated by the two antisera (Columns E and F) or is unaffected (Column C) = Column G. Difficulties in the calculation of the component activity are encountered in two extracts from spleen, where the sums of supernatant activities exceed the control considerably. This may be due to interference of endogeneous 6-phosphogluconate dehydrogenase (EC 1.1.1.44) of the extract with the assay of hexokinase activity via the production of NADPH by glucose-

TABLE II

QUANTITATION OF HEXOKINASE ISOENZYME CONCENTRATION IN TISSUE EXTRACTS BY IMMUNOPRECIPITATION

The specific hexokinase activity of the extracts is presented in Table I. The case numbers are those of Table I. Immunoprecipitation was done as described in Materials and Methods. Activity is given as percent of control (sample incubated with preimmunization serum) Definition of sums is given in the text.

Tissue	Case No.	Supernatant activity after incubation with			Sum (D)	Loss of activity after incubation with		Sum (G)
		Anti-hexokinase I (A)	Anti-hexokinase III (B)	Anti-hexokinase I + anti-hexokinase III (C)		Anti-hexokinase I (E)	Anti-hexokinase III (F)	
Liver	I	72	45	20	97	28	55	103
Spleen	II	51	57	10	98	49	43	102
	III	50	64	6	108	50	36	92
	IV	42	76	5	113	58	24	87
	V	61	53	0	114	39	47	86
Lung	II	21	82	0	103	79	18	97
	IV	23	84	4	103	77	16	97
Heart	II	2	99	0	101	98	1	99
	IV	2	102	0	104	98	0	98
Kidney	I	19	99	19	99	81	1	101

6-phosphate dehydrogenase (EC 1.1.1.49). If endogenous 6-phosphogluconate dehydrogenase is present in excess the formation of NADPH per time unit is to be divided by two to give the velocity of the hexokinase reaction (15). However, if 6-phosphogluconate dehydrogenase is present in low concentrations, it may effect the calculation of supernatants with varying hexokinase activity to different extents. The effect is demonstrated in Table III which gives the supernatant activities tested in the

TABLE III

QUANTITATION OF HEXOKINASE ISOENZYME CONCENTRATION IN SPLEEN EXTRACT BY IMMUNOPRECIPITATION

Hexokinase activity in the supernatants was assayed in the absence (A) and presence of 0.5 I.U. 6-phosphogluconate dehydrogenase per ml of assay mixture (B)

Serum addition	A			B			Relative activity B/A
	Activity		Loss of activity (%)	Activity		Loss of activity (%)	
	$\Delta A_{366\ nm}$ 10 min	%		$\Delta A_{366\ nm}$ 10 min	%		
Preimmunization serum	0.1414	100	0	0.1788	100	0	1.27
Anti-hexokinase I	0.0860	61	39	0.0955	53	47	1.11
Anti-hexokinase III	0.0743	53	47	0.0855	48	52	1.15
Anti-hexokinase I + anti-hexokinase III	0	0	100	0	0	100	—
Sum of the component activity (% of control)		114	86		101	99	

presence or absence of 6-phosphogluconate dehydrogenase from yeast. A more pronounced enhancement of $NADP^+$ reduction is found in the control than in the supernatants. It is concluded that the endogenous enzyme may lead to an overestimation of the component concentrations in the supernatants unless the activity in the assay is standardized by addition of 6-phosphogluconate dehydrogenase in excess.

DISCUSSION

This report is concerned with additional proof for lack of immunological cross-reactivity of two low K_m hexokinases from human tissues and the quantitation of their concentrations in tissue extracts by immunological methods. The enzymes are termed hexokinase I and III according to the nomenclature of Katzen and Schimke [2]. The classification is based on their electrophoretical and chromatographical properties and the affinity towards glucose [11]. Evidence for lack of similar immunological determinants comes from electroimmunodiffusion experiments with the Laurell technique, as shown in detail in the other paper [11]. The specificity of the precipitation is also demonstrated by the results of the precipitin experiments (Fig. 3). After an incubation period as short as 30 min in the presence of excess of antiserum insoluble immune complexes are formed, and these can be readily removed by low speed centrifugation. When mixtures of the two enzymes are treated in this way, a high correlation is found between the concentration of a component and the loss of activity after incubation with the homologous antiserum. Similarly, the concentration of the second form present is in agreement with the supernatant activity. There is no indication of loss of sample activity exceeding the concentration of the hexokinase component which is expected to react with the antiserum.

In agreement with the specificity of the precipitin reaction enzymatic inhibition is observed with the homologous antiserum only (Fig. 2). The isoenzyme neutralization was found to be incomplete both with hexokinase I and anti-hexokinase I and hexokinase III and anti-hexokinase III serum. Residual activities of approx. 5% (hexokinase III) and 29% (hexokinase I) were seen. No further inhibition of hexokinase I could be obtained by prolonged incubation with the antiserum. Also no obvious deviation from linearity in residual activity is seen when different concentrations of hexokinase I are incubated with the antiserum (Fig. 2, Curve a). Therefore the conditions of the neutralization experiments, i.e. length of incubation period and concentration of antiserum appear to be near the optimal. The differences in residual activities of hexokinase I and III in this method may reflect different structural features of these enzymes or different inactivating properties of the antibody populations in the two antisera.

Considerable experimental evidence has accumulated up to now to argue in favor of a genetic mechanism as underlying the generation of the hexokinase isoenzymes I to III and glucokinase rather than an epigenetic one [1]. Whereas in yeast the modification of the native Forms A and B by proteolysis leads to the formation of Forms A′ and B′, respectively [16], it was demonstrated that the low K_m-hexokinases from rat could not be interconverted by proteolysis experiments [17]. Data for molecular weight, approx. 96 000 daltons, were reported for the low K_m hexokinases of rat by the latter authors. Hexokinase from pig heart has a molecular weight of 97 000 as determined by sedimentation equilibrium experiments [18], with

a tendency to slow polymerisation reactions, however. By gel filtration we have found molecular weights of approx. 111 000 and 116 000 for hexokinase I and III respectively from man [11]. The enzyme from pig heart was shown to consist of a single polypeptide chain as demonstrated by electrophoresis on sodium dodecylsulphate–polyacrylamide gels [18]. There is no evidence for hybrids as judged by zymograms of tissue extracts [11] or the kinetics of precipitin reactions of mixtures of hexokinases I and III.

The complete absence of immunological crossreactivity of the hexokinases I and III from man prove that these enzymes constitute a family of not very closely related proteins with similar catalytic specifity. According to the definition given by the IUPAC-IUB Commission on Biochemical Nomenclature [19] they represent true isoenzymes, i.e. heterogenzymes in the nomenclature proposed by Theorell [20]. The exact nature of hexokinase form II is not known, but it was shown that anti-rat muscle hexokinase II did not neutralize hexokinase I from rat adipose tissue or brain nor glucokinase (hexokinase IV_f) from rat liver [10]. Cross-reactivity was observed with glucokinase (hexokinase IV_s) from rat kidney.

Glucokinase from rat liver was reported to be a distinct protein with no cross-reactivity with the low K_m hexokinases of this species [9].

As a synopsis of the available data it may be suggested that the low K_m hexokinases and glucokinase of mammalian tissues are all products of different genes. The cross-reactivity of glucokinase from rat kidney (hexokinase IV_s) with hexokinase II [10] does not fit into the scheme and should be reinvestigated in order to shed some light on the genetic relationship of these two forms. Splitting of hexokinase II [4, 5] and glucokinase [21] into two subfractions in starch gel electrophoresis under certain conditions may (or may not) result from some enzyme modification the exact mechanisms of which remain to be explored.

The distinct immunological properties of hexokinases I and III and perhaps of all mammalian low K_m hexokinases offer a new tool for the quantitation of tissue concentrations of hexokinase isoenzymes. Analysis of hexokinase I and III concentration by the Laurell technique proved unsuccessful in experiments not shown here because of the low activities found in the extracts. The neutralization technique also gave poor results because of interference of precipitate formation with the hexokinase assay system. But it may be applicable when antiserum directed against the hexokinase protein alone is available. In the precipitin experiments a direct quantitation of hexokinases I and III was achieved in mixtures of partially purified enzymes. The technique allowed the direct quantitation of cross-reacting hexokinase protein in the extracts (Table II, Columns E and F). There is evidence for a different hexokinase activity which can not be removed by precipitation in the presence of both antisera. The tissue concentrations of hexokinase III suggested by the data in Table II were not expected as judged by the staining intensity of hexokinase III in the zymograms after starch gel electrophoresis. Hexokinase III may account for more than 50% of hexokinase activity at 0.5 mM glucose in liver extract, near 50% in spleen and nearly 20% in lung. These data are consistent with the diagramatic representation of hexokinase band staining intensity in extracts from rat tissues [4], but quite new as to the level of relative concentration of this form.

The differences of the apparent tissue concentrations seen in electrophoresis and immunochemical analysis may be due to different thermal stability of the hexo-

kinase isoenzymes [17]. This then strongly argues for the reevaluation of hexokinase isoenzyme tissue concentrations by less destructive analytical methods such as the precipitin reaction. The immunochemical technique also offers a new approach to the quantitative analysis of of hexokinase isoenzyme concentrations in tissue under different physiological conditions or in pathological sera.

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