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Glucose Metabolism in the Mucosa of the Small Intestine

A STUDY OF HEXOKINASE ACTIVITY

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1. The intracellular distribution of hexokinase activity was studied in the mucosa of rat and guinea-pig small intestine. In the rat 60% and in the guinea pig 45% of the hexokinase activity of homogenates were recovered in a total particulate fraction that contained only 5–17% of the homogenate activity of hexose phosphate isomerase, pyruvate kinase, lactate dehydrogenase and overall glycolysis (formation of lactate from glucose). 2. Fractionation of homogenates from guinea-pig small intestine showed that the particulate hexokinase activity was chiefly in the mitochondrial fraction with a small proportion in the nuclei plus brush-border fraction. 3. After chromatography of the particle-free supernatants on DEAE-cellulose, hexokinase types I and II were determined quantitatively. No evidence was obtained for the presence of hexokinase type III or glucokinase. In the preparations from guinea pigs, hexokinase types I and II amounted to 69% and 31% respectively of the eluted activity; the corresponding values for preparations from rats were 5·8% and 94·2%. 4. Total and specific hexokinase activities decreased significantly in homogenates and particle-free supernatants prepared from the intestinal mucosa of rats starved for 36hr. and increased again after re-feeding. The decrease in hexokinase activity in the particle-free supernatant from starved rats was chiefly due to a decrease in the type II enzyme.

Hexokinase (EC 2.7.1.1) activity of rat intestinal mucosa has been studied in several Laboratories (Long, 1952; Hele, 1953; Sols, 1956; Hänninen & Hartiala, 1964). Special aspects investigated were the effects of diet (Long, 1953; De Torrontegui, 1961), of thyroxine (Nishikawara, 1961; Nishikawara & Gabrielson, 1961) and of insulin (Broquet, 1954) on hexokinase activity. Further, it has been reported that the hexokinase of rat intestinal mucosa was mainly localized in unidentified particulate subcellular structures (Long, 1952; Crane & Sols, 1953; Lange & Kohn, 1961). Hexokinase was found to be the rate-limiting enzyme when the formation of lactate from glucose was studied with subcellular preparations of the intestinal mucosa of the cat and rat (Srivastava & Hübscher, 1966). It was also shown that the glycolytic activity resided predominantly in the particle-free supernatant, that this activity decreased during starvation and that this decrease was mainly due to a diminished hexokinase activity (Srivastava & Hübscher, 1966).

The object of the present work was to study the subcellular distribution of hexokinase activity in the intestinal mucosa of the guinea pig and rat, and to compare its distribution with that of other glycolytic enzymes. Also, the types of hexokinase present in the mucosa of small intestine were quantitatively assessed and studied with respect to the effect of starvation and re-feeding. Preliminary observations of this work have been published (Shakespeare, Srivastava & Hübscher, 1967).

MATERIALS AND METHODS

Animals. Male rats (Wistar) weighing 200–250g. and adult guinea pigs of either sex were used. All animals had free access to water. The rats were maintained on the Autoclaved Laboratory Small Animals Diet supplied by Spillers Ltd., Gainsborough, Lincs. The guinea pigs were fed on a mixture (1:1, w/w) of Spillers Intensive Laying Pellets and Hills R.G.P. pellets supplied by L. E. Graston, Birmingham. They had also free access to cabbage and hay.

Chemicals and enzymes. Fructose 6-phosphate (sodium salt), fructose 1,6-diphosphate (sodium salt), ATP, NAD^+, NADH, $NADP^+$, nitro blue tetrazolium and phenazine methosulphate were purchased from Sigma (London) Chemical Co., London, S.W. 6, and phosphoenolpyruvate (potassium salt), glucose 6-phosphate dehydrogenase (EC 1.1.1.49; 140 units/mg. of protein), lactate dehydrogenase (EC 1.1.1.27; 360 units/mg. of protein), glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) and triose phosphate isomerase (EC 5.3.1.1) from Boehringer Corp., London, W.5. The two enzymes last named were supplied as a mixture. A starch hydrolysate was obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada. Purified 6-phosphogluconate dehydrogenase (decarboxylating)

(EC 1.1.1.44) was prepared from rat liver by the method of Glock & McLean (1953).

All other chemicals were of A.R. grade.

Preparation of subcellular fractions from the intestinal mucosa. The collection of intestinal mucosa and preparation of homogenates and subcellular fractions were performed as described by Hübscher, West & Brindley (1965). In some experiments, when the homogenates were fractionated only into a total particulate and a supernatant fraction, the medium usually employed for homogenization (0·3M-sucrose) was replaced by 0·3M-mannitol in 50mM-tris–HCl buffer, pH 7·4.

Ammonium sulphate fractionation of the particle-free supernatant. When it was intended to fractionate the particle-free supernatant with $(NH_4)_2SO_4$, the mucosal scrapings were homogenized in a medium containing 0·15M-KCl, 50mM-tris–HCl buffer, pH 7·4, 4mM-$MgCl_2$, 8mM-EDTA and 4mM-*N*-acetylcysteine. The final pH value of the medium was adjusted to 7·4 by addition of a few drops of NaOH solution. This medium was chosen to stabilize the hexokinase activity during fractionation.

Starch-gel electrophoresis. Vertical starch-gel electrophoresis of the particle-free supernatants and subsequent staining of the gel slices were carried out by the method of Katzen & Schimke (1965), except that mercaptoethanol was replaced by 5mM-*N*-acetylcysteine (Parry & Walker, 1966). Electrophoresis was performed at 4° for 18hr. with a voltage gradient of 6v/cm. of gel.

Column chromatography on DEAE-cellulose. The method used was essentially that described by Grossbard & Schimke (1966). The columns (1·4cm. × 6·0cm.) were equilibrated with 10mM-potassium phosphate buffer, pH 7·4, containing 10mM-glucose and 5mM-*N*-acetylcysteine. After application of the particle-free supernatant, the column was washed with approx. 100ml. of the equilibrating buffer to remove non-adsorbed protein. Elution was carried out by raising the concentration of KCl in the above buffer linearly from 0 to 0·6M-KCl (total elution volume 1500ml.). The flow rate was between 25 and 50ml./hr. and the volume of the individual fractions collected varied between 7·5 and 9·0ml.

Enzyme determinations. All determinations were carried out at 30° and the rates were measured continuously over a period of 3–5min. by following the change in E_{340}. In initial experiments it was established for all assay systems employed that the enzyme was the rate-limiting factor. Optimum conditions for the assay of each enzyme are given below. Unless otherwise stated, the reaction rate was recorded with a reaction mixture without substrate as control.

(*a*) Hexokinase. The assay was based on the method of McLean & Brown (1966) and involved the determination of the glucose 6-phosphate formed with glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44). The reaction mixture contained, in a final volume of 2·5ml., the following (final concns.): tris–HCl buffer, pH 7·8 (40mM); KF (24mM); $MgCl_2$ (2mM); ATP (2mM); *N*-acetylcysteine (4mM); $NADP^+$ (0·2mM); glucose (10mM); glucose 6-phosphate dehydrogenase (0·3i.u.); 6-phosphogluconate dehydrogenase (decarboxylating) (0·25i.u.); 0·2–0·8mg. of protein of the enzyme preparation to be assayed. The reaction was started by the addition of glucose.

(*b*) Hexose phosphate isomerase (EC 5.3.1.9). The method of assay was based on that of Slein (1955). The reaction mixture contained, in a final volume of 3·0ml., the following (final concns.): tris–HCl buffer, pH 9·0 (33mM); $NADP^+$ (0·2mM); $MgCl_2$ (6·6mM); fructose 6-phosphate (3·3mM); glucose 6-phosphate dehydrogenase (EC 1.1.1.49; 0·3i.u.); 0·05–0·3mg. of protein of the preparation to be assayed. The reaction was started by the addition of substrate.

(*c*) Pyruvate kinase (EC 2.7.1.40). The assay was based on the method of Bücher & Pfleiderer (1955). The reaction mixture contained, in a final volume of 3·0ml., the following (final concns.): potassium glycine buffer, pH 7·6 (180mM); ADP (5mM); phosphoenolpyruvate (2mM); $MgCl_2$ (13·3mM); NADH (0·11mM); lactate dehydrogenase (EC 1.1.1.27; 0·3i.u.); 0·2–0·5mg. of protein of the preparation to be assayed. The reaction was started by addition of phosphoenolpyruvate.

(*d*) Lactate dehydrogenase. The assay method was essentially that described by Kornberg (1955). The assay contained, in a final volume of 3ml., the following (final concns.): potassium phosphate buffer, pH 7·4 (33mM); sodium pyruvate (2mM); NADH (0·11mM); 0·02ml. of an ethanolic solution of rotenone (saturated at room temperature); 0·05–0·2mg. of protein of the preparation to be assayed. The reaction was started by the addition of pyruvate.

(*e*) Fructose diphosphate aldolase (EC 4.1.2.13). The method of assay was essentially that of Taylor (1955). The assay contained, in a final volume of 3ml., the following (final concns.): potassium glycine buffer (180mM); fructose 1,6-diphosphate (8mM); NADH (0·11mM); glycerol 1-phosphate dehydrogenase and triose phosphate isomerase (10µg. of protein); 0·2–0·8mg. of protein of the preparation to be assayed. The reaction was started by the addition of substrate.

(*f*) Other enzymes. These as well as overall glycolysis were assayed as described previously (Hübscher *et al.* 1965; Srivastava & Hübscher, 1966).

EXPERIMENTAL AND RESULTS

In initial experiments, optimum conditions were established for the assay of hexokinase activity with homogenates or subcellular fractions from rat or guinea-pig intestinal mucosa. Typical plots of reaction rate versus concentration were obtained for glucose, ATP and magnesium chloride. From these plots, the K_m values were calculated (Lineweaver & Burk, 1934). Maximum reaction rates were obtained at 2·0–2·5mM-glucose. No further increase in reaction rate was seen when the substrate concentration was raised to 120mM, indicating the absence of glucokinase (EC 2.7.1.2) from intestinal preparations. The K_m values for glucose are summarized in Table 1. With the supernatant fraction from mucosal homogenates of rat and guinea-pig small intestine the K_m values for ATP were found to be $2{\cdot}0 \times 10^{-4}$M and $1{\cdot}1 \times 10^{-4}$M respectively. At 2mM-ATP maximum reaction rates were obtained when the concentration of magnesium chloride was also 2mM. The variation of reaction rate with pH value of the assay system is shown in

Table 1. *K_m values determined with subcellular preparations from the intestinal mucosa*

Preparation used	K_m (glucose) (M)
Rat, total particulate fraction	$1\cdot9\times10^{-4}$
Rat, particle-free supernatant	$1\cdot1\times10^{-4}$
Guinea pig, mitochondrial fraction	$8\cdot0\times10^{-4}$
Guinea pig, particle-free supernatant	$1\cdot7\times10^{-5}$

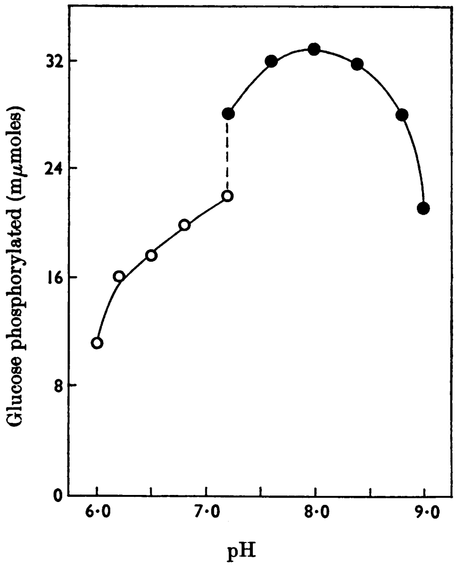

Fig. 1. Variation of hexokinase activity with pH value of incubation medium. ○, Tris phosphate buffer; ●, tris–HCl buffer.

Fig. 1. There was a broad optimum between pH values 7·6 and 8·4, which was similar to that previously noted for glycolysis as determined by lactate formation from glucose (Srivastava & Hübscher, 1966).

On the basis of these studies, the assay system for hexokinase activity described in the Materials and Methods section was defined.

Subcellular distribution of hexokinase activity and of other glycolytic enzymes. It was reported that about 90% of the glycolytic activity of homogenates from the intestinal mucosa of rats, guinea pigs and other species was recovered in the particle-free supernatant (Srivastava & Hübscher, 1966; Clark & Sherratt, 1967). However, a large proportion of the hexokinase activity of homogenates from rat intestinal mucosa has been reported to be localized in unidentified particulate subcellular structures (Long, 1952; Crane & Sols, 1953; Lange & Kohn, 1961). In view of these observations, which were not made under identical conditions of preparation of subcellular fractions, the subcellular distribution of hexokinase activity was studied in the intestinal mucosa of the guinea pig and rat. This was compared with the subcellular distribution of hexose phosphate isomerase, pyruvate kinase and lactate dehydrogenase and of overall glycolysis (formation of lactate from glucose). The results of these experiments are summarized in Table 2.

The homogenates were fractionated only into a total particulate fraction and a supernatant fraction (6000000*g*-min.) and the enzymic activities recovered in these two fractions were expressed as a function of the original homogenate activity. The distribution of 6-phosphogluconate dehydrogenase was also measured to assess the contamination of the total particulate fraction by the particle-free supernatant, which was always less than 5%. In both species there was a discrepancy between the intracellular distribution of hexokinase activity and that of other glycolytic enzymes, as shown most clearly when the recoveries in the total particulate fractions are compared. In the rat 60% of the hexokinase activity was recovered in the total particulate fraction, but only 5·1–16·1% of other glycolytic enzymes or of overall glycolysis. The recovery of lactate dehydrogenase was rather low, but, even if all the lactate dehydrogenase activity not accounted for had been in the particulate fraction, its distribution would still have been significantly different from that of hexokinase activity. In the guinea-pig experiments overall recoveries of enzymic activities were far better and a similar, but less pronounced, localization of hexokinase activity in the total particulate fraction was obtained.

The medium used for the preparation of the subcellular fractions in the experiment described above was 0·3M-mannitol in 50mM-tris–hydrochloric acid buffer, pH 7·4. The ionic strength of this medium should have been high enough to prevent adsorption of some glycolytic enzymes on particulate subcellular components as reported by Roodyn (1957) and Hultin & Westort (1966). To check this possibility further, homogenates of rat intestinal mucosa were prepared in 0·3M-mannitol in 5mM-tris–hydrochloric acid buffer, pH 7·4, or in 0·15M-potassium chloride in 50mM-tris–hydrochloric acid buffer, pH 7·4. There was no significant difference observed in the proportion of hexokinase activity recovered in the particle-free supernatants with the medium of low ionic strength ($25\cdot3\pm10\cdot3\%$; mean ± S.D.; three rats) or high ionic strength ($26\cdot9\pm4\cdot9\%$; mean ± S.D.; four rats).

The next step was to determine if the hexokinase activity present in the total particulate fraction was localized mainly in one subcellular fraction. Because homogenates of the intestinal mucosa from guinea pigs are known to be more easily fractionated than those from rats (Hübscher *et al.* 1965) only the

Table 2. *Subcellular distribution of glycolytic enzymes in the mucosa of the small intestine*

Particle-free supernatant and total particulate fractions derived from the mucosa of the whole small intestine were assayed. Values quoted are means ± S.D. of six rats and three guinea pigs.

	Rat intestinal mucosa				Guinea-pig intestinal mucosa			
		% of total activity present				% of total activity present		
Activity	Total homogenate activity (μmoles/min.)	Particle-free supernatant	Total particulate fraction	Recovery (%)	Total homogenate activity (μmoles/min.)	Particle-free supernatant	Total particulate fraction	Recovery (%)
Hexokinase	6·4 ± 0·7	36·4 ± 2·0	60·0 ± 3·0	96·4	3·3 ± 0·4	60·3 ± 13·0	45·3 ± 3·0	105·6
Hexose phosphate isomerase	52·5 ± 9·5	80·1 ± 14·0	5·1 ± 2·4	85·2	94·7 ± 18·0	79·1 ± 2·6	10·1 ± 1·4	89·2
Pyruvate kinase	230 ± 22·5	96·1 ± 2·3	6·2 ± 1·0	102·3	51·0 ± 6·0	79·7 ± 9·0	11·5 ± 2·0	91·2
Lactate dehydrogenase	1190 ± 70	61·7 ± 1·0	16·1 ± 2·5	78·2	98·2 ± 11·0	86·0 ± 4·5	17·1 ± 3·2	103·1
Formation of lactate from glucose	1·43 ± 0·51	81·9 ± 9·6	11·8 ± 3·5	93·7	1·03 ± 0·03	105·0 ± 1·0	Not detected	105·0

Table 3. *Subcellular location of hexokinase in the mucosa of the small intestine*

The mucosa of the whole small intestine was used. Values quoted are mean percentages (± S.D.) of the homogenate amount or activity of results obtained from five guinea pigs. Numbers in parentheses are total enzymic activities (μmoles/min./total homogenate) or total amounts (mg. of protein or DNA/total homogenate). Other details are given in the text.

	Amount or activity (% of that of homogenate)						
Fraction	Protein	DNA	Invertase	Succinate dehydrogenase	Glucose 6-phosphatase	Hexokinase	Particulate hexokinase
Homogenate	100 (389 ± 124)	100 (14·8 ± 7·3)	100 (10·8 ± 2·3)	100 (7·3 ± 0·2)	100 (13·8 ± 4·8)	100 (10·1 ± 2·2)	—
Nuclear plus brush-border	18·0 ± 4·1	101·4 ± 14·0	65·5 ± 5·7	10·9 ± 5·7	15·6 ± 7·2	10·4 ± 6·0	23·7 ± 12·0
Mitochondrial	14·8 ± 1·5	6·0 ± 6·0	14·2 ± 2·9	84·1 ± 18·0	22·9 ± 7·0	28·5 ± 10·0	65·3 ± 12·0
Microsomal	13·4 ± 5·1	2·5 ± 2·5	10·0 ± 2·9	7·8 ± 6·0	53·3 ± 12·5	4·8 ± 2·5	11·0 ± 5·5
Particle-free supernatant	44·1 ± 7·6	7·2 ± 7·2	13·6 ± 12·0	Absent	10·9 ± 9·8	69·9 ± 13·0	—
Recovery	90·3 ± 3·4	116·4 ± 18·0	103·3 ± 20·0	102·8 ± 19·0	102·7 ± 20·0	113·6 ± 25·0	—

former were investigated. The results of these experiments are shown in Table 3. DNA and various marker enzymes were also assayed to assess the cross-contamination of the various subcellular fractions. If the hexokinase activity present in each fraction was expressed as a percentage of the total particulate hexokinase activity (see last column, Table 3), the subcellular distribution of the particulate hexokinase activity was similar to that of succinate–2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride oxidoreductase. However, the amount of hexokinase activity present in the nuclei plus brush-border fraction was higher than could be accounted for by contamination with the mitochondrial fraction. The results therefore indicate that the particulate hexokinase activity was mainly in the mitochondrial fraction with a small proportion in the nuclei plus brush-border fraction.

A mitochondrial fraction prepared from the intestinal mucosa of the guinea pig that phosphorylated 24·5 mμmoles of glucose/min./mg. of protein did not form lactate or glycerol phosphate from glucose under the standard conditions of assay of glycolysis. In agreement with these results is the observation that hexose phosphate isomerase and fructose diphosphate aldolase could not be detected in these mitochondrial preparations.

Occurrence of multiple hexokinases in the supernatant fraction of mucosal homogenates. Particle-free supernatants of homogenates obtained from rat and guinea-pig intestinal mucosa were used for starch-gel electrophoresis. Two bands of hexokinase activity were identified, with mobilities similar to

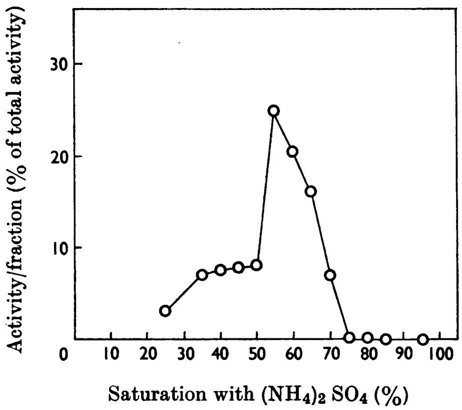

Fig. 2. Precipitation of hexokinase activity from the particle-free supernatant of rat intestinal mucosa by $(NH_4)_2SO_4$. The activity present in each fraction is expressed as a percentage of the total activity precipitated.

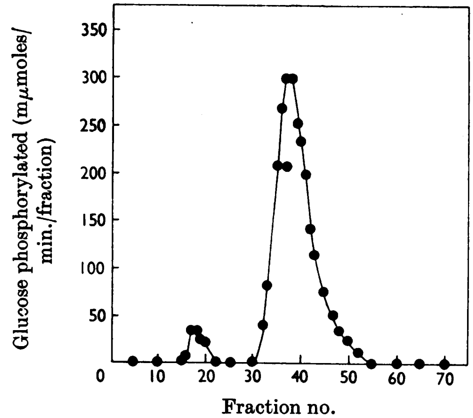

Fig. 3. Elution of hexokinase activity from a DEAE-cellulose column. A particle-free supernatant preparation from rat intestinal mucosa was applied; the buffer used for elution contained glucose (see the Materials and Methods section).

those reported by Katzen & Schimke (1965) for hexokinase types I and II.

Stepwise ammonium sulphate fractionation of the particle-free supernatant from rat intestinal mucosa resulted in 12 fractions, nine of which contained hexokinase activity. The results are shown in Fig. 2 and indicate that there may be two types of hexokinase, one of which was precipitated between 25 and 50% saturation and the other between 50 and 75% saturation with ammonium sulphate. The total recovery was 64% of the hexokinase activity present in the original particle-free supernatant. All the fractions lost considerable amounts of activity on storage for 1 day at $-8°$. However, the hexokinase activity precipitated at the lower ammonium sulphate concentration decreased only to 37·3% of its original value, whereas that precipitated at the higher ammonium sulphate concentrations was decreased to 18·9%.

For a more accurate determination of the types of hexokinase, chromatography of the proteins of the particle-free supernatant from rat intestinal mucosa on DEAE-cellulose was employed. Two bands of hexokinase activity were eluted from the column when a supernatant preparation was applied (see Fig. 3). As essentially the same method as that described by Grossbard & Schimke (1966) was used, the relative positions of the bands of hexokinase activity indicated the occurrence of hexokinase types I and II. The total recovery of hexokinase activity from the columns was 55–65% of the activity applied. Hexokinase type I amounted to only $5{\cdot}8 \pm 1{\cdot}4\%$ (mean ± S.D.; four rats) of the total activity eluted, the remainder being type II enzyme.

Hexokinase type III, which has a low K_m (glucose)

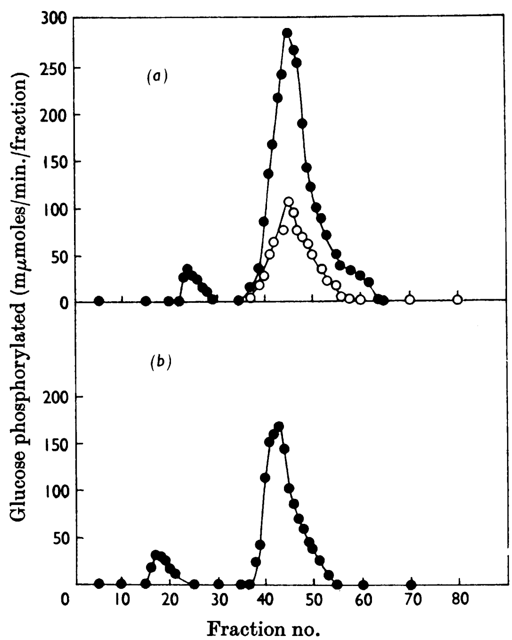

Fig. 4. Elution of hexokinase activity from DEAE-cellulose columns. (a) The particle-free supernatant preparation from rat intestinal mucosa was applied; the buffer used for elution did not contain glucose. Individual fractions were assayed at 10mM-glucose (●) and 0·1mM-glucose (○) respectively. (b) The particle-free supernatant from the intestinal mucosa of a starved rat was applied.

value, is known to be eluted together with or overlapping with the type II enzyme under the conditions employed in these experiments (Grossbard &

Schimke, 1966). As the buffer used for the elution contained 10mM-glucose it was not possible to test directly for the occurrence of hexokinase type III or to determine the K_m (glucose) value. In subsequent experiments elution was therefore carried out with the same buffer system except that glucose was omitted. The recoveries of hexokinase activity from the columns were 52–58% of the activity applied. The K_m (glucose) values were determined with pooled samples of each band and were found to be $5{\cdot}6 \times 10^{-5}$ and $1{\cdot}4 \times 10^{-4}$M for the type I and the type II enzyme respectively. The type II hexokinase band was assayed at both 10mM- and 0·1mM-glucose (see Fig. 4a). As hexokinase type III has been reported to have a higher affinity for glucose than the type II enzyme, the reaction rate of the former enzyme should have remained unchanged at 0·1mM-glucose whereas that of hexokinase type II should have been greatly decreased. From the K_m value of the type II enzyme, the expected reaction rate at 0·1mM-substrate was calculated and was 11% lower than the reaction rate actually obtained at 0·1mM-glucose (see Fig. 4a). This result indicates that the type III enzyme was not present in the second band eluted from DEAE-cellulose columns.

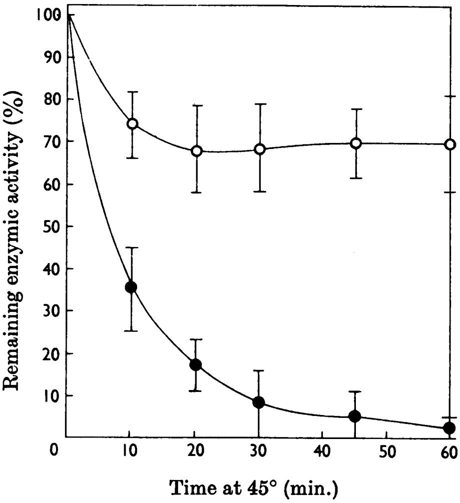

Fig. 5. Effect of heating on hexokinase activity. Particle-free supernatant preparations from the intestinal mucosa of guinea pigs (○) and rats (●) were kept for the periods indicated at 45°. The values shown are means ± S.D. of three animals.

After chromatography of the proteins of the particle-free supernatant from guinea-pig intestinal mucosa, two bands of hexokinase activity were obtained. Their positions were the same as those of the hexokinase types I and II from rat intestinal mucosa, but the type I and type II enzymes constituted 69 and 31% respectively of total eluted activity. Thus the relative amounts of the type I and type II enzymes differed sharply from those observed in preparations from rat small intestine. Similar conclusions were drawn from experiments where the particle-free supernatants from the small intestine of the guinea pig and rat respectively were kept for various times at 45° (see Fig. 5). The type II enzyme from fat pads and muscle is heat-labile whereas the type I enzyme from liver and brain is heat-stable (Katzen & Schimke, 1965). More recently, this difference in thermal stability has been employed to determine the relative amounts of hexokinase types I and II in mammary gland (Walters & McLean, 1967) and adipose tissue (McClean, Brown, Walters & Greenslade, 1967). The results summarized in Fig. 5 indicate that in the particle-free supernatant from guinea-pig intestine

Table 4. *Effect of starving and re-feeding on hexokinase activity of rat intestinal mucosa*

Particle-free supernatants derived from the mucosa of the whole small intestine were assayed. The control group was given rat cubes *ad lib.* and was killed between 9 and 10 a.m. The starved group was deprived of food for 36hr. The re-fed group was starved for 36hr., then given rat cubes *ad lib.* and killed 22hr. later. Values quoted are means ± S.D. of results obtained from eight rats.

	Hexokinase activity					
	Control fed		Starved (36hr.)		Re-fed	
	Total activity (μmoles of glucose phosphorylated/min.)	Sp. activity (mμmoles of glucose phosphorylated/min./mg. of protein)	Total activity (μmoles of glucose phosphorylated/min.)	Sp. activity (mμmoles of glucose phosphorylated/min./mg. of protein)	Total activity (μmoles of glucose phosphorylated/min.)	Sp. activity (mμmoles of glucose phosphorylated/min./mg. of protein)
Homogenate	9·83 ± 1·62	44·2 ± 5·0	5·85 ± 0·84	37·0 ± 4·6	10·87 ± 2·60	53·4 ± 8·3
Particle-free supernatant	4·92 ± 1·58	37·3 ± 9·4	2·03 ± 0·58	21·6 ± 6·1	3·50 ± 0·70	33·3 ± 7·9

hexokinase type I accounted for 70·7% of the total hexokinase activity, whereas in the same subcellular fraction from rat small intestine it amounted to only 2·1% of the total activity.

Effect of starvation on hexokinase activity. With subcellular preparations from the mucosa of rat small intestine, the formation of lactate from glucose was greatly decreased after starvation for 36hr., though the formation of lactate from glucose 6-phosphate remained almost unchanged (Srivastava & Hübscher, 1966). This indicated that starvation affected hexokinase activity in the intestinal mucosa. Hexokinase was therefore assayed in homogenates and particle-free supernatants prepared from the intestinal mucosa of starved and re-fed rats. The decrease in total hexokinase activity after starvation was more pronounced than the decrease in specific activity because the amount of total protein obtained from the mucosa of a whole rat small intestine fell slightly after starvation (see Table 4). A *t* test showed that the total activity measured in preparations from starved rats was significantly different from those of control fed rats ($P < 0{\cdot}001$) and re-fed rats ($P < 0{\cdot}001$). Similarly, the specific activity of hexokinase in preparations from starved rats was significantly different from those of control fed rats ($P < 0{\cdot}01$) and re-fed rats ($P < 0{\cdot}01$).

Chromatography of the proteins of the particle-free supernatant from rat intestinal mucosa on DEAE-cellulose columns (see Fig. 4*b*) indicated that the ratio of the amounts of type I and type II hexokinase was changed after starvation. In preparations from control fed rats hexokinase type I was $5{\cdot}8 \pm 1{\cdot}4\%$ (mean ± S.D.; four rats) of the total activity eluted, whereas with preparations from starved rats the corresponding value was 9·4% (average; two rats). If this result is considered in conjunction with the values reported in Table 4, then the decrease in hexokinase activity observed in the particle-free supernatant prepared from the intestinal mucosa of starved rats is mainly and possibly wholly due to a decrease in hexokinase type II.

DISCUSSION

The observation that a high proportion of the hexokinase activity of rat and guinea-pig intestinal mucosa was associated with the total particulate fraction confirms and extends earlier reports on rat intestinal mucosa (Long, 1952; Crane & Sols, 1953; Lange & Kohn, 1961). The localization of part of the total hexokinase activity in particulate subcellular structures is not only confined to the intestinal mucosa, as rat brain, heart, kidney and liver (Crane & Sols, 1953) and frog skeletal muscle (Simon, 1967) also possess, though to various degrees, particulate hexokinase activity.

In the present study, the particulate hexokinase activity was localized chiefly in the mitochondrial fraction. A mitochondrial localization of hexokinase has also been reported in rat brain (Johnson, 1960; Bachelard, 1967; Biesold & Teichgräber, 1967), liver (Fomina, 1963) and mammary gland (Walters & McLean, 1967) and in Ehrlich ascites-tumour cells (Li & Ch'ien, 1965). The amounts of hexokinase associated with the mitochondrial fraction vary greatly from tissue to tissue: 52–75% of brain homogenates was recovered in the mitochondrial fraction (Johnson, 1960; Biesold & Teichgräber, 1967), whereas with liver preparations the corresponding value was only 10–15% (Fomina, 1963). Allowing for the cross-contamination of the various subcellular fractions as determined by the distribution of succinate dehydrogenase, about 33% of the hexokinase activity of homogenates from the mucosa of guinea-pig small intestine was associated with the mitochondrial fraction.

The role of mitochondrial hexokinase in glycolysis is difficult to understand in view of the observation that the glycolytic activity of mucosal homogenates is almost completely recovered in the particle-free supernatant, even though the hexokinase reaction is the rate-limiting step in overall glycolysis when mucosal homogenates are assayed (Srivastava & Hübscher, 1966; Clark & Sherratt, 1967). It has been suggested that mitochondrially bound hexokinase of Ehrlich ascites-tumour cells acts as a trap for ATP formed by oxidative phosphorylation (Li & Ch'ien, 1965).

Katzen & Schimke (1965) detected after starch-gel electrophoresis hexokinase types I and II and also small amounts of the type III enzyme in a high-speed supernatant from rat intestine. The occurrence of type I and type II hexokinase was confirmed in the present study, but no evidence in support of occurrence of the type III enzyme was obtained.

Long (1952) and De Torrontegui (1961) studied the effect of diet on the hexokinase activity of rat intestinal mucosa. They observed an increased hexokinase activity after a high-carbohydrate low-fat diet compared with a low-carbohydrate high-fat diet. De Torrontegui (1961) also noticed that starvation for 24hr. did not bring about a change in the hexokinase activity of rat intestinal mucosa. This is in contrast with the present observations, which showed a significant decrease in total as well as specific hexokinase activity after starvation. However, the decrease was not as marked as might have been expected from the sharp decrease in glycolysis observed previously with subcellular preparations from the small intestine of starved rats (Srivastava & Hübscher, 1966). It is noteworthy that in the high-speed supernatant from rat fat pads, which, like the supernatant fraction from rat intestinal mucosa, contain mainly type II hexokinase, a relative decrease of this enzyme was observed after starvation for 48hr. (Katzen & Schimke, 1965). Adipose tissue is known to be an insulin-sensitive tissue and the activities of type II hexokinase were shown to be significantly lowered in fat pads from alloxan-diabetic rats (McLean *et al.* 1967). A lack of this hormone was also reported to prevent an increase of hexokinase type II, which is developing in rat mammary glands during lactation (Walters & McLean, 1967). It is not yet known whether the type II hexokinase of rat intestinal mucosa is also influenced by insulin and whether the changes of the type II enzyme observed during starvation are in some way mediated by this hormone. According to Broquet (1954), the hexokinase activity of the intestinal mucosa of rats is higher in diabetic rats. Thus the hexokinase of the intestinal mucosa seems to respond differently to a lack of insulin from hexokinase of adipose tissue and mammary gland.

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