Mitochondrial Hexokinase from Small-Intestinal Mucosa and Brain

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1. The submitochondrial localization of hexokinase activity in preparations of mitochondria from the small intestine of the guinea pig was studied by conventional methods. 2. Hexokinase activity in this tissue was predominantly associated with the outer mitochondrial membrane. 3. The inactivation of mitochondrial enzymes by trypsin in iso-osmotic and hypo-osmotic conditions was also used to determine the submitochondrial localization of hexokinase activity. 4. Hexokinase activity was found to be on the outside of the outer mitochondrial membrane. 5. It was shown that both type I and type II hexokinase activities are bound to the outside of the outer mitochondrial membrane. The types are present in the same ratio as that in which they occur in the cytosol of the cell. 6. Mitochondrial hexokinase from the small intestine did not show the latency phenomenon demonstrated by mitochondrial hexokinase from brain when subjected to a variety of treatments. However, hexokinase activity was solubilized from preparations of mitochondria from the small intestine by the same treatments as for mitochondrial hexokinase from brain. 7. The submitochondrial distribution of hexokinase activity in mitochondrial preparations from rat brain was determined by the trypsin inactivation method. 8. Hexokinase activity in preparations of mitochondria from rat brain was found on the outside of the outer membrane, between the mitochondrial membranes, and within the inner mitochondrial membrane. 9. Hexokinase from rat brain showed latency properties irrespective of its submitochondrial location.

In previous work it was observed that the ratio of hexokinase (EC 2.7.1.1) activity in the total particulate fraction to that in the particle-free supernatant was significantly higher in the intestinal mucosa of starved rats than in the intestinal mucosa of fed rats (Mayer, Shakespeare & Hübscher, 1970). Perfusion of the proximal half of the small intestine of starved rats with a medium containing glucose caused a significant decrease in the ratio of hexokinase activity in total particulate fraction to that in the particle-free supernatant. This indicated, but did not prove, that the glucose-dependent increase of hexokinase activity in the particle-free supernatant was due to a release of hexokinase from subcellular particulate structures.

Before a thorough characterization of the redistribution of hexokinase activity within the cell under various metabolic conditions can be attempted, it is necessary to understand the binding of hexokinase to subcellular particulate structures. The major portion of particulate hexokinase activity in the small intestinal mucosa of the

guinea pig is associated with the mitochondrial fraction (Srivastava, Shakespeare & Hübscher, 1968). The properties of mitochondrial hexokinase from the small-intestinal mucosa of the guinea pig were therefore studied. Preparations from the mucosa of the small intestine of the rat were not used owing to the difficulty in fractionating mucosa from this source (Hübscher, West & Brindley, 1965).

MATERIALS AND METHODS

Animals. Adult male guinea pigs (350-500 g) of mixed strain and male Wistar rats (150-200 g) were obtained from the Nottingham School of Agriculture, Sutton Bonington, Notts., U.K.

Chemicals. Trypsin (EC 3.4.4.4), ATP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Soya-bean trypsin inhibitor was purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Triton X-100 was obtained from Lennig Chemicals Ltd., Croydon, London CR9 3NB, U.K. Dithiothreitol was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other chemicals were of A.R. grade.

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Preparation of subcellular fractions. Angle-head (8×50 ml, 10×10 ml) or swing-out (3×23 ml) rotors were used in MSE centrifuges. All operations were carried out at $0-4^{\circ}\text{C}$. (1) Mitochondrial preparations were obtained from the small-intestinal mucosa of the guinea pig by modifications of the method of Hübscher *et al.* (1965). The homogenization medium used was 0.88M -sucrose, containing 2mM -EDTA, 1mM -NaF and 1mM -dithiothreitol. The pH of this and all subsequent sucrose solutions was adjusted to 7.4 with KHCO_3 . Each homogenate was centrifuged at $1000g_{\text{av}}$ for 10 min. The sediment was washed twice with 30ml of homogenization medium. The combined supernatants were centrifuged at $30000g$ for 15 min. The sediment was washed twice and the final sediment was suspended in homogenization medium.

(2) Particle-free supernatants were prepared from the mucosa of rat and guinea-pig small intestine. Homogenates (in 0.88M -sucrose, containing 2mM -EDTA, 1mM -NaF, 1mM -dithiothreitol) were centrifuged for 2 h at $104000g$.

(3) In experiments in which the properties of mitochondrial hexokinase from the small intestine of the guinea pig and from rat brain were compared, mitochondrial preparations were obtained from both sources by a slight modification of the method of Katzen, Soderman & Wiley (1970). Homogenates were prepared in 0.25M -sucrose.

Preparation of mitochondrial subfractions. (1) Mitochondrial preparations were subjected to water lysis by a slight modification of the method of Shephard & Hübscher (1969). After the final lysis treatment, the aqueous suspension (6ml) was layered over 6ml of 0.3M -sucrose containing 2mM -EDTA and 1mM -NaF and centrifuged for 30 min at $59000g$. The final supernatant was combined with the lysis supernatants and the pellet was resuspended in 0.3M -sucrose, containing 2mM -EDTA, 1mM -NaF and 1mM -dithiothreitol. This was layered over a discontinuous sucrose density gradient consisting of 10ml of 1.5M -sucrose (bottom) and 5ml of 1.2M -sucrose (top). Both sucrose solutions contained 2mM -EDTA and 1mM -NaF. After centrifugation for 2 h at $98000g$ in a swing-out rotor three fractions (top, middle and bottom, approx. 6ml each) were collected and diluted with water to give a concentration of approx. 0.3M -sucrose. These fractions were designated O_1 , O_2 and O_3 . Each fraction was centrifuged for 30 min at $306000g$ and the pellets were resuspended in 0.88M -sucrose, containing 2mM -EDTA and 1mM -NaF. Enzyme assays were carried out on the supernatants and resuspended pellets.

Mitochondrial preparations were also lysed by two alternative procedures.

(2a) Mitochondrial preparations were isolated in either 0.88M -sucrose or 0.25M -sucrose and centrifuged for 15 min at $30000g$. The pellets were resuspended in 2mM -EDTA, containing 1mM -dithiothreitol (pH 7.4) and dialysed for 1 h at 4°C against a solution of the same composition. Samples of each preparation of lysed mitochondria or samples of the supernatant prepared by centrifugation of the lysed mitochondrial suspension for 30 min at $104000g$ were used.

(2b) Preparations of brain mitochondria were lysed by adding water ($1:1$, v/v) followed by incubation for 3 min at 20°C .

Enzyme assays. These were carried out in duplicate at 30°C either immediately (for hexokinase and isocitrate dehydrogenase, EC 1.1.1.42) or on fractions that had been stored at -20°C . Enzyme activities are expressed as μmol of substrate converted/min and specific activities as $\mu\text{mol}/\text{min}$ per mg of protein.

Hexokinase was assayed as described by Srivastava *et al.* (1968). Succinate dehydrogenase (EC 1.3.99.1), monoamine oxidase (EC 1.4.3.4) (with kynuramine as substrate) and arylesterase (EC 3.1.1.2) were assayed as described by Shephard & Hübscher (1969). Arylsulphatase B (EC 3.1.6.1) was assayed by a modification of the method of Roy (1953). The fractions were pre-incubated for 30 min at 0°C with Triton X-100 (final concentration 0.1% , v/v). The assay system contained 10mM -barium acetate. Isocitrate dehydrogenase and adenylate kinase (EC 2.7.4.3) were assayed as described by Sottocasa, Kuylenskierna, Ernster & Bergstrand (1967).

Tryptic digestion. Samples of mitochondrial preparations, lysed mitochondrial preparations and solutions of proteins solubilized from mitochondrial preparations were incubated with trypsin under the conditions described below. Each incubation mixture contained, in a final volume of 1ml , the following (final concentrations): tris-HCl buffer, pH 7.8 (20mM); EDTA (2mM); dithiothreitol (1mM); sucrose (0.3M in iso-osmotic conditions); trypsin and a sample of the preparation to be treated. Each incubation was for 15 min at 37°C in duplicate or triplicate and was terminated by the addition of soya-bean trypsin inhibitor ($2:1$, w/w, with trypsin).

Treatments of mitochondrial preparations. Mitochondrial suspensions were diluted $1:1$ (v/v) with the following solutions and with the exception of solutions 7, 8, 9, 10 and 13 were incubated for 3 min at 20°C . Incubations with solutions 7 and 8 were carried out for 20 min at 20°C . Incubation mixtures contained in a final volume of 2ml (final concentrations): (1) sucrose (0.25M) previously adjusted to pH 7.4 with KHCO_3 ; (2) NaCl (0.9M); (3) KCl (0.9M); (4) sucrose (0.25M), pH 7.4, containing Triton X-100 (0.5% , w/v); (5) NaCl (0.9M) containing Triton X-100 (0.5% , w/v); (6) KCl (0.9M) containing Triton X-100 (0.5% , w/v); (7) sucrose (0.25M) containing glucose 6-phosphate (2mM); this solution was previously adjusted to pH 7.4 with KHCO_3 ; (8) sucrose (0.25M) containing ATP (5mM); this solution was previously adjusted to pH 7.4 with KHCO_3 ; (9) sucrose (0.25M), pH 7.4, followed by sonication for 30 s at 21kHz in an MSE sonicator; (10) sucrose (0.25M), pH 7.4, followed by freezing and thawing the samples four times; (11) sucrose (0.25M), pH 7.4, containing bovine serum albumin (fatty acid-poor, 1% , w/v); (12) water previously adjusted to pH 7.4 with KHCO_3 ; (13) water previously adjusted to pH 7.4 with KHCO_3 (incubated for 3 min at 20°C) followed by Triton X-100 (0.5% , w/v) (3 min at 20°C).

Determinations of protein. These were carried out as described by Hübscher *et al.* (1965) or by the method of Lowry, Rosebrough, Farr & Randall (1951).

EXPERIMENTAL AND RESULTS

The results of preliminary fractionations of homogenates of small-intestinal mucosa from the guinea pig gave a subcellular localization of hexo-

kinase essentially the same as that obtained by Srivastava *et al.* (1968). Most of the particulate hexokinase was associated with the mitochondrial fraction. The final mitochondrial suspension contained 7% of the arylsulphatase B activity (lysosomal marker) of the homogenate and less than 1% of the arylesterase activity (microsomal marker) of the homogenate.

The submitochondrial location of hexokinase activity was determined and the results are shown in Table 1. With the exception of the supernatant from subfraction O₁ no enzymic activities were found in the supernatants.

In preliminary experiments approx. 50% of the hexokinase activity, 75% of the adenylate kinase activity and 90% of the isocitrate dehydrogenase activity in subfraction O₁ was in the supernatant prepared from this subfraction. Less than 10% of the monoamine oxidase activity was in this supernatant. When the final lysed mitochondrial suspension was centrifuged through 0.3M-sucrose and then layered over the discontinuous sucrose gradient, less than 10% of the hexokinase activity and no monoamine oxidase activity was present in the supernatant derived from subfraction O₁. However, 80% of the isocitrate dehydrogenase activity and 75% of the adenylate kinase activity were still present in the supernatant. This procedure was used in the experiments reported in Table 1. The final lysed mitochondrial pellet (membrane vesicle preparation) was not in a hypo-osmotic environment and therefore lysis with concomitant enzyme leakage or solubilization from the membrane vesicles was prevented.

Subfraction O₁ was enriched with hexokinase activity to a greater extent than any other fraction. However, approximately one-third of hexokinase activity in unlysed mitochondrial preparations was found in the combined lysis supernatants and subfraction O₁ also contained some material derived from inner membranes. Therefore an alternative technique was chosen to study the localization of hexokinase activity more exactly.

The experimental approach used was the tryptic inactivation of mitochondrial enzymes in iso-osmotic and hypo-osmotic conditions (Kuylenstierna, Nicholls, Hovmöller & Ernster, 1970). In a preliminary experiment, the sensitivity of mitochondrial hexokinase and adenylate kinase activities to tryptic digestion was determined. The experiment was carried out with a particle-free supernatant containing hexokinase and adenylate kinase activities obtained by centrifugation of a lysed mitochondrial preparation. Adenylate kinase activity was extremely sensitive to tryptic digestion. All activity was lost at 20 µg of trypsin/mg of protein. Hexokinase activity was much more resistant to the tryptic digestion, complete loss of

Table 1. Recovery of mitochondrial activity in subfractions

A sample of a lysed mitochondrial preparation obtained from intestinal mucosa was applied to a discontinuous sucrose density gradient. Subfractions O₁, O₂ and O₃ represent the top, middle and bottom layers of the discontinuous sucrose density gradient. Activities of enzymes in these subfractions are expressed as percentages of the activity in the mitochondrial sample or as specific activity relative to the mitochondrial sample. Combined lysis supernatant activity is given as a percentage of the activity in the mitochondrial sample and as specific activity relative to the mitochondrial sample.

Enzyme assayed	Preparation no.	Combined lysis supernatants						Subfraction O ₁		Subfraction O ₂		Subfraction O ₃		% recovery of mitochondrial sample (O ₁ + O ₂ + O ₃)
		Activity		Sp. activity		Activity	Sp. activity	Activity	Sp. activity	Activity	Sp. activity			
Hexokinase	1	43.5	1.26	49.0	2.09	20.0	0.45	10.0	0.25	79				
	2	49.5	1.91	58.0	2.26	13.0	0.42	7.0	0.12	78				
Isocitrate dehydrogenase	1	110.0	3.30	28.0	1.18	41.0	0.9	9.0	0.54	78				
	2	81.0	2.92	33.0	1.26	36.0	1.2	20.0	0.36	89				
Monoamine oxidase	1	0.0	—	59.0	2.48	15.0	0.34	11.0	0.24	85				
	2	0.0	—	61.0	2.33	15.0	0.46	10.0	0.16	86				
Succinate dehydrogenase	1	0.0	—	8.0	0.32	70.0	1.54	25.0	1.61	103				
	2	0.0	—	8.0	0.32	52.0	1.66	27.0	1.50	87				
Adenylate kinase	1	480	14.0	76.0	2.42	16.0	0.36	2.0	0.2	94				
	2	410	15.0	57.0	2.21	12.0	0.36	11.0	0.18	80				

A sample of a lysed mitochondrial preparation obtained from intestinal mucosa was applied to a discontinuous sucrose density gradient. Subfractions O₁, O₂ and O₃ represent the top, middle and bottom layers of the discontinuous sucrose density gradient. Activities of enzymes in these subfractions are expressed as percentages of the activity in the mitochondrial sample or as specific activity relative to the mitochondrial sample. Combined lysis supernatant activity is given as a percentage of the activity in the mitochondrial sample and as specific activity relative to the mitochondrial sample.

activity occurring at 400 μ g of trypsin/mg of protein. Further experiments with mitochondrial preparations were carried out with 0.5mg of trypsin/mg of protein.

The tryptic sensitivity of hexokinase and adenylate kinase activities in whole mitochondrial preparations is as shown in Table 2. Adenylate kinase was used as a marker enzyme for inter-membrane space. In iso-osmotic conditions all the hexokinase activity was lost whereas adenylate kinase activity remained almost unchanged. In hypo-osmotic conditions 80% of the adenylate kinase was lost.

The particle-free supernatant prepared from the mucosa of the guinea-pig small intestine contains only types I and II hexokinase in the ratio of 7:3 (Srivastava *et al.* 1968). These two types of hexokinase can be distinguished in both crude (Srivastava *et al.* 1968) and partially purified (Katzen & Schimke, 1965) preparations by their selective heat instability. This difference in properties was utilized to study the types of hexokinase which are bound to the outer membrane in preparations of mitochondria from the small intestine. The results are presented in Fig. 1. The ratio of type I/type II hexokinase activities in preparations of hexokinase solubilized from mitochondria with 0.9M-sodium chloride were not different from the ratio of hexokinase types in a particle-free supernatant prepared from the mucosa of guinea-pig small intestine. Only 80% of mitochondrial hexokinase from small-intestinal mucosa is solubilized by 0.9M-sodium chloride. Although there is no obvious basis for assuming heat stability to be identical for soluble and particulate activities, the heat sensitivity of hexokinase activity associated with preparations of whole mitochondria and preparations of mitochondria previously treated with 0.9M-sodium chloride was measured. The behaviour of hexokinase activity was the same as with the solubilized enzyme and the ratio type I/type II hexokinase activities in both these preparations was identical with that obtained for preparations of solubilized hexokinase.

The experiments reported here show that some of the properties of mitochondrial hexokinase from the mucosa of guinea-pig small intestine were different from those of mitochondrial hexokinase observed in other tissues (Wilson, 1968; Craven, Goldblatt & Basford, 1969; Vallejo, Marco & Sebastian, 1970; Katzen *et al.* 1970). Most studies have been carried out with mitochondrial hexokinase from rat and bovine brain. Mitochondrial hexokinase activity in these tissues is measured in two forms, i.e. exposed hexokinase activity and latent hexokinase activity, which is activated by various metabolites and membrane disruptive agents. It was decided to compare the response of hexokinase activity in mitochondrial preparations from intestinal mucosa and rat brain in conditions known to expose hexokinase activity in mitochondrial preparations from brain. Mitochondrial preparations from these tissues were obtained by the same method to ensure that any difference in properties could not result from alternative methods of preparation. The results of these experiments (Table 3) indicated that mitochondrial hexokinase from intestinal mucosa does not exhibit the latency properties shown by mitochondrial hexokinase from brain, but that hexokinase activity from both sources is solubilized by the same treatments.

To explain the differences in the response of hexokinase from the two sources to the various treatments it was decided to examine the sub-mitochondrial localization of hexokinase activity in rat brain. In preliminary experiments the sensitivity of mitochondrial hexokinase and adenylate kinase activities to tryptic digestion was determined. The sensitivity of brain adenylate kinase activity to tryptic digestion was the same as that of the enzyme in preparations of mitochondria from small intestinal mucosa. Mitochondrial hexokinase from brain was much less sensitive to tryptic digestion than the enzyme from intestinal mucosa. A complete loss of activity occurred at 4mg of trypsin/mg of protein. Further experiments with mitochondrial preparations from rat brain

Table 2. *Susceptibility of mitochondrial hexokinase and adenylate kinase activities from small-intestinal mucosa to tryptic digestion in iso-osmotic and hypo-osmotic conditions*

Mitochondrial preparations were lysed by the prolonged dialysis procedure (method 2a) and incubated with 0.5mg of trypsin/mg of protein or in controls trypsin+soya-bean trypsin inhibitor (1:2, w/w). Hexokinase and adenylate kinase activities are expressed as percentages of their activities in preparations of iso-osmotic mitochondria incubated with trypsin plus trypsin inhibitor. Each value represents the mean $\pm$ 2 s.e.m. of four animals.

		Hexokinase activity	Adenylate kinase activity
Iso-osmotic conditions	No tryptic activity	100	100
	Tryptic activity	0	97 $\pm$ 4.0
Hypo-osmotic conditions	No tryptic activity	100 $\pm$ 2.0	103 $\pm$ 8.0
	Tryptic activity	0	20 $\pm$ 1.0

Fig. 1. Effect of heating on small-intestinal hexokinase activity. (a) Particle-free supernatant preparations from intestinal mucosa of guinea pigs; (b) particle-free supernatants prepared from mitochondrial preparations after dilution (1:1, v/v) with 1.8 M-NaCl; (c) particle-free supernatant preparations from intestinal mucosa of rats. For experimental details see the text. The values shown are the means ± 2 S.E.M. for three animals.

were carried out with 5 mg of trypsin/mg of protein.

The susceptibility of hexokinase and adenylate kinase activities in mitochondrial preparations to tryptic digestion is shown in Table 4. In iso-osmotic conditions adenylate kinase activity was essentially unchanged after tryptic digestion. However, the exposed hexokinase activity fell from 54 to 30% of the 'maximum assayable activity' in controls. The 'maximum assayable activity' after tryptic digestion was 60% of that in controls.

In hypo-osmotic conditions the adenylate kinase activity was almost completely lost after tryptic digestion, although in controls the adenylate kinase activity was not different from that in iso-osmotic controls. After tryptic digestion the exposed hexokinase activity was 18% of the 'maximum

assayable activity' of iso-osmotic controls, whereas the 'maximum assayable activity' was approx. 32% of its value in iso-osmotic controls.

After lysis by method (2a) the exposed hexokinase activity was not different from its value in iso-osmotic controls but after lysis by method (2b) the exposed hexokinase activity had increased 1.2-fold. The 'maximum assayable activity' in preparations of mitochondria lysed by method (2a) was 76% of that in iso-osmotic controls, whereas it was 89% of that in iso-osmotic controls in preparations lysed by method (2b).

DISCUSSION

The distribution of the marker-enzyme activities among the various subfractions allowed the following interpretation. The combined lysis supernatants represent material derived from between the outer and inner mitochondrial membranes and material which may have leaked from or been solubilized from membrane vesicles. Subfraction O_1 contained mainly outer mitochondrial membranes together with some inner membranes and material which may have leaked or been solubilized from inner-membrane vesicles, subfractions O_2 and O_3 contained inner-membrane vesicles and unbroken mitochondria. Approx. 30% of the mitochondrial hexokinase activity is found in the combined lysis supernatants. It is difficult to infer the source of hexokinase found in this fraction since it represents material from various submitochondrial compartments.

If lysed mitochondrial preparations were sedimented through a cushion of 0.3M-sucrose before application to discontinuous sucrose density gradients, almost all of the hexokinase activity in the mitochondrial sample (90%) was subsequently found to be associated with outer-membrane vesicles prepared from subfraction O_1 . By contrast, isocitrate dehydrogenase and adenylate kinase activities were predominantly in the supernatant fraction prepared from subfraction O_1 . This showed that hexokinase activity in the mitochondrial sample applied to the discontinuous sucrose density gradient was bound to the outer-membrane vesicles whereas isocitrate dehydrogenase and adenylate kinase activities are in solution. Hexokinase may be solubilized from the outer-membrane vesicles during the water-lysis procedure.

Previously the submitochondrial location of hexokinase activity has been studied predominantly by using preparations of brain mitochondria. There are conflicting reports indicating either an association of the greater part of the hexokinase activity with the outer mitochondrial membrane (Craven *et al.* 1969) or with the inner mitochondrial membrane

Table 3. *Effect of various treatments on the activity of hexokinase in mitochondrial fractions prepared from guinea-pig intestinal mucosa and rat brain*

Mitochondrial fractions from both tissues were diluted (1:1, v/v) with either isolation medium or the solutions indicated in the table. Incubations were for 3 min at 20°C with the exception of treatments 7 and 8, which were for 20 min at 20°C. Values given are related to the activities in preparations treated only by dilution with isolation medium. Values are the mean $\pm$ 2 s.e.m. of results obtained from three animals. Descriptions of treatments and concentrations of solutions are given in the Materials and Methods section.

Treatment no.	Mitochondrial fractions diluted with the following solutions	Mitochondrial hexokinase from intestinal mucosa				Mitochondrial hexokinase from brain			
		(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
		Total activities in mitochondrial suspensions (% of 1a)	Activity solubilized (% of total activity)	Activity resuspended pellets (% of total activity)	Recoveries $\left(\frac{(b+c) \times 100}{(a)}\right)$	Total activities in mitochondrial suspensions (% of 1a)	Activity solubilized (% of total activity)	Activity in resuspended pellets (% of total activity)	Recoveries $\left(\frac{(b+c) \times 100}{(a)}\right)$
1	Isolation medium	100	10 $\pm$ 1.8	83 $\pm$ 3.4	93 $\pm$ 3.6	100	8 $\pm$ 3.6	89 $\pm$ 5.2	98 $\pm$ 2.4
2	NaCl	80 $\pm$ 6.4	80 $\pm$ 6.0	22 $\pm$ 4.4	104 $\pm$ 4.6	124 $\pm$ 2.0	76 $\pm$ 3.6	30 $\pm$ 2.0	105 $\pm$ 2.0
3	KCl	84 $\pm$ 8.0	65 $\pm$ 5.0	38 $\pm$ 10.0	100 $\pm$ 6.4	128 $\pm$ 4.8	74 $\pm$ 3.8	28 $\pm$ 2.4	102 $\pm$ 2.4
4	Triton X-100	78 $\pm$ 11.0	28 $\pm$ 6.0	58 $\pm$ 3.0	86 $\pm$ 3.0	174 $\pm$ 3.6	25 $\pm$ 4.4	72 $\pm$ 6.2	97 $\pm$ 8.6
5	Triton X-100-NaCl	54 $\pm$ 4.2	100	0	100	180 $\pm$ 14.0	100 $\pm$ 4.6	0	104 $\pm$ 3.0
6	Triton X-100-KCl	54 $\pm$ 4.2	100	0	100	186 $\pm$ 9.2	96 $\pm$ 4.0	0	98 $\pm$ 5.0
7	Glucose	75 $\pm$ 4.2	70 $\pm$ 4.0	30 $\pm$ 10.4	100 $\pm$ 13.6	120 $\pm$ 11.6	67 $\pm$ 4.2	28 $\pm$ 4.6	96 $\pm$ 4.2
8	ATP	85 $\pm$ 4.8	33 $\pm$ 5.0	72 $\pm$ 6.0	105 $\pm$ 10.2	116 $\pm$ 4.0	60 $\pm$ 11.4	40 $\pm$ 13.4	100 $\pm$ 12.4
9	Isolation medium followed by sonication	84 $\pm$ 5.4	17 $\pm$ 8.0	72 $\pm$ 9.6	88	144 $\pm$ 11.2	20 $\pm$ 6.0	68 $\pm$ 4.4	88 $\pm$ 6.4
10	Isolation medium followed by freezing-thawing	85 $\pm$ 8.4	17 $\pm$ 4.0	71 $\pm$ 11.6	89 $\pm$ 4.8	129 $\pm$ 2.6	21 $\pm$ 6.0	70 $\pm$ 4.2	91 $\pm$ 2.0
11	Bovine serum albumin	85 $\pm$ 8.0	11 $\pm$ 3.2	81 $\pm$ 3.2	92 $\pm$ 1.2	103 $\pm$ 4.8	7 $\pm$ 2.4	91 $\pm$ 16.0	99 $\pm$ 14.0
12	Water	85 $\pm$ 8.0	13 $\pm$ 6.0	80 $\pm$ 4.6	93 $\pm$ 5.2	129 $\pm$ 8.6	7 $\pm$ 3.0	91 $\pm$ 1.2	99 $\pm$ 2.0
13	Water followed by Triton X-100	—	—	—	—	180	—	—	—

Table 4. *Susceptibility of mitochondrial hexokinase and adenylate activities from rat brain to tryptic digestion in iso-osmotic and hypo-osmotic conditions*

Mitochondrial preparations were lysed by the prolonged dialysis procedure (method 2a) or by diluting the mitochondrial preparation (method 2b). The lysed mitochondrial preparations were incubated with 5 mg of trypsin/mg of protein or in controls with trypsin plus soya-bean trypsin inhibitor (1:2, w/w). The enzyme activities of the preparations were determined. Each preparation was then incubated for 3 min at 20°C with an equal volume of a solution of Triton X-100 (final concentration 0.5%, w/v). Hexokinase activity was again determined. Hexokinase activities are expressed as percentages of the 'maximum assayable activity', i.e. hexokinase activity in preparations of iso-osmotic mitochondria incubated with trypsin plus trypsin inhibitor followed by treatment with a solution of Triton X-100. Results are corrected for dilution by Triton X-100. Adenylate kinase activities are expressed as percentages of the activity in preparations of iso-osmotic mitochondria incubated with trypsin plus trypsin inhibitor. Where indicated each value is the mean $\pm$ 2 S.E.M. of three animals.

		Hexokinase activity		Adenylate kinase activity
		Before Triton X-100 treatment	After Triton X-100 treatment	
Iso-osmotic conditions	No tryptic activity	54 $\pm$ 5.0	100	100
	Tryptic activity	30 $\pm$ 3.0	60 $\pm$ 10.4	95 $\pm$ 10.0
Hypo-osmotic conditions (method 2a)	No tryptic activity	60 $\pm$ 3.6	76 $\pm$ 4.2	97 $\pm$ 12.0
	Tryptic activity	18 $\pm$ 3.0	32 $\pm$ 5.8	0
Hypo-osmotic conditions (method 2b)	No tryptic activity	67	89	100
	Tryptic activity	18	30	10

(Vallejo *et al.* 1970). In mitochondrial preparations from liver the binding sites for hexokinase appear to be mostly associated with the outer membrane (Kropp & Wilson, 1970). As in the experiments reported here, a considerable amount of hexokinase activity was found associated with the soluble subfraction from mitochondrial preparations, thus making it difficult to allot the submitochondrial location of hexokinase precisely.

The study of the submitochondrial location of enzymes can be approached in a different way. This approach involves the use of trypsin to inactivate enzymes in iso-osmotic and hypo-osmotic conditions, and therefore distinguish between enzymes located on the outside of the outer mitochondrial membrane and inside the outer membrane. This technique has been used to substantiate existing evidence on the location of mitochondrial rotenone-insensitive NADH-cytochrome *c* reductase activity (EC 1.6.99.-) and adenylate kinase activities in relation to the outer mitochondrial membrane (Kuylenstierna *et al.* 1970).

Table 2 shows that all the mitochondrial hexokinase activity was lost after tryptic digestion in iso-osmotic conditions. Since adenylate kinase activity remained unchanged it was inferred that the outer mitochondrial membrane was not punctured and therefore that hexokinase activity lies on the outside of the outer mitochondrial membrane, thus substantiating evidence from the water lysis experiments that hexokinase was

associated with the outer mitochondrial membrane in mitochondrial preparations from small-intestinal mucosa of the guinea pig.

In spite of the large amount of trypsin (1:2, w/w, with protein) in each incubation its high specificity resulted in no lesion of the outer mitochondrial membrane in iso-osmotic conditions, at least when measured by leakage of adenylate kinase activity. In hypo-osmotic conditions only 80% of the adenylate kinase activity was lost on tryptic digestion. This was somewhat surprising in view of the high sensitivity of adenylate kinase activity to tryptic digestion. It may be that not all the mitochondria were lysed, or alternatively that some adenylate kinase activity in mitochondrial preparations from the intestinal mucosa lies within the inner membrane.

Particulate hexokinase activity has been studied in detail in various tissues. It has been suggested that type I hexokinase is responsible for the latency properties observed with particle-bound hexokinase (Katzen *et al.* 1970). This hypothesis is supported by the observation that in adipose tissue there is little type I hexokinase activity (Borrebaek & Spydevold, 1967) and no latency properties can be demonstrated with mitochondrial hexokinase from epididymal fat-pads (Borrebaek, 1970).

Type II hexokinase may undergo a redistribution between particulate and soluble parts of the cell as an acute manifestation of the lack of insulin in lactating mammary gland (Walters & McLean, 1968) and as a chronic manifestation of lack of

insulin in muscle (Katzen *et al.* 1970). Type III hexokinase may only be present in the soluble part of the cell (Katzen *et al.* 1970).

In view of the supposed differences in properties of the types of hexokinase it was decided to determine the relative amounts of these two forms of hexokinase which are bound to mitochondrial preparations obtained from the small intestine of the guinea pig. Only type I and type II hexokinase activities are present in a soluble fraction prepared from the intestinal mucosa of the guinea pig (Srivastava *et al.* 1968). Fig. 1 shows that the ratio of type I/type II hexokinase activity in material solubilized from mitochondrial preparations is 7:3, which is the same as that in the particle-free supernatant prepared from intestinal mucosa. This suggests that no distinction is made between the different types of hexokinase in terms of affinity for the binding sites on the outer mitochondrial membrane.

The behaviour of hexokinase activity associated with mitochondrial preparations from the mucosa of the small intestine has been compared with the behaviour of mitochondrial hexokinase activity from brain in conditions known to expose and solubilize the mitochondrial hexokinase of brain (Table 3). The experimental conditions were maintained as far as possible as indicated by other authors and, in agreement with these authors (Biesold & Teichgräber, 1967; Wilson, 1968; Craven *et al.* 1969; Thompson & Bachelard, 1970; Katzen *et al.* 1970), mitochondrial hexokinase from brain exhibits latency and solubilization properties in response to various chemicals or treatments.

Although 70% of mitochondrial hexokinase activity is type I it does not show latency properties; in fact some hexokinase activity is lost after treatment with various agents. However, similar amounts of hexokinase activity were solubilized from mitochondrial preparations obtained from intestinal mucosa and brain when exposed to the same treatments. This suggests that the binding affinity of hexokinase for sites on the mitochondrial surface may be similar in both preparations.

The submitochondrial location of hexokinase activity in mitochondrial preparations from brain was determined by means of the trypsin-sensitivity technique. Treatment with a solution of Triton X-100 was used to distinguish exposed and latent hexokinase and therefore the trypsin-sensitivities of the two forms could be determined. Adenylate kinase activity was again used as a marker for the intermembrane space and the results indicated that only in hypo-osmotic conditions was adenylate kinase activity lost on trypsin treatment.

The trypsin-sensitivity of hexokinase activity in preparations of brain mitochondria was one-tenth

of that in preparations of mitochondria from the small intestine. This difference in sensitivity could be due to different amounts of 'inert' protein, i.e. protein which is not a substrate for trypsin, in the two preparations (Kuylenstierna *et al.* 1970) or to a greater substrate protection against trypsin activity (Grossbard & Schimke, 1966) afforded to the mitochondrial hexokinase from brain.

Mitochondrial preparations employed in the trypsin-sensitivity experiments were lysed in two different ways, the prolonged dialysis procedure (method 2a) or dilution with water (method 2b). After the latter treatment it was possible to observe an activation of mitochondrial hexokinase activity and a subsequent further activation by Triton X-100 (see Table 3, treatments 12 and 13). After the former treatment less activation of hexokinase activity occurred. The prolonged dialysis procedure may result in the loss of some latent hexokinase activity associated with either the inter-membrane space or the outer mitochondrial membrane.

The amount of exposed and latent hexokinase activity associated with the inner mitochondrial membrane is the same irrespective of the method of mitochondrial lysis (Table 4). This indicates that the hexokinase activity associated with the inner membrane is membrane bound or that it cannot leak from the inner membrane, even after prolonged exposure in a hypo-osmotic environment. The integrity of the inner mitochondrial membrane in the conditions employed is nevertheless very important in the use of the trypsin-susceptibility technique.

The work of Kuylenstierna *et al.* (1970) indicated that inner-membrane functions such as respiration or respiratory control, with succinate or β -hydroxybutyrate as substrates, were resistant to tryptic digestion for 10 min at 25°C in iso-osmotic and hypo-osmotic media containing 0.1 mM-EDTA. Further, incubation of mitochondrial preparations at 30°C for periods up to 30 min in a hypo-osmotic medium containing 1 mM-EDTA caused very little release of nicotinamide nucleotides, glutamate dehydrogenase (EC 1.4.1.2) or malate dehydrogenase (EC 1.1.1.37) from the inner membrane (Pfaff & Schwalbach, 1967).

The results presented in Table 1 show that approx. 50% of mitochondrial isocitrate dehydrogenase activity is found in the combined lysis supernatants after repeated lysis in water containing 2 mM-EDTA. The prolonged dialysis (method 2a) used in the trypsin-sensitivity experiments to lyse preparations of mitochondria from guinea-pig mucosa or rat brain resulted in a 20 or 30% leakage respectively of isocitrate dehydrogenase activity from the inner mitochondrial membrane. The short lysis procedure

(method 2b) used in trypsin-susceptibility experiments with preparations of brain mitochondria showed negligible loss of isocitrate dehydrogenase activity from the inner membrane.

The hexokinase activity which is lost after tryptic digestion in iso-osmotic conditions is considered to lie on the outside of the outer mitochondrial membrane, that which is lost in hypo-osmotic conditions is on the outside of the outer mitochondrial membrane and between the mitochondrial membranes, and that remaining after tryptic digestion in hypo-osmotic conditions is within the inner membrane. Table 4 shows that the hexokinase activity associated with untreated mitochondrial preparations from rat brain can be increased approximately twofold with Triton X-100. Similarly the hexokinase activities within the outer membrane and within the inner membrane could also be increased approximately twofold. Thus, irrespective of the submitochondrial location, hexokinase activity could be increased with Triton X-100. Further, after tryptic digestion the same proportion of exposed and 'maximum assayable hexokinase' activity was lost in iso-osmotic conditions and the same proportion of each form of activity was lost in hypo-osmotic conditions. Therefore it is not possible to make submitochondrial location a reason for the latency phenomenon. The latency phenomenon is not restricted to any submitochondrial compartment and is presumably the property of a single form of hexokinase which is inactivated by trypsin.

From the information contained in Table 4 the submitochondrial distribution of exposed and latent hexokinase activity given in Table 5 was deduced. Four pools of hexokinase activity have been suggested in mitochondrial preparations from brain (Wilson, 1968). The exposed pool of hexokinase activity constituted 43% of the 'maximum assayable hexokinase' activity, which compares favourably with the 40% of the exposed (and total) hexokinase activity on the outside of the outer mitochondrial membrane found in the experiments reported above. Other authors have indicated that approx. 50% of the exposed hexokinase activity is on the outer mitochondrial membrane (Craven *et al.* 1969).

The use of the trypsin-sensitivity method has made it possible to deduce the location of exposed and latent hexokinase activity without the complications of conventional fractionation procedures. Hexokinase activity was measured on the outside of the outer mitochondrial membrane in iso-osmotic conditions without the disruption of the membrane, and possible redistribution of hexokinase activity. Redistribution can occur in hypo-osmotic conditions but the prolonged dialysis procedure (method 2a) and the alternative method of lysis of brain mitochondrial preparations (method 2b) did not affect the activity of hexokinase within the inner membrane (Table 4). Therefore the effect of redistribution of hexokinase activity on the final interpretation must be minimal. The fundamental difference between mitochondrial hexokinase from small intestine and brain is that the former was only found on the outside of the outer mitochondrial membrane. It does not have latency properties in spite of showing similar solubilization behaviour to the mitochondrial hexokinase from brain.

From the results presented in this paper and the findings of other authors (Craven *et al.* 1969; Vallejo *et al.* 1970) it would appear that mitochondrial hexokinase from brain can be bound to any side of both mitochondrial membranes, although that bound to the inner mitochondrial membrane is external to the atractyloside barrier, and may be active only on extramitochondrial substrates (Vallejo *et al.* 1970).

The binding of hexokinase to mitochondrial membranes may either bring about some conformational change to the enzyme or may result in the occlusion of the enzyme in the membrane structure, which results in its partial inactivation. It may be reactivated in the presence of various metabolites and membrane-disruptive agents and is solubilized by metabolites and salts (Biesold & Teichgräber, 1967; Wilson, 1968; Katzen *et al.* 1970).

The soluble-particulate distribution of hexokinase in the brain has been implicated as a possible physiological control mechanism for glycolysis which occurs in the cytosol of the cell (Thompson & Bachelard, 1970). However, further control could be superimposed on the system by the partial inactivation of hexokinase caused by binding to

Table. 5. *Submitochondrial location of exposed and latent hexokinase activity*

The results are expressed as percentages of the 'maximum assayable activity'.

Submitochondrial location	Exposed hexokinase activity	Latent hexokinase activity	Total hexokinase activity
Outer mitochondrial membrane	24	16	40
Inter-membrane space	12	16	28
Inner mitochondrial membrane	18	14	32

mitochondrial membranes. Such a mechanism could provide two advantages: first, hexokinase could be stored to meet any physiological contingency that may arise, and secondly, such stored enzyme would not be exposed to the normal degradation processes within the cytosol of the cell.

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REFERENCES

- Biesold, D. & Teichgräber, P. (1967). *Biochem. J.* **103**, 13c.
- Borrebaek, B. (1970). *Biochem. Med.* **3**, 485.
- Borrebaek, B. & Spydevold, Ø. (1967). *Excerpta med. int. Cong. Ser. no. 140: Abstr. 6th int Cong. International Diabetes Federation*, p. 138.
- Craven, P. A., Goldblatt, P. J. & Basford, R. E. (1969). *Biochemistry, Easton*, **8**, 3525.
- Grossbard, L. & Schimke, R. T. (1966). *J. biol. Chem.* **241**, 3546.
- Hübscher, G., West, G. R. & Brindley, D. N. (1965). *Biochem. J.* **97**, 629.
- Katzen, H. M. & Schimke, R. T. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1218.
- Katzen, H. M., Soderman, D. D. & Wiley, C. E. (1970). *J. biol. Chem.* **245**, 4081.
- Kropp, E. S. & Wilson, J. E. (1970). *Biochem. biophys. Res. Commun.* **38**, 74.
- Kuylenstierna, B., Nicholls, D. G., Hovmöller, S. & Ernster, L. (1970). *Eur. J. Biochem.* **12**, 419.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Mayer, R. J., Shakespeare, P. & Hübscher, G. (1970). *Biochem. J.* **116**, 43.
- Pfaff, E. & Schwalbach, K. (1967). In *Mitochondrial Structure and Compartmentation*, p. 346. Ed. by Quagliariello, E., Papa, S., Slater, E. C. & Tager, J. M. Bari: Adriatica Editrice.
- Roy, A. B. (1953). *Biochem. J.* **53**, 12.
- Shephard, E. H. & Hübscher, G. (1969). *Biochem. J.* **113**, 429.
- Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967). In *Methods in Enzymology*, vol. 10. p. 448. Ed. by Eastabrook, R. W. & Pullman, M.E. New York: Academic Press.
- Srivastava, L. M., Shakespeare, P. & Hübscher, G. (1968). *Biochem. J.* **109**, 35.
- Thompson, M. F. & Bachelard, H. S. (1970). *Biochem. J.* **118**, 25.
- Vallejo, C. G., Marco, R. & Sebastian, J. (1970). *Eur. J. Biochem.* **14**, 478.
- Walters, E. & McLean, P. (1968). *Biochem. J.* **109**, 737.
- Wilson, J. E. (1968). *J. biol. Chem.* **243**, 3640.