Cerebral-Cortex Hexokinase

COMPARISON OF PROPERTIES OF SOLUBILIZED MITOCHONDRIAL AND CYTOPLASMIC ACTIVITIES

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1. Cerebral-cortex mitochondria, after purification by using high-density sucrose solutions, were extracted with Triton X-100. The total hexokinase activity of the intact mitochondria was increased by 50-80% in the Triton extracts. 2. Triton X-100 was removed from mitochondrial extracts by a combination of ammonium sulphate fractionation and DEAE-cellulose chromatography. Mitochondrial hexokinase remained soluble after removal of extractant. 3. The behaviour of solubilized mitochondrial hexokinase was compared with soluble cytoplasmic hexokinase from the same samples of cerebral cortex on identical columns of DEAE-cellulose. Two peaks were eluted from each source of hexokinase. The distribution between hexokinase peaks was similar for the two sources. Peak I (approx. 80% of the total hexokinase) from each was eluted at identical concentrations of potassium chloride and slight differences were observed in the elution profiles for peak II. 4. The purified mitochondrial hexokinase showed the following kinetic properties: peak I, $K_m(\text{ATP})$ 0.60 mM, $K_m(\text{glucose})$ 0.042 mM; peak II, $K_m(\text{ATP})$ 0.66 mM, $K_m(\text{glucose})$ 0.043 mM. The purified cytoplasmic hexokinase Michaelis constants were: peak I, $K_m(\text{ATP})$ 0.56 mM, $K_m(\text{glucose})$ 0.048 mM; peak II, $K_m(\text{ATP})$ 0.68 mM, $K_m(\text{glucose})$ 0.062 mM. 5. Although no significant differences between mitochondrial and cytoplasmic hexokinases were noted in chromatographic behaviour or in the kinetic properties studied, the purified mitochondrial enzyme was activated slightly (approx. 20%) by Triton X-100, in contrast with the cytoplasmic enzyme, which was not affected. 6. The results, taken to indicate basic similarity between mitochondrial and cytoplasmic hexokinases, are discussed in relation to the role of the two sources of enzyme in the metabolism of the tissue.

Most of the glycolytic enzymes in the brain are found mainly if not exclusively in the cytoplasm; an exception is hexokinase, which occurs partly cytoplasmic and partly bound to mitochondria (Bachelard, 1967; Newsholme, Rolleston & Taylor, 1968). It has been argued that sufficient cytoplasmic enzyme is available to account for the normal rates of glycolysis known to be required, and that adequate control of hexokinase in relation to regulatory mechanisms of glycolysis can most satisfactorily be explained on that basis (Bachelard & Goldfarb, 1969). If such is the case, the function of the particulate activity remains an open question. Cerebral glycolytic rates, normally operating at about 20 μmol of glucose consumed/h per g of tissue, can be stimulated, for very brief periods, to rates greatly in excess of this and may approach ten times the normal unstimulated rate (McIlwain,

1966; King, Lowry, Passonneau & Venson, 1967). Under these conditions, even if the product inhibitions and substrate relations that may normally be effective in controlling hexokinase at the glycolytic rate were completely overcome, the potential maximal rate (120-150 $\mu\text{mol}/\text{h}$ per g, 20-25% of the total) of the cytoplasmic hexokinase is likely to be insufficient; the activity of the non-cytoplasmic enzyme (70% or more of the total) may well be relevant.

Before this can be assessed, information is required on the relationship between and possible identity of the particulate and cytoplasmic systems, for which purpose a truly solubilized particulate enzyme is desirable. The particulate activity, which has been shown to be relatively tightly bound and essentially mitochondrial by density-gradient analysis, has been extracted from crude primary

mitochondrial preparations by methods involving osmotic shock or detergents (Bachelard, 1967; Rose & Warms, 1967; Schwartz & Basford, 1967; Wilson, 1967; Teichgräber & Biesold, 1968).

Preliminary studies in our laboratory showed the 'osmotic shock' methods to be unsatisfactory in releasing the activity from purified mitochondria prepared by sucrose-density-gradient centrifugation; only 20–25% of the original activity was rendered non-particulate. On the other hand, use of Triton X-100 as described by Teichgräber & Biesold (1968) proved perfectly satisfactory on both sources of mitochondria, crude or purified. One problem, rarely tackled, is the difficulty of removing detergent from detergent-solubilized proteins. Certain attempts that have been made have illustrated the need for caution. Partial removal of anionic detergents from a variety of solubilized proteins or microsomes may result in precipitation (Martonosi, 1968; Pitt-Rivers & Impiombato, 1968). Pig brain mitochondrial L-3-glycerophosphate dehydrogenase, extracted with Triton X-100, was also reported to become insoluble on removal of the detergent (Dawson & Thorne, 1969). It is likely that solubility of a protein in a detergent involves micelle formation; the conformation of the protein in the micelle could be quite different from that of the native protein with consequent differences in kinetic properties.

During the course of this work, it was reported that the K_m (ATP) of bovine particulate hexokinase was increased tenfold from 0.4 to 4 mM as a result of solubilization with Triton X-100 and chromatography on DEAE-cellulose in the presence of the detergent (Schwartz & Basford, 1967; Copley & Fromm, 1967). It is not clear whether the kinetic behaviour was altered by changes induced by the solubilization or was due directly to the presence of detergent in the solubilized preparation.

The present paper reports the removal of Triton X-100 from extracts of bovine cerebral-cortex hexokinase prepared from purified mitochondria. A careful comparison has been made of the chromatographic behaviour and kinetic properties of the detergent-free solubilized mitochondrial activity with the cytoplasmic activity prepared from the same samples of cerebral cortex. A preliminary account of the extraction of the mitochondrial activity and of attempts to remove the detergent has been given (Thompson & Bachelard, 1969).

MATERIALS AND METHODS

Preparation of subcellular fractions. Unless stated otherwise the Spinco model L2-65 ultracentrifuge was employed; centrifugal forces are expressed as g_{av} values calculated to the centres of the tubes. Ox brain was placed in a chilled plastic bag immediately after its removal

from the animal at a slaughterhouse and transported to the laboratory on ice. The dura and adhering blood vessels were removed, and the cerebral cortex was scraped off and homogenized in 5 vol. of 0.32 M-sucrose in a Teflon-pestle homogenizer (type C, clearance 0.15–0.23 mm; A. H. Thomas Co., Philadelphia, Pa., U.S.A.) Dispersion involved ten passages of the pestle rotating at 1500 rev./min during 30–40 s with intermittent periods of cooling in ice. The homogenate was centrifuged as previously described (Bachelard, 1967) and the primary mitochondrial and cytoplasmic fractions were retained.

Purification of the primary mitochondrial fraction. Method 1. The primary mitochondrial fraction, prepared from 150 g of ox cerebral cortex, was washed twice by resuspension of the pellet in 0.32 M-sucrose (5 ml/g of original tissue) and centrifugation at 15000 g for 15 min. The resuspended pellets were purified by sucrose-density-gradient centrifugation in the B4 zonal rotor of the Spinco model L4 ultracentrifuge (Rodnight, Weller & Goldfarb, 1969). Two preparations were made; the mean recovery of protein was 53.5% and of hexokinase 56.0% of that originally present in the primary mitochondrial fractions.

Method 2. The primary mitochondrial fraction, originally 0.32 M with respect to sucrose, was adjusted by addition of sucrose to a final concentration of 1.2 M and purified by the method of Løvtrup & Svennerholm (1963). The white upper portion of the pellet was removed by gentle aspiration. The residual tan-coloured pellet was resuspended in 0.32 M-sucrose to a final concentration of 34 mg of protein/ml. It contained 79.5% of the hexokinase originally present in the primary mitochondrial fraction and 50.5% of the protein.

Triton extraction of mitochondrial hexokinase. The mitochondria (primary or purified) were centrifuged at 78000 g for 20 min and resuspended by brief homogenization in 'Triton medium'. This contained 0.5% (w/v) Triton X-100 in M-KCl and 10 mM-potassium phosphate buffer, pH 6.5 (Teichgräber & Biesold, 1968). In certain of the preliminary studies, indicated in the text, 0.32 M-sucrose replaced the phosphate buffer. The mitochondrial suspensions (usually 1–4 mg of protein/ml) in Triton medium were kept at 0°C for 60 min and centrifuged at 78000 g for 20 min. The clear straw-coloured supernatants were retained as the extracts; the pellets were resuspended in the original volume of 0.32 M-sucrose.

Ammonium sulphate fractionation. Saturated $(NH_4)_2SO_4$ solutions at 0°C, adjusted to pH 6.5 with a few drops of NH_3 solution (approx. 5 M), were added to a final saturation of 50%. For concentrations above 50% saturation, solid $(NH_4)_2SO_4$ was added at 0°C according to the calculation of Nicholas & Bachelard (1969). The pH was maintained at 6.5 during all additions. To prevent loss of activity during fractionation of hexokinase in the mitochondrial extracts, 100 mM-glucose, 5 mM-EDTA and 10 mM-potassium phosphate buffer, pH 6.5 (final concentrations) were added to the extracts before the addition of $(NH_4)_2SO_4$. In order to achieve strictly comparable conditions similar additions were made to cytoplasmic preparations. With fractionation of mitochondrial Triton extracts a calculated 90% saturation seemed to give full saturation. The reason is uncertain, but possibly the presence of Triton or KCl in the Triton medium affected the solubility of $(NH_4)_2SO_4$.

DEAE-cellulose chromatography. DEAE-cellulose (Whatman DE 52, microgranular, pre-swollen; W. and R. Balston Ltd., Maidstone, Kent, U.K.) was suspended (200 g/l) in column buffer (10 mM-potassium phosphate buffer, pH 7.4, 5 mM-EDTA and 10 mM-glucose; Grossbard & Schimke, 1966) and prepared and packed as recommended in Whatman Technical Bulletin no. I E2, with a value for n of 1.85. Final equilibration was performed at a flow rate of 45 ml/h per cm² until the pH and conductivity of the eluate and buffer were identical. Column dimensions were 30 cm × 1.2 cm diam. for pilot runs and 30 cm × 1.8 cm diam. for larger-scale preparations. All operations were performed at 1–2°C.

The hexokinase fractions from (NH₄)₂SO₄ treatment were dialysed against the column buffer. The loaded columns were then washed with the column buffer until the eluates were free of unexchanged inactive protein (usually 2–5 column volumes). Elution of enzyme was effected by the inclusion of KCl in the same buffer as linear or concave gradients where indicated. Concave gradients were produced with a Technicon Autograd gradient mixer. Fractions (2 or 5 ml) were collected with an LKB UltroRac fraction collector and protein contents (E_{280}) were recorded with an LKB Uvicord 11 photometer and LKB 6520H recorder. Hexokinase was measured in all fractions of each eluted protein peak and in every fourth tube of unexchanged protein peaks. Protein determinations were made (Lowry, Rosebrough, Farr & Randall, 1951) on all tubes of each fraction detected by the Uvicord instrument. Selected fractions from the mitochondrial columns were assayed also for Triton X-100 content.

Determination of Triton. Method (i) of Stevenson (1954) was used. It was found that high concentrations of (NH₄)₂SO₄, KCl, sucrose and phosphate all interfered with the measurement. This was appreciable only in fractions where the Triton concentrations were low and the sample volumes had therefore to be large to allow accurate measurement. (NH₄)₂SO₄ fractions were dialysed against the column buffer before Triton assays were performed. Determination of Triton in column eluates was carried out by using standards containing equivalent amounts of column buffer. The modified procedure was as follows: to samples (2 ml) in tapered centrifuge tubes were added 2 M-HCl (75 μ l), 10% (w/v) BaCl₂ (50 μ l) and 10% (w/v) phosphomolybdic acid (50 μ l). The contents were thoroughly mixed and centrifuged at 3000 rev./min for 10 min. The supernatants were removed by decanting and the tubes drained for 1–2 min by inversion over filter paper. The precipitates were dissolved in conc. H₂SO₄ (1 ml) and the E_{520} was measured exactly 40 min later.

Determination of protein. This was carried out by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin as standard. When sucrose or column buffer was present in samples analysed, the standards were prepared to contain equivalent concentrations.

Hexokinase activity measurements. These were done in duplicate at 37°C in semi-micro cuvettes in a Unicam SP.800 recording spectrophotometer as described previously (Bachelard & Goldfarb, 1969).

Reagents. Triethanolamine hydrochloride, glucose 6-phosphate dehydrogenase (EC 1.1.1.49, from yeast), ATP and NADP⁺ (sodium salts) were purchased from Boehringer Corp. (London) Ltd., London W.5., U.K.

Triton X-100 was obtained from Lennig Chemicals Ltd., London W.C.1., U.K. All other chemicals were of the highest purity available from British Drug Houses Ltd., Poole, Dorset, U.K. Measurements of pH were made with a pH-meter (Radiometer, Copenhagen, Denmark) with a glass electrode (type G 202C).

RESULTS

Extraction of mitochondrial hexokinase. The presence of non-mitochondrial elements (nerve-ending particles and myelin fragments) in crude mitochondrial fractions renders doubtful the validity of using the primary fractions as sources of a mitochondrial enzyme for purposes of comparison with enzymes having similar catalytic properties in the cytoplasm, since the nerve-ending particles contain entrapped cytoplasm. So it was considered preferable to use mitochondria purified relatively free from the non-mitochondrial contaminants by high-density-sucrose centrifugation. Preliminary experiments showed the yield of hexokinase from Triton extraction of the purified mitochondria to be slightly higher than from primary mitochondrial fractions. Treatment of mitochondria with Triton in sucrose at pH 6.5 gave a higher yield of extractable hexokinase than in phosphate buffer at the same pH (Table 1). Nevertheless the yields from subsequent steps involving attempts to remove the detergent from the extracts indicated that the use of phosphate buffer was preferable. In confirmation of previous reports (Teichgräber & Biesold, 1968; Thompson & Bachelard, 1969), an elevation of hexokinase activity resulted from exposure to the detergent. Both Triton X-100 and M-potassium chloride were required for maximum activity in the extracts.

No appreciable difference was observed in the yields of hexokinase resulting from extraction of the mitochondria purified by the two methods (1) and (2) involving centrifugation of primary fractions. In view of the higher preparative yield of hexokinase and the convenience technically, method (2) of Løvtrup & Svennerholm (1963) was preferred.

Ammonium sulphate fractionation. The use of ammonium sulphate resulted in removal of most of the Triton together with some of the inactive protein from the extracts. Preliminary studies had shown that the bulk of the Triton separated as a floating pellicle after high-speed centrifugation of extracts treated with ammonium sulphate to 25% saturation. Only low hexokinase activity was observed in this fraction (Table 2). The major portion of the total hexokinase recovered from ammonium sulphate fractionation was precipitated between 25% and 90% saturation. Fractions precipitated between 75% and 90% saturation

Table 1. *Extraction of hexokinase from purified mitochondria with Triton X-100 at pH 6.5*

Mitochondria, purified by methods (1) and (2), were extracted with Triton X-100 (0.5%, w/v) at pH 6.5 in the presence of (a) 0.32 M-sucrose (four preparations) or (b) 10 mM-phosphate buffer (five preparations) as described in the Materials and Methods section. Results are expressed as mean $\pm$ S.D.

	(a)			(b)		
	Protein		Hexokinase activity	Protein		Hexokinase activity
	(mg/ml)	(% of original)	(μ mol/min per mg of protein)	(mg/ml)	(% of original)	(μ mol/min per mg of protein)
Intact mitochondria	1.16 $\pm$ 0.09					
Triton extract		77.7 $\pm$ 3.9	0.36 $\pm$ 0.02	4.03 $\pm$ 0.25	(100)	0.40 $\pm$ 0.02
Triton residue		19.1 $\pm$ 0.5	0.79 $\pm$ 0.07		134.6 $\pm$ 17.0	0.79 $\pm$ 0.03
Total recovered		96.8	0.05 $\pm$ 0.005		5.6 $\pm$ 0.65	0.06 $\pm$ 0.007
			175.6		140.2	

were found to contain significant amounts of enzyme, in contrast with reports on the precipitation of soluble hexokinase at 55% saturation (Grossbard & Schimke, 1966) and in the present work, when the cytoplasmic enzyme was all precipitated by 70% saturation. It seems possible that the Triton present might be affecting the solubility of the ammonium sulphate and of protein, particularly in view of the observation that the extracts appeared to be saturated with ammonium sulphate at a calculated 90% saturation. The fractionation of the mitochondrial extracts of the present study was designed primarily to separate hexokinase in maximum yield as free as possible from Triton rather than to achieve maximum separation from non-enzymic protein. In view of the major objective, i.e. to compare the chromatographic and kinetic properties of mitochondrial and cytoplasmic enzymes, it has been considered preferable to use as much as possible of the total hexokinase originally present in the mitochondria.

The advantage obtained from the use of phosphate buffer over sucrose as the medium for purification after Triton extraction is apparent from the results presented in Table 2. When the procedure was carried out in the presence of 10 mM-phosphate buffer throughout, the hexokinase activity/mg of protein was higher whereas the mg of Triton/mg of protein was less than when sucrose was used.

The total recovery of hexokinase (87–100%) was considerably below that present in the Triton extract and not more than 100% of that originally present in the intact purified mitochondria. It is difficult to distinguish between two possibilities: (1) that this represents a loss of true activity present in the Triton extract, i.e. that the elevated activity was due to exposure and solubilization of 'latent' enzyme in the intact mitochondria or (2) that the elevated activity present in the Triton extracts was due to the activation by Triton and disappeared on removal of the Triton. Inclusion of 20% (w/v) glycerol had no effect on the recoveries from ammonium sulphate fractionation, and the inclusion of 5 mM-N-acetylcysteine resulted in complete loss of activity.

To test whether Triton had a direct activating effect on the enzyme, Triton medium was added to the Triton-depleted enzyme fraction from the ammonium sulphate purification step. The 16% elevation that resulted (Table 5) was consistent but lower than the elevation observed in the original Triton extract. This may have been due to the small amounts of Triton still present in the fraction. Similar tests described below were therefore made on more highly purified preparations obtained by column chromatography.

DEAE-cellulose chromatography of mitochondrial and cytoplasmic hexokinase. Enzymes from the two

Table 2. *Ammonium sulphate fractionation of mitochondrial Triton extracts*

Triton extracts, prepared from purified mitochondria in the presence of 0.32 M-sucrose or 10 mM-phosphate buffer and 100 mM-glucose at pH 6.5 (Table 1), were fractionated with $(\text{NH}_4)_2\text{SO}_4$ in the buffer-glucose mixture as described in the Materials and Methods section. The results from five preparations are expressed as mean $\pm$ S.D.

	Protein (% of original*)	Hexokinase activity		Triton/protein ratio (mg/mg)
		(% of original*)	($\mu\text{mol}/\text{min}$ per mg of protein)	
Triton extract (prepared in sucrose, <i>a</i> in Table 1)	80.7 $\pm$ 4.3	174.2 $\pm$ 8.9	0.79 $\pm$ 0.07	4.3
$(\text{NH}_4)_2\text{SO}_4$				
0–25% satn.	53.3 $\pm$ 2.2	13.1 $\pm$ 0.2	0.10 $\pm$ 0.01	8.2 $\pm$ 0.3
25–80% satn.	17.0 $\pm$ 1.2	59.1 $\pm$ 2.5	1.28 $\pm$ 0.05	0.9 $\pm$ 0.15
80% supernatant	18.5 $\pm$ 3.1	28.0 $\pm$ 4.7	0.66 $\pm$ 0.17	1.0 $\pm$ 0.15
Total recovered*	88.8	100.2		
Triton extract (prepared in phosphate, <i>b</i> in Table 1)	65.8 $\pm$ 1.8	134.6 $\pm$ 17.0	0.79 $\pm$ 0.03	1.2
$(\text{NH}_4)_2\text{SO}_4$				
0–24% satn.	27.5 $\pm$ 2.0	14.7 $\pm$ 1.5	0.21 $\pm$ 0.03	4.3 $\pm$ 0.15
24–90% satn.	17.2 $\pm$ 1.2	63.9 $\pm$ 4.1	1.53 $\pm$ 0.18	0.5 $\pm$ 0.06
90% supernatant	5.6 $\pm$ 1.6	8.6 $\pm$ 3.4	0.34 $\pm$ 0.10	0.7 $\pm$ 0.25
Total recovered*	50.3	87.2		

* Original protein or hexokinase activity refers to that present in the intact mitochondria before extraction.

sources after ammonium sulphate fractionation were chromatographed on identical columns of DEAE-cellulose under identical conditions. Fig. 1 shows that the elution profiles obtained with a linear gradient were very similar for the enzymes from the two sources. The major peak was eluted at identical points on the potassium chloride gradient (0.04 M), but slight differences were noted in the flat diffuse minor peaks. Peak II of the cytoplasmic enzyme was clearly demarcated and was eluted at about 0.30–0.35 M-potassium chloride. The second peak from the mitochondrial column, not completely distinguishable from the 'tailing' of the first peak, was eluted at about 0.23 M-potassium chloride. In order to sharpen the second peaks, the procedure was repeated with a concave elution gradient (Fig. 2) and resulted in considerable sharpening of peak II from the cytoplasmic enzyme. With the mitochondrial enzyme, however, only a slight improvement over the result with the linear gradient was achieved. The use of the concave gradient resulted in elution at slightly higher concentrations of potassium chloride.

The major hexokinase peaks (peak I) for the two sources were eluted at identical potassium chloride concentrations (0.05 M), but differences were again noted in the potassium chloride concentrations that eluted the minor peaks. Peak II of the cytoplasmic enzyme was eluted at 0.35 M whereas that for the mitochondrial enzyme emerged at about 0.19 M.

It appears therefore that the enzymes from the two sources exhibited identical behaviour on DEAE-cellulose columns in the case of the major peak with minor differences in the concentration of potassium chloride at which the minor peaks were eluted. The results from three identical column pairs are summarized in Table 3 and demonstrate that the percentage distribution of hexokinase between the two peaks (approx. 80% for peak I and 16% for peak II) was similar for the enzyme from the two sources. The general similarity of elution profile and distribution between peaks serves to indicate that the hexokinase in the mitochondria exhibits no gross difference in chromatographic behaviour from the cytoplasmic activity. Similar specific activities ($\mu\text{mol}/\text{min}$ per mg of protein) were also obtained (Table 3).

The Triton/protein ratio in the eluted peaks of the mitochondrial extract was less than 0.02, which is the limit of sensitivity of the method used.

The reactivation by Triton of the Triton-depleted enzyme was 22% (Table 5) which is slightly higher than the reactivation observed in the ammonium sulphate fraction and below that obtained as a result of Triton extraction of the intact mitochondria (Table 1).

Kinetic properties. The apparent K_m values for ATP and for glucose of the enzymes from the two sources at various stages of the purification procedure are summarized in Table 4. The K_m (ATP),

Fig. 1. DEAE-cellulose chromatography of mitochondrial and cytoplasmic hexokinase with a linear KCl gradient. The preparations used were those after $(\text{NH}_4)_2\text{SO}_4$ purification as indicated in the text. Fractions of volume 5 ml were collected. (a) Mitochondrial extract; (b) cytoplasmic preparation. —, Hexokinase activity;, protein; ----, KCl gradient.

0.38 mM in the intact mitochondria, is very close to the values previously reported (Fromm & Zewe, 1962; Bachelard, 1967). This value was increased to almost double that value (0.72 mM) in the Triton extracts and remained consistently close to the higher value during subsequent purification. It is also notable that the soluble enzyme from the cytoplasm behaved in a similar fashion; from K_m 0.37 mM in the original tissue supernatant fraction the value also increased in the purified enzyme, so the elevation of K_m does not appear to be a property peculiar to the particulate enzyme.

The K_m values for glucose did not change appreciably in the cytoplasmic enzyme, although the value for the second peak from the column was slightly

higher. This cannot be regarded as significant, however; glucose was added to the material before chromatography for stabilization and it proved impossible to remove this completely by repeated dialysis. This was no problem in the more active peak I as the activity was sufficiently high to allow measurements on small samples. In this case the control cuvettes containing all assay constituents except added glucose gave ΔE changes of 0. However, with peak II kinetic measurements, small control activity was present, indicating that the concentration of endogenous glucose was significant.

In the intact mitochondria the apparent K_m for glucose was consistently slightly higher than for the cytoplasmic enzyme, as reported by Bachelard

Fig. 2. DEAE-cellulose chromatography of mitochondrial and cytoplasmic hexokinase with a concave KCl gradient. The preparations used were those after $(\text{NH}_4)_2\text{SO}_4$ purification as indicated in the text. (a) Mitochondrial extract; fractions of volume 5.2 ml were collected; (b) cytoplasmic preparation; fractions of volume 5.6 ml were collected. —, Hexokinase activity;, protein; ----, KCl gradient.

(1967). This remained at the higher value (0.07 mM) in the Triton extracts, but in the purified peaks from the column was similar (0.042–0.043 mM) to that of peak I and the original preparation of the cytoplasmic enzyme.

So except for the possibility of peak II of the cytoplasmic enzyme having a slightly higher K_m for glucose, the kinetic properties studied were apparently similar for the purified mitochondrial and cytoplasmic enzymes.

DISCUSSION

Solubilization and activation of mitochondrial hexokinase. The criteria for solubilization of particulate protein are often based on non-sedimentation during high-speed centrifugation. This can be misleading, as the techniques used to achieve extraction may produce mixed micelles of protein and extractant in which the solubility of the protein

depends on the presence of the extractant. Difficulties in retaining many proteins in soluble form on removal of extractant have received comment in the introduction. Assessment of solubility is uncertain even with techniques that do not depend on the use of chemical agents; microsomal protein, rendered non-sedimentable by ultrasonic irradiation, was considered to remain in particulate form (Brackenridge & Bachelard, 1969).

The preparation of cerebral mitochondrial hexokinase described in the present study remained soluble after removal of detergent by a combination of ammonium sulphate fractionation and DEAE-cellulose chromatography. The Triton content of the purified enzyme was decreased from about 2 mg/mg of protein in the original extract to 0.5 mg/mg of protein after ammonium sulphate precipitation and to less than 0.02 mg/mg of protein after chromatography. It is not possible to state that Triton has been completely removed from the

Table 3. *DEAE-cellulose chromatography of hexokinase preparations*

Triton extracts of mitochondria prepared by method (2) and treated with $(\text{NH}_4)_2\text{SO}_4$ were used. The cytoplasmic enzyme was that precipitated between 25% and 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. Chromatography on DEAE-cellulose was performed as described in the Materials and Methods section and in Figs. 1 and 2. The percentage recovered refers to the material placed on the columns. Specific activity is expressed as $\mu\text{mol/min}$ per mg of protein. The results are expressed as the mean (range) from three column pairs.

Preparation	Source of hexokinase	
	Mitochondria	Cytoplasm
$(\text{NH}_4)_2\text{SO}_4$ fraction		
Hexokinase	(100)	(100)
% recovered		
Sp. activity	1.41 (1.13–1.89)	0.12 (0.10–0.15)
Triton/protein ratio (mg/mg)	0.54 (0.42–0.61)	—
Peak I		
Hexokinase		
% recovered	80.4 (70.9–87.5)	82.9 (74.0–89.2)
Sp. activity	3.82 (3.47–4.03)	3.13
Triton/protein ratio (mg/mg)	<0.02	—
Peak II		
Hexokinase		
% recovered	16.9 (12.1–21.1)	15.1 (10.8–26.0)
Sp. activity	0.90 (0.11–1.56)	0.75
Triton/protein ratio (mg/mg)	<0.02	—

Table 4. *Michaelis constants of hexokinase preparations*

Source	Preparation	No. tested	K_m (ATP) (mM)	K_m (glucose) (mM)
Cytoplasm	Original	2	0.37	0.043
	Peak I*	2	0.56	0.048
	Peak II*	2	0.68	0.062
Mitochondria	Intact	4	0.38	0.072
	Triton extract*	1	0.72	0.072
	$(\text{NH}_4)_2\text{SO}_4$ fraction*	1	0.63	0.066
	Peak I*	2	0.60	0.042
	Peak II*	1	0.66	0.043

* Preparations were dialysed exhaustively against 0.32M-sucrose before kinetic studies were performed. The Triton extract was as in (b), Table 1 and the $(\text{NH}_4)_2\text{SO}_4$ fraction was that precipitated from this Triton extract between 24% and 90% saturation (Table 2).

Table 5. *Effect of added Triton X-100 on hexokinase activities in Triton-depleted extracts of mitochondria*

The hexokinase extracts were treated with 'Triton medium' at 0°C for 30 min under the conditions used for the Triton extraction of intact purified mitochondria as described in the Materials and Methods section.

Preparation	Source	No. of preparation	Hexokinase activity ($\Delta E/\text{min}$)			
			No Triton	With Triton	% increase	Mean
$(\text{NH}_4)_2\text{SO}_4$ 24–90% satn.*	Mitochondria	1	0.034	0.039	15	16
		2	0.020	0.025	25	
		3	0.031	0.034	10	
		4	0.030	0.034	13	
		5	0.023	0.027	18	
Peak I, DEAE-cellulose†	Mitochondria	1	0.0175	0.021	20	22
		2	0.0145	0.018	24	
Peak I, DEAE-cellulose‡	Cytoplasm	1	0.022	0.022	0	

* The $(\text{NH}_4)_2\text{SO}_4$ fraction of Table 4.

† The major peak eluted from DEAE-cellulose (Fig. 2a).

‡ The major peak eluted from DEAE-cellulose (Fig. 2b).

enzyme protein as the concentration reached was at the limits of sensitivity of the method employed. However, from kinetic and chromatographic behaviour it seems unlikely that any traces of residual detergent were affecting the solubility or other properties of the enzyme.

The increase of activity of hexokinase in Triton extracts of cerebral mitochondria has been suggested to be due to the release of 'latent' activity present in the intact organelle (Teichgräber & Biesold, 1968), presumably by exposure of previously inaccessible active centres. It could equally well be due to changes in enzyme conformation induced directly by the detergent. The loss of activity observed during removal of Triton could be due either to reversal of a direct activation effect or to loss of enzymic activity during purification. The reactivation of the Triton-depleted enzyme resulting from readdition of Triton does not resolve this completely, but does indicate that at least part of the original activation on extraction has been due to a direct effect of the Triton itself. The increase in activity due to added Triton was less in the ammonium sulphate fraction (Triton/protein ratio = 0.5) than in the column peak (Triton/protein ratio < 0.02), so the extent of 'reactivation' by Triton is inversely related to the residual Triton present. In the column-purified activity the extent of reactivation was still less than that observed as a result of the original Triton extraction from the mitochondria.

Comparison of cytoplasmic and solubilized mitochondrial hexokinase. Previous comparative studies on the properties of cytoplasmic and particulate hexokinases have indicated some differences. The enzyme in intact unpurified mitochondria was reported to exhibit a slightly higher K_m for glucose than the cytoplasmic enzyme (Bachelard, 1967) and differed also in extent of inhibition by glucose 6-phosphate (Newsholme *et al.* 1968). Such differences either could be due to differences in the properties of the enzymes in the two sites or could merely reflect impedance of transport of substrates or products across the mitochondrial membrane. The identical behaviour during chromatography on DEAE-cellulose columns of the cytoplasmic and solubilized mitochondrial activities strongly suggests identity of the enzymes from the two sources. This is also seen in the kinetic properties studied; no appreciable differences were noted in the K_m values for glucose or ATP in the chromatographically purified activity peaks. A slight but consistent elevation in the K_m for ATP (from 0.37 mM in the crude fractions to 0.6–0.7 mM in the purified preparations) was observed during purification; this occurred with both cytoplasmic and mitochondrial activities. No evidence was found for a large elevation of the

K_m for ATP such as the tenfold increase reported as a result of detergent extraction of mitochondria (Copley & Fromm, 1967) in which the chromatographic purification of the enzyme was performed in the presence of the extractant. The observed elevated K_m in that study may have been due to the continued presence of the detergent.

A further indication of the identity of the enzymes from the two subcellular sources is the similarity of enzymic specific activity in the purified peaks after chromatography in view of major differences in the specific activities of the primary fractions from the tissue disruption. Whereas the cytoplasmic and extracted mitochondrial enzymes exhibited almost identical behaviour during chromatography and very similar substrate kinetic properties, one point of dissimilarity remains, i.e. the effects of Triton on the purified enzymes. The purified mitochondrial enzyme was activated by Triton but the purified cytoplasmic activity was not affected under strictly comparable conditions. The identity of the particulate and cytoplasmic activities is relevant to the regulatory mechanisms of cerebral carbohydrate metabolism. From a consideration of the release of mitochondrial hexokinase as a result of osmotic shock in the presence of low concentrations of ATP or glucose 6-phosphate, Wilson (1968) has suggested that intracellular concentrations of ATP, glucose 6-phosphate and inorganic phosphate may be involved in the regulation of hexokinase distribution. As discussed above it is also possible that physiological factors (electrical activity and hormone action) may influence the hexokinase available for rapid glucose utilization. The validity of investigations on these questions is increased by evidence on the similarity or identity of the two sources of enzyme.

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