The Subcellular Distribution and Properties of Hexokinases in the Guinea-Pig Cerebral Cortex

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1. Hexokinase activities were estimated in primary subcellular fractions from guinea-pig cerebral cortex and in sucrose-density-gradient subfractions of the mitochondrial and microsomal fractions. 2. Appreciable activities were observed in mitochondrial, microsomal and soluble fractions. The activity in the mitochondrial fraction was associated with the mitochondria rather than with myelin or nerve endings and that in the microsomal fraction was associated with membrane fragments. 3. Most of the mitochondrial activity was extracted in soluble form by osmotic 'shock'. The activity of the mitochondrial extract differed from the soluble activity in kinetic properties and in electrophoretic behaviour. 4. No evidence was obtained for the presence of a high- K_m glucokinase in the brain. 5. The results are discussed in terms of relevance to considerations of glucose utilization by the brain.

Since the studies by Crane & Sols (1953) on brain hexokinase (ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1), it has been generally accepted that this enzyme activity is associated mainly with the mitochondria, in contrast with other tissues where hexokinase is found mainly in the cytoplasmic or soluble fraction. In view of the special requirement for glucose by the brain and its rapid utilization therein, the distribution of glucose-phosphorylating systems is of particular interest, particularly as it has been suggested (Lowry & Passonneau, 1964) that hexokinase is one of the control points of glycolysis in the brain.

Most of the investigations subsequent to the report of Crane & Sols (1953) have involved the use either of whole homogenates (Kerly & Leaback, 1957; Bennett, Drori, Krech, Rosenzweig & Abraham, 1962; Lowry & Passonneau, 1964) or of mitochondrial preparations (Fromm & Zewe, 1962; Nesbakken & Eldjarn, 1963); an examination of possible glucose-phosphorylating systems in all subcellular fractions would seem relevant, especially in view of the report by Butterfield, Abrams, Sells, Sterky & Whichelow (1966) that brain glucose utilization is insulin-sensitive. An insulin-inducible 'glucokinase' (ATP-D-glucose 6-phosphotransferase, EC 2.7.1.2) has been demonstrated in insulin-sensitive tissues (Walker, 1963; Walker & Rao, 1964; Salas, Viñuela & Sols, 1963; Sharma, Manjeshwar & Weinhouse, 1963; Gromova & Il'in, 1965), but an analogous brain glucokinase has not been detected.

The present paper reports the results of tissue-fractionation and sucrose-density-gradient studies of glucose phosphorylation in brain. Kinetic experiments and electrophoretic separations of multiple forms of the enzyme have been carried out on different subfractions. A preliminary communication has been given (Bachelard, 1967).

EXPERIMENTAL

Tissue fractionation

Centrifugal forces are expressed as g_{av} values calculated to the centres of the tubes. A Spinco model L ultracentrifuge was used throughout, and 0.32 M-sucrose, adjusted to pH 7 with KOH, was used except where stated otherwise.

Guinea pigs (350–400 g.) were stunned by a sharp blow on the back of the neck and bled. The cerebral hemispheres were removed and freed from white matter. The grey matter, usually 1.8–2.0 g./animal, was placed immediately in 9 vol. of ice-cold 0.32 M-sucrose, pH 7, in a Teflon-pestle homogenizer (A. H. Thomas Co., Philadelphia, Pa., U.S.A.; type B; internal diam. 20 mm., clearance 0.13–0.18 mm.). Grinding involved six passages of the pestle, rotating at 2000 rev./min., during 20–30 sec. with intermediate periods of cooling in ice. The homogenate was centrifuged at 1000g for 10 min. in the no. 40 head. The pellet was washed once by resuspension in the original volume of 0.32 M-sucrose, pH 7, and recentrifugation. The washed pellet (nuclear fraction N) was suspended in 0.32 M-sucrose, pH 7 (5 ml./g. of original tissue). The combined supernatant and washings were centrifuged at 10000g for 15 min. The pellet (mitochondrial fraction Mt) was washed twice by resuspension in the original volume of 0.32 M-sucrose, pH 7, and

recentrifugation, and was finally suspended in 0.32M-sucrose, pH7 (5ml./g. of original tissue). The combined supernatant and washings were centrifuged at 105000g for 1 hr. and the pellet (microsomal fraction M_{cs}) was suspended in 0.32M-sucrose, pH7 (5ml./g. of original tissue); the supernatant was retained as the soluble fraction S.

For density-gradient centrifugation of the mitochondrial fraction, a discontinuous gradient (24ml.) of sucrose was prepared with the following steps: 1.4M, 1.2M, 1.0M and 0.8M. After storage of the gradient for 5–6 hr. at 3°, the mitochondrial fraction (2.5ml.) was layered on top and centrifuged for 2 hr. at 53500g in the no. SW 25 head by the procedure described by De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicof (1962). The resulting four suspended subfractions (A, B, C and D from the top) that appeared as layers in the sucrose density gradient were withdrawn with fine pipettes and were diluted to a sucrose concentration of 0.32M. The pellet (E) was suspended in 0.32M-sucrose, pH7. Each subfraction was centrifuged at 30000g for 15 min. in the no. 40 head and the pellets were suspended in 0.32M-sucrose, pH7 (5ml./g. of original tissue).

For density-gradient centrifugation of the microsomal fraction, a discontinuous gradient of sucrose was prepared with the following steps: 1.5M, 1.2M, 0.9M and 0.6M. After storage of the gradient for 3–4 hr. at 3°, the microsomal fraction was carefully layered on top and centrifuged at 125000g for 2 hr. in the no. SW 39 head by the procedure described by Schwartz, Bachelard & McIlwain (1962). The four subfractions (F, G, H and I, from the top) that appeared as layers in the sucrose density gradient were withdrawn with fine pipettes and diluted to a sucrose concentration of 0.32M. The pellet (J) was suspended in 0.32M-sucrose, pH7. Each subfraction was centrifuged at 105000g for 1 hr. in the no. 40 head and the pellets were suspended in 0.32M-sucrose, pH7 (2.5ml./g. of original tissue).

All fractions and subfractions were stored as 0.5ml. samples at –20°.

Reagents and analyses

Hexokinase. Assays of hexokinase activity were performed in glycylglycine–KOH buffer, pH8.0, essentially as described by Bennett *et al.* (1962). The incubation mixture (total vol. 3.0ml.) contained (final concentrations): glycylglycine–KOH buffer, pH8.0 (150mm), MgCl₂ (10mm), ATP (5mm), NADP⁺ (0.25mm), glucose 100mm, (or as indicated in the text) and glucose 6-phosphate dehydrogenase (0.2 international unit). After equilibration of the mixture at 37° the reactions were started by the addition of tissue subfractions (usually 20–50μl.) and followed at 37° at 340mμ in either a Hilger and Watts Uvispek spectrophotometer or a Unicam SP.800 recording spectrophotometer, both of which were equipped with constant-temperature cell housings. The reference cells contained the same constituents except for glucose and ATP. Rates were linear for at least 15 min. and were not affected by the presence of 100mm-KCl and 5mm-cysteine (Salas *et al.* 1963). Negligible activity (less than 0.001 E unit/min.) was observed in the presence of all constituents except the tissue fraction. There was found to be no need to correct for endogenous 6-phosphogluconate-dehydrogenase (EC 1.1.1.44) activity, unlike the case with liver preparations

(Walker, 1963; Salas *et al.* 1963). Direct estimations of NADPH formation in all fractions in the presence of 5mm-sodium 6-phosphogluconate showed no activity (less than 0.002 E unit/min.). This is to be expected since the 6-phosphogluconate-dehydrogenase activity in brain, 20 μmoles of substrate oxidized/g./hr. (McIlwain, 1966), is only 2% or less of the activity of brain hexokinase. Such a relatively low 6-phosphogluconate-dehydrogenase activity would not be detected in brain samples chosen to show a hexokinase activity of 0.02–0.04 E unit/min.

Acetylcholinesterase. Assays for acetylcholinesterase were performed with a pH-stat (Radiometer, Copenhagen, Denmark). The acid produced during hydrolysis of acetylcholine at 37° and constant pH (7.4) was measured in the following mixture: NaCl (100mm), MgCl₂ (10mm) and acetylcholine perchlorate (15mm). After the rate of non-enzymic hydrolysis (usually 5–10% of the enzymic rate) had been followed for 5 min., tissue extract was added (20–100μl.) and the enzymically catalysed rate of hydrolysis was followed for a further 5–10 min. and corrected by subtraction of the non-enzymic rate.

Succinate dehydrogenase. Estimations for succinate-dehydrogenase activity were carried out manometrically according to the method of Aldridge & Johnson (1959) with the modifications described by Schwartz *et al.* (1962).

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as standard. When sucrose was present in samples analysed it was included in similar concentrations in the blank and albumin standards, as it diminished colour formation.

Electrophoresis. Starch-gel electrophoresis was performed on microscope slides according to the technique described by Marsh, Joliff & Payne (1966) in the following buffer systems: (a) tris buffer (gel buffer: 300mm-tris–HCl, pH8.6; electrode buffer: 5mm-EDTA–25mm-tris–HCl, pH8.6; Moore, Chandler & Tettenhorst, 1964); (b) barbitone buffer (gel buffer: 1mm-EDTA–20mm-sodium barbitone, pH8.6; electrode buffer: 1.7mm-EDTA–60mm-sodium barbitone, pH8.6; McLean, Brown, Greenslade & Brew, 1966). Electrophoresis was carried out at 3° for 3–5 hr. (current 15–20mA, potential difference 4–5v/cm.). The gels were stained for hexokinase activity at 37° in a moist oven for 1–2 hr. with a solution based on that described by Katzen & Schimke (1965) and containing: tris–HCl buffer, pH8.0 (100mm), glucose (100mm), MgCl₂ (10mm), ATP (10mm), NADP⁺ (0.2mm), glucose 6-phosphate dehydrogenase (0.2 international unit/ml.), nitro-blue tetrazolium (0.3 mg./ml.) and phenazine methosulphate (0.03 mg./ml.). Background staining was usually slight and was further diminished by immersion in the solution of methanol–acetic acid–water (5:1:5, by vol.) used as preservative. Controls contained all constituents except glucose and ATP, and showed no bands corresponding to the hexokinase ones.

Reagents. ATP (disodium salt), NADP⁺ (sodium salt), 6-phosphogluconate (sodium salt) and tris were obtained from Sigma (London) Chemical Co. Ltd., London, S.W. 6. Glucose 6-phosphate dehydrogenase (from yeast, EC 1.1.1.49) was obtained from Boehringer Corporation (London) Ltd., London, W. 5. Hydrolysed starch, for gel electrophoresis (Smithies, 1955), was purchased from Connaught Medical Research Laboratories, Toronto, Canada. All other chemicals, including glycylglycine, acetylcholine perchlorate, sodium barbitone, nitro-blue

tetrazolium and *N*-methylphenazonium methosulphate, were from British Drug Houses Ltd., Poole, Dorset.

RESULTS

Enzyme distribution in tissue fractions from differential centrifugation. The distribution of glucose-phosphorylating activity in primary tissue fractions from guinea-pig cerebral cortex is compared in Table 1 with the distributions of succinate-dehydrogenase and acetylcholinesterase activities. The total glucose-phosphorylating activity of the cerebral cortex was 1366 μ moles of NADPH produced/g./hr. at 37°, in confirmation of the results of Bennett *et al.* (1962), who reported activities of 1270–1400 μ moles/g./hr. for areas of rat cerebral cortex. In the present study, the crude mitochondrial fraction (Mt) contained 35–40% of the total activity.

The results in Table 1 show that about 65% of the total activity was present in the combined particulate fractions; the activity in the microsomal fraction (Mcs) was not likely to be due to contamination by mitochondria since the succinate-dehydrogenase activity in fraction Mcs was very low. The activity in the non-particulate or soluble fraction S, about 25% of the total present, was similarly not likely to be due to fragments of disrupted mitochondria. No succinate-dehydrogenase activity was present in fraction S, and freezing and thawing the mitochondrial fraction in 0.32 M-sucrose did not release the hexokinase activity. Johnson (1960) also reported that the hexokinase activity of his mitochondrial preparations was not released by freezing and thawing in 0.3 M-sucrose.

Density-gradient centrifugation. The distribution of hexokinase was similar to that of succinate dehydrogenase, but different from that of acetylcholinesterase, in the mitochondrial subfractions (*A*, *B*, *C*, *D* and *E*) prepared by sucrose-density-gradient centrifugation (Table 2), suggesting that the hexokinase is localized in the mitochondria, in accord with the results reported by Johnson (1960), rather than in the nerve-ending particles, which are enriched in acetylcholinesterase (De Robertis *et al.* 1962). The possibility that the hexokinase activity of the microsomal fraction was due to contamination by cytoplasmic enzyme is rendered unlikely by its distribution in the microsomal subfractions (*F*, *G*, *H*, *I* and *J*) prepared by sucrose-density-gradient centrifugation (Table 3).

The hexokinase distribution in these subfractions was not identical with that of acetylcholinesterase, and suggested that the hexokinase was associated with membrane structures that may not be the same as those containing the acetylcholinesterase (Hanzon & Toschi, 1959). The hexokinase distribution was more similar to that found for the Na⁺-

Table 1. Glucose-phosphorylating activities of primary fractions from guinea-pig cerebral cortex

Fraction	Protein (mg./g. of tissue)	Hexokinase		Succinate dehydrogenase		Acetylcholinesterase	
		(μ moles/g. of tissue/hr.)	(μ moles/mg. of protein/hr.)	(μ moles/g. of tissue/hr.)	(μ moles/mg. of protein/hr.)	(μ moles/g. of tissue/hr.)	(μ moles/mg. of protein/hr.)
Whole homogenate	94.0 (89.4–97.8)	1366 (1275–1600)	14.5	595 (570–625)	6.4	590 (540–620)	6.3
Nuclear	16.0 (12.6–19.4)	141 (118–164)	8.8	132 (110–140)	8.3	11 (8–12)	0.7
Mitochondrial	27.8 (26.3–33.6)	485 (380–638)	17.5	370 (358–391)	13.3	100 (79–118)	3.6
Microsomal	19.6 (14.2–25.2)	238 (155–260)	12.2	36 (30–48)	1.8	410 (380–435)	21.0
Supernatant	22.0 (18.5–24.0)	335 (325–426)	15.2	0	0	0	0

Tissue fractions were prepared and assayed for enzymic activities as described in the Experimental section. Results from the enzyme assays are expressed in terms of μ moles of substrate utilized. Mean values from three preparations are given. The ranges of results are given in parentheses.

Table 2. *Glucose-phosphorylating activities of subfractions prepared by sucrose-density-gradient centrifugation of the mitochondrial fraction*

Subfractions were prepared and assayed for enzymic activities (expressed in terms of μ moles of substance utilized/hr.) as described in the Experimental section. Mean values from two preparations are given.

Subfraction of fraction	Protein (mg./g. of original tissue)	Hexokinase		Succinate dehydrogenase		Acetylcholinesterase	
		(% of original mitochondrial activity)	(μ moles/mg. of protein/hr.)	(% of original mitochondrial activity)	(μ moles/mg. of protein/hr.)	(% of original mitochondrial activity)	(μ moles/mg. of protein/hr.)
Mt							
A	4.1	5.1	6.0	0	0	18.1	4.5
B	2.2	2.6	5.7	3.7	6.4	21.5	10.0
C	4.5	6.0	6.5	6.8	5.8	47.0	10.6
D	6.1	32.5	25.8	23.0	14.6	8.3	1.4
E	4.9	40.4	40.1	34.1	26.8	0	0

Table 3. *Glucose-phosphorylating activities of subfractions prepared by sucrose-density-gradient centrifugation of the microsomal fraction*

Subfractions were prepared and assayed for enzymic activities (expressed in terms of μ moles of substrate utilized/hr.) as described in the Experimental section. Mean values from two preparations are given.

Subfraction of fraction	Protein (mg./g. of original tissue)	Hexokinase		Acetylcholinesterase	
		(% of original microsomal activity)	(μ moles/mg. of protein/hr.)	(% of original microsomal activity)	(μ moles/mg. of protein/hr.)
Mcs					
F	4.2	13.5	6.2	55.0	52.7
G	3.0	29.8	19.0	25.5	34.7
H	4.4	36.0	15.7	11.9	11.3
I	2.3	11.5	9.7	4.1	7.1
J	0.9	2.1	6.6	0.5	2.2

stimulated adenosine triphosphatase (Schartz *et al.* 1962).

Extraction of mitochondrial hexokinase. Attempts to extract the hexokinase activity from the mitochondrial fraction (Mt) by successively freezing and thawing the suspension as prepared in 0.32M-sucrose were unsuccessful: over 90% of the activity remained in the pellet after centrifugation at 80000g for 20min. Freezing and thawing in 100mM-tris-hydrochloric acid buffer, pH 7.0, resulted in extraction of the enzymic activity, but this disappeared on storage overnight. A stable solubilized preparation was made as follows: the mitochondrial fraction as originally prepared in 0.32M-sucrose, pH 7 (Experimental section), was centrifuged immediately at 10000g for 15min. The pellet was resuspended in the following buffer system to a final volume of 3ml./g. of original tissue: glycylglycine (50mM), potassium chloride (5mM), magnesium chloride (5mM), cysteine (2mM) and ATP (5mM), made up immediately before use and adjusted with hydrochloric acid to pH 7.0. The suspension was rapidly frozen in an acetone-solid carbon dioxide bath at about -50° , and thawed at $0-3^{\circ}$. This procedure was repeated twice and the suspension was centri-

fuged at 80000g for 20min. The clear supernatant (MtS₁) was removed and the pellet (MtP₁) was resuspended in the original volume of the same glycylglycine buffer. Half of the suspension was retained for subsequent analyses and the other half was treated in an MSE ultrasonic disintegrator (current 1.5A) for 10sec. periods (total 60sec.) in a bath at 0° . The suspension was centrifuged at 80000g for 10min. to yield a clear supernatant (fraction MtS₂) and a pellet (fraction MtP₂), which was suspended in the glycylglycine buffer, pH 7.0 (3ml./g. of original tissue). The osmotic 'shock' in hypo-osmotic glycylglycine buffer resulted in a slight enhancement of the hexokinase activity originally present in the mitochondrial fraction (Table 4). Of the activity in fraction Mt₁, over 70% was released (in fraction MtS₁), and of the residual activity in the pellet (fraction MtP₁) over 80% was released by ultrasonic disintegration (fraction MtS₂). Over 90% of the total activity was rendered soluble. The enzyme extract (fraction MtS₁) was stable on storage at -20° for at least 5 weeks. Negligible 6-phosphogluconate-dehydrogenase activity was found.

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Table 4. *Extraction of mitochondrial glucose-phosphorylating activity*

Conditions for the extraction of the mitochondrial fraction (Mt) were as described in the text. Hexokinase activity was estimated as described in the Experimental section. Mean values from two preparations are given.

Sample	Treatment	Hexokinase		
		(μ moles of NADPH formed/g. of tissue/hr.)	(% of that in Mt ₁)	(μ moles of NADPH formed/mg. of protein/hr.)
Mt		490		17.5
Mt ₁	After freezing and thawing Mt	547	(100)	19.6
MtS ₁	After centrifuging Mt ₁	389	71	49.0
MtP ₁		130	24	5.8
MtS ₂	After ultrasonic treatment of MtP ₁ and centrifuging	104		
MtP ₂		20		1.4

Fig. 1. Reciprocal plot of hexokinase activities versus glucose concentration in the presence of 5mm-ATP. ●, Mitochondrial extract (MtS₁); ○, supernatant fraction (S). The constant amounts of protein used were within the ranges: fraction MtS₁, 0.04–0.05 mg.; fraction S, 0.08–0.10 mg.

Fig. 3. Reciprocal plot of hexokinase activities versus ATP concentration in the presence of 100mm-glucose. ●, Mitochondrial extract (MtS₁); ○, supernatant fraction (S). The constant amounts of protein used were within the ranges: fraction MtS₁, 0.04–0.05 mg.; fraction S, 0.08–0.10 mg.

Fig. 2. Reciprocal plot of hexokinase activities versus glucose concentration in the presence of 0.2mm-ATP. ●, Mitochondrial extract (MtS₁); ○, supernatant fraction (S). The amounts of protein used were: fraction MtS₁, 0.04 mg.; fraction S, 0.08 mg.

Fig. 4. Hexokinase activity of two preparations of brain extracts after starch-gel electrophoresis in barbitone buffer, pH 8.6, followed by staining as described in the text. (a) Mitochondrial extract (MtS₁), preparation I; (b) supernatant fraction (S), preparation I; (c) mitochondrial extract (MtS₁), preparation II; (d) supernatant fraction (S), preparation II.

apparent Michaelis constants for glucose, derived from reciprocal plots (Fig. 1), were different for the enzymes from different sources at high (5 mM) ATP concentration. The non-particulate system S had K_m 0.074 mM (six preparations, range 0.061–0.085 mM) and the mitochondria K_m 0.136 mM (six preparations, range 0.110–0.155 mM). These differences were not observed at low (0.2 mM) ATP concentration (Fig. 2). No difference was observed in the K_m for ATP (0.4 mM; Fig. 3).

Two forms of hexokinase in mitochondrial extracts and three in the supernatant fraction were detected after separation by starch-gel electrophoresis (Fig. 4). The two buffer systems tested gave similar results.

DISCUSSION

Brain hexokinase has generally been accepted as being mainly particulate in occurrence, and most studies have used a particulate fraction prepared by centrifugation of homogenates at 18 000 g for 30 min. based on the work of Crane & Sols (1953). However, the results of Stern (1954) indicated that not all of the glucose-phosphorylating activity is associated with particulate fractions. Kerly & Leaback (1957) found that only one-third of the hexokinase was sedimented by centrifuging as above, and Johnson (1960) found that 20% was non-particulate. In the present study, of the activity recovered in sub-cellular fractions from sucrose homogenates, 40% appeared in the primary mitochondrial fraction, over 25% in the non-particulate soluble fraction and nearly 20% in the microsomal fraction. The results from density-gradient centrifugation of the microsomal fraction indicated that the activity was associated with membrane structures. The greater part of the microsomal activity would be likely to have appeared in the particulate fraction obtained by Crane & Sols (1953), prepared at 18 000 g for 20 min., since a large proportion of the membrane fragments of microsomal preparations have been shown to be sedimented under similar conditions (Schwartz *et al.* 1962).

The results from density-gradient centrifugation of the primary mitochondrial fraction suggested that the activity is associated with the mitochondria rather than with the other constituents that occur there, such as myelin or nerve endings. The distribution of acetylcholinesterase (enriched in nerve endings; De Robertis *et al.* 1962) differed markedly from the hexokinase distribution in the mitochondrial subfractions. The ready release, by osmotic 'shock', of the hexokinase activity from the mitochondria suggests that the enzyme is relatively weakly bound, and in the nomenclature suggested by Egger & Rapoport (1963) is 'non-sessile'.

That the soluble hexokinase activity was not an

artifact of preparation is supported by the differences in properties observed between the enzymes in the mitochondrial and soluble fractions. Not only were the Michaelis constants for glucose different, but the soluble system was also found to contain a third form of hexokinase, not present in the mitochondria and not previously described in the brain. The relatively high affinity of the soluble system for glucose may result in the contribution of cytoplasmic enzymes to the overall rate of glucose phosphorylation in the intact cell being of considerable significance. Such factors are of relevance in consideration of the access of glucose to possible phosphorylating sites in the brain.

The concentration of intracellular glucose in the brain is particularly difficult to assess in view of the conflicting reports of the overall glucose content of brain. Gey (1956) separated glucose from other reducing substances by paper chromatography of extracts prepared from unanaesthetized rat brain frozen *in situ*, and reported the glucose content to be 8 mg./100 g. of tissue. Lowry, Passonneau, Hasselberger & Schultz (1964) found the brain glucose content to be 20–27 mg./100 g. in anaesthetized mice, also after freezing *in situ*. The blood volume of whole brain has been estimated to be about 3% of the tissue volume (Everett, Simmons & Lasher, 1956; Rosomoff, 1961) and the cerebrospinal-fluid volume to be about 9% (Rosomoff, 1961). Assuming a mean blood glucose content of 90 mg./100 ml. and a cerebrospinal-fluid glucose content of 70 mg./100 ml. (Davson, 1955; Cooper & Archdeacon, 1960), the contribution of these fluids to the apparent glucose content of whole brain would be about 9 mg./100 g. Any glucose present in the extracellular non-vascular space of the brain (4–25% of the tissue volume; Barlow, Domek, Goldberg & Roth, 1961; Gibson & Mellwain, 1965) has also to be taken into account, so it seems possible that the net intracellular glucose concentration of the brain may be very low, and if the estimated glucose content of Gey (1956) is accepted, may even approach zero. Though such a calculation is based on considerations of the brain as a whole, without regard to differences in cell types, it does indicate the possibility that all of the glucose available for glycolysis in the brain is phosphorylated immediately on entry, which would be difficult to envisage if the phosphorylating sites were essentially mitochondrial. However, the enzyme in the microsomal fraction (presumably derived from the plasma membrane and the endoplasmic reticulum) and particularly in the cell cytoplasm together total nearly half of the hexokinase activity present (i.e. about 550–600 μ moles/g./hr.), and should be more than sufficient to account for all of the glucose phosphorylated (Lowry & Passonneau, 1964).

The three forms of hexokinase observed in the

soluble supernatant fraction exhibit electrophoretic behaviour similar to the three forms of hexokinase found (Katzen & Schimke, 1965; Grossbard & Schimke, 1966) in liver, kidney and intestine. According to their nomenclature, types I and II were found in all tissues examined (including brain), type III was the additional form found in kidney and intestine, and liver contained, in addition to types I, II and III, another type IV (of high K_m), which was suggested to be glucokinase. Type III hexokinase was found to exhibit a very low K_m for glucose (0.005–0.007 mM in liver) in comparison with the K_m values for type I (0.030–0.045 mM in brain, 0.025 mM in liver, 0.049 mM in kidney) and type II (0.20–0.23 mM in muscle, 0.14–0.28 mM in fat pad). The presence of a third low- K_m type, analogous to type III (Katzen & Schimke, 1965; Grossbard & Schimke, 1966), in the brain supernatant fraction would account for the K_m recorded for that fraction being lower than that for the mitochondrial extract, which contained only two forms of the enzyme.

No evidence, kinetic or electrophoretic, has been found that would indicate the presence of a high- K_m glucokinase-type enzyme in the brain. Since the glucokinase of liver (Walker & Rao, 1964; Salas *et al.* 1963) and epididymis (Gromova & Il'in, 1965) has been shown to be insulin-inducible, the difficulty of detecting this enzyme in brain is of interest in view of the demonstration that the human brain is insulin-sensitive. Butterfield *et al.* (1966) reported that the response of human brain to insulin is slower than that of peripheral tissues, so that any analogy to an insulin-induced glucokinase might be very difficult to demonstrate; also, human brain may be more sensitive to insulin than that of the guinea pig. An insulin-sensitive process in the brain could be localized in a particular area, and might not easily be detected in studies on cerebral cortex or the whole tissue.

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