Katzen, H. M., and Schimke, R. T. (1965), *Proc. Natl. Acad. Sci. U. S. 54*, 1218.
Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exptl. Biol. Med. 103*, 250.
Lillie, R. D. (1954), Histopathologic Technic and Practical Histochemistry, New York, N. Y., McGraw-Hill, p 183.
Lovtrup, S., and Svennerholm, L. (1963), *Exptl. Cell Res. 29*, 298.
Misaka, E. (1966), *J. Biochem. (Tokyo) 60*, 609.
Nairn, R. C. (1964), Fluorescent Protein Tracing, London, E. & S. Livingstone Ltd., p 22.
Nakane, P. K., and Pierce, G. B. (1967), *J. Cell Biol. 33*, 307.
Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.
Ruscak, M., Macejova, P., Ruscakova, D., and Mrova, E. (1967), *Federation Proc. 24*, T641.
Schultz, R. L., Maynard, E. A., and Pease, D. C. (1957), *Am. J. Anat. 100*, 369.
Schwartz, G. P., and Basford, R. E. (1967), *Biochemistry 6*, 1070.
Söderholm, U. (1965), *Acta Physiol. Scand. 65*, Suppl. 256.
Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967), *J. Cell Biol. 32*, 415.
Tomasi, T., and Kunkel, H. G. (1964), *Methods Med. Res. 10*, 80.
Walker, D. G., and Seligman, A. M. (1963), *J. Cell Biol. 16*, 455.

Brain Hexokinase. The Preparation of Inner and Outer Mitochondrial Membranes*

Patricia A. Craven,† Peter J. Goldblatt,‡ and R. E. Basford

ABSTRACT: Three different methods were used to prepare inner and outer membranes of brain mitochondria. The digitonin and phospholipase A methods were relatively unsuccessful. In the former case, the outer membrane marker enzyme, monoamine oxidase, was not released into the outer membrane fraction over a range of concentrations from 0.11 to 0.66 mg of digitonin per mg of mitochondrial protein. In the latter case, 60% of the outer membrane marker rotenone-insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase was lost during the incubation with phospholipase A and the portion of the enzyme which was recovered remained in the inner membrane fraction even after prolonged digestion. On the other hand, the combined swelling, shrinking, and sonication method gave more satisfactory results. The sonication procedure resulted in a 1.5–2-fold activation of hexokinase activity which could be duplicated by diluting the mitochondrial suspension in 1% bovine serum albumin. Biochemical assay revealed that between 70 and 75% of the outer membranes were removed. Approximately 63% of the total hexokinase activity was associated with the outer membrane fraction while 12% of the activity was solubilized during the process. The increase in the specific activity of hexokinase was the same as that for the outer membrane marker, rotenone insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase. Biochemical and electron microscopic data are presented which indicate that rotenone insensitive reduced diphosphopyridine nucleotide–cytochrome *c* reductase is not a microsomal contaminant. The results are discussed with reference to the problems and limitations of enzyme localization.

The presence of hexokinase on the outer membrane of brain mitochondria is indicated by the success of the fluorescent antibody technique in revealing the sites of hexokinase activity in thin sections of cortex (Craven and Basford, 1969) and by the ease with which it can be solubilized and rebound (Rose and Warms, 1967; Wilson, 1968). Wilson (1968) has suggested the presence of a latent hexokinase activity on the inner membrane of brain mitochondria based on the harsh treatment needed to activate this activity. However, as demonstrated in the present report, the same activity can be revealed by dilution of the mitochondria in 1% bovine serum albumin as by sonication or detergent treatment. Fractionation of serum albumin with methanol at −10° to remove fatty acids and decanol, a detergent used in the preparation of serum albumin, had no effect on its ability to activate latent hexokinase activity. It seemed desirable to separate the mitochondria into inner and outer membranes and assay these fractions under conditions of maximal hexokinase activity.

Methods have been described for the separation of the inner and outer membranes of rat liver (Sottocasa *et al.*, 1967;

* From the Biochemistry and Pathology Departments, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. *Received March 27, 1969.* This work was supported by Research Grant NB 1984 from the National Institute of Neurological Diseases and Blindness, National Institutes of Health, U. S. Public Health Service. P. A. C. also received funds from the U. S. Public Health Service, Division of General Medical Sciences, under Training Grant 2TIG149.

† The data presented are taken from the dissertation of Patricia A. Craven offered in partial fulfillment of the requirements for the Ph.D. degree.

‡ During this work, Dr. Goldblatt was a Post doctoral Research Scholar of the American Cancer Society, Inc., Grant PRS-23.

Schnaitman *et al.*, 1967; Parsons *et al.*, 1967) and beef heart mitochondria (Bachmann *et al.*, 1966); however no report has appeared describing their successful separation in brain. Tipton (1967) has compared the relative effectiveness of large amplitude swelling, first described by Parsons *et al.* (1967), and the phospholipase A method of Bachmann *et al.* (1966). Neither of these techniques resulted in a high recovery of enzymatic marker activity nor an effective separation of the inner and outer membrane marker enzymes of brain mitochondria. The present report describes the application of three of these methods to brain mitochondria. It is tentatively concluded that brain hexokinase is localized on the outer michondrial membrane.

Materials

The following materials were obtained commercially: glucose 6-phosphate, glucose 6-phosphate dehydrogenase (type 5), cytochrome *c* (type 3), rotenone, 2,6-dichlorophenol-indophenol, *Crotalus atrox* venom, ATP, ADP, TPN^+, DPNH, creatine phosphate, hexokinase (type 3), and EDTA from Sigma Chemical Co., St. Louis, Mo.; bovine serum albumin from Armour Pharmaceutical Co., Chicago, Ill.; Triton X-100 from Ruger Chemical Co., Irvington, N. Y.; and Tris from General Biochemicals, Chagrin Falls, Ohio. All other reagents of analytical grade were obtained from Fisher Scientific Co., Pittsburgh, Pa.

Methods

All enzyme assays, unless otherwise stated, were carried out at room temperature in a Zeiss Model M4Q spectrophotometer. Samples were diluted in 1% (w/v) bovine serum albumin unless otherwise stated.

Hexokinase activity was measured in a system in which glucose 6-phosphate production was coupled to TPNH formation in the presence of glucose 6-phosphate dehydrogenase. The procedure used is described by Schwartz and Basford (1967). One unit of enzyme activity is defined as the formation of 1 μmole of glucose 6-phosphate/min under the above conditions.

Monoamine oxidase activity was determined with benzylamine as substrate according to the procedure outlined by Schnaitman *et al.* (1967). One unit of activity is defined as the oxidation of 1 mμmole of benzylamine/min.

Succinic dehydrogenase activity was estimated by following the succinate-dependent reduction of indophenol to leucoindophenol at 600 mμ according to the method of Bachmann *et al.* (1966). One unit of enzyme activity is defined as the oxidation of 1 mμmole of succinate/min.

RIDCR[1] *and TPNH–cytochrome c reductase activities* were followed by the reduction of cytochrome *c* at 550 mμ using the method described by Sottocasa *et al.* (1967). One unit of enzyme activity is defined as the reduction of 1 mμmole of cytochrome *c*/min.

Creatine kinase activity was determined by a spectrophotometric assay in which ATP production was coupled to TPNH formation in the presence of hexokinase and glucose 6-phosphate dehydrogenase. The procedure is described by Eppenberger *et al.* (1967). One unit of enzyme activity is defined as the formation of 1 μmole of ATP/min.

Protein was determined by the biuret method of Gornall *et al.* (1949) or by the method of Lowry *et al.* (1951) as described by Schwartz and Basford (1967).

Beef brain mitochondria were prepared according to method II described by Stahl *et al.* (1963).

Rat brain mitochondria were prepared by the method of Ozawa *et al.* (1966).

Preparation of Light Microsomes. The first postmitochondrial supernatant was centrifuged at 30,000*g* for 20 min in the Spinco Model L centrifuge (rotor no. 30[2]). The microsomes were then sedimented from the 30,000*g* supernatant by centrifugation at 78,480*g* in the no. 30 rotor.

Pure soluble beef brain hexokinase was prepared by the method of Schwartz and Basford (1967). It had a specific activity of 80 units/mg.

Preparation of Phospholipid. The supernatant obtained after the centrifugation of mitochondria in medium F,[3] according to the procedure of Stahl *et al.* (1963), was diluted with ten volumes of acetone. The lipid was sedimented at 10,300*g* (Servall SS34[4] rotor), collected on a Buchner funnel, and allowed to air dry. After weighing, the amount of phospholipid phosphorus was calculated by assuming that phospholipid contains 4% phosphorus. A solution was made by homogenization in 0.01 M Tris–0.002 M EDTA (pH 8) and clarified by sonication in a Branson probe sonifier, Model S 75 (Fleischer and Fleischer, 1967).

Defatted serum albumin was prepared by methanol fractionation at −10° according to the procedure described by Vogt and Basford (1968).

Preparation of Tissue for Electron Microscopy. A cellulose nitrate centrifuge tube was carefully cut so as to remove the pellet containing either mitochondria, inner membranes, or outer membrane vesicles. The pellet was fixed for 5 hr in phosphate-buffered glutaraldehyde (pH 7.2). The washed pellet was postfixed in 2% osmium tetroxide and embedded in Epon Araldite. Thin sections, cut on a Reichert OMU_2 microtome using glass knives, were picked up on uncoated 300 mesh copper grids, given a light carbon backing, and stained with lead citrate and uranyl acetate. Negatively stained preparations of whole mitochondria and inner and outer membranes were made with silicotungstic acid at pH 7.0 and spread on 300-mesh copper grids previously coated with carbon. All grids were examined in a Phillips 100 B or 200 electron microscope at an acceleration of 60 or 80 kV.

Treatment of Mitochondria with Phospholipase A. Phospholipase digestion was carried out according to the method of Bachmann *et al.* (1966). The incubation time was varied between 30 and 90 min and additions of bovine serum albumin and phospholipid were made to the incubation medium in an effort to prevent large losses of enzyme activity.

Treatment of Mitochondria with Digitonin. The method previously described by Schnaitman *et al.* (1967) was used.

[1] Abbrevation used is: RIDCR, rotenone-insensitive DPNH–cytochrome *c* reductase.

[2] All centrifugal forces are given as the average gravitational force. For calculations using the no. 30 rotor, a radius of 7.8 cm was used.

[3] Medium F contained 0.4 M sucrose–10^{-3} M EDTA–0.02% polyethylene sulfonate–8% Ficoll (pH 6.8).

[4] For calculations using the SS34 rotor, a radius of 3 in. was used.

TABLE I: The Addition of Soluble Hexokinase to Beef Brain Mitochondria and Homogenates.

Starting Material	Original Hexokinase Act. (μmoles/min)	Soluble Hexokinase Act. Added (μmoles/min)	Column 2 + Column 3 (μmoles/min)	Act. in Mitochondrial Pellet (μmoles/min)	Act. in Mitochondrial Supernatant (μmoles/min)	% of Added Hexokinase Recovd in Supernatant[c]	Total Act. Recovd (μmoles/min)
Mitochondria[a]	0.61	0.33	0.94	0.50	0.48	145	0.98
Brain homogenate[b]		5.6		24	11	97	35
Brain homogenate		0		27	5.6		32.6

[a] Mitochondria, suspended in 0.4 M sucrose–10^{-3} M EDTA–0.02% polyethylene sulfonate (pH 6.8, medium A), were mixed with 0.1 ml of pure soluble beef brain hexokinase. The suspension was diluted to 5 ml with 1% bovine serum albumin–0.002 M EDTA and incubated for 30 min at 0–4°. [b] Pure soluble beef brain hexokinase was added to half a brain homogenate containing 25 g of cortex in 50 ml of medium A. Both halves were treated in the same manner and the mitochondria were isolated. [c] The per cent of the hexokinase added to the brain homogenate which was recovered in the supernatant was determined by subtracting the amount of hexokinase in the control supernatant, dividing by the number of units of hexokinase added, and multiplying by 100.

The concentration of digitonin was varied from 0.11 to 0.66 mg per mg of mitochondrial protein.

The Combined Swelling, Shrinking, and Sonication of Mitochondria. The method previously described by Sottocasa *et al.* (1967) was used. The addition of solid bovine serum albumin to a concentration of 1% by weight just before the sonication helped prevent the release of enzymes into the supernatant fraction and, by increasing the viscosity of the sonication medium, prevented frothing.

Results

Addition of Soluble Hexokinase to Mitochondria and Brain Homogenates. Intact mitochondria and brain homogenates were examined for their ability to bind pure soluble beef brain hexokinase. If the presence of hexokinase on isolated mitochondria is a result of the nonspecific adsorption of the enzyme to the mitochondria during the process of cell breakage, it might be possible to demonstrate a similar binding of added soluble hexokinase to brain homogenates or intact mitochondria. All of the hexokinase added to the mitochondrial suspension was recovered in the supernatant obtained by centrifuging the incubation mixture at 3520*g* for 15 min (Servall SS34 rotor). Some solubilization of enzyme from the mitochondrial membrane was also observed (Table I). Similarly, 97% of the soluble hexokinase added to the homogenate was recovered in the first mitochondrial supernatant. The fact that no binding was observed does not necessarily mean that no binding occurred, since it is possible that the mitochondria were already saturated with bound hexokinase.

Effect of Diluent on the Activity of Hexokinase. Mitochondrial hexokinase activity varies depending upon the dilution medium. Dilution of the enzyme with 1% bovine serum albumin, human serum albumin, or 0.5% Triton X-100 results in a twofold increase in activity as compared with dilution in a variety of other buffers (Table II). Under the present experi-

TABLE II: Latent Hexokinase Activity.[a]

Diluting Buffer	Hexokinase Act. (μmole/min)
0.3 M mannitol–0.1 mM EDTA (pH 7.4)	0.28
0.25 M sucrose–0.01 M Tris (pH 7.4)	0.28
0.2 M phosphate (pH 7.6)	0.28
1 M mercaptoethanol–0.01 M Tris (pH 7.4)	0.28
0.25 M sucrose–0.01 M Tris–30 mM oleic acid (pH 7.4)	0.28
0.5% Triton X-100	0.56
1% bovine or human serum albumin	0.59
1% human serum albumin, with fatty acids and decanol removed	0.56

[a] Beef brain mitochondria, suspended in medium A, were diluted 20 times with the buffers indicated. The reaction was started by adding the diluted enzyme samples, without preincubation, to the standard assay cuvet.

TABLE III: Activation of Mitochondrial Hexokinase by Sonic Oscillation and Bovine Serum Albumin.

Diluting Buffer[a]	Hexokinase Act. (μmoles/min)
Before treatment[b]	
0.3 M mannitol–0.1 mM EDTA (pH 7.4)	2.8
1% bovine serum albumin	4.5
After treatment	
0.3 M mannitol–0.1 mM EDTA (pH 7.4)	4.5
1% bovine serum albumin	4.7

[a] Rat brain mitochondria, suspended in 0.3 M mannitol–0.1 mM EDTA, were diluted 20 times with the indicated buffer. The reaction was started by adding the diluted enzyme samples, without preincubation, to the standard assay cuvet. [b] Mitochondria were swollen, shrunk, and sonicated exactly as described for the preparation of inner and outer membranes.

TABLE IV: The Effect of Increasing Concentrations of Digitonin on the Distribution of Enzyme Activities in Beef Brain Mitochondria.

	mg of Digitonin/mg of Protein	Inner Membrane (%)	Outer Membrane (%)	P_2[a] (%)	Supernatant (%)
Succinic dehydrogenase	0.11	78.5	0	11.9	9.6
	0.33	62.5	0	26.5	11.0
	0.50	47.0	0.4	43.4	9.2
	0.66	51.4	8.8	9.7	30.1
Monoamine oxidase	0.11	78.5	0	21.5	0
	0.33	49.5	7	38.6	4.9
	0.50	25.4	10.6	26.8	37.2
	0.66	21.9	11.8	24.1	42.2
Hexokinase	0.11	91.0	2	0	7.0
	0.33	51.0	5.0	31.0	13.0
	0.50	42.9	7.1	40.3	9.7
	0.66	41.0	16.0	32.0	11.0

[a] P_2 refers to the 40,000*g* pellet or fragmented inner membrane fraction.

mental conditions, dilution of mitochondria in 1% bovine serum albumin does not result in mitochondrial swelling as measured by light scattering at 520 mμ. Fractionation of serum albumin with methanol at −10° followed by dialysis to remove fatty acids and decanol, a detergent used in its preparation, had no effect on the ability of human serum albumin to activate mitochondrial hexokinase. The addition of oleic acid to mitochondria suspended in the sucrose–Tris buffer did not inhibit the enzyme. Wilson (1967) has postulated the existence of a latent hexokinase activity in rat brain based on the observations that treatment with detergent, sonic oscillation, or freezing and thawing, procedures which might be expected to result in the disruption of the phospholipid membrane, have an activating effect on hexokinase. This does not however explain the similar effect of serum albumin (Table III). The activation by serum albumin is the same as that observed after swelling, shrinking, and sonication of mitochondria and no further activation occurs if the sonicated mitochondria are then diluted with 1% bovine serum albumin. A more general explanation might be that fatty acids, released from mitochondria during their isolation, are inhibitory to hexokinase and all of these procedures have the effect of either removing fatty acids or disrupting their association with the enzyme. Recently, it has been reported that soluble liver hexokinase is inhibited by a variety of fatty acids (Lea and Weber, 1968). Although the addition of oleic acid to mitochondria did not result in an inhibition of hexokinase, fatty acid inhibition was observed during the course of work with phospholipase A.

Treatment of Beef Brain Mitochondria with Phospholipase A. Succinic dehydrogenase was used as a marker for the inner and RIDCR for the outer membrane. After 30-min digestion, all the hexokinase and succinic dehydrogenase activities were recovered, but only 40% of the RIDCR activity. The recovery was not improved by the addition of 0.4 mg of phospholipid phosphorus/mg of mitochondrial protein, immediately after the digestion, or by the inclusion of 1% bovine serum albumin in the digestion mixture. The outer membrane fraction had no hexokinase or RIDCR activity. After 60-min digestion, only 10% of the hexokinase was recovered. The inclusion of 1% bovine serum albumin in the digestion mixture, to remove fatty acids released by the action of phospholipase A, raised the recovery to 40%. Increasing the concentration of bovine serum albumin to 2% resulted in an 80% recovery of hexokinase activity. In agreement with the results of Fleischer *et al.* (1964), after 60-min digestion of mitochondria with phospholipase A, succinate was oxidized as long as lysophosphatides remained in the mitochondrial suspension. If they were removed by the addition of serum albumin, it was necessary to add phospholipid back to the mixture in order to recover the activity. Although the digestion was twice as long as that used by Bachmann *et al.* (1966) to fractionate beef heart mitochondria, only 20% of the RIDCR and 18% of the hexokinase activity were found in the beef brain outer membrane preparation. Lengthening the time of incubation to 90 min resulted in the irreversible loss of succinic dehydrogenase activity. Although 80% of the hexokinase activity was recovered, only 10% of this was in the outer membrane fraction.

Treatment of Mitochondria with Digitonin. Beef brain mitochondria were treated with digitonin according to the procedure of Schnaitman *et al.* (1967) (Table IV). Monoamine oxidase, the enzyme used as a marker for the outer mitochondrial membrane, did not appear in the outer membrane fraction but was 37% solubilized when the concentration of digitonin reached 0.5 mg/mg of mitochondrial protein. The inner membrane marker, succinic dehydrogenase, remained with the inner membrane until the concentration of digitonin reached 0.66 mg/mg of mitochondrial protein, whereupon 30% was solubilized. Hexokinase, however, remained associated with the inner membrane and fragmented inner membrane (40,000*g* pellet) fraction in the range 0.11–0.66 mg of digitonin/mg of mitochondrial protein.

The Combined Swelling, Shrinking, and Sonication of Beef

TABLE V: The Preparation of Inner and Outer Mitochondrial Membranes.[a]

Enzyme	Outer Membrane Sp Act. (1)	Inner Membrane Sp Act. (2)	Mitochondria Sp Act. (3)	Column 1/ Column 3	Outer Membrane (%)	Inner Membrane (%)	Supernatant (%)	Recov (%)
Hexokinase	1.6	0.26	0.53	3	63	25	12	97
Succinic dehydrogenase	50	56	40		18	80	2	120
RIDCR[b]	36	6	12	3	61	29	9	93
Creatine kinase					60	10	30	80

[a] Inner and outer membranes (in units per milligram) were prepared from beef brain mitochondria by the combined swelling, shrinking, and sonication method of Sottocasa *et al.* (1967). Bovine serum albumin was omitted from the sonication medium. Enzymes were assayed after the sonication step. [b] All RIDCR activities were corrected for the conversion of rotenone-sensitive into rotenone-insensitive activity as described in the text.

Brain Mitochondria. The procedure which resulted in both good recovery and separation of inner and outer membrane marker activities of brain mitochondria was based on that described by Sottocasa *et al.* (1967) for rat liver mitochondria. It involved the combined swelling and shrinking of mitochondria followed by controlled sonication. The optimal time and amperage used in the sonication, 3 A for 15 sec, was found to be the same for beef brain as for rat liver mitochondria. Between 70 and 75% of the outer membrane marker enzyme, RIDCR, was associated with the outer membrane fraction with only 20% contamination by the inner membrane marker enzyme succinic dehydrogenase (Table V). A correction was made for the conversion of rotenone sensitive to insensitive activity which occurs when the assay is carried out in phosphate buffer (D. W. Allmann, personal communication). There is a 55% increase in the activity of brain mitochondrial RIDCR after the sonication step, which is not observed if the phosphate buffer is replaced by glycylglycine buffer. In making the correction it was assumed that all of the rotenone sensitive activity which had been exposed by the sonication procedure appeared associated with fragmented inner membrane in the outer membrane fraction. Hexokinase was associated with the outer membrane fraction. The increase in the specific activity observed in the outer membrane fraction as compared with that in the original mitochondria was the same for hexokinase as for RIDCR. Since all the enzyme assays were carried out after the sonication step and the enzyme samples were diluted in 1% bovine serum albumin, the data reported for hexokinase are applicable to both the latent and assayable forms. Preincubation of the outer membrane fraction with antiserum to hexokinase resulted in a 66% inhibition of enzyme activity. Creatine kinase, which has been shown to be present in the mitochondria of brain and muscle (Jacobs *et al.*, 1964), was also assayed and found to be associated with the outer mitochondrial membrane fraction (Table V).

Recently, evidence has been presented which indicates that RIDCR is a strictly microsomal enzyme and therefore not suitable for use as a marker for the outer mitochondrial membrane (Green *et al.*, 1968; Allmann *et al.*, 1968). If it is assumed that RIDCR is a microsomal enzyme, then the brain mitochondria used in the preparation of inner and outer membranes contain 60% microsomal contamination, calculated on a weight basis. A tenfold increase in the concentration of DPNH had no effect on the microsomal RIDCR activity. On the other hand, assay of the exclusively microsomal enzyme, TPNH–cytochrome *c* reductase, revealed that washed brain mitochondria do not contain this enzyme, whereas the microsomes do contain a sizeable amount (Table VI). An attempt was made to wash the RIDCR activity from the mitochondria (Table VII). Only 64% of the RIDCR activity found in the first mitochondrial pellet could be removed after six washes with 20 volumes of 0.3 M mannitol–0.1 mM EDTA. This is in contrast to the microsomal enzyme TPNH–cytochrome *c* reductase which is nearly completely removed after only two washes. A small increase in the specific activity of mitochondrial RIDCR was observed during the washing procedure. This could be due to partial conversion of the rotenone sensitive into insensitive activity which has been observed when the assay is conducted in phosphate buffer. The conversion does not significantly affect the results, however, since intact brain mitochondria exhibit only a 10% increase in DPNH–

TABLE VI: Comparison of TPNH–Cytochrome *c* Reductase and RIDCR in Rat Brain Mitochondria and Microsomes.

Enzyme	Sp Act. of Microsomes (units/mg) (1)	Sp Act. Mitocondria (units/mg) (2)	Microsomal Contamination[a] (%)
TPNH–cytochrome *c* reductase	21	0	0
RIDCR	148	89	60

[a] Assuming that RIDCR and TPNH–cytochrome *c* reductase are strictly microsomal enzymes, the per cent contamination of mitochondria by microsomes was calculated as the specific activity of the mitochondria/specific activity of the microsomes × 100.

TABLE VII: The Washing Off of Enzyme Activity from Mitochondria.[a]

	RIDCR		Hexokinase		TPNH–Cytochrome *c* Reductase	
	% of Residual Act. Washed Out	Sp Act. (units/mg)	% of Residual Act. Washed Out	Sp Act. (units/mg)	% of Residual Act. Washed Out	Sp Act. (units/mg)
First mitochondrial pellet		67.3		0.30		4.5
Washed twice at 5000*g*	39	72.3	43	0.29	89	1.7
Washed twice at 10,000*g*	35	68.8	23	0.34	100	0
Washed twice at 15,000*g*	0	88.6	2	0.41		
Microsomes		147.5		0.06		21

[a] A crude rat brain mitochondrial pellet was washed a total of six times with 20 volumes of 0.3 M mannitol–0.1 mM EDTA (pH 7.4) at the indicated centrifugal forces.

cytochrome *c* reductase activity when rotenone is omitted from the assay medium. This is in contrast to an eightfold increase when mitochondria are first subjected to sonication for 3 min at the highest setting in a Branson probe sonifier. Controlled sonication, as used in the preparation of inner and outer membranes, resulted in a 55% increase in RIDCR activity.

Electron Microscopy of Submitochondrial Fractions Prepared by the Combined Swelling, Shrinking, and Sonication of Rat Brain Mitochondria. The electron micrographs are of rat brain mitochondria prepared by the method of Ozawa *et al.* (1966). The inner and outer membranes were prepared in the same manner as described for beef brain, and the distribution of enzyme activities among the fractions was found to be identical with beef brain mitochondria.

Inspection of three separate mitochondrial fractions with the electron microscope, after fixation and embedding, revealed that the principal contamination consisted of nerve endings. Some of these contained small mitochondria and small vesicular profiles, while others contained only the small synaptic vesicles (Figure 1). In negatively stained preparations the whole mitochondria were composed of an outer membrane which was free of projections, and an inner membrane, disposed in folds, along which numerous projections could be seen. The two inner membrane preparations examined consisted predominantly of large membranous vesicles, some of which contained inner protrusions suggestive of cristae (Figure 2). The majority of these vesicles were devoid of contents, but some contained flocculent material of the density of mitochondrial matrix. After negative staining, membranes with regularly arranged projections were seen. The outer membrane fractions examined consisted of smaller membrane-bound vesicles (Figure 3). There were occasional large myelin figures and scattered small electron dense particles. These latter were not attached to the membranes, and were smaller than ribosomal particles (75–100 Å). In negative stain the majority of the membranes were devoid of projections. The microsomal fraction contained similar small vesicles, but many had attached electron dense particles approximately 150°–200 Å in diameter and these particles were also seen free of membranes, both singly and in clusters.

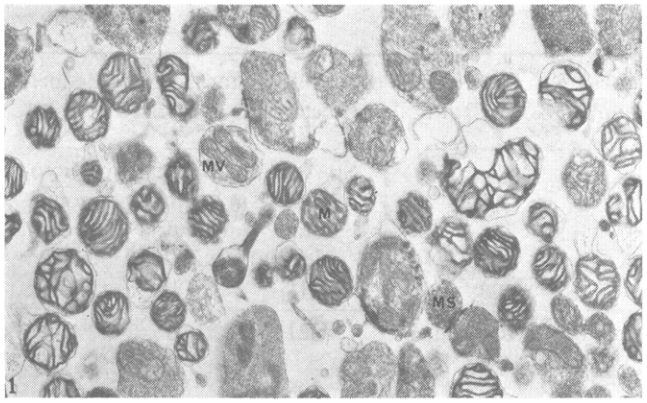

FIGURE 1: Whole mitochondrial pellet derived from rat brain. Mitochondria (M) show some dilitation of the intermembranous and intercritsal compartments with occasional blebbing of the outer membrane. In addition to individual mitochondria, mitochondria within membrane-limited sacs containing small vesicles (MV) and membrane-limited sacs containing only vesicles (SV), interpreted as synaptic vesicles, are also present, ×11,300.

Discussion

The results obtained using three different methods for the isolation of inner and outer membranes of brain mitochondria will be discussed with reference to the problem of enzyme localization in these membranes. The main difficulty has been in the assignment of properties, both morphological and biochemical, which would define the outer mitochondrial membrane fraction regardless of the method used to isolate it.

In the present report, the digitonin method of Schnaitman *et al.* (1967) which is said to rely on the presence of larger amounts of cholesterol in the outer than in the inner membrane of rat liver mitochondria did not result in the release of the outer membrane marker, monoamine oxidase, nor the enzyme under study, brain mitochondrial hexokinase. However, it cannot be concluded from these results that separation did not occur since the submitochondrial location of monoamine oxidase is the subject of a great deal of controversy. For example, using the digitonin method for the preparation of

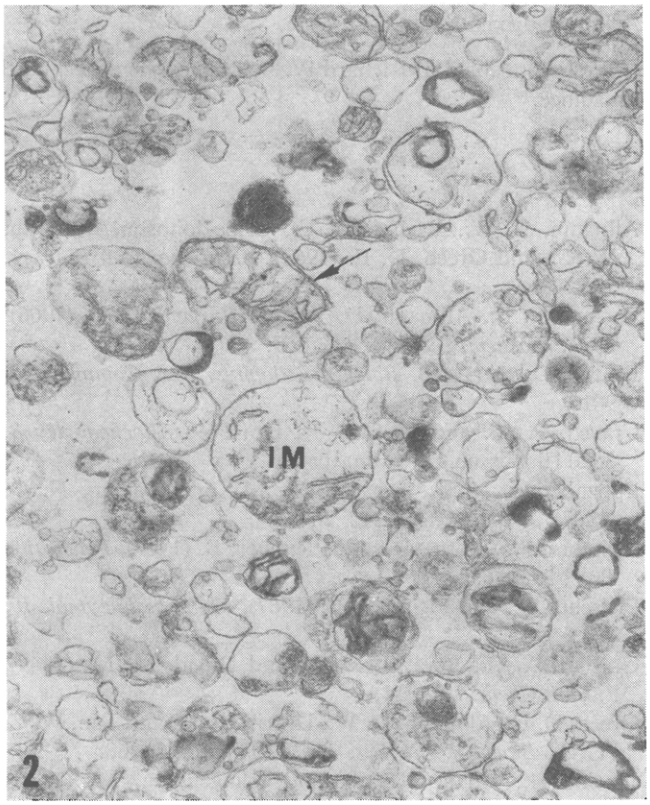

FIGURE 2: Inner membrane fraction derived from rat brain. Membrane-limited vesicular images predominate (IM). Many of these are identifiable as mitochondrial in origin by infoldings resembling cristae. The majority of these are devoid of matrix, and most are surrounded by a single membrane, although some still have segments of the outer membrane (arrow); ×18,800.

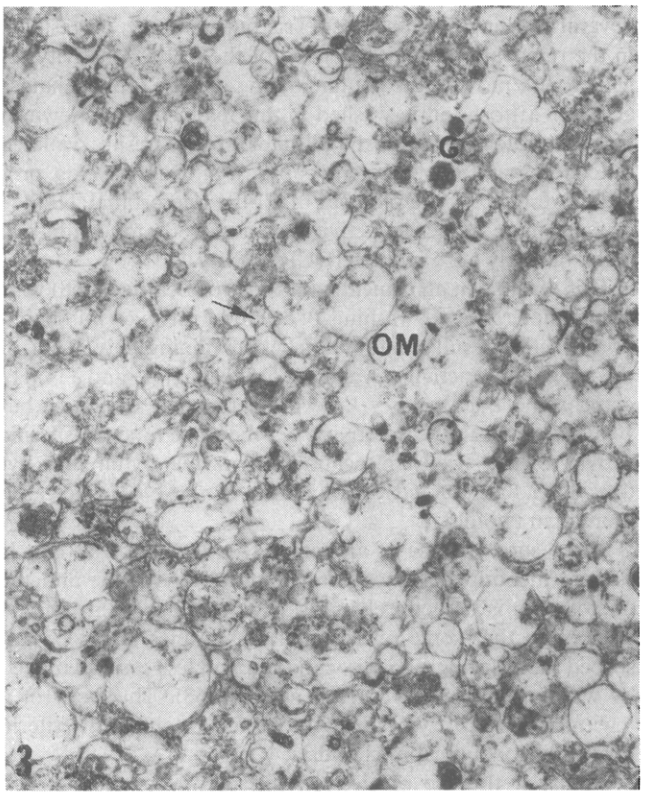

FIGURE 3: Outer membrane fraction derived from rat brain. Small and larger vesicles devoid of cristae predominate (OM). Some dense granules (G) and flocculent material are also seen. A small particle (arrow) 75–100 Å in diameter is also scattered throughout the fraction, but is not membrane bound; ×18,800.

inner and outer membranes of rat liver mitochondria and benzylamine as substrate for the assay of monoamine oxidase, Schnaitman *et al.* (1967) have localized this enzyme on the outer mitochondrial membrane, while Rendon and Waksman (1969) have localized it on the inner membrane. In addition, using phospholipase A digestion for the preparation of inner and outer membranes and [^{14}C]tyramine as substrate for the assay of monoamine oxidase, Green *et al.* (1968) have localized this enzyme on the inner mitochondrial membrane. The use of [^{14}C]tyramine eliminates the complication arising from the further oxidation of bezaldehyde to benzoic acid or its reduction to benzyl alcohol when benzylamine is used as substrate (Green *et al.*, 1968). However, Schnaitman and Greenawalt (1968) have reported that the localization of monoamine oxidase in the outer membranes of their preparations is not affected by the use of the assay involving [^{14}C]tyramine. Finally, Tipton (1967) has also assayed monoamine oxidase by the oxidation of tyramine and has found it in the outer membrane of rat liver mitochondria prepared by phospholipase A digestion.

A second enzyme, RIDCR, has also been used as a marker for the outer mitochondrial membrane (Sottocasa *et al.*, 1967). In the present report this enzyme was used as a marker for the outer membrane of brain mitochondria prepared by phospholipase A digestion (Bachmann *et al.*, 1966) and the combined swelling, shrinking, and sonication method (Sottocasa *et al.*, 1967). Phospholipase A acts by releasing fatty acids from the A position of phospholipid molecules. When beef heart mitochondria are treated with this enzyme only the outer membrane is removed while the inner membrane remains intact (Bachmann *et al.*, 1966). This method has also been applied to rat liver mitochondria (Tipton, 1967; Allmann *et al.*, 1968). Although both workers claim to have separated inner and outer membranes of mitochondria by the same method, they do not agree on the localization of monoamine oxidase in these membranes. Phospholipase A digestion has also been applied unsuccessfully to rat brain mitochondria (Tipton, 1967). In the present report, phospholipase A digestion could not be used to separate inner and outer membranes of brain mitochondria because of the poor recovery of the outer membrane marker enzyme, RIDCR. The small amount which was recovered was found in the inner membrane fractions even after prolonged digestion.

At any rate the use of RIDCR as a marker for the outer mitochondrial membrane has also been criticized. Sottocasa *et al.* (1967) assayed three microsomal enzymes in the mitochondria and microsomes, including glucose 6-phosphatase, and concluded from the ratios of the specific activities of these enzymes in the microsomes to that in the mitochondria that RIDCR is both a mitochondrial and a microsomal enzyme. However, other evidence has been presented by Green *et al.* (1968) and Allmann *et al.* (1968) to indicate that the mitochondrial RIDCR activity is due to microsomal contamination.

Several washings with sucrose–Tris buffer at pH 7.8 resulted in the removal of a major portion of the RIDCR activity together with most of the contaminating glucose 6-phosphatase activity. Mitochondria washed in this manner were shown to be morphologically indistinguishable from freshly isolated mitochondria. In the present paper, a similar washing procedure resulted in the removal of all the microsomal TPNH–cytochrome *c* reductase activity from a crude mitochondrial pellet but only 64% of the RIDCR activity.

This result is in agreement with those of Beattie (1968) who found that repeated washing of a crude rat liver mitochondrial fraction results in a final pellet containing only 3–4% of the microsomal activity of glucose 6-phosphatase and TPNH–cytochrome *c* reductase, known microsomal enzymes, but 17–24% of the RIDCR activity. At the same time the specific activity of the strictly mitochondrial enzyme, kynurenine hydroxylase, first studied by Okamoto *et al.* (1967), increased more than twofold.

To be sure, the use of an enzyme marker which at best is present in both the outer mitochondrial membrane and the microsomes is not entirely satisfactory. It has been pointed out before that some microsomal membranes might be adsorbed more strongly to mitochondria and less easily removed by washing procedures (Green *et al.*, 1968). The assay of a few microsomal enzymes would then not be sufficient criteria for the purity of the mitochondria.

Similarly, the use of electron microscopy to characterize submitochondrial fractions has been complicated by the fact that microsomal membranes are not easily distinguished from outer mitochondrial membranes. (Compare, for example, the work of Green *et al.*, 1968, and Parsons *et al.*, 1967.) Therefore, it is important to use a mitochondrial preparation which is relatively free from microsomal contamination.

Although the procedure used to isolate rat brain mitochondria resulted in a preparation contaminated with nerve ending particles and encapsulated nonmitochondrial particulate material, as demonstrated in Figure 1, the beef brain mitochondrial preparation with which most of the numerical data were obtained has previously been shown to be quite pure (Stahl *et al.*, 1963).

In view of the numerous difficulties associated with enzyme localization, a few of which have been presented here, it is not possible to draw a firm conclusion regarding the subcellular localization of brain hexokinase. However, we feel that the swelling, shrinking, and sonication method gave the most satisfactory results of the three procedures tried for the following reasons. (1) The outer membrane marker enzyme was shown not to be washed off by an extensive procedure which resulted in the removal of all of the TPNH–cytochrome *c* reductase activity, a result consistent with the idea that RIDCR is not a microsomal contaminant. (2) The mitochondrial preparation used as the starting material has been shown to be nearly free of contaminating nonmitochondrial particulate material. (3) The outer membrane marker enzyme, RIDCR, was recovered in high yield in the outer mitochondrial membrane fraction. (4) The increase in specific activity of the RIDCR was the same as that for hexokinase in the outer membrane as compared with the intact mitochondria. From these results it is tentatively concluded that hexokinase is located on the outer mitochondrial membrane.

Acknowledgments

We are indebted to Richard W. Bell for excellent technical assistance.

References

Allmann, E. W., Bachmann, E., Orme-Johnson, M., Tan, W. C., and Green, D. E. (1968), *Arch. Biochem. Biophys. 125*, 981.

Bachmann, E., Allmann, D. W., and Green, D. E. (1966), *Arch. Biochem. Biophys. 115*, 153.

Beattie, D. S. (1968), *Biochem. Biophys. Res. Commun. 31*, 901.

Craven, P. A., and Basford, R. E. (1969), *Biochemistry 8*, 3520 (this issue, preceding paper).

Eppenberger, H. E., Dawson, D. M., and Kaplan, N. O. (1967), *J. Biol. Chem. 242*, 204.

Fleischer, S., Causa, A., and Fleischer, B. (1964), *Federation Proc. 23*, 486.

Fleischer, S., and Fleischer, B. (1967), *Methods Enzymol. 10*, 423.

Gornall, A. G., Barawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Green, D. E., Allmann, D. W., Harris, R. A., and Tan, W. C. (1968), *Biochem. Biophys. Res. Commun. 31*, 368.

Jacobs, H., Heldt, H. W., and Klingenberg, M. (1964), *Biochem. Biophys. Res. Commun. 16*, 516.

Lea, M. A., and Weber, G. J. (1968), *J. Biol. Chem. 243*, 1096.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Okamoto, H., Yamamoto, S., Nozaki, M., and Hayaishi, O. (1967), *Biochem. Biophys. Res. Commun. 26*, 309.

Ozawa, K., Seta, K., Takeda, H., Ando, K., Harida, H., and Araki, C. (1966), *J. Biochem. (Tokyo) 59*, 501.

Parsons, D. F., Williams, G. R., Thompson, W., Wilson, D. F., and Chance, B. (1967), *in* Symposium on Mitochondrial Structure and Compartmentation, Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., Ed., Bari, Italy, Adriatric Enditrice, p 29.

Rendon, A., and Waksman, A. (1969), *Biochem. Biophys. Res. Commun. 35*, 324.

Rose, I. A., and Warms, J. V. B. (1967), *J. Biol. Chem. 242*, 1635.

Schnaitman, C., Erwin, V. G., and Greenawalt, J. W. (1967), *J. Cell Biol. 32*, 719.

Schnaitman, C., and Greenawalt, J. W. (1968), *J. Cell Biol. 38*, 158.

Schwartz, G. P., and Basford, R. E. (1967), *Biochemistry 6*, 1070.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. (1967), *J. Cell Biol. 32*, 415.

Stahl, W. L., Smith, J. C., Napolitano, L. M., and Basford, R. E. (1963), *J. Cell Biol. 19*, 293.

Tipton, K. F. (1967), *Biochim. Biophys. Acta 135*, 910.

Vogt, M. T., and Basford, R. E. (1968), *J. Neurochem. 15*, 1313.

Wilson, J. E. (1967), *Biochem. Biophys. Res. Commun. 28*, 123.

Wilson, J. E. (1968), *J. Biol. Chem. 243*, 3640.