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Preparation and Properties of Mitochondria Derived from Synaptosomes

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1. A method has been developed whereby a fraction of rat brain mitochondria (synaptic mitochondria) was isolated from synaptosomes. This brain mitochondrial fraction was compared with the fraction of 'free' brain mitochondria (non-synaptic) isolated by the method of Clark & Nicklas (1970). (*J. Biol. Chem.* **245**, 4724–4731). Both mitochondrial fractions are shown to be relatively pure, metabolically active and well coupled. 2. The oxidation of a number of substrates by synaptic and non-synaptic mitochondria was studied and compared. Of the substrates studied, pyruvate plus malate was oxidized most rapidly by both mitochondrial populations. However, the non-synaptic mitochondria oxidized glutamate plus malate almost twice as rapidly as the synaptic mitochondria. 3. The activities of certain tricarboxylic acid-cycle and related enzymes in synaptic and non-synaptic mitochondria were determined. Citrate synthase (EC 4.1.3.7), isocitrate dehydrogenase (EC 1.1.1.41) and malate dehydrogenase (EC 1.1.1.37) activities were similar in both fractions, but pyruvate dehydrogenase (EC 1.2.4.1) activity in non-synaptic mitochondria was higher than in synaptic mitochondria and glutamate dehydrogenase (EC 1.4.1.3) activity in non-synaptic mitochondria was lower than that in synaptic mitochondria. 4. Comparison of synaptic and non-synaptic mitochondria by rate-zonal separation confirmed the distinct identity of the two mitochondrial populations. The non-synaptic mitochondria had higher buoyant density and evidence was obtained to suggest that the synaptic mitochondria might be heterogeneous. 5. The results are also discussed in the light of the suggested connexion between the heterogeneity of brain mitochondria and metabolic compartmentation.

A variety of observations, both *in vivo* and *in vitro* suggest that the metabolism of the tricarboxylic acid-cycle intermediates and related metabolites such as glutamate and 4-aminobutyrate may be compartmented in the mammalian brain. The evidence for proposing the metabolic compartmentation of these metabolites has been extensively and comprehensively reviewed (Berl & Clarke, 1969; Balázs & Cremer, 1973). Metabolic compartmentation may reflect at one level preferential penetration of certain cells or subcellular regions by the administered tracer substrate and at another level the distribution of enzymes in the cytosol and various particulate fractions (Berl, 1973). The latter manifestation of metabolic compartmentation can be readily discerned when one examines the numerous observations that suggest that brain mitochondria may be heterogeneous (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Balázs *et al.*, 1966; Neidle *et al.*, 1969; Blokhuis & Veldstra, 1970; Lai *et al.*, 1975). As the metabolism of the tricarboxylic acid-cycle intermediates and related metabolites is closely associated with mitochondria it is therefore pertinent to examine whether or not the metabolism of these organelles *in vitro* reflects the metabolic compartmentation of these compounds observed both *in vivo* and *in vitro*.

Studies on the heterogeneity of brain mitochondria have been largely confined to centrifugation of brain homogenates on sucrose density gradients by either swing-out or zonal rotor. These investigations have led to the recognition of at least two populations of mitochondria; those which appear in the density gradient at the generally accepted banding density of 'free' mitochondria (i.e. nonsynaptic mitochondria) and which are associated with the highest activities of glutamate dehydrogenase, succinic semialdehyde dehydrogenase and 4-aminobutyrate transaminase, and those associated with the banding density of synaptosomes (synaptic mitochondria) which appear to be associated with the highest activities of NAD–isocitrate dehydrogenase and glutaminase (Salganicoff & De Robertis, 1965; Van Kempen *et al.*, 1965; Balázs *et al.*, 1966; Salganicoff & Koeppe, 1968; Neidle *et al.*, 1969). More recent studies have sug-

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gested even more complex patterns of mitochondrial enzyme distributions (Blokhuis & Veldstra, 1970; Reijnierse, 1973).

Although these studies have been useful they do, however, suffer from two major drawbacks; (*a*) the mitochondria have been centrifuged for considerable periods in very hyperosmotic sucrose media, which leads to their isolation in a very poor metabolic state (Bradford, 1969; Lai, 1975) (e.g. they possess very poor respiratory control); (*b*) the measurement of mitochondrial enzyme specific activities, particularly in the synaptosomal fraction, is obscured by the presence of considerable protein contamination of a non-mitochondrial origin (Whittaker, 1968; Barondes, 1974; Kornguth, 1974). In the present paper we report studies in which an attempt to overcome these problems has been made. A preparative procedure has been devised, derived in part from the previously published methods of Clark & Nicklas (1970) and Cotman & Matthews (1971), whereby a fraction of metabolically active, well coupled and relatively pure rat brain mitochondria may be isolated from an osmotically lysed synaptosomal fraction. The ability of these 'synaptic' mitochondria to metabolize tricarboxylic acid-cycle and related metabolites is compared with similar properties of a rat brain mitochondrial population of non-synaptic origin ('free'). The results are discussed in relation to the metabolic compartmentation of the tricarboxylic acid cycle in brain.

Experimental

Materials

All laboratory chemicals were AnalaR grade and, except where otherwise stated, were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., including bovine plasma albumin and Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid)]. ADP, CoA, NAD^+, NADH, $NADP^+$, NADPH, 2-oxoglutarate and succinate were purchased from Boehringer Corp. (London) Ltd. (Bell Lane, Lewes, E. Sussex BN7 1LG, U.K.). Acetylthiocholine iodide, glutamic acid, glutamine, DL-isocitrate, mannitol, malic acid and Tris were obtained from Sigma (London) Chemical Co. (Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, U.K.). Ficoll was obtained from Pharmacia, Uppsala, Sweden, and purified by dialysis against double-glass-distilled water for at least 5h before use. Pyruvate was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and was twice distilled under vacuum and stored at −20°C before use. All reagents were made up in double-glass-distilled water.

Animals

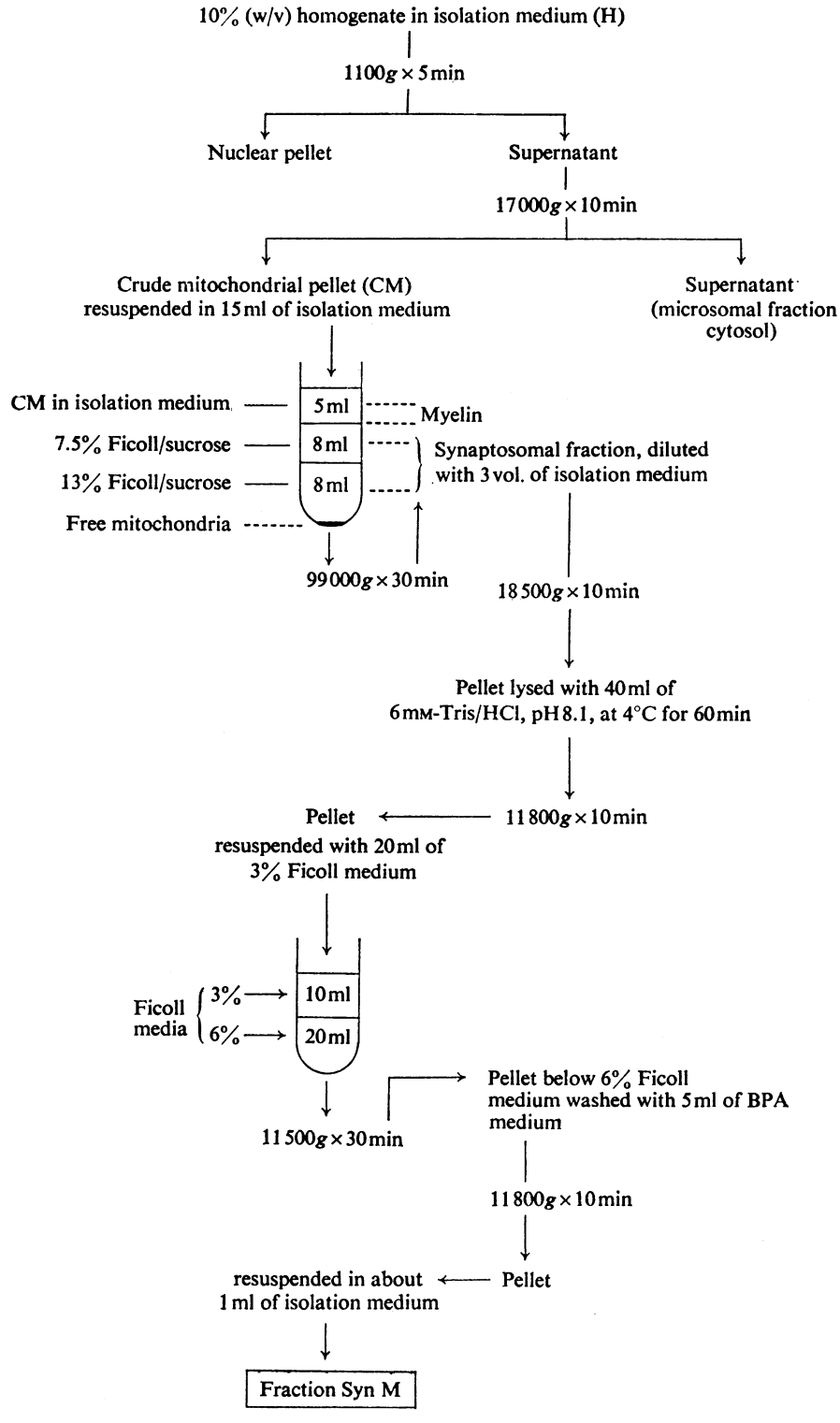
Adult male rats (150–190g) of the Wistar strain were used in all the experiments. Rats were killed by decapitation. The forebrain of the animal was rapidly removed by transecting the brain at the level of the two colliculi and that part of the brain rostral to this transection, except the olfactory bulbs, was taken.

Preparation of non-synaptic mitochondria from rat brain

A fraction of non-synaptic rat brain mitochondria was prepared by the method of Clark & Nicklas (1970) with the following minor modification. After centrifuging a 15% (w/v) homogenate of eight forebrains at 2000*g* for 3min, the supernatant was decanted and this supernatant was re-centrifuged at 2000*g* for 3min. The latter supernatant was then centrifuged at 12500*g* for 8min. The crude mitochondrial pellet was resuspended with 12ml of the 3% Ficoll medium (see below) and 6ml of this suspension was layered on to 25ml of the 6% Ficoll medium and centrifuged at 11500*g* for 30min. The rest of the procedure was essentially the same as described by Clark & Nicklas (1970).

Preparation of a fraction of synaptosomally derived rat brain mitochondria

The forebrains of eight adult male rats were used in each experiment. The following homogenization and fractionation procedures were carried out at 4°C. A 20% (w/v) homogenate in isolation medium [0.32M-sucrose, 1mM-EDTA (potassium salt), 10mM-Tris/HCl, pH7.4] was made in a motor-driven Potter homogenizer (total clearance 1mm) with ten up-and-down strokes, diluted to about 10% (w/v) with isolation medium and fractionated as outlined in Scheme 1. The 7.5 or 13% Ficoll/sucrose medium contained: 7.5% or 13% (w/v) Ficoll, 0.32M-sucrose, 1mM-EDTA (potassium salt) and 10mM-Tris/HCl, pH7.4. The 3% Ficoll medium contained: 3% (w/w) Ficoll, 0.12M-mannitol, 30mM-sucrose, 25μM-EDTA (potassium salt) and 5mM-Tris/HCl, pH7.4, whereas the 6% Ficoll medium contained: 6% (w/w) Ficoll, 0.24M-mannitol, 60mM-sucrose, 50μM-EDTA (potassium salt) and 10mM-Tris/HCl, pH7.4. The BPA medium consisted of 1ml of bovine plasma albumin (containing 10mg of albumin/ml) and 19ml of isolation medium (see above), giving a final concentration of 0.5mg of albumin/ml of the BPA medium. With one exception all centrifugation was carried out in an MSE 18 high-speed centrifuge; the separation of the crude mitochondrial fraction on the 7.5% Ficoll/sucrose/13% Ficoll/sucrose gradient was carried out in an MSE 50 ultracentrifuge with a 3×23ml swing-out rotor. Other fractions, e.g. non-synaptic mitochondria, microsomal fraction or myelin, may also be isolated, if required, by this technique (see Scheme I).



Scheme 1. *Preparation of synaptosomally derived rat brain mitochondria (fraction Syn M)*

Comparison of two fractions of rat brain mitochondria by zonal separation

Preparation of samples for rate-zonal separation. Two samples were used for rate-zonal separation.

One fraction of non-synaptic rat brain mitochondria was prepared from 16 rats by the method of Clark & Nicklas (1970). The washed mitochondrial material was resuspended in 20.5ml of 0.32M-sucrose buffered with 10mM-Tris/HCl, pH7.4, and 18.5ml of this suspension was used as one of the samples for rate-zonal centrifugation.

A crude semi-purified preparation of synaptosomally derived mitochondria was made from eight rats essentially as Scheme 1, except that the last Ficoll-gradient procedure and wash was left out. The mitochondrial pellet was resuspended in 23.5ml of 0.32M-sucrose/10mM-Tris/HCl, pH7.4, and 20ml of this was used as the sample for rate-zonal separation.

Separation with the HS-zonal rotor. Rate-zonal separation was carried out in a MSE HS zonal rotor in a MSE 18 centrifuge at 4°C. The discontinuous sucrose gradient used consisted of 100ml of 15% (w/w) and 50ml each of 20, 22.5, 30, 32.5, 35 and 38% (w/w) sucrose; 5% (w/w) sucrose was the overlay and 40% (w/w) sucrose was the cushion. All sucrose was buffered with 10mM-Tris/HCl, pH7.4. The introduction of gradient, sample, cushion and overlay was done with a peristaltic pump. The gradient was introduced via the peripheral inlet with the rotor spinning at 1000rev./min. The sample was introduced via the centre inlet followed by the overlay (about 170ml). The rotor was then accelerated to 10000rev./min (8400*g*) and maintained at this speed for 60min. The rotor was decelerated to 1000rev./min and the separated fractions were forced through the centre inlet by pumping 50% (w/w) sucrose into the rotor via the peripheral inlet. Usually 40–45 fractions (15ml each) were collected.

The sucrose densities of fractions were checked by determining their refractive index with a refractometer. Protein in fractions was assayed by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard. Blanks with appropriate sucrose concentrations were used where applicable.

Enzyme assays

These were carried out at 25°C by using a Unicam SP.800 recording spectrophotometer. All enzyme assays were carried out in the presence of excess of substrate and cofactor concentrations and rates were proportional to amount of enzyme protein. Where Triton is present, the concentration used has been established to be that which gives maximal enzyme activity with no inhibition.

Pyruvate dehydrogenase (EC 1.2.4.1) and citrate synthase (EC 4.1.3.7) were measured as described by Clark & Land (1974).

NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase (EC 1.1.1.41 and EC 1.1.1.42) were assayed by a method essentially similar to that of Plaut (1969). The reaction mixture contained (final concns.): 0.7mM-$MnCl_2$, 0.1% (v/v) Triton X-100, 35mM-Tris/HCl, pH7.2, 0.71mM-ADP, 0.7mM-NAD^+ or 0.2mM-$NADP^+$ and 64mM-DL-isocitrate. The rate of NADH or NADPH formation was measured at 340nm with 0.2–1.0mg of mitochondrial protein.

Fumarase (EC 4.2.1.2) activity was determined by a method which had been modified from that of Racker (1950). The assay mixture contained (final concentrations) 100mM-potassium phosphate buffer, pH7.4, 0.1% (v/v) Triton X-100, 50mM-potassium malate and about 0.4mg of mitochondrial protein. The reaction was started with the addition of malate and the increase in E_{240} was measured.

NAD^+-linked malate dehydrogenase (EC 1.1.1.37) activity was measured by a modification of the method of Ochoa (1955). The reaction mixture contained (final concentrations) 0.1M-potassium phosphate buffer, pH7.4, 0.16mM-NADH, 0.16% (v/v) Triton X-100, 133μM-oxaloacetate (freshly prepared just before use, pH adjusted to 6.7 with $NaHCO_3$) and about 20μg of mitochondrial protein. The reaction was commenced with the addition of oxaloacetate and the first minute of linear NADH oxidation was measured at 340nm.

NAD^+-linked and $NADP^+$-linked glutamate dehydrogenase (EC 1.4.1.3) activities were assayed by a modification of the method of Schmidt (1963), in the direction of glutamate formation, in a reaction mixture containing (final concentrations) 0.1M-phosphate/Tris, pH7.7, 162mM-ammonium acetate, 1mM-EDTA (potassium salt), 0.167mM-NADH or 0.33mM-NADPH, 1.7mM-ADP, 0.16% (v/v) Triton X-100 and 10mM-2-oxoglutarate. The initial linear rate of oxidation of NADH or NADPH in the first minute was measured at 340nm.

Lactate dehydrogenase (EC 1.1.1.27) activity was assayed as described by Clark & Nicklas (1970), and acetylcholinesterase activity (EC 3.1.1.7) was determined by the method of Ellman *et al.* (1961).

Proteins were determined either by the biuret method (Gornall *et al.*, 1949) or by the method of Lowry *et al.* (1951) with bovine plasma albumin as standard.

Mitochondrial respiration experiments

Oxygen uptakes were measured polarographically with a Clark-type micro-electrode as described by Clark & Nicklas (1970) and Clark & Land (1974). The media used as a routine contained either 5mM- or 100mM-K^+. The 5mM-K^+ medium consisted of 225mM-mannitol, 75mM-sucrose, 5mM-phosphate/Tris, pH7.4, 10mM-Tris/HCl, pH7.4, 0.05mM-EDTA (potassium salt) and 5mM-KCl. The 100mM-K^+ medium contained: 75mM-mannitol, 25mM-sucrose,

5mM-phosphate / Tris, pH7.4, 10mM-Tris / HCl, pH7.4, 0.05mM-EDTA (potassium salt) and 100mM-KCl.

State 3 conditions were initiated by the addition of 0.5mM-ADP in the presence of substrate(s). The respiratory control ratio was the ratio of the state-3 rate to the state-4 rate (Chance & Williams, 1956).

Results

Electron micrographs of rat brain mitochondria prepared by the Clark & Nicklas (1970) method revealed that this fraction consisted predominantly of mitochondria with well-defined cristal structure; very few synaptosomes were present and myelin-like particles were apparently absent (cf. Clark & Nicklas, 1970). The fact that the mitochondria of synaptic origin may be separated so definitely from the 'free' mitochondria (fraction M) in the zonal-separation experiments discussed below strongly suggests that the 'free' mitochondria (fraction M) are derived from alternative cellular localizations such as the neuronal and glial cell bodies.

The survey of a number of electron micrographs of the fraction of synaptosomally derived rat brain mitochondria suggests that mitochondria with distinct condensed cristae comprised between 85 and 95% of the total particles in this fraction; the only contaminants appeared to be membrane vesicles.

These observations by electron microscopy are consistent with the assessment of the degree of purity of these fractions by marker-enzyme assays discussed below.

Protein and enzyme distribution

Table 1 shows the distribution of protein and certain marker enzymes in the synaptosomally derived and nonsynaptic rat brain mitochondria relative to those in the homogenate. Lactate dehydrogenase may be considered as a synaptoplasmic (cytosolic) marker enzyme (Johnson & Whittaker, 1963) and acetylcholinesterase as a marker for synaptic and other membranous material (Cotman & Matthews, 1971). The low recovery of these two enzymes (less than 0.5% of the homogenate activity) in the mitochondrial fractions suggest that these fractions are minimally contaminated with synaptosomal or membranous materials. Further, both the high mitochondrial to non-mitochondrial enzyme ratios (Table 1) and the low specific activities of lactate dehydrogenase and acetylcholinesterase in both mitochondrial fractions (Table 4) confirm the relative purity of these preparations.

Substrate oxidation by synaptic and non-synaptic mitochondria

The oxidation of a variety of substrates by non-synaptic mitochondria has been extensively studied

Table 1. *Distribution of enzymes and protein in rat brain mitochondria prepared by the Clark & Nicklas (1970) method (fraction M) and synaptosomally derived rat brain mitochondria (fraction Syn M) as compared with the homogenate*

Homogenates were prepared from eight rat brains and enzyme assays were performed as described in the Experimental section. Protein was measured by the method of Gornall *et al.* (1949). All values are the means of duplicates in each individual preparation with the number of separate preparations in parentheses. The results are expressed as mean±S.D.

$$\frac{\%\text{Enzyme}}{\%\text{LDH(AChE)}} = \frac{\text{Total enzyme activity in fraction M (Syn M) expressed as \% of homogenate value}}{\text{cholinesterase) activity of same fraction expressed as a \% of homogenate value.}}$$

	Homogenate	Fraction M				Fraction Syn M			
	Total activity (μmol/min)	Total activity (μmol/min)	(% of) homogenate)	% Enzyme / % LDH	% Enzyme / % AChE	Total activity (μmol/min)	(% of homogenate)	% Enzyme / % LDH	% Enzyme / % AChE
NAD^+-isocitrate dehydrogenase	52.3±4.4 (2)	2.7±0.2 (4)	5.2	43.4	12.1	2.0±0.2 (2)	3.9	97.5	43.1
$NADP^+$-isocitrate dehydrogenase	21.5±0.9 (2)	0.7±0.1 (4)	3.2	26.6	7.4	0.5±0.02 (2)	2.3	57.5	25.5
NAD^+-malate dehydrogenase	4568±199 (3)	154±20 (4)	3.4	28.3	7.9	114±6 (3)	2.5	62.5	27.8
NAD^+-glutamate dehydrogenase	233±17 (3)	11.2±0.8 (9)	4.81	20.4	11.2	13.2±0.4 (3)	5.7	142.0	63.1
Lactate dehydrogenase	1286±120 (6)	1.5±0.3 (5)	0.12	1.0	0.3	0.6±0.04 (2)	0.04	1.0	0.4
Acetylcholinesterase	114±1 (2)	0.5±0.06 (3)	0.43	3.6	1.0	0.1±0.01 (2)	0.09	2.3	1.0
Protein (mg)	1888±120 (8)	19.4±2.8 (26)	1.02			14.0±2.6 (10)	0.74		

Table 2. *Oxidation of substrates by non-synaptic mitochondria (fraction M) and synaptosomally derived brain mitochondria (fraction Syn M)*

All values are the means of at least two measurements in at least two distinct experiments. The state 3 respiration was induced by the addition of ADP (see the Experimental section). Respiratory control ratio = state 3 respiration/state 4 respiration.

		Respiration rate (ng-atoms of O/min per mg of protein)					
		Fraction M			Fraction Syn M		
Substrates used	K^+ concn. (mM)	State 4	State 3	Respiratory control ratio	State 4	State 3	Respiratory control rate
2.5mM-Malate+5mM-pyruvate	5	9	99	11	7	81	11.6
	100	36	166	4.6	30	143	4.8
2.5mM-Malate+	5	22	97	4.4	9	55	6.1
2.5mM-glutamate	100	36	113	3.1	30	58	1.9
2.5mM-Malate+	5	31	32	1.03	14	36	2.6
2mM-4-aminobutyrate	100	30	40	1.3	32	41	1.3
2.5mM-Malate+	5	18	47	2.6	9	43	4.8
2.5mM-glutamine	100	24	57	2.4	29	47	1.6

Table 3. *Oxidation of substrates by synaptosomally derived rat brain mitochondria*

All values are means, with the numbers of experiments in parentheses; at least two determinations were carried out in each experiment.

	Oxygen uptake (ng-atoms of O/min per mg of protein)					
	5mM-K^+			100mM-K^+		
Substrates used	State 4	State 3	Respiratory control ratio	State 4	State 3	Respiratory control ratio
5mM-Pyruvate (1)	0	3	∞	12	60	5
2.5mM-Malate (1)	11	34	3.1	27	64	2.4
3.7mM-2-Oxoglutarate (2)	18	23	1.3	26	78	3.0
2.5mM-Glutamate (2)	7	24	3.5	26	51	1.9
2.5mM-Glutamine (2)	11	16	1.5	18	35	2.0
10.0mM-Succinate (2)	56	130	2.3	89	148	1.7
2.5mM-Malate+5mM-citrate (2)	13	80	6.2	34	110	3.2
2.5mM-Malate+3.7mM-2-oxoglutarate (2)	6	59	9.8	27	103	3.8
2.5mM-Malate+10mM-acetylcarnitine (2)	17	42	2.5	29	50	1.7
2.5mM-Malate+4mM-isocitrate (2)	18	55	3.1	36	65	1.8
2.5mM-Malate+1mM-3-hydroxybutyrate (2)	11	39	3.5	26	60	2.3
2.5mM-Glutamate+3mM-4-aminobutyrate (2)	11	25	2.3	22	61	2.8
2.5mM-Malate+10mM-acetate (2)	12	20	1.7	23	27	1.2
2.5mM-Malate+10mM-acetate+5mM-pyruvate (1)	9	85	9.4	86	171	2.0

(Clark & Nicklas, 1970; Nicklas *et al.*, 1971). However, as a means of comparison of the metabolic integrity of the synaptic mitochondria with that of the non-synaptic mitochondria the results in Table 2 are presented. Both types of mitochondria are metabolically active and tightly coupled, showing respiratory-control ratios well above 4 with pyruvate and malate as substrates. Although the oxidation of most substrates by both mitochondrial types was slightly stimulated by an increase in K^+ concentration from 5mM to 100mM, this was most marked for pyruvate and malate, where in both mitochondrial populations the state-3 respiration was increased by some 70%. Insignificant differences in the ability to oxidize glutamine or 4-aminobutyrate were apparent between the two mitochondrial types. However, the non-synaptic mitochondria showed consistently higher rates of oxygen uptake in the presence of either pyruvate or glutamate plus malate than did the synaptic mitochondria.

Further indications of the variety of substrates oxidized by the synaptic mitochondria are shown in Table 3. As with the non-synaptic mitochondria (Clark & Nicklas, 1970) these mitochondria utilized

Table 4. *Enzyme activities in non-synaptic (fraction M) and synaptic (fraction Syn M) rat brain mitochondria*

The values quoted are the means±S.D. with the numbers of separate experiments in parentheses. In each experiment the enzyme activity was measured at at least two different enzyme concentrations. The significance of differences was calculated by using the Student's *t* test (*P* values are indicated in the Table). N.S., Not significant ($P>0.05$).

Enzymes	Activity (nmol/min per mg of mitochondrial protein) Fraction M		Fraction Syn M		*P* values
Pyruvate dehydrogenase	72±6	(14)*	49±4	(2)	<0.001
Citrate synthase	1070±104	(14)†	1063±105	(2)	N.S.
NAD^+–isocitrate dehydrogenase	141±13	(4)	145±29	(2)	N.S.
$NADP^+$–isocitrate dehydrogenase	34±7	(4)	34±1.3	(2)	N.S.
Fumarase	372±23	(2)	332±11	(2)	N.S.
NAD^+–malate dehydrogenase	7919±1055	(4)	8150±419	(3)	N.S.
NAD^+–glutamate dehydrogenase	578±44	(9)	945±28	(3)	≪0.001
$NADP^+$–glutamate dehydrogenase	490±39	(4)	741±37	(2)	0.002
Lactate dehydrogenase	79±17	(5)	41±3	(2)	—
Acetylcholinesterase	25±3.3	(3)	6.8±0.4	(2)	—

* Land (1974)
† Land & Clark (1973)

pyruvate plus malate and succinate most rapidly. Further, the synaptosomally derived mitochondria in the presence of malate oxidized citrate, 2-oxoglutarate, isocitrate, 3-hydroxybutyrate, glutamate and acetylcarnitine at rates 77, 72, 45, 42, 41 and 35% respectively of the rate with pyruvate and malate. Mitochondria of non-synaptic origin have been shown also to oxidize these substrates (Clark & Nicklas, 1970), but at rates which were approx. 50% of that for pyruvate and malate or succinate. Neither the synaptic mitochondria (Table 3) nor the non-synaptic mitochondria (Clark & Nicklas, 1970) utilized acetate very readily.

Tricarboxylic acid-cycle and related enzymes in synaptic and non-synaptic mitochondria

The activities of a number of enzymes of the tricarboxylic acid cycle in the two populations of mitochondria are shown in Table 4. These were assessed in order to have available some estimate of the total potential capabilities of both mitochondrial populations so as to relate this to flux measurements observed both in mitochondrial experiments *in vitro* and in compartmentation experiments *in vivo*.

As shown in Table 4, the activities of citrate synthase, NAD^+ and $NADP^+$–isocitrate dehydrogenase, fumarase and malate dehydrogenase in the two mitochondrial populations were similar. However, pyruvate dehydrogenase activity in non-synaptic mitochondria was significantly higher (approx. 47%, $P<0.001$) than in synaptic mitochondria, whereas the glutamate dehydrogenase (both NAD^+ and NADP-linked) activities were significantly lower (39 and 34% respectively $P<0.001$).

Rate-zonal centrifugation of synaptic and non-synaptic mitochondria

Rate-zonal centrifugation of both populations of mitochondria was carried out on a discontinuous sucrose gradient (15–40%, w/w) on an MSE HS zonal rotor as a means of (*a*) differentiating between the mitochondrial populations and (*b*) establishing their degree of purity and homogeneity. Fig. 1 shows the distribution of NAD^+–glutamate dehydrogenase, NAD^+–malate dehydrogenase, citrate synthase and protein in various fractions collected after zonal centrifugation of a non-synaptic mitochondrial preparation. The bulk of the activities of the enzymes studied and protein were localized in the region of the gradient where the sucrose concentration was 37.5% (w/w). Further, no detectable lactate dehydrogenase was present in any of the fractions. The sharpness and coincidence of the enzyme distribution, together with the lack of lactate dehydrogenase activity indicate that the non-synaptic mitochondrial population is one of a fair degree of homogeneity which is minimally contaminated with cytosol.

Zonal centrifugation under the same conditions as in Fig. 1 of a partially purified synaptic population of mitochondria, prepared from lysed synaptosomes as indicated in the methods section, is shown in Fig. 2. Most of the isocitrate dehydrogenase, citrate synthase and malate dehydrogenase enzyme activities were found in the gradient where the sucrose concentration was 28% (w/w), that is at a considerably lower density than the non-synaptic mitochondria. However, the glutamate dehydrogenase activity reached a peak at a sucrose concentration of 35% (w/w), suggesting a second mitochondrial population.

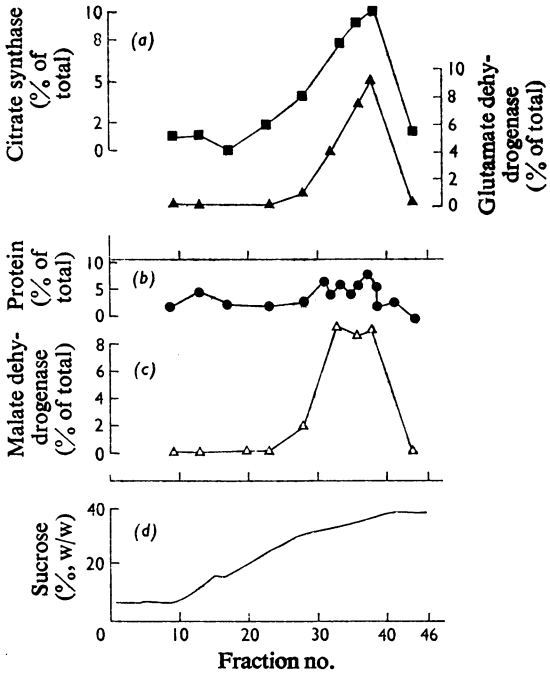


Fig. 1. *Rate-zonal separation of non-synaptic mitochondria*

Rat brain mitochondria were prepared by the method of Clark & Nicklas (1970) and centrifuged in a zonal rotor as outlined in the Experimental section. Fractions were collected and enzymes, protein and sucrose concentration assayed as indicated in the Experimental section. All values are the means of at least two determinations and are expressed as a percentage of the total enzyme (or protein) present in the mitochondrial sample before centrifugation. Recoveries were as follows: protein (●), 116%; NAD^+-glutamate dehydrogenase (▲), 87%; citrate synthase (■), 118%; malate dehydrogenase (△), 103%. Lactate dehydrogenase was not detectable in any of the fractions examined.

Despite the similarities between the positions on the sucrose gradient where this latter mitochondrial population and the non-synaptic mitochondria come to rest, it is unlikely that they are one and the same population, since the non-synaptic mitochondria possess a much lower glutamate dehydrogenase activity (Table 4). It is also noteworthy that some protein and lactate dehydrogenase activity was found at lower sucrose concentrations (6–22%, w/w). This suggests that the small amount of contamination associated with the preparation (Table 1) has been separated from the main synaptic-mitochondrial population, thus suggesting that the synaptic-mitochondrial population is relatively uncontaminated.

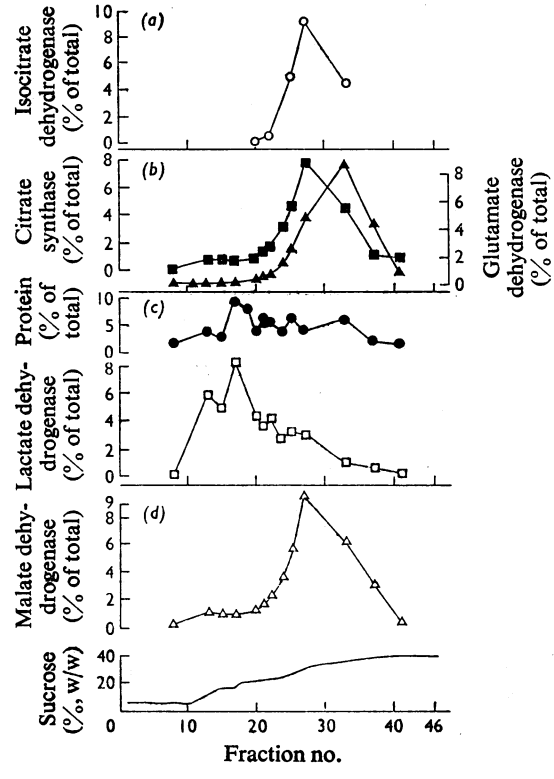


Fig. 2. *Rate-zonal separation of synaptic mitochondria derived from osmotically shocked synaptosomal fraction*

Rat brain mitochondria derived from synaptosomes were prepared as outlined in the Experimental section and subjected to zonal centrifugation. Fractions were collected, enzymes and protein assayed as previously indicated. All values are the means of at least two determinations and are expressed as a percentage of the total enzyme (or protein) present in the mitochondrial sample before centrifugation. Recoveries were: protein (●), 120%; NAD^+-glutamate dehydrogenase (▲), 95%; citrate synthase (■), 85%; malate dehydrogenase (△), 110%; NAD^+-isocitrate dehydrogenase (○), 75%; lactate dehydrogenase (□), 98%.

Thus under the conditions used in the zonal centrifugation the non-synaptic and synaptic mitochondria may be differentiated, the former having a higher buoyant density than the latter. Further, although the non-synaptic preparation is fairly homogeneous, the synaptic mitochondria may be less homogeneous and may include a sub-population of higher buoyant density and particularly high glutamate dehydrogenase activity. Indeed preliminary evidence (J. C. K. Lai and J. B. Clark, unpublished work) suggest that these synaptic mitochondria may be further resolved into at least two subpopulations.

Discussion

It has been a recognized problem to prepare brain mitochondria which are in a good metabolic state and which are essentially free from non-mitochondrial contaminants (for a detailed discussion see Clark & Nicklas, 1970). The Clark & Nicklas (1970) preparation of non-synaptic mitochondria is metabolically active and relatively pure. Thus it is highly suitable for metabolic studies. The position as regards the isolation of intraterminal mitochondria, is, however, far less satisfactory. A number of procedures have been used for subfractionating the synaptosomal fraction with the view of isolating various subsynaptosomal elements such as synaptic vesicles, synaptic plasma membranes, junctional complexes and intraterminal mitochondria (Whittaker *et al.*, 1964; De Robertis, 1967; Cotman & Matthews, 1971; Morgan *et al.*, 1971); consequently, any of these methods may be theoretically applicable for the isolation of intraterminal mitochondria. However, all these techniques involve sucrose-density-gradient centrifugation for the separation of mitochondria, and the latter were only partially characterized by using marker-enzyme assays. No attempt to assess the metabolic integrity of these mitochondria has been reported. In any event, as previously discussed (Clark & Nicklas, 1970; Lai, 1975), such a procedure as centrifugation in a hyperosmotic sucrose gradient invariably results in mitochondria which show little or no metabolic activity. For instance, mitochondria prepared by the Gray & Whittaker (1962) technique (Fraction C) showed, with pyruvate and malate as substrates, a respiration of 20 ± 1.3 ng-atoms of O/min per mg of mitochondrial protein ($n = 6$) in a 100mM K^+ medium and a respiratory control ratio of unity (i.e. uncoupled) (J. C. K. Lai & J. B. Clark, unpublished observations). It is therefore obvious that there is a need for the development of a method whereby metabolically active, well-coupled and relatively pure intraterminal mitochondria may be isolated in yields large enough for metabolic studies. Accordingly, a method is described here which does meet this requirement.

When the metabolism of tricarboxylic acid-cycle and related metabolites in synaptic mitochondria are compared with that in non-synaptic mitochondria, the following picture emerges. Although both populations of mitochondria are metabolically active and well coupled, non-synaptic mitochondria appear to oxidize pyruvate with malate at higher rates than those obtained with synaptic mitochondria. This is consistent with the fact that the activity of pyruvate dehydrogenase in non-synaptic mitochondria is higher than that in synaptic mitochondria (see Tables 2 and 4). In spite of the higher glutamate dehydrogenase activity in synaptic as compared with non-synaptic mitochondria, the latter oxidize glutamate plus malate at rates greater than those of the former. The suggestion that brain mitochondria oxidize glutamate primarily via the glutamate–oxaloacetate transaminase route may be relevant here (Balázs, 1965). Apart from glutamate and pyruvate, both synaptic and non-synaptic mitochondria also oxidize a wide range of substrates (see Table 3 and Clark & Nicklas, 1970). Whereas synaptic mitochondria oxidize citrate and 2-oxoglutarate, in the presence of malate, at rates slightly above those of non-synaptic mitochondria, the latter oxidize isocitrate at rates somewhat higher than those of the former (see Table 3 and Clark & Nicklas, 1970). Nonetheless, the activities of citrate synthase, isocitrate dehydrogenase and malate dehydrogenase in these two fractions of brain mitochondria are apparently similar: thus they do not appear to reflect the difference observed in the rates of oxidation of citrate and isocitrate by these two mitochondrial fractions. This suggests that caution must be exerted in relating $V_{max.}$ values for enzymes *in vitro* to metabolic compartmentation effects *in vivo* seen in whole brain (cf. Van den Berg, 1973).

The data presented here suggest that the mitochondria derived from synaptosomes are a distinct population from the mitochondria derived by the Clark & Nicklas (1970) method, particularly with respect to the metabolism of pyruvate and glutamate. There is, however, considerable difficulty in relating these two mitochondrial populations to the compartmentation of glutamate seen in brain (see Balázs & Cremer, 1973). Some correlation between the heterogeneity of brain mitochondria and metabolic compartmentation has been attempted (Van den Berg, 1973). As acetate, ammonia and 4-aminobutyrate are precursors of the 'small' glutamate pool (see Balázs & Cremer, 1973, for full discussion) and the distribution of the activities of acetyl-CoA synthetase, 4-aminobutyrate transaminase and glutamate dehydrogenase were similar in a sucrose gradient, it was postulated that these enzymes were preferentially localized in the mitochondria of the small compartment, which was correlated with the 'free' mitochondria (Van den Berg, 1973). In a similar fashion the 'large' glutamate compartment was assigned to those mitochondria derived from nerve endings containing relatively high activities of fumarase, citrate synthase and NAD^+–isocitrate dehydrogenase. One problem with the assigning of enzyme specific activities to the nerve ending mitochondria as separated in the experiments of Van den Berg (1973) and others is the probability of a considerable amount of non-mitochondrial protein in these fractions leading to artificially low enzyme specific activities. Thus Van den Berg (1973) reported that synaptic mitochondria have a low glutamate dehydrogenase activity relative to the 'free' mitochondria whereas the results reported here (Table 4)

show the opposite. By using the same approach as Van den Berg (1973), the data reported here would lead one to assign the 'small' glutamate compartment to the synaptic mitochondria. It is pertinent to notice that for the 'free' mitochondria there is considerable agreement both in this paper and elsewhere (Balázs, 1965; Salganicoff & De Robertis, 1965; Balázs *et al.*, 1966; Salganicoff & Koeppe, 1968; Van den Berg, 1973) for the actual specific activities for both glutamate dehydrogenase and other enzymes. The latter may be largely attributable to the fact that the methods for isolating 'free' brain mitochondria do not lead to excessive contamination by non-mitochondrial protein material.

However, possibly a more fundamental problem lies in the relationship between $V_{max.}$ enzyme activities and the metabolic fluxes in brain mitochondria. Although the use of $V_{max.}$ enzyme activities as being indicative of metabolic fluxes may be useful for constructing model systems, one must treat these correlations with care because mitochondrial metabolism and its control is influenced by a number of factors, including substrate availability and the phosphate and redox potential (Van Dam, 1973; Meijer & Van Dam, 1974). The availability of metabolically active tightly coupled and relatively pure brain mitochondrial preparations such as those described in the present paper renders it practicable to study this multitude of control points with the ultimate view of clarifying the connexion between the heterogeneity of brain mitochondria and metabolic compartmentation.

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