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HIGHLY PURIFIED MITOCHONDRIA FROM RAT BRAIN PREPARED BY PHASE PARTITION

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Summary

Mitochondria and synaptosomes from adult rat forebrain can easily be separated by counter-current distribution in an aqueous two phase system composed of Dextran T500 and poly(ethylene glycol) 4000. Both particles may also be separated by a batch procedure in which the same phase system is used. Electron micrographs and enzymatic activities show a high purity of the mitochondria obtained from the dextran-rich lower phase. Electron micrographs and enzymatic activities also show that intact synaptosomes can be obtained from the poly(ethylene glycol)-rich upper phase.

The mitochondria purified by this method show good ADP/O ratios, respiratory control ratios, and state 3 rates. Synaptosomes showed a state 2-state 3 transition with no recuperation to state 4.

Introduction

Centrifugation has remained the principal method for the separation of synaptosomes and mitochondria from brain tissue homogenates. Discontinuous sucrose gradients have been popular and the original method of Gray and Whittaker [1] has been repeatedly employed. However, mitochondria prepared by sucrose gradients do not show good respiratory control, probably due to the hypertonicity of the gradient. Some other preparation procedures employing sucrose gradients, like the one developed by Ozawa et al. [2], yield brain mitochondria with good respiratory control, but contaminated with synaptosomes [3]. High purity fractions lacking respiratory control have also been obtained with CsCl gradients [4]. To avoid the effects of hypertonicity, isotonic gra-

dients generated by Ficoll have been introduced [5—7]. The most frequently used Ficoll preparations are the one described by Clark and Nicklas [3] and the one of Lai and Clark used to obtain free and synaptic mitochondria [8] and pure synaptosomes [9]. These mitochondrial preparations show good respiratory control but are still contaminated with synaptosomes, as observed by their lactate dehydrogenase and acetylcholinesterase activities. Highly active brain mitochondria have recently been obtained [10] by differential centrifugation of 'salt-washed' mitochondria. This preparation is, however, more contaminated with lactate dehydrogenase than the one described by Clark and Nicklas [3].

Partition in two-phase systems created by dextran and poly(ethylene glycol) has been used for the preparation of cell particles, such as mitochondria and chloroplasts [11—15]. Leaf mitochondria purified by phase partition show a higher integrity and are less contaminated than those obtained by centrifugation alone [12]. This work describes the isolation of free brain mitochondria and synaptosomes by phase partition. The mitochondria obtained are highly purified and have a good metabolic performance.

Materials and Methods

Chemicals. Dextran T500 (M_r 500 000) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden, and poly(ethylene glycol) 5000 (M_r 3350) was purchased from Union Carbide, New York, U.S.A., as Carbowax PEG 4000 (now available as Carbowax PEG 3350).

Stock solutions of the polymers were prepared as follows:

20% (w/w) Dextran T500: dextran powder contains 5—10% water; therefore the exact concentration of the solution had to be determined [11,14,15]. 220 g of dextran was layered on 780 g of water, and heated on a waterbath under gentle stirring until all dextran was dissolved. A known amount of the solution (approx. 5 g) was diluted to 25.0 ml with water and the optical rotation measured with a polarimeter. After calculation of the exact concentration using the specific rotation $[\alpha]_D^{25} = +199$ degree $\cdot$ ml $\cdot$ g^{-1} $\cdot$ dm^{-1} the stock solution was adjusted to 20.0% with water.

40% (w/w) poly(ethylene glycol): 400 g was made up to 1000 g with water.

Isolation of crude mitochondria. Unless stated otherwise, 12 forebrains from male rats weighing from 150 to 200 g were finely chopped with scissors and washed with cold isolation medium to remove as much blood as possible. The chopped brain tissue was resuspended in 10 vols. of 0.32 M sucrose, 1 mM EDTA, 0.1% fatty acid-free bovine serum albumin, 1 mM Tris-HCl, pH 7.4 (medium) and homogenized with a Dounce homogenizer fitted with a Teflon pestle at 400 rev./min with five up-down strokes. The homogenate was centrifuged at 1100 $\times g$ for 10 min giving a '11 000 $g \cdot$ min pellet'. The supernatant was further centrifuged at 19 000 $\times g$ for 20 min to produce a '380 000 $g \cdot$ min supernatant' and a pellet. This pellet was resuspended in 5 ml of medium A ('380 000 $g \cdot$ min pellet') and layered on top of 20 ml of 6% Ficoll (w/w) in medium A and centrifuged at 19 000 $\times g$ for 30 min. The supernatant and the fluffy layer were carefully removed and discarded. The pellet was resuspended in 1.5 ml of medium B consisting of 0.32 M sorbitol, 0.1 mM EDTA,

0.1% fatty acid-free bovine serum albumin, 5 mM potassium phosphate, pH 7.8 ('crude mitochondria'). All operations were carried out at 4°C.

Selection of a suitable phase composition. The optimum phase composition for separation of mitochondria and synaptosomes was determined by systematic variation of the polymer concentrations in a series of single tube experiments [14,15]. 0.50-g aliquots of a suspension of 'crude mitochondria' in medium B were added to 3.50-g phase mixtures (see below) to produce 4.00-g phase systems with Dextran T500/poly(ethylene glycol) 4000 concentrations of: 5.8 : 5.8, 6.0 : 6.0, 6.2 : 6.2, 6.4 : 6.4, 6.6 : 6.6% (w/w), also containing 0.32 M sorbitol, 0.1 mM EDTA, 0.1% bovine serum albumin (fatty acid free), and 5 mM potassium phosphate, pH 7.8. Temperature, 4°C. Each system was mixed by 20 inversions of the tube, and centrifuged in a Christ swinging-bucket centrifuge at 600 × *g* for 2 min to decrease the time for phase settling. The upper phase of each system was carefully removed with a Pasteur pipette without disturbing the interface. Material left at the interface was always collected together with the lower phase. All phases were diluted with medium B to a final volume of 4.0 ml and used for assays of marker enzymes. The phase system containing 6.4% (w/w) of each polymer was found to give the best separation of mitochondria and synaptosomes, and this composition was used for all further experiments.

Counter-current distribution. An automatic thin-layer counter-current distribution apparatus [11,15] with 60 chambers was used. Preparation of phase systems and handling of the apparatus was as described elsewhere [11,13,15]. 'Crude mitochondria' from three brains suspended in a sample system [13] were added to chambers 0—2 of the counter-current distribution apparatus. After 57 transfers the fractions were collected and diluted with medium B to 2 ml. The absorbance at 280 nm was measured on fractions with even numbers, and fractions with odd numbers were used for enzyme assays.

Batch procedure. A large (50 g) phase system was prepared in a separatory funnel by weighing up: 16.0 g 20% (w/w) Dextran T500, 8.0 g 40% (w/w) poly(ethylene glycol) 4000, 16.0 g 1.0 M sorbitol, 0.25 g 20 mM sodium EDTA, 5.0 g 1.0% bovine serum albumin, 1.25 g 0.20 M potassium phosphate, pH 7.8, and 3.50 g distilled water. After temperature equilibration at 4°C the phase system was well mixed and allowed to settle overnight. The lower and upper phases were collected (the interface discarded) and stored separately in

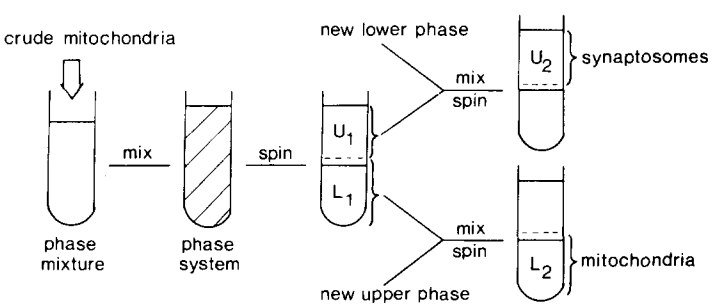


Fig. 1. Preparation of rat brain mitochondria and synaptosomes by phase partition. For details see text.

the freezer. A 7.00-g phase mixture was prepared by weighing up: 2.56 g 20% (w/w) Dextran T500, 1.28 g 40% (w/w) poly(ethylene glycol) 4000, 2.24 g 1.0 M sorbitol, 0.035 g 10 mM sodium EDTA, 0.70 g 1.0% bovine serum albumin, 0.175 g 0.20 M potassium phosphate, pH 7.8, and 0.01 g water.

When 1.00 g of a suspension of 'crude mitochondria' in medium B was added to such a 7.00 g phase mixture, this gave a 8.00-g phase system with the composition found to be optimum for separation (see above). The phase system was mixed by 20 inversions of the tube, and centrifuged in a Christ swinging-bucket centrifuge for 2 min at 600 × *g* to shorten the time for phase settling. The upper phase (U_1 in Fig. 1) was carefully removed with a Pasteur pipette and added to a tube containing 3 ml lower phase (obtained from the 50-g phase system). To the remaining lower phase plus interface (L_1 in Fig. 1) 3 ml upper phase was added. The two phase systems so obtained were treated as above. The final upper and lower phases (U_2 and L_2 in Fig. 1), containing synaptosomes and mitochondria, respectively, were diluted with 15 ml of medium B and centrifuged at 19 000 × *g* for 30 min. The pellets were suspended in 30 ml of medium B and recentrifuged under the same conditions. The final pellets were suspended in 0.5 ml of medium B giving 'lower phase material' (mitochondria) and 'upper phase material' (synaptosomes), respectively.

Enzyme assays. Lactate dehydrogenase was assayed according to the method of Clark and Nicklas [3], fumarase according to the method of Racker [16], cytochrome *c* oxidase according to the method of Turner [17], acetylcholinesterase according to the method of Ellmann et al. [18], and citrate synthetase according to the method of Lai and Clark [19]. Protein was determined by the biuret method [20], or (for respiratory activities) by the Coomassie Blue method [21].

Respiratory activities. Respiration was measured polarographically at 25°C in a final volume of 1 ml with 50—300 μg of material. Incubation media containing 5 mM or 100 mM KCl were used according to the method of Lai et al. [22]. State III was induced by addition of ADP, and calculations were carried out according to Chance and Williams [23].

Electron microscopy. Electron microscopy was essentially as previously described [13].

Results and Discussion

Separation of mitochondria and synaptosomes

Fig. 2 shows the result of a counter-current distribution experiment with crude mitochondria. Three main peaks (A_{280}) can be seen. Lactate dehydrogenase and acetylcholinesterase, used as markers for synaptosomes, are located in the third peak from the left. Fumarase and cytochrome *c* oxidase, used as mitochondrial markers, were either found in the first peak or in the third one. These results suggest that the first peak correspond to free mitochondria and the third one to synaptosomes. The nature of the middle peak remains unknown.

Separation by batch procedure

The counter-current distribution experiment shows that mitochondria par-

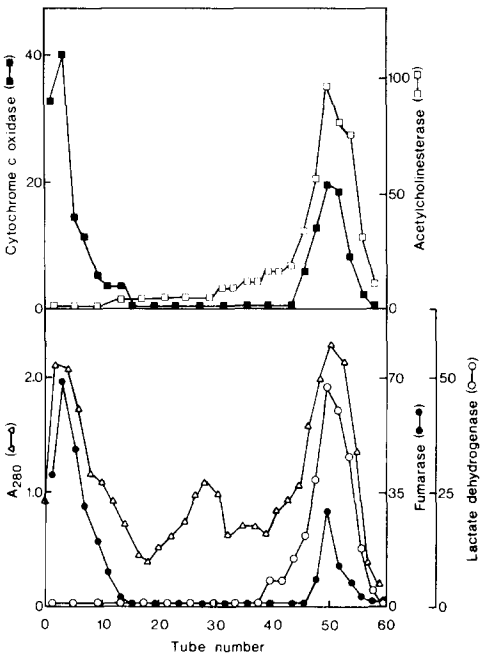


Fig. 2. Counter-current distribution of crude mitochondria. A_{280} nm was determined after 1 : 10 dilution. Enzymatic activities are expressed as $\mu mol \cdot min^{-1}$ of the whole fraction.

tition with a high affinity to the lower dextran-rich phase while synaptosomes partition extremely to the poly(ethylene glycol)-rich upper phase. Thus, separation of these particles from each other should be possible by a simple batch procedure employing a phase system with the same composition.

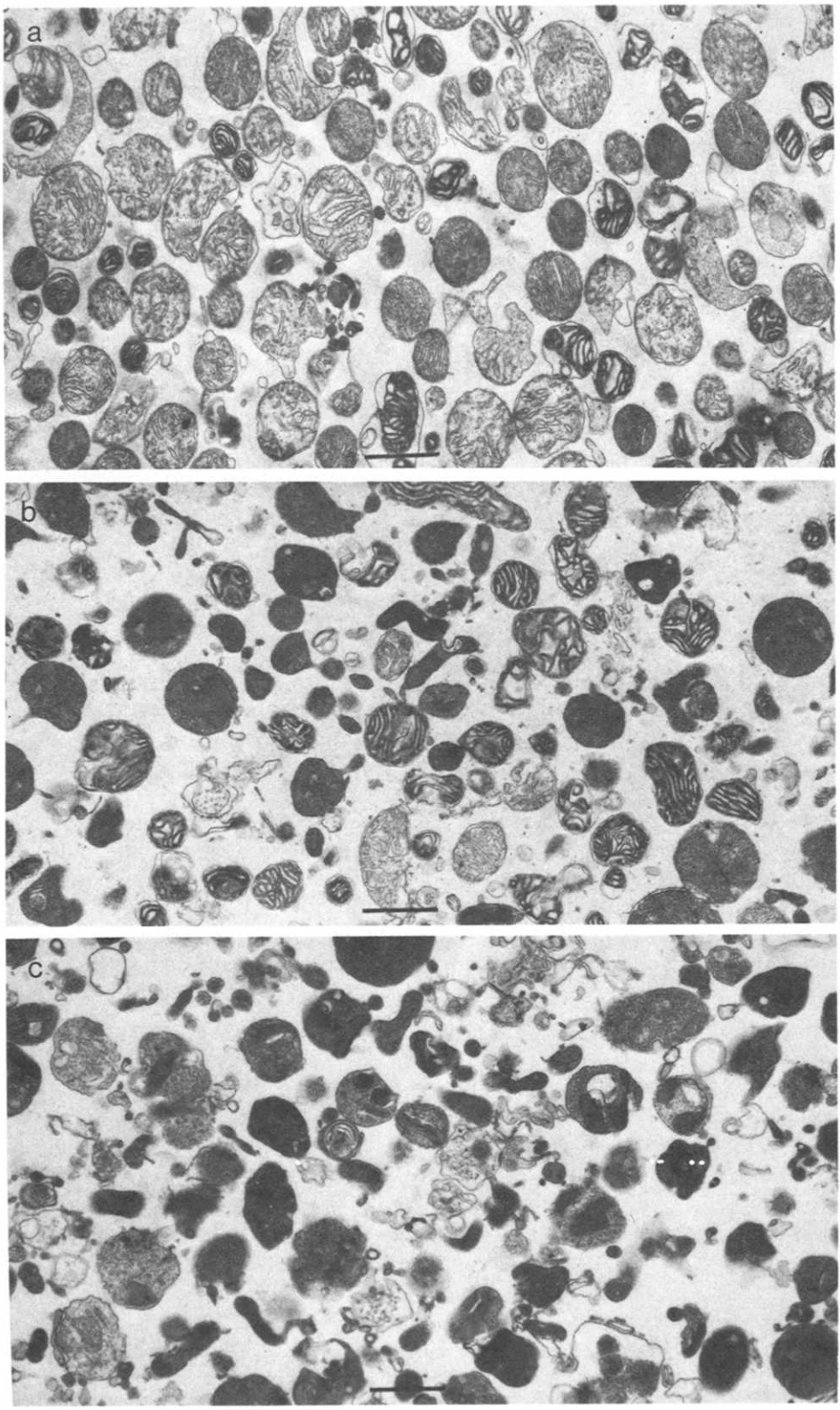
The results obtained are summarized in Table I. Particles obtained from the lower phase showed high specific activities of the mitochondrial markers fumarase, cytochrome *c* oxidase and citrate synthetase, in good agreement with data for preparations obtained with other methods [3,4,8,22,24]. Lactate dehydrogenase (spec. act. 0.014 ± 0.006 unit), acetylcholinesterase (spec. act. 0.010 ± 0.002 unit), markers of synaptosomal contamination, are much lower than those obtained by methods using sucrose [2,10,25] or Ficoll [4,8,22] gra-

TABLE I

SPECIFIC ENZYME ACTIVITIES OF THE LOWER AND UPPER PHASE MATERIAL OBTAINED BY THE BATCH PROCEDURE

Activities are expressed as $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein. Results are the average of the number of experiments expressed within parentheses.

	Lactate dehydrogenase		Acetyl-cholin-esterase	Citrate synthetase	Fumarase	Cyto-chrome *c* oxidase
	— Triton	+ Triton				
Lower phase material	0.005 (6)	0.014 (4)	0.010 (6)	0.633 (2)	0.340 (3)	0.320 (3)
Upper phase material	0.104 (2)	0.695 (2)	0.061 (2)	0.309 (1)	0.068 (3)	0.042 (3)


a
b
c

dients as the main step of purification. Still purer preparations can be obtained by further extractions of the lower phase with fresh upper phase, but this also lowers the yield (data not shown).

Further confirmation of the purity of the mitochondria can be observed in Fig. 3a which shows an electron micrograph of the lower phase material. The preparation contains pure mitochondria without any synaptosomal contamination. Fig. 3b shows the composition of the crude mitochondrial preparation (previous to phase partition) in which mitochondria and synaptosomes are the most abundant particles.

The values for lactate dehydrogenase and acetylcholinesterase of the upper phase material are in good agreement with specific activities of the pure synaptosomal preparations previously described [6]. The increase of lactate dehydrogenase activity on addition of Triton X-100 is a good indication of the integrity of the synaptosomes [6].

Fig. 3c shows an electron micrograph of the upper phase material containing mainly synaptosomes without contamination of free mitochondria.

The ratio lactate dehydrogenase plus Triton to lactate dehydrogenase without Triton increased during the process (Table II) reaching the highest value (approx. 7) in the upper phase material suggesting a progressive enrichment of intact synaptosomes. However, the highest specific activities of lactate dehydrogenase during the process was found in the homogenate and 380 000 $g \cdot$ min supernatant, as expected by the elimination of the free (not particle-bound) lactate dehydrogenase. In the same way, the high activity of acetylcholinesterase during the first stages of purification, decreasing after sedimentation through Ficoll, suggests the elimination of the synaptic membranes at this purification step.

With respect to citrate synthetase and cytochrome *c* oxidase the specific activities of the lower phase material is about twice the value of that of the crude mitochondria showing the high efficiency of the two phase system in the purification process.

Respiratory activities

The respiratory behaviour of the 'crude mitochondria' (previous to phase partition), mitochondria and synaptosomes are presented in Fig. 4, using malate plus glutamate as substrates in a medium containing 5 mM $MgCl_2$. Crude mitochondria showed a good state 2-state 3 transition, but recuperation to state 4 was very poor with an ADP/O ratio of 1.0—1.2. Synaptosomes did not show any recuperation to state 4 but mitochondria presented a good respiratory control with ADP/O ratios of 2.6—2.7. The absence of state 4 in synaptosomes can be explained by the presence of an ATP-consuming reaction in the cytoplasmic layer and maintainance of the ADP level in the particles. Contamination of synaptosomal material was probably responsible for the poor respiratory control of the crude mitochondria (Fig. 4).

The ADP/O ratios, respiratory control and state 3 rates of purified mito-

Fig. 3. Electron micrographs of the particles obtained from the lower phase (a), crude mitochondria (b) and particles of the upper phase (c).

TABLE II

TOTAL PROTEIN, TOTAL ENZYMATIC ACTIVITIES AND SPECIFIC ACTIVITIES OF THE DIFFERENT FRACTIONS OF THE PURIFICATION PROCESS

For details see Material and Methods. Total activity is expressed as $\mu mol \cdot min^{-1}$ of the whole fraction and specific activities as $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of protein.

	Protein (mg)	Lactate dehydrogenase				Acetyl-cholinesterase		Citrate synthetase		Cytochrome *c* oxidase	
		Total act.		Spec. act.							
		+ Triton	— Triton	+ Triton	— Triton	Total act.	Spec. act.	Total act.	Spec. act.	Total act.	Spec. act.
Homogenate	2266	1626	1626	0.717	0.717	211	0.093	453	0.200	183	0.081
11 000 *g* · min pellet	897	529	218	0.589	0.243	79	0.088	152	0.169	65	0.073
380 000 *g* · min supernatant	882	712	561	0.807	0.636	93	0.105	70	0.079	8	0.009
380 000 *g*· min pellet	387	101	25	0.261	0.064	38	0.098	119	0.308	41	0.107
Crude mitochondria	69	40.8	10.9	0.584	0.156	2.5	0.035	25.5	0.365	11	0.159
Lower phase material	12	0.2	0.1	0.020	0.005	0.1	0.012	7.8	0.679	4.7	0.395
Upper phase material	36	25.6	3.8	0.695	0.104	2.3	0.062	11.3	0.307	2.5	0.069

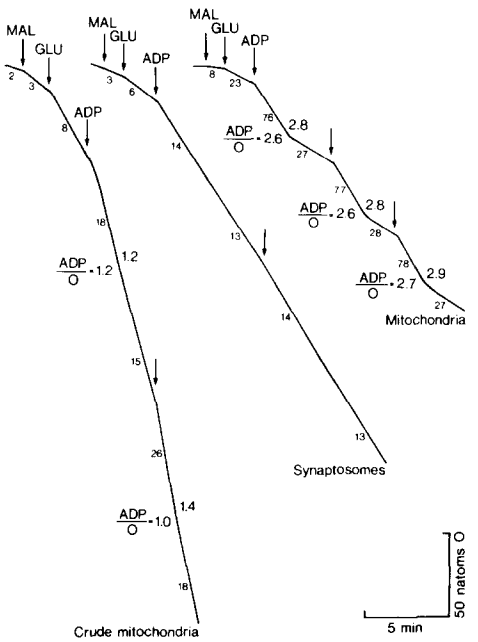


Fig. 4. Oxygen electrode traces of crude mitochondria, mitochondria (lower phase material) and synaptosomes (upper phase material). The figures to the left show rates as natoms O · min^{-1} of protein. The figures to the right show the respiratory control ratios. Respiration was carried out in 100 mM KCl medium (see Materials and Methods) also containing 5 mM $MgCl_2$, and with 5 mM glutamate plus 2.5 mM malate as substrate.

chondria when succinate, malate plus glutamate, and malate plus pyruvate were used as substrates in the absence of Mg^{2+} are summarized in Table III. ADP/O ratios were close to the theoretical values in all cases. With our mitochondrial preparations we did not observe a significant stimulation of the state 3 rates by high potassium concentration in the medium, as described for mitochondria purified by Ficoll methods [3,8].

TABLE III

ADP/O RATIOS, RESPIRATORY CONTROL RATIOS AND STATE 3 RATES OF MITOCHONDRIA OBTAINED BY PHASE PARTITION

State 3 rates are expressed as natoms O · min^{-1} · mg^{-1} of protein. Except in the case of succinate, the experiments were carried out in the presence of 2.5 mM malate. The medium did not contain Mg^{2+} in this case. ADP/O ratios and respiratory control ratios (RCR) average of two determinations on sucessive additions of ADP.

	KCl (mM)	ADP/O	RCR	State 3
10 mM succinate	5	1.5	2.6	106
	100	1.8	3.4	118
5 mM glutamate	5	2.4	3.0	70
	100	2.4	3.4	59
5 mM pyruvate	5	3.0	5.3	94
	100	2.8	3.0	70

With purified mitochondria the presence of 5 mM $MgCl_2$ did not significantly affect the ADP/O ratios for glutamate (Fig. 4, Table III), but resulted in a slight decrease of the respiratory control ratio as already described for brain mitochondria by Moore and Jöbsis [25]. The same effect of Mg^{2+} on the respiratory control was found using succinate and pyruvate as substrates (data not shown).

Concluding remarks

Centrifugation methods separate cell particles according to size and density. Phase partition, on the other hand, separates according to membrane surface properties. A combination of these two methods should therefore yield preparations with high purity, as shown for the rat brain mitochondria. Especially mitochondria and plasma membranes (or particles limited by the plasma membrane) are easily separated by phase partition, since plasma membranes have an extreme affinity for the upper phase, while mitochondria prefer the lower phase. This seems to be a general phenomenon and can be observed with both animal and plant material [15].

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References

1 Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96, 79—87
2 Ozawa, K., Seta, K., Takeda, H., Ando, K., Manda, H. and Araki, C. (1966) J. Biochem. (Tokyo) 59, 501—510
3 Clark, J.B. and Nicklas, W.J. (1970) J. Biol. Chem. 245, 4724—4731
4 Levitan, I.B., Mushynski, W.E. and Ramirez, G. (1972) J. Biol. Chem. 247, 5376—5381
5 Kurokawa, M., Sakamoto, T. and Kato, M. (1965) Biochem. J. 97, 833—844
6 Autilio, L.A., Appel, S.H., Pettis, P. and Gambetti, P.L. (1968) Biochemistry 7, 2615—2622
7 Cotman, C.W. and Matthews, D.A. (1971) Biochim. Biophys. Acta 249, 380—394
8 Lai, J.C.K. and Clark, J.B. (1976) Biochem. J. 154, 423—432
9 Booth, R.F.G. and Clark, J.B. (1978) Biochem. J. 176, 365—370
10 Bernard, P.A. and Cockrell, R.S. (1979) Biochim. Biophys. Acta 548, 173—186
11 Albertsson, P.Å. (1971) Partition of Cell Particles and Macromolecules, Wiley, New York
12 Gardeström, P., Ericson, I. and Larsson, C. (1978) Plant Sci. Lett. 13, 231—239
13 Larsson, C., Collin, C. and Albertsson, P.Å. (1971) Biochim. Biophys. Acta 245, 425—438
14 Larsson, C. and Andersson, B. (1979) in Plant Organelles, Methodological Surveys (b) (Reid, E., ed.) Vol. 9, pp. 35—46, Ellis Horwood, Chichester
15 Albertsson, P.Å., Andersson, B., Larsson, C. and Akerlund, H.E. (1981) in Methods of Biochemical Analysis (Glick, D., ed.), Vol. 28, Wiley-Interscience, New York, in the press
16 Racker, E. (1950) Biochim. Biophys. Acta 4, 211—214
17 Turner, G. (1973) Eur. J. Biochem. 40, 201—206
18 Ellmann, G.L., Courtney, K.D., Andres, V., Jr. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
19 Lai, J.M. and Clark, J.B. (1973) Biochem. J. 134, 545—555
20 Gornall, A.G., Bardewill, C.S. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
21 Spector, T. (1978) Anal. Biochem. 86, 142—146
22 Lai, J.M., Booth, R.F.G., Berger, R. and Clark, J.B. (1977) Biochem. J. 164, 339—348
23 Chance, B. and Williams, G.K. (1972) Adv. Enzymol. 17, 65—134
24 Jones, L.R., Mahler, H.K. and Moore, W.J. (1973) J. Biol. Chem. 250, 975—983
25 Moore, C.L. and Jöbsis, F.F. (1970) Arch. Biochem. Biophys. 138, 295—305