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A STUDY OF PREPARATIVE PROCEDURES FOR BRAIN MITOCHONDRIA

FRANS F. JÖBSIS*

Laboratory of Physiological Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

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SUMMARY

1. A new preparative procedure for the isolation of mitochondria from rabbit or rat brain is described.

2. The preparations were found to be superior to those previously described with respect to the P:O ratio and the degree of stimulation of the ATPase activity by dinitrophenol.

INTRODUCTION

In the last few years several methods have been described for the isolation of mitochondria from brain tissue[1-19]. The method to be described in the present paper was designed to yield a preparation with a sufficiently low ATPase activity to show respiratory control[20] in the presence of ATP.

METHODS

One young (4–7 months old) rabbit was used for most experiments. After a satisfactory procedure had been developed with rabbit brain, preparations were also made from rat brain (4 rats per experiment). The animal was stunned by a blow on the head and the skull split sagittally with a sharp clean chisel. The cerebrum, cerebellum and brain stem were removed, immediately placed in a tared beaker with ice-cold isotonic sucrose and quickly chopped with scissors.

The tissue was weighed by difference and then homogenized at 0° in approx. 7 times its volume of isolation medium using a Potter–Elvehjem homogenizer with Teflon pestle (clearance approx. 0.3 mm). The separation of various fractions was performed at 0–4° in a refrigerated International centrifuge with a high-speed attachment. The mitochondria were washed at least once in a wash medium and finally taken up in a minimal volume of suspension medium.

The dinitrophenol-stimulated ATPase activity was determined according to MYERS AND SLATER[21] with a reaction mixture containing 50 mM Tris–acetate buffer (pH 7.4), 75 mM KCl, 0.5 mM EDTA, 1.0 mM $MgCl_2$, 183 mM sucrose and (where

* Present address: Johnson Foundation, University of Pennsylvania, Philadelphia 4, Pa. (U.S.A.)

added) 0.1 mM 2,4-dinitrophenol. The O_2 uptake and oxidative phosphorylation were measured in differential manometers (1-ml vessels). The phosphate esterification was determined by measurement of the disappearance of inorganic phosphate[22] or by the determination of the amount of glucose 6-phosphate formed[23,24]. Though the latter method is much the more sensitive, no significant difference was found between the two methods at the levels of phosphate employed in this study, provided that, when using the phosphate-disappearance method, the contents of the manometric vessels were cooled immediately after the end of the run and kept at 0° until color development.

Protein was determined by the biuret method as described by CLELAND AND SLATER[25]. For some fractions it proved to be valuable to place the samples, after the addition of trichloroacetic acid, in a boiling-water bath for 5 min and to repeat the extraction with ethanol. This procedure tends to prevent the occurrence of cloudiness during color development.

RESULTS

The routine test for the quality of the preparations was the ratio of the ATPase activity with dinitrophenol to that without dinitrophenol, both measurements being carried out in the presence of 1 mM Mg^{2+}. Refinements were tested also by their effect on the P:O ratio and on the degree of metabolic control (respiratory rate in the presence of ADP/respiratory rate after the esterification of the added ADP).

Preparative procedure

It is not necessary to report all modifications of the centrifugation scheme and of the various media tested during this study. The method which was finally adopted as giving the best and most consistent results will be described.

Isolation medium. Sucrose, 250 mM; ATP, 2 mM; citrate, 5 mM; phosphate, 1 mM; cysteine, 0.01% (w/v). Potassium salts and water twice-distilled from glass were used. The pH was adjusted to 7.4 with 1 N KOH. It was found that the procedure could be slightly simplified by using 250 mM sucrose with 1 mM ATP as both the *wash* and the *suspension medium.*

Centrifugation. The homogenate is spun for 5 min at 600 × *g* and the supernatant is poured off and saved. The debris is resuspended in about 30 ml of isolation medium and spun once more at 600 × *g* for 5 min and the supernatant added to that of the previous run. The debris is rather voluminous and care must be taken not to disturb its surface during handling and decanting. It is advisable to remove the belt of the centrifuge during both decelerations.

The supernatant is spun at 10000 × *g* for 10 min. The resulting pellet is solid and the supernatant can be easily poured off. The precipitate consists of two layers which are not sharply delineated, the lower being somewhat darker in color.

The pellets are stirred up in a few millilitres of wash medium and then disintegrated further by sucking up and down a few times in a pipette with a small orifice. The volume is made up to about half the original volume of the homogenate. This is again centrifuged for 10 min at 10000 × *g*. The resulting pellet has a solid brown bottom layer and a large, slightly loose top layer, while the supernatant is definitely cloudy. This difference in the aspect of the tubes after the first and second

high-speed spins is caused by the differences in composition of the isolation and wash media.

Two procedures can now be followed: (a) The supernatant is carefully poured off, the precipitate stirred up and transferred to a small glass Potter–Elvehjem homogenizer and gently homogenized by hand. The transfer from the centrifuge tubes is made quantitative by rinsing with suspension medium (which is the same as wash medium unless otherwise noted). The total volume is kept to a minimum, about 5 ml for 8–10 g of tissue. The yield is 9–10 mg protein/g tissue. This preparation, in which the solid and loose layers are combined, is designated preparation C. (b) Alternatively, the solid, brown pellet can be separated from the white layer. First the supernatant is decanted, after which the whitish top layer is removed by gentle shaking with a small amount of wash medium and poured into a clean centrifuge tube. This is repeated several times. This procedure can also be applied to the precipitate of the first 10000 $\times$ g spin, though the separation will be less clean-cut. The white layers and the brown pellets are separately dispersed in 1/4 the volume of the original homogenate and spun once more at 10000 $\times$ g for 10 min which results in a new layering of brown and white components. A small white layer on top of the pellet made up of the previous brown fractions is rather solidly attached to the brown material. It can not be removed without considerable loss of the latter. Washing the surface may, however, yield a small amount of white material. The pellet precipitated from the previous white fractions yields again a brownish button, underneath a white layer. This brown pellet is combined with the other one. The two final fractions are homogenized by hand and are designated B (brown) and W (white). They can conveniently be made up to about 2.0 and 3.5 ml, respectively. The protein yield is approx. 2.5–3.0 mg/g tissue for the brown and 5–7 mg/g tissue for the white fraction.

ATPase tests

The three fractions C, B and W are the final, standard preparations which emerged from this study. The average values for the ATPase ratio (amount of ATP split in the presence of Mg^{2+} and dinitrophenol/the amount split in presence of Mg^{2+} alone) were 4.2, 7.1 and 2.7. Preparation B is, therefore, by far the best and comes close to equaling liver mitochondria in this test. This ratio can be increased to values greater than 10 if the small, white layer adhering tenaciously to the surface of the brown pellets is rigorously discarded. This can be done by scraping it off with a small spatula. The concurrent loss of material is, however, so great that the resulting pure fraction B is too small for most metabolic tests.

The low ATPase ratio of the white layer, W, in the absence of dinitrophenol could be due to the presence of either microsomes or mitochondrial fragments. Recently the usefulness of oligomycin in distinguishing between these two ATPases has been pointed out by HUIJING AND SLATER[26]. This antibiotic is a true inhibitor of oxidative phosphorylation[27] and also of the ATPase activity of the uncoupled respiratory chain, while ATPases of other origin are not affected. To ascertain the contribution of non-mitochondrial enzymes the ATPase activity of the three fractions was tested in the presence and absence of various amounts of oligomycin. A typical experiment is shown in Table I. Preparation B′ was derived from B by mechanically removing and discarding the solid white layer adhering to the top of one of the brown pellets (see above). The level of oligomycin employed is sufficient for more than 90 %

inhibition of the mitochondrial ATPases. The remaining activity must, therefore, be derived from other sources. The results show that, in all cases, the Mg^{2+}-stimulated ATPase measured in the absence of dinitrophenol is largely of non-mitochondrial origin. The contamination is greatest in the W preparation.

TABLE I

EFFECT OF OLIGOMYCIN ON ATPASE ACTIVITY OF PREPARATIONS OF RABBIT-BRAIN MITOCHONDRIA

Addition	*ATPase activity (μmoles P/mg protein/h)*			
	Preparation C	*Preparation B*	*Preparation W*	*Preparation B'*
None	1.5	1.1	2.0	0.7
Dinitrophenol (0.1 mM)	5.8	7.9	4.7	8.8
Oligomycin (2.5 μg/ml)*	1.2	0.8	1.8	0.5

* Equivalent to 22, 19, 27 and 20 μmoles/g protein, respectively, assuming a molecular weight of oligomycin of 333.

Brain homogenates contain a considerable activity of ($Na^+ + K^+$)-stimulated ATPase[28–30]. This enzyme appears to be located on particles derived from membranous structures and is strongly implicated in the mechanism of sodium extrusion from the cell[31–33]. The high activity in the presence of 100 mM NaCl and 20 mM KCl is inhibited approx. 90% by 10 μM strophantin[30]. The efficacy of this inhibitor was tested on the present B and W fractions. The results (Table II) show clearly that a considerable contaminationw ith the ($Na^+ + K^+$)-stimulated ATPase is found in the W but practically none in the B fraction. Although the activity of this enzyme would be low at the

TABLE II

THE ($Na^+ + K^+$)-STIMULATED ATPASE OF PREPARATIONS OF RABBIT-BRAIN MITOCHONDRIA

The reaction was allowed to proceed for 15 min at 37° in the presence of 30 mM histidine buffer (pH 7.2), 6 mM $MgCl_2$, 3 mM ATP (Sigma Tris ATP) and further additions as shown[30].

Addition	*ATPase activity (μmoles P/mg protein/h)*	
	Preparation B	*Preparation W*
NaCl (125 mM), KCl (25 mM)	8.4	28.8
KCl (150 mM)	5.8	9.9
NaCl (125 mM), KCl (25 mM), strophantin (0.13 mM)	6.1	10.7
KCl (150 mM), strophantin (0.13 mM)	5.8	9.2

cationic levels of the standard dinitrophenol-stimulated ATPase test, it is clear that a considerable and equal liberation of P_i must have occurred in both the control and dinitrophenol reaction mixtures. This causes a lowering of the ATPase ratio and supplies a partial reason for the lower values of this ratio in the W fraction.

Effects of variations in procedure

The large number of components in the isolation medium is the result of a careful

study of a number of additions, most of which were unsuccessful in promoting a higher ATPase ratio. A simpler but more expensive medium consisting of 0.25 M sucrose + 5 mM ATP can, however, be substituted with little loss of quality or yield. The beneficial influence of ATP at low levels is thought to lie in its ability to prevent the swelling of the mitochondria. In higher concentration it probably has a chelating function especially of Ca^{2+}. In keeping with this, good results were obtained in 2 mM ATP with citrate but not with other calcium binders. It was found that EDTA at all concentrations tested has a deleterious effect. The white material in the mitochondrial fraction becomes more voluminous and it is impossible to separate it from the brown pellet, while very low ATPase ratios were obtained. MYERS AND SLATER[21] have reported that the addition of EDTA to a sucrose isolation medium hinders the separation of rat-liver mitochondria from the "fluffy layer".

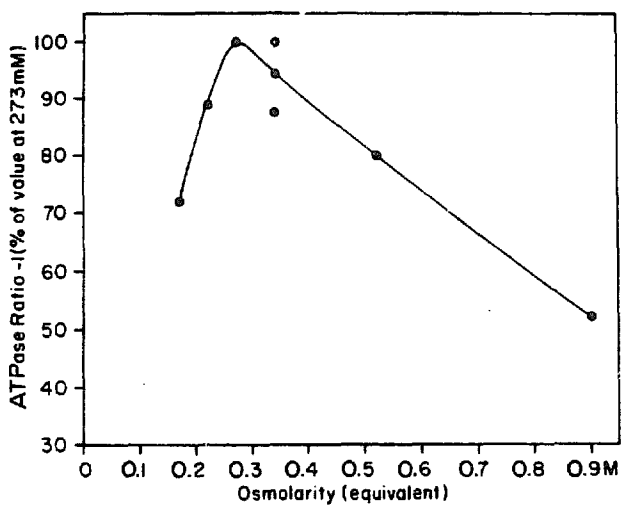

Fig. 1. The influence of the osmolarity of isolation medium on the ATPase ratio of brain mitochondria.

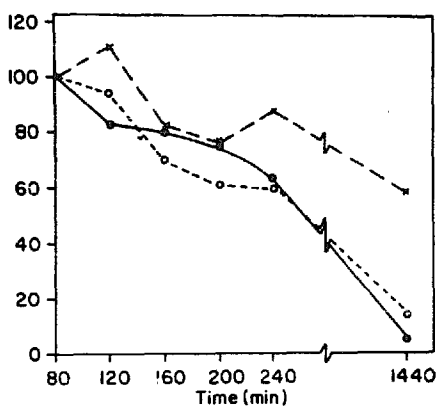

Fig. 2. Stability of preparation C. ———, ATPase ratio *minus* 1; – – – –, ATPase in presence of dinitrophenol; — — —, ATPase in absence of dinitrophenol. The time given is that elapsing after the death of the animal. The preparation was completed at 80 min and was kept at 0°. All values are given as percentage of value at 80 min.

A satisfactory explanation can not be given for the slight but consistently beneficial effects of the inclusion of small amounts of phosphate and cysteine in the isolation medium. At higher concentrations both exert a deleterious effect.

Fig. 1 shows that the osmolarity of the isolation and wash media is also a significant parameter in the procedure, a definite optimum being found around 300 mequiv.

The standard procedure described above can be completed in 80 min. Fig. 2 shows that the preparation is rather unstable, both ATPases, but particularly the dinitrophenol-induced, declining with time. It may well be that proteolytic enzymes are present in the preparation. It is clearly advisable to prepare and use the mitochondrial preparations as quickly as possible.

Some metabolic characteristics

Respiration and oxidative phosphorylation of the mitochondria were studied with various substrates in a number of different phosphorylation media.

The reaction medium used in most experiments contained the following components (the concentrations are those in the reaction vessels after addition of the mitochondrial preparation): sodium glycylglycine, 15 mM; substrate, 13 mM; sodium potassium phosphate buffer, 15 mM; ATP, 0.1 mM; glucose, 30 mM; EDTA, 0.1 mM; KCl, 25 mM; hexokinase; $MgCl_2$, 5 mM. Preparations C and W were added to provide approx. 6–8 mg protein/ml in the vessel. Because of the smaller yield and higher activity, preparation B was used at a final level of 3–4 mg protein/ml.

The addition of DPN^+ or of cytochrome *c* had no effect on either the respiratory rate or the P:O ratio. They were, therefore, not included in the standard phosphorylation medium. In a small number of experiments the glycylglycine and KCl were replaced by 50 mM Tris–HCl buffer without noticeable effect. The results of these experiments are included among the averaged data reported in the tables.

The present preparation was compared with some others which have been described in the literature.

Preparation 1 was the one described by BRODY AND BAIN[1] as their preparation II. The fraction used was collected between 1500 × *g* for 10 min and 18000 × *g* for 20 min. As medium 0.32 M sucrose was used throughout. The fraction consisted of a brown pellet and a large white, fluffy layer. For some experiments this whole preparation was used (called C below), for others it was divided as well as possible into a brown (B) and a white fraction (W).

Preparation 2, made according to SACKTOR[17], was collected between 2000 × *g* for 1 min and 25000 × *g* for 25 min. A solution of 0.25 M sucrose + 5 mM EDTA was used throughout. In this procedure the tissue is homogenized considerably longer (1 min after all big pieces had disappeared) than in the procedure described in this paper (approx. 20 sec). A bulky and fluffy precipitate was obtained which could not be separated into brown and white fractions.

Preparation 3 was isolated according to the method given by MCKHANN AND TOWER[12] which is a modification of preparation 1 insofar as 5 mM EDTA is added to the 0.25 M sucrose solution. This preparation was homogenous and could not be subdivided.

Preparation 4 was the one described in the present paper.

The results for the complete fractions (C) are listed in Table III. The differences between the preparations with respect to the P:O ratios are correlated with the ATPase ratios. In general, the phosphorylating efficiency of preparation 4 may be considered reasonably satisfactory.

For all practical purposes γ-aminobutyrate was not a substrate for oxidative metabolism by any of the preparations. Even in the presence of 1 mM α-ketoglutarate, the Q_{O_2} with γ-aminobutyrate was only 17, well below the value reached with glutamate. These results are at variance with those of MCKHANN AND TOWER[12,19], who found that γ-aminobutyrate + α-ketoglutarate was oxidized at rates equaling those for glutamate using cat-cortex mitochondria. A species difference is not excluded.

The differences between the entire preparations (C) and the B and W fractions are illustrated in Table IV for those preparations in which separation could be accomplished (preparations 1 and 4). The brown material (B) is obviously the more active and better fraction. It is not unlikely that the activity of fraction W depends for a large part on remaining brown material and perhaps on mitochondrial fragments contained in it. (Note the relatively higher Q_{O_2} values for succinate in fraction W.)

TABLE III

COMPARISON OF VARIOUS PREPARATIONS OF BRAIN MITOCHONDRIA

Preparation 1, ref. 1; preparation 2, ref. 17; preparation 3, ref. 12; preparation 4, preparation C is described in text.

Substrate		*Rabbit*				*Rat*			
		Preparation				*Preparation*			
		1	*2*	*3*	*4*	*1*	*2*	*3*	*4*
Glutamate	Q_{O_2}	40.4	28.3	38.2	50.1	34.0	28.8	34.4	40.9
	P:O	2.0	1.8	2.1	2.2	1.6	1.3	2.0	2.3
	No.*	(6)	(3)	(4)	(11)	(4)	(2)	(3)	(7)
Succinate	Q_{O_2}	44.1		45.5	51.6	33.0	29.4		36.0
	P:O	1.6		1.5	1.9	1.2	1.1		1.6
	No.	(3)		(2)	(9)	(3)	(2)		(5)
α-Ketoglutarate	Q_{O_2}		19.2	26.8	37.9		20.4		31.4
	P:O		1.9	2.2	2.6		2.0		2.7
	No.		(1)	(2)	(4)		(2)		(3)
γ-Aminobutyrate	Q_{O_2}		5.4	4.7	5.2		5.4	7.6	7.4
	No.		(3)	(3)	(7)		(2)	(3)	(4)
Glucose	Q_{O_2}	4.2	4.5	2.8	1.9	4.8	5.8	7.2	5.3
	No.	(4)	(2)	(2)	(9)	(2)	(1)	(3)	(4)
Endogenous**	Q_{O_2}			2.0	1.5				
	No.			(2)	(4)				
ATPase ratio		2.8	2.7	3.2	4.2	1.6	2.2	2.2	3.1
	No.	(6)	(3)	(3)	(15)	(5)	(2)	(3)	(6)

* Number of preparations tested.
** No glucose added.

TABLE IV

COMPARISON OF WHITE AND BROWN FRACTIONS

Preparations 1 and 4 as in Table III.

Substrate	*Rabbit*					*Rat*			
	Preparation	*1*		*4*		*1*		*4*	
	Fraction	*W*	*B*	*W*	*B*	*W*	*B*	*W*	*B*
Glutamate	Q_{O_2}	35.2	68.3	26.2	86.5	19.7	40.1	21.4	49.5
	P:O	1.8	2.2	2.0	2.4	1.7	2.0	1.9	2.3
	No.*	(4)	(4)	(8)	(11)	(3)	(3)	(4)	(3)
Succinate	Q_{O_2}	46.5	58.1	33.0	63.1				40.6
	P:O	1.5	1.8	1.5	1.9				1.9
	No.	(4)	(2)	(7)	(9)				(1)
ATPase ratio		3.0	4.1	2.7	7.1	1.4	2.4	1.6	2.5
	No.	(4)	(4)	(19)	(19)	(3)	(3)	(4)	(4)

* Number of preparations tested.

The material giving fraction W its white appearance is apparently a contamination with another cellular component. Attempts to increase the yield of fraction B by further extraction of the mitochondria from fraction W would serve a worthwhile purpose.

The P:O ratios were also calculated from the increased respiration produced by the addition of ADP from the side-arm. After the ADP has become phosphorylated the respiratory rate decreases again. The P:O ratio is then calculated from the amount of oxygen taken up during the rapid phase of respiration and the amount of ADP added[20]. In this way the P:O ratios could be measured satisfactorily only with the present preparations C and B. They agree closely with those found with the other method, *i.e.*, 2.1 and 2.3 with glutamate for C and B, respectively. The amount of deceleration after the ADP had been phosphorylated was about 3-fold. With other preparations, this ratio never exceeded 1.8.

NOTE ADDED IN PROOF

Recently we have had considerable success in increasing the yield and quality of the B fraction by treatment with proteinase before homogenization and by using 0.22 M mannitol and 0.07 M sucrose to maintain osmolarity[34]. The diced tissue is incubated for 5 min at 0° with 20 ml proteinase solution (0.4 mg/ml) in the presence of 20 mM Tris–HCl buffer (pH 7.6) and 0.5 mM EDTA. After a short homogenization in a homogenizer with large clearance, the incubation is continued for a further 5–10 min and the tissue is finally homogenized in the usual homogenizer. The volume is then brought up to approx. 10 times the tissue volume with a medium containing 0.5 mM EDTA and 2 mM ATP. The centrifugation scheme is the same as that described above, using the same wash medium.

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