THE PREPARATION OF MITOCHONDRIA FROM MUSCLE WITHOUT THE USE OF A HOMOGENISER

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1. Introduction

Skeletal muscle from adult rats is difficult to homogenise and the excessive shearing forces needed raise the possibility that isolated subcellular organelles and particles are not truly representative of the *in vivo* state. In the case of ribosomes this is clear when sucrose density gradients show a preponderance of single ribosomes [1] although polyribosomes predominate in the intact tissue. It also seems likely to be the case with mitochondria since these organelles cover a wide range of sizes and preferential rupture of the larger particles during homogenisation could easily lead to enrichment with respect to the smaller members of the subcellular population.

In the past, attempts have been made to overcome this difficulty by adding proteolytic enzymes to the tissue before homogenisation in order to make the cells less resistant to mechanical breakdown [2].

We have now shown that functional skeletal muscle mitochondria can be released in satisfactory yield by the use of proteolytic enzymes alone and that a mechanical homogeniser need not be used.

2. Materials and methods

Three proteolytic enzymes were used. Nagarse (Teikoku Chemical Industry Ltd., 100 P.U.N./mg); Trypsin (Worthington Biochemical Corporation, 220 units/mg) and Pronase B (Calbiochem., 45 P.U.K. units/g).

Hind leg and back muscles of adult male rats (250 g) were cleaned of fat and connective tissue and placed in an ice cold isolation medium containing tris (0.1 M, pH 7.4), mannitol (0.21 M), sucrose (0.07 M) and EDTA (0.01 M).

The muscle was finely minced with a hand mincer (Gallenkamp) and 10 g was stirred with 100 ml of medium and one of the enzymes for 30 min either in a cold room (+ 4°) or in an ice bath. The muscle suspension, in a 400 ml beaker, contained 2.0 mg of Nagarse, 2.4 mg of trypsin or 14.3 mg Pronase, these enzyme concentrations having been shown to be proteolytically equivalent through the use of Azocoll (Calbiochem.). A magnetic stirrer was used with a 5.0 cm follower rotating at 100–120 rpm. After cell lysis the suspension was centrifuged for 10 min at 650 *g* to remove fibre and cell debris and the supernatant was filtered through a double layer of muslin before centrifuging at 10,000 *g* for 5 min to sediment the mitochondria. When the supernatant was decanted from the mitochondria a thin whitish layer, presumably composed of membranes, also slid away from the surface of the pellet and was removed. The particles were resuspended prior to testing in 2.5 ml of isolation medium. Their functional characteristics were determined with the use of a biological oxygen monitor (Yellow Springs Instrument Co. Inc.) using the buffered medium described by Dow [3].

3. Results

The activity of these skeletal muscle mitochondria with respect to oxidative capacity and energy-coupling compare very favourably with activities quoted by Ernster [4] and by Dow [3] in the absence of heparin. Some values, e.g. respiratory control ratios on α-ketoglutarate, pyruvate, and succinate are very similar to

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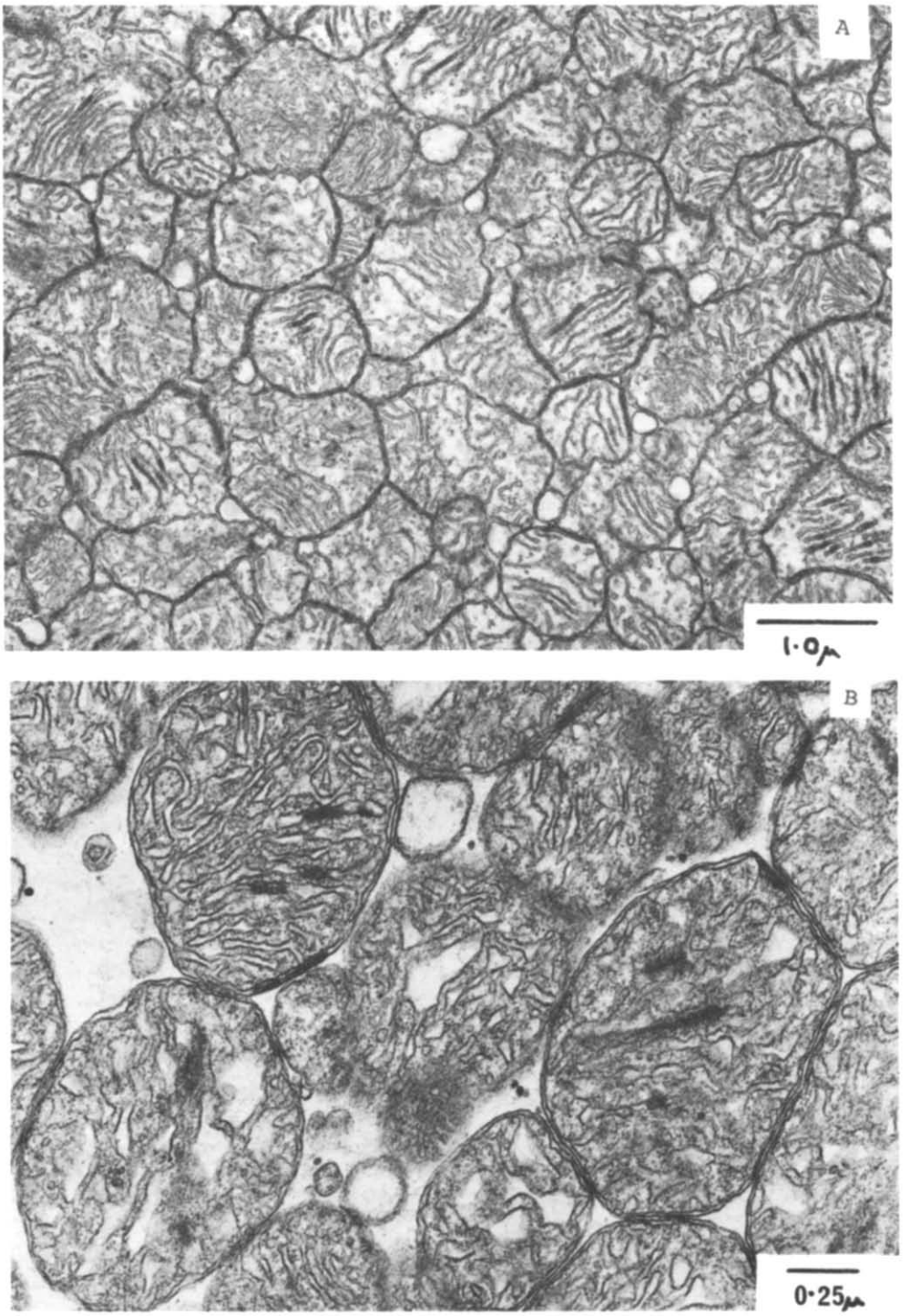

Fig. For electron microscopy the mitochondrial pellet was fixed *in situ* with Millonig's OsO_4 solution and embedded in Epikote 812. Sections were cut from representative areas on diamond knives and stained with uranyl acetate and lead citrate. (A) × 20,000; (B) × 48,000.

Table
Functional characteristics of skeletal muscle mitochondria prepared with proteolytic enzymes.

Function	Enzyme	Substrates				
		Glutamate (10 mM)	α-Ketoglutarate (15 mM)	Pyruvate/malate (5 mM/0.5 mM)	Succinate/amytal (10 mM/2 mM)	NADH (0.2 mM)
Respiratory	Pronase	17.6 ± 0.66	12.1 ± 0.55	6.8 ± 0.10	3.4 ± 0.19	–
Control ratio	Trypsin	19.2 ± 1.9	13.5 ± 0.53	9.7 ± 0.63	3.3 ± 0.10	–
state 3 → state 4	Nagarse	12.4 ± 1.3	10.3 ± 0.70	7.1 ± 0.70	2.6 ± 0.20	–
QO_2	Pronase	215 ± 10	159 ± 13	192 ± 8	328 ± 26	63 ± 5
(natoms oxygen/min	Trypsin	271 ± 24	216 ± 13	177 ± 10	321 ± 21	69 ± 2
/mg mitochondrial protein)	Nagarse	255 ± 16	193 ± 8	171 ± 13	188 ± 8	67 ± 4
	Pronase	3.00 ± 0.15	3.2 ± 0.19	3.09 ± 0.10	1.81 ± 0.06	
ADP:O ratio	Trypsin	2.81 ± 0.04	2.77 ± 0.09	2.78 ± 0.08	1.72 ± 0.05	
	Nagarse	3.00 ± 0.03	2.82 ± 0.04	2.89 ± 0.08	1.85 ± 0.12	

Each figure represents the mean of determinations made on muscle from six individual animals ± S.E.M. The yields of mitochondria (mg mitochondrial protein/g muscle) were: pronase 0.84 ± 0.07; trypsin 1.27 ± 0.14; nagarse 0.91 ± 0.02.

those quoted by Dow [3] when heparin was used in the isolation medium. We were not able to detect any improvement in the performance of the mitochondria prepared by the present method when heparin was used.

The preparations had low spontaneous ATPase activity which was stimulated about ten-fold by the addition of 2,4-dinitrophenol in 0.12% albumin. The respiratory control ratio could always be measured correctly by dividing the maximum respiration rate in the presence of ADP by the rate when ADP is used up. The presence of cytochrome c made no difference to the functional characteristics of the mitochondria except with NADH as substrate. In the absence of cytochrome c the rate then dropped to about 20% of the values quoted in the table.

Under the electron microscope the pelleted material showed a range of structures (see fig.). There was a large central zone of well preserved mitochondria covering a wide range of sizes. These were characterized by well-packed cristae and sharply-defined outer membranes. Outside this there was a zone of broken mitochondria with little matrix leading into an outer zone which also contained some fibres. The organelles produced with Pronase appeared to be slightly inferior to those produced with the aid of trypsin or Nagarse.

We have no explanation as to the nature of the densely staining material in some of the inter-cristal spaces.

4. Discussion

All aspects of the procedure are critical and must be strictly adhered to for reproducible preparations of mitochondria. Taking into account morphological and functional aspects, it appears that of the three enzymes tried, trypsin is superior to the others.

There seems no reason why the method should not be applied to other tissues with suitable changes in enzyme concentration and time of contact. We have applied the method successfully to heart muscle without modification. The method has obvious advantages where tissue is difficult to homogenise reproducibly or where hormone or drug treatment have made some of the mitochondria more fragile than others.

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