Biochimica et Biophysica Acta, 292 (1973) 350–359
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 46474

THE ANALYSIS OF RAT SKELETAL MUSCLE MITOCHONDRIA ISOLATED BY THE METHOD OF TRYPTIC LYSIS

GILLIAN R. BULLOCK, ELIZABETH E. CARTER and A. M. WHITE

CIBA Laboratories, Horsham, Sussex, RH12 4AB (Great Britain)

(Received July 31st, 1972)

SUMMARY

1. Rat skeletal muscle mitochondria were isolated by the method of tryptic lysis developed by Bullock, Carter and White which does not utilise mechanical homogenisation.

2. These mitochondria were subjected to centrifugation on a discontinuous sucrose density gradient at $105000 \times g$ for 45 min. Broken organelles (Band 1) did not penetrate into the gradient. The remaining mitochondria collected into three bands at the interfaces of the following sucrose molarities: 1.07 M/1.12 M, Band 2, 1.12 M/1.17 M, Band 3, and 1.17 M/1.22 M, Band 4.

3. Band 2 mitochondria had a respiratory control ratio of 16 and were smaller than those in Band 3 and Band 4. They also contained the lowest proportion of broken and swollen organelles.

4. Some mitochondria in all bands showed a shift from an aggregated to an open-mesh form during transition from State 4 to State 3. Band 2 contained the highest proportion of mitochondria capable of changing in this way.

INTRODUCTION

Previous work in this laboratory has been concerned with the enlargement of skeletal muscle mitochondria which can be seen as early as 2 h after the administration of triamcinolone acetonide (20 mg/kg) to rats and which reaches a maximum 12 h after administration of this drug[1]. In order to commence an investigation of the functional properties of enlarged mitochondria produced by this method, a procedure which does not utilise a mechanical homogeniser[2] was devised for isolating mitochondria from rat skeletal muscle. This method routinely gives mitochondria with intact membranes as judged by lack of penetration of NADH, a respiratory control ratio of 19 and a range of diameters from 1 to 6 μm (ref. 3).

This paper now reports a further study of mitochondria prepared by the method of tryptic lysis using the technique of discontinuous sucrose density centrifugation. A preliminary communication has appeared[4].

MATERIALS AND METHODS

Reagents

The monosodium salt of α-glutamic acid and the dipotassium salt of ADP were obtained from Sigma (London) Chemical Co., London, S.W.6. Lyophilised

trypsin (220 units/mg) and Azocol were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Isolation and fractionation of mitochondria

Non-fractionated mitochondria were prepared from hind limb skeletal muscle of white rats using trypsin digestion as previously described[2] with Azocol standardisation[3]. The isolation medium contained, as before, 0.1 M Tris–HCl (pH 7.4), 0.21 M mannitol, 0.07 M sucrose and 0.01 M EDTA.

Fractionated mitochondria were derived from a suspension of mitochondria isolated from muscle of 3 animals and pooled in 1.5 ml of isolation medium. Such a suspension usually contained 40–50 mg mitochondrial protein. This suspension was layered on a discontinuous density gradient, containing Tris, mannitol and EDTA at the same concentration as in the isolation medium and ranging in 6 stages (2.5 ml) from 1.07 M sucrose to 1.32 M sucrose over a cushion of 1.97 M sucrose (2.5 ml). Centrifugation was carried out at $105000 \times g$ for 45 min using an MSE superspeed 50 and the 3×23 ml swing-out rotor. After centrifugation the bands (1 to 4) were clearly separated, without smearing. They were removed with a hypodermic syringe using a long needle, diluted to 20 ml with isolation medium, made 10 mM with respect to glutamate and centrifuged at $11000 \times g$ for 10 min. The pellets containing mitochondria from different bands were suspended in isolation medium (4 ml) containing glutamate (10 mM) and divided into 2 ml portions for 5 min incubation at 37 °C in the presence or absence of ADP (1 mM).

Respiratory control ratios and R.Q., of fractionated mitochondria were measured by previously described methods[2].

Electron microscopy was carried out on mitochondria fixed by the method of Hirsch and Fedorko[5]. The mitochondria were resuspended in the fixative as described by Hirsch and Fedorko[5] and all subsequent centrifugation was carried out at 3000 rev./min for 10 min. The only difference between our method and the one described by Hirsch was that the acetate buffer was pH 5.6 and not 6.3. The mitochondria tended to clump after the fixing and dehydration processes and therefore they were embedded directly in Epon without using the agar intermediate.

Chemical methods

Phospholipids were extracted from mitochondria by the method of Folch *et al.*[6]. Phosphorus determinations were carried out according to Martin and Doty[7]. Protein was measured by the method of Lowry *et al.*[8].

RESULTS

Fixation and morphology of non-fractionated mitochondria

When muscle is fixed for electron microscopic examination by the method involving perfusion of glutaraldehyde as described by Bowes *et al.*[9] a variety of mitochondrial forms is seen (Fig. 1). In the same way non-fractionated mitochondria prepared by tryptic lysis and lightly pelleted also showed a variety of forms when fixed simultaneously with glutaraldehyde and osmium according to the method of Hirsch and Fedorko[5] (Fig. 2).

It is true that because the mitochondria are gently teased from the tissue

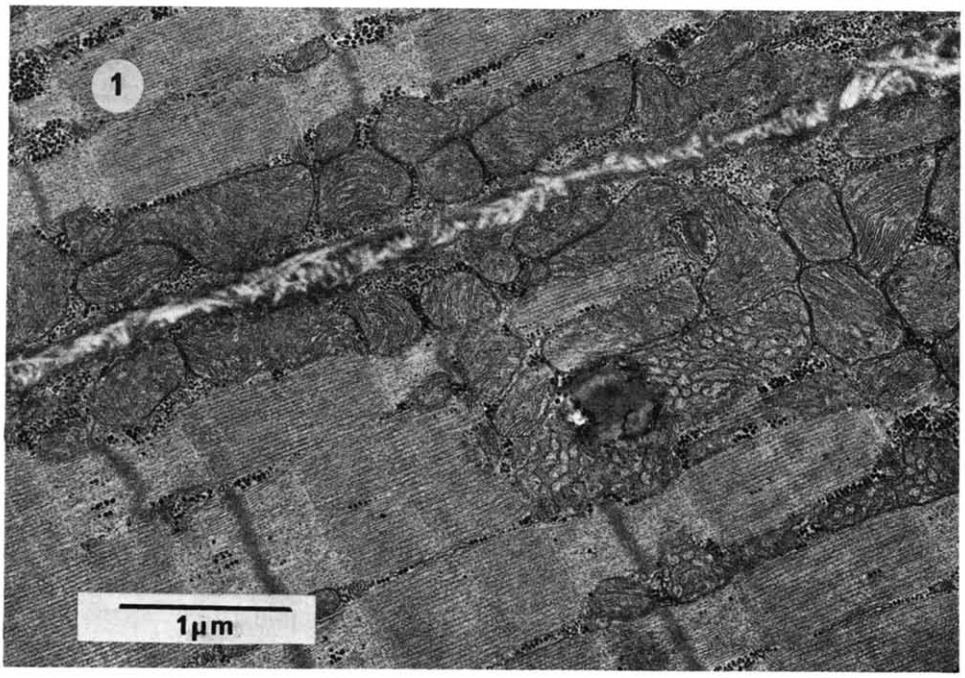

Fig. 1. Rat hind limb skeletal muscle fixed by perfusion of glutaraldehyde at 34 °C. A proportion of the mitochondria are seen to be in the open-mesh form frequently associated with lipid droplets. The remainder are non-energised and non-aggregated.

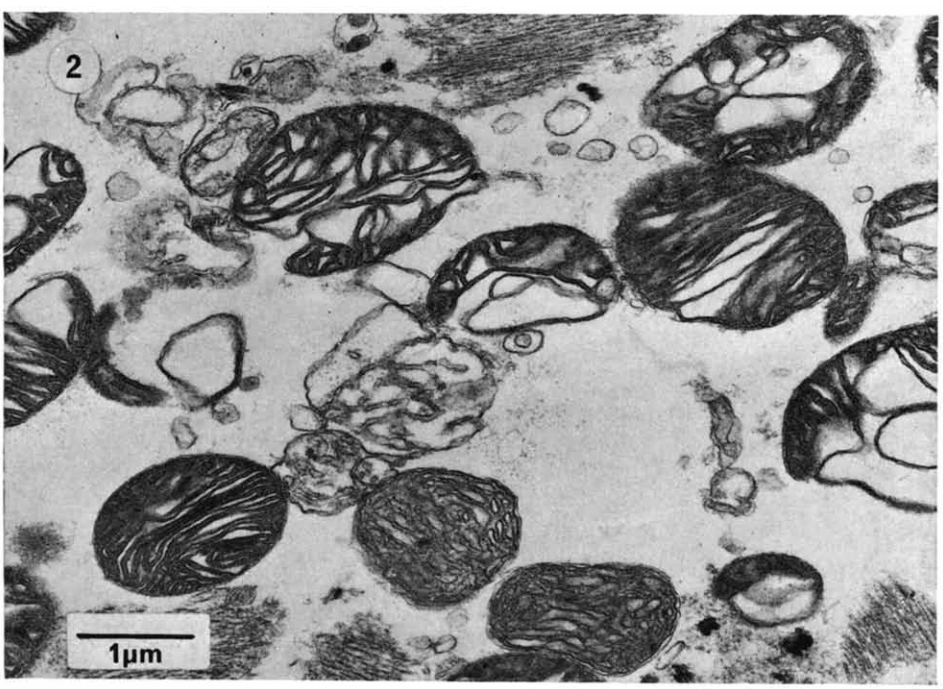

Fig. 2. Range of mitochondrial structure seen in the non-fractionated pellet.

some fibrous and membranous material is always present in the original pellet but previous experience shows that there is not enough contamination to appreciably decrease the respiratory control ratio of organelles prepared in this way. The fact that even after prolonged centrifugation the mitochondria can still be isolated with a respiratory control ratio as high as 16 (Fig. 3) is additional evidence on this point.

Morphology, chemical composition and function of fractionated mitochondria

After centrifugation of the non-fractionated mitochondria on the discontinous sucrose-density gradient, four bands were detectable (Fig. 6). Band 1 which did

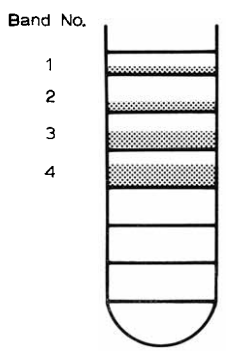

Fig. 3. Position of bands of mitochondria after centrifugation on a discontinuous sucrose gradient.

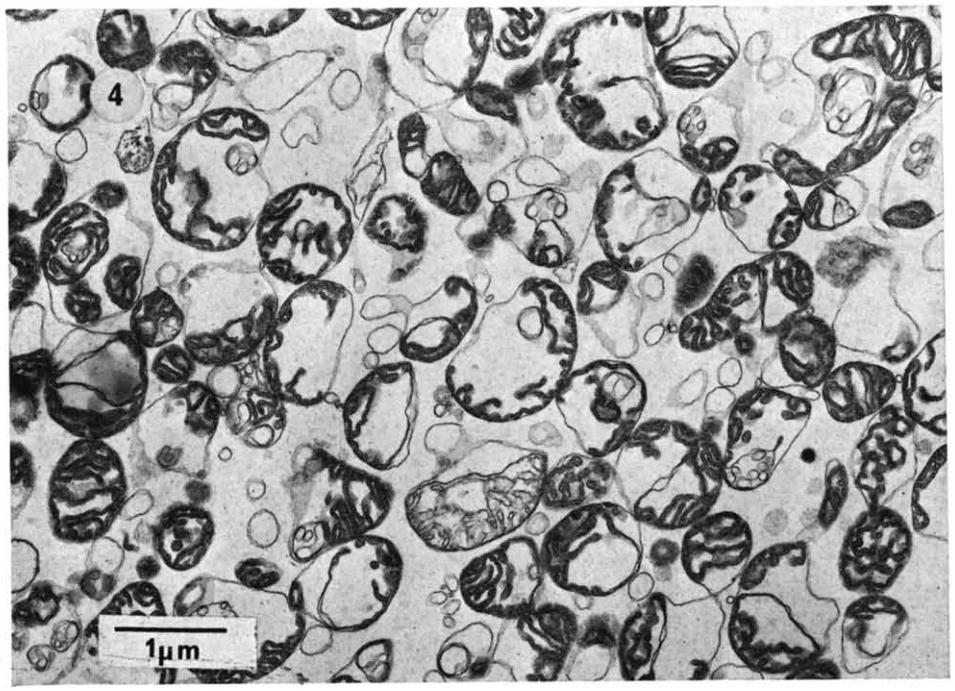

Fig. 4. Band 2 mitochondria seen in a highly condensed state and still containing sucrose from the gradient.

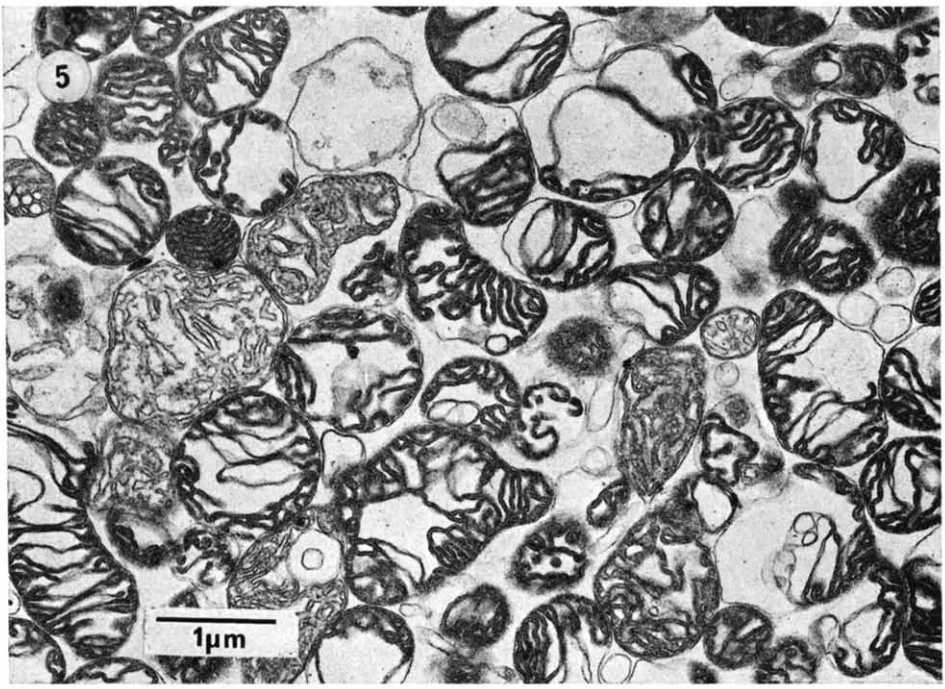

Fig. 5. Band 3 showing a greater variety of mitochondrial structures. The cristae appear less swollen and the organelles generally larger than in Band 2.

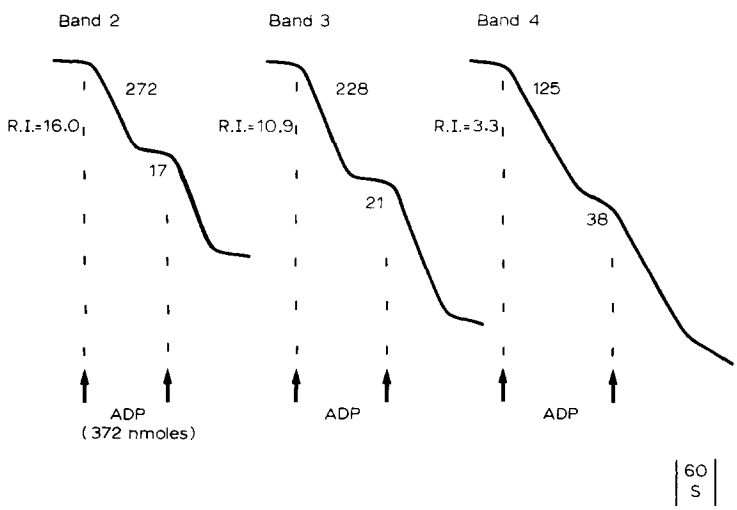

Fig. 6. Representative oxygen electrode tracing of fractionated rat muscle mitochondria. Rates of oxygen utilization in ng atoms of oxygen/mg protein per h were measured with 10 mM glutamate as substrate and 0.2 to 0.5 mg mitochondrial protein in medium previously described[2]. Respiratory index (R.I.) is the rate of oxygen utilisation in the presence of ADP divided by the rate after ADP exhaustion.

not penetrate the gradient contained mostly broken mitochondria and membranous fragments. Band 2 (Fig. 4) at the 1.07 M to 1.12 M interface contained smaller organelles than Band 3 (Fig. 5) at the 1.12 M to 1.17 M interface. Band 4 at the 1.17 M to 1.22 M interface contained some very large organelles and others which were very swollen, together with aggregated mitochondrial debris from other bands which had been pulled down through the gradients.

The respiratory control ratio of mitochondria in Band 2 was still high at 16 (Fig. 6) and that of Band 3 was lower at 10.9. The respiratory control ratio of Band 4 was very low (3.3) and these mitochondria were not studied further.

The protein (μg/ml) to phospholipid (μg/ml) ratios in suspensions of the Bands 2, 3 and 4 were 7.4, 6.8, and 7.1, respectively. The measurements were made in duplicate and fractions were examined for purity under the electron microscope before chemical analysis.

State 4 morphology of fractionated mitochondria

It was confirmed that mitochondria from Band 2 (Fig. 7) were smaller than those in Band 3 (Fig. 8). They had a very condensed matrix and a few (arrows) were seen in the form described as energised-twisted by Green and Baum[10]. However this form is now known to be a fixation artifact[11].

Mitochondria in Band 3 (Fig. 8) showed greater heterogeneity of form in State 4 than those in Band 2. Aggregated (A) and non-aggregated (N) mitochondria

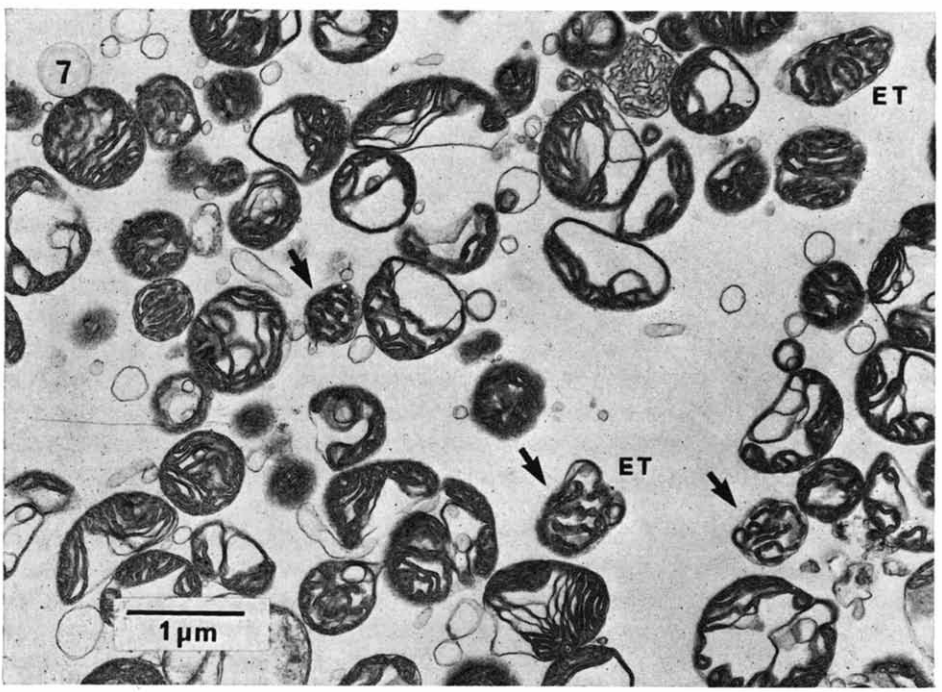

Fig. 7. Band 2 mitochondria in respiratory State 4. The cristae are less swollen than in Fig. 4 but the matrices are still aggregated. Some are in the energised-twisted (ET) form.

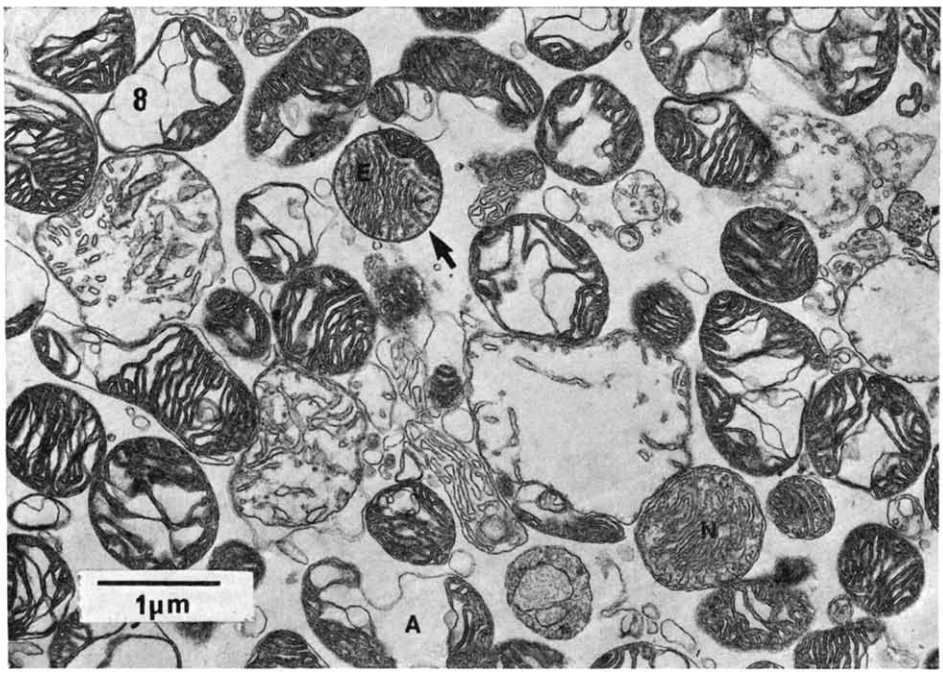

Fig. 8. Band 3 mitochondria in respiratory State 4. Most mitochondria similar in appearance to Band 3 (Fig. 5) with a proportion in the non-energised non-aggregated state (N). Also illustrated are mitochondria in the aggregated (A) and energised (arrow) forms.

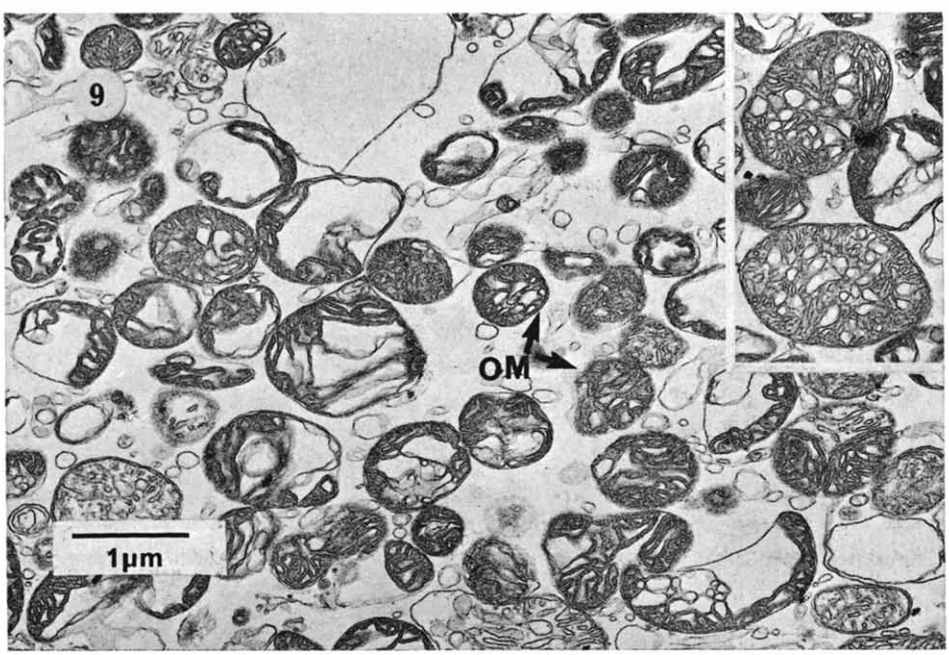

Fig. 9. Band 2 mitochondria in respiratory State 3. The predominantly aggregated open mesh (OM) appearance of the cristae produced by the ADP in the incubation medium.

were seen, also some which were remarkable for the number of angulations on the cristae. A mitochondrion (arrow) which shows these angulations in one-half and an aggregated structure in the other half is seen in Fig. 8.

State 3 morphology of fractionated mitochondria

Band 2 mitochondria (Fig. 9), whilst containing a very small proportion with a structure indistinguishable from those seen after incubation in the absence of ADP, also contained a larger proportion in which the cristae were no longer in parallel or near-parallel array but have very frequent anastomoses. A vesiculated appearance results which we have called open-mesh (OM). These mitochondria with open mesh cristae ranged from small organelles with fairly dense matrix to a few with evidence of swelling and reduced matrix density. A pair of well preserved, slightly swollen open-meshed forms are seen in the insert to Fig. 9. Band 3 had a higher proportion of mitochondria in this swollen open-mesh form than Band 2 and again different morphological forms in the same mitochondrion (Fig. 10 arrows).

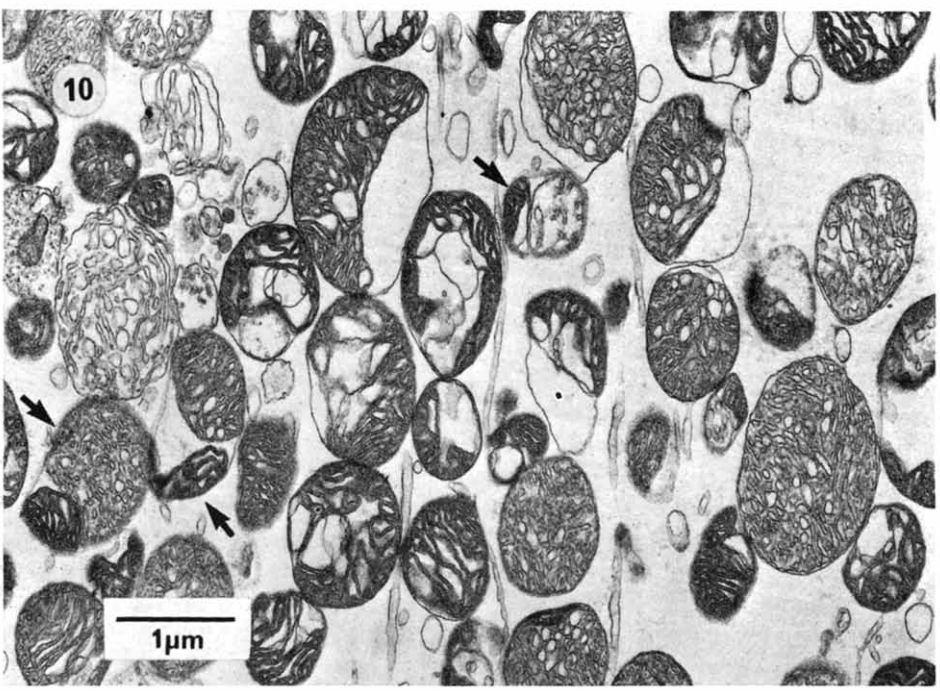

Fig. 10. Band 3 mitochondria in respiratory State 3. A high percentage of these mitochondria are in the swollen open-mesh state. Some mitochondria showed a dual response (arrows).

DISCUSSION

The use of simultaneous fixation by glutaraldehyde and osmium together has not generally received much support until recent times since a chemical reaction occurs when these substances are mixed, detectable by the development of a brown colour, and a reduction in the effective concentration of the two fixatives.

However, by following the method of Hirsch and Fedorko[5], in which the two component solutions are stored and mixed at 4 °C it was possible to complete fixation of the samples before chemical change in the fixatives had occurred. The wide variety of structures seen after the method of fixation is consistent with our finding that rapid fixation of muscle by perfusion with glutaraldehyde at 34 °C also revealed a variety of mitochondrial structures (Fig. 1). Also fewer broken mitochondria were apparent than when either 1% (w/v) OsO_4 (Millonigs) or 2% (w/v) potassium permanganate was used as described by Butler and Judah[12]. Not only did the latter method give a uniform appearance to all the mitochondria but it also resulted in a considerable loss of matrix material during fixation.

Mitochondria depicted in Figs 4, 7 and 9 are from Band 2 and those in Figs. 5, 8 and 10 are from Band 3. By comparing these sets of figures it can be seen that mitochondria in Band 2 were smaller than those in Band 3. It could be construed that the mitochondrial population of apparently lowest density (Band 2) had a lowered protein/phospholipid ratio because of excessive destruction, and removal of mitochondrial protein during incubation of the muscle mince with trypsin. However, this view seems unlikely because these were the mitochondria which retained the highest respiratory control ratio and the highest protein/phospholipid ratio. This would not have been so if their integrity had been partially destroyed through loss of protein. It is possible that the mitochondria were separating as a result of artifacts introduced as a result of elevated hydrostatic pressures produced during centrifugation[13]. In our case this seems unlikely since blurring of enzyme distribution described by Wattiaux *et al.*[13] did not take place until centrifugal forces of over $164000 \times g$ were used. The maximum g used in the work described here was $105000 \times g$.

Pollak and Munn[14] used different fixation techniques in their work on liver mitochondria but it would appear that Bands 2 and 3 from muscle have similar morphology to B2 from liver, particularly since mitochondria in these bands did not swell or lose their condensed form when centrifuged through isotonic medium *plus* glutamate. From this we infer that our methods do not induce artifactual changes in membrane permeability which would be the basis of strict segregation into organelles in which the matrix space was either completely accessible or inaccessible to sucrose. Therefore, since the protein/phospholipid ratio was lower in Band 3 than in Band 2, mitochondrial size was mainly responsible for the separation. This conclusion is supported by the electron micrographs. It seems probable that Band 2 and Band 3 mitochondria are representative of different *in vivo* populations. This view is supported by studies on their enzyme content and rates of oxygen uptake in the presence of butyrate (Bullock, G. R., Carter, E. E., Gillam, E. A. and White, A. M., unpublished). From knowledge of the morphology of red and white muscle the smallest mitochondria (Band 2) probably come from the inner zones of white fibres and the larger mitochondria from the outer zones of white fibres and from red muscle.

The morphology of the mitochondria after incubation with glutamate as substrate, should correspond to respiratory State 4. It was found that in general the mitochondria in Band 2 and most of those in Band 3 took on a characteristic appearance with cristae in more or less parallel array and a fairly dense matrix. This would appear to be equivalent to the orthodox form of Hackenbrock[15] with

a dense matrix, or perhaps a better description would be the aggregated non-energised form described by Green[10].

The addition of ADP and glutamate to the incubation medium and an incubation period of 5 min so that excess of substrate was always present ensured mitochondria which were actively phosphorylating and in respiratory State 3. Morphologically this was seen as a shift of a large proportion of the mitochondria from the non-energised aggregated form to an open-mesh pattern. This was characteristic for all bands although only a small percentage changed over in Band 4. Mitochondria from Band 2 which were predominantly small and which mostly retained an aggregated matrix even in the open-mesh form possessed the highest respiratory control ratio.

Our results differ from those of Kuner and Beyer[16] who were unable to show morphological changes as a result of State 3 to State 4 respiratory changes. We have shown that mitochondria from muscle, do, in fact, undergo conformational changes as a response to changes in energy state and are therefore similar to mitochondria from liver where this has been previously clearly demonstrated. It is possible that the absence of mechanical homogenisation is an advantage in this respect.

ACKNOWLEDGMENTS

Thanks are expressed to Dr P. Simpson for estimating the protein/phospholipid ratios and to Dr W. Stäubli for valuable comment and criticism.

REFERENCES

1 Bullock, G. R., Christian, R. A., Peters, R. F. and White, A. M. (1971) *Biochem. Pharmacol.* 20, 943–953
2 Bullock, G. R., Carter, E. E. and White, A. M. (1970) *FEBS Lett.* 8, 109–111
3 Bullock, G. R., Carter, E. E., Elliot, P., Peters, R. F., Simpson, P. and White, A. M. (1972) *Biochem. J.* 127, 881–892
4 Bullock, G. R., Carter, E. E. and White, A. M. (1971) *Biochem. J.* 125, 107P
5 Hirsch, J. G. and Fedorko, M. E. (1968) *J. Cell Biol.* 38, 615–627
6 Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* 266, 497–509
7 Martin, J. B. and Doty, D. M. (1949) *Anal. Chem.* 21, 965–967
8 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
9 Bowes, D., Bullock, G. R. and Winsey, N. J. P. (1970) in *Microscopie Electronique* (Favard, P., ed.), p. 397, Société C.I.B., Paris
10 Green, D. E. and Baum, H. (1970) *Energy and the Mitochondrion*, p. 114, Academic Press, New York and Londen
11 Hunter, G. R. and Brierley, G. P. (1971) *J. Cell Biol.* 50, 250–255
12 Butler, W. H. and Judah, J. D. (1970) *J. Cell Biol.* 44, 278–287
13 Wattiaux, R., Wattiaux-de Coninck, S. and Ronveaux-Dupal, M. -F. (1971) *Eur. J. Biochem.* 22, 31–39
14 Pollak, J. K. and Munn, E. A. (1970) *Biochem. J.* 117, 913–919
15 Hackenbrock, C. R. (1966) *J. Cell Biol.* 30, 269–297
16 Kuner, J. M. and Beyer, R. E. (1970) *J. Membrane Biol.* 2, 17–84