CHARACTERISTICS OF SKELETAL MUSCLE MITOCHONDRIA ISOLATED BY A NEW, IMPROVED TECHNIQUE

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Relatively few studies of mitochondria isolated from normal and diseased mammalian skeletal muscle have been reported (Azzone and Carafoli, 1960; Azzone et al, 1961; Hedman et al, 1962; Tata et al, 1963; Bode and Klingenberg, 1964; Klingenberg, 1964; Klingenberg and Schollmeyer, 1965; Hedman, 1965; Gustafsson et al, 1965). In large part this stems from the difficulty, unreliability and inefficiency of the isolation techniques available and from the low content of mitochondria in mammalian skeletal muscle relative to liver, kidney, heart or pigeon breast (Klingenberg, 1964). The purpose of this paper is to describe a new, simple, reliable technique for isolation of rat or human skeletal muscle mitochondria and to comment on certain interesting metabolic characteristics of the mitochondria so isolated. The technique allows preparation of skeletal muscle mitochondria from small biopsies of normal humans, and data will be presented comparing such mitochondria with those of patients having severe hyperthyroidism and muscle weakness. Because of the increased efficiency of mitochondrial isolation this technique should stimulate the study of mitochondria in human muscle in a variety of diseases.

Standard precautions for preparation of mitochondria were used throughout this study. Approximately 5 grams of muscle from the hind limbs of Wistar male rats (weighing 300-400 grams) were excised, cooled (0-4°), dissected free of gross fibrous and adipose tissue, scissors minced and suspended in ice-cold modified Chappell-Perry media (Chappell and Perry, 1954) containing 0.05M TES (N-Tris (hydroxynethyl) Methyl-2-

Amino-Ethane Sulfonic Acid), 0.1M KCl, 0.005M $MgSO_4$, 0.001M ATP, 0.001M EDTA and 5 mg bovine serum albumin (Pentex)/ml, pH 7.4. Respiratory control ratio (RCR) is the ratio of Q_{O_2} in state 3 to Q_{O_2} in state 4 after added ADP is converted to ATP. When P_i is omitted from the medium both the Q_{O_2} and respiratory control ratio or RCR of the resultant mitochondria are generally lower than with P_i present during preparation, although final incubation conditions are identical. Albumin increases the RCR and the length of time these mitochondria are functional.

Homogenization was accomplished in a CO_2-cooled Bronwill mechanical cell homogenizer (Braun Model MSK, Van Waters and Rogers, Inc., Braun Division, Los Angeles). This method of homogenization is preferable to the Potter-Elvehjem or similar instruments because it allows precise control of the temperature and degree of homogenization. In our experience, glass beads of 0.5 mm diameter provide excellent homogenization of minced rat or human skeletal muscle in 30 seconds at 4,000 cycles per minute with a bead/tissue ratio (w/w) of 7:1 and a tissue concentration of 18% (w/v). The mitochondria are then isolated by differential centrifugation. Two low speed runs (70 x g, 10 minutes) are performed to remove debris, after which the mitochondria are sedimented at 3,500 x g, 10 minutes. The mitochondria are then washed twice with 20 mls of the same medium described above minus ATP. An aliquot is washed in albumin-free medium for subsequent protein determination. A final suspension of mitochondria (10 to 20 mg protein/ml) is then made in the ATP-free medium for oxygraph work. Speeds higher than 70 x g for the low speed runs result in significant loss of mitochondria. More mitochondria can be isolated from the 3,500 x g supernatant but as noted previously (Hedman, 1965) these are damaged and exhibit poor respiratory control.

The yield of mitochondria at 3,500 x g averages 4.2 mg of mitochondrial protein per gram wet-weight rat skeletal muscle and approaches three fold that reported with other homogenization techniques (Hedman, 1965). Using

Potter-Elvehjem homogenization our yields of rat skeletal muscle mitochondria average only about 1.8 mg mitochondrial protein per gram, a yield slightly higher than that reported previously (Hedman, 1965). The precise control of temperature and degree of homogenization attainable in the Braun cell homogenizer probably accounts for the greatly increased yield of intact mitochondria.

All determinations of respiratory control were carried out at pH 7.4, 26° in media containing 25 mM TES, 30 mM P_i, 8.0 mM $MgSO_4$, 0.5 mM EDTA, 50 mM KCl, 5 mg bovine serum albumin/ml and about .75 mg mitochondrial protein/ml. Substrates were present in concentrations given in Table I.

TABLE I

CHARACTERISTICS OF RAT SKELETAL MUSCLE MITOCHONDRIA

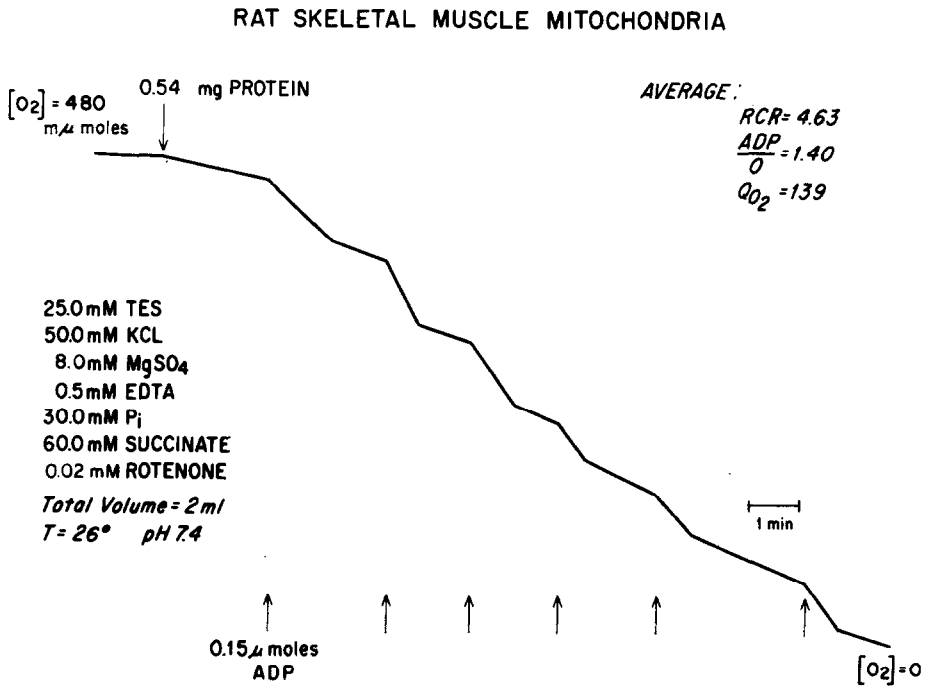
Substrate	Q_{O_2} µl/hr/mg protein	RCI*	ADP/O
Pyruvate (15mM) + Malate (15mM)	142	5.7	2.1
Succinate (60mM) + Rotenone (0.02mM)	139	4.6	1.4
α-Glycerophosphate (22.5 mM)	42	1.5	-
NADH (15mM)	0	-	-
NADH + Cytochrome c (.015mM)	23	-	-
Palmitate (1mM)	0	-	-
Palmitate + Carnitine (1mM) + CoA (.02mM)	30	-	-

*Average Value: $\frac{Q_{O_2} \text{ with ADP}}{Q_{O_2} \text{ after added ADP forms ATP}}$

Table I shows certain metabolic characteristics of mitochondria obtained with this method. As expected, the Q_{O_2}'s of these mitochondria vary with different substrates but are consistent enough to allow detection of any significant differences which might occur in diseases of animals or humans. The Q_{O_2}'s in state 3 with pyruvate-malate, succinate and

α-glycerophosphate equal or exceed those reported previously for rat skeletal muscle mitochondria which averaged 72, 40 and 35 μl/mg protein/hr for pyruvate-malate, succinate and α-glycerophosphate respectively (Hedman, 1965; Hedman et al, 1962; Azzone and Carafoli, 1960; Klingenberg, 1964). With our preparation the Q_{O_2}'s are only slightly stimulated by cytochrome c. Previously reported cytochrome c-stimulated oxidation of pyruvate-malate, succinate, and α-glycerophosphate (Hedman, 1965; Hedman et al, 1962) probably manifested damage of the mitochondria with loss of cytochrome c. Respiratory control ratios (RCR) and Q_{O_2}'s obtainable with succinate in the presence of rotenone (Figure 1) are as good or better than those seen with pyruvate-malate in preparations obtained by previous techniques including Potter-Elvehjem homogenization (Hedman, 1965) and grinding with or without quartz-sand (Azzone and Carafoli, 1960). Presumably this indicates a degree of purity as well as biochemical and morphologic integrity not previously obtainable with skeletal muscle mitochondria, nor, for that matter, with many preparations of liver mitochondria. Oxidation of succinate in the presence of rotenone far exceeds that of α-glycerophosphate in contrast to previous preparations of rat skeletal muscle mitochondria (Klingenberg and Schollmeyer, 1965). Our preparations oxidize α-glycerophosphate relatively slowly and this is not a manifestation of purity or concentration of the α-glycerophosphate used. Other metabolic and morphologic characteristics of rat skeletal muscle mitochondria prepared by these techniques will be reported in detail elsewhere.

In contrast to previous reports (Hedman, 1965; Hedman et al, 1962), NADH is not oxidized by these mitochondria in the absence of added cytochrome c despite the fact that other experiments (Peter and Lee, 1967) show that the concentration of P_i present in the isolation medium is sufficient to cause significant mitochondrial swelling. The inability to oxidize NADH most likely indicates a degree of membrane integrity or purity of the mitochondria not heretofore observed with mitochondria isolated from rat or human skeletal muscle.



Previous reports indicated that rat skeletal muscle mitochondria do not undergo the swelling and contraction characteristic of liver mitochondria (Tata et al, 1963). Skeletal muscle mitochondria which are similar to liver mitochondria with respect to swelling and contraction may be isolated using the technique and media described herein (Peter and Lee, 1967). Likewise, skeletal muscle mitochondria prepared by this method catalyze CoA-carnitine-dependent fatty acid oxidation (Table I) whereas skeletal muscle mitochondria isolated by previous methods fail to show palmitate oxidation unless the fatty acid-carnitine ester was used (Bode and Klingenberg, 1964; Klingenberg, 1964). This inability to oxidize fatty acids was perplexing because fatty acids are thought to serve as major energy sources for skeletal muscle (Rabinowitz and Zierler, 1962). With our preparations the rates of fatty acid oxidation vary with the incubation medium employed but this is not likely to explain previous failure to observe fatty acid oxidation by rat skeletal muscle mitochondria.

TABLE II

CHARACTERISTICS OF SKELETAL MUSCLE MITOCHONDRIA FROM NORMAL AND HYPERTHYROID HUMANS

Substrate	Parameter Studied	Normal**	Hyperthyroid †
Pyruvate (15 mM)	Q_{O_2}*	87	63
+	RCR ††	3.28	3.66
Malate (15 mM)	ADP/O	2.53	2.24
Succinate (45 mM)	Q_{O_2}	100	74
+	RCR	3.05	4.32
Rotenone (0.02.5 mM)	ADP/O	1.46	2.03
α -glycerophosphate (22.5 mM)	Q_{O_2}	34	16

* µl O_2/mg protein/hour
** Average values from 4 individuals
† Average values from 2 individuals
†† Respiratory control ratio

Having gathered considerable data on rat skeletal muscle, attention is now focused on mitochondria from normal and diseased human muscle using biopsies as small as 2 grams of muscle in contrast to the 10-20 gram biopsies employed in previous studies (Azzone et al, 1961). The Q_{O_2}'s reported here for pyruvate-malate, succinate, and α-glycerophosphate are considerably higher than previously reported values of 68.4, 29.2, 17.9, for normals (Ernster and Nordenbrand, 1967). Mitochondria from skeletal muscle of two patients with severe hyperthyroidism and muscle weakness showed no significant differences from normal in Q_{O_2}, RCR, and ADP/O ratio (Table II). Q_{O_2} of α-glycerophosphate was not enhanced in contrast to what would be expected from previously reported data on thyroxin-treated animals (Tata et al, 1963) and data showing induction of α-glycerophosphate oxidase by thyroid hormone (Sellinger and Lee, 1964). Further studies of human skeletal muscle mitochondria under a variety of conditions are now quite reasonable with the technique described herein, and are being pursued in this laboratory.

REFERENCES

G.F. Azzone, O. Eeg-Olofsson, L. Ernster, R. Luft, G. Szabolcsi, Experimental Cell Research, 22, 415 (1961).

G.F. Azzone, E. Carafoli, Experimental Cell Research, 21, 447 (1960).

C. Bode, M. Klingenberg, Biochimica et Biophysica Acta, 84, 93 (1964).

J.B. Chappell, S.V. Perry, Nature, 173, 1094 (1954).

L. Ernster and K. Nordenbrand in Methods in Enzymology, R.W. Estabrook and M.E. Pullman, Ed. (Academic Press, New York, 1967), vol x.

R. Gustafsson, J.R. Tata, O. Lindberg, and L. Ernster, The Journal of Cell Biology, 26, 555 (1965).

R. Hedman, E.M. Suranyi, R. Luft, L. Ernster, Biochemical and Biophysical Research Communications, 8, 314 (1962).

R. Hedman, Experimental Cell Research, 38, 1 (1965).

M. Klingenberg, Ergeb. Physiol. Biol. Chem. Exptl. Pharmakol., 55, 131 (1964).

M. Klingenberg, P. Schollmeyer, Biochemische Zeitschrift, 341, 271 (1965).

J.B. Peter, L.D. Lee, Unusual Effects of a Substituted Throphen on Mitochondrial Swelling, Contraction and Oxidative Phosphorylation, Biochem. Biophys. Res. Comm., 26, 1053 (1967).

D. Rabinowitz, K. Zierler, Journal of Clinical Investigation, 41, 2191 (1962).

O.Z. Sellinger, K.L. Lee, Biochimica et Biophysica Acta, 91, 183 (1964).

J.R. Tata, L. Ernster, O. Lindberg, E. Arrhenius, S. Pedersen, R. Hedman, Biochemical Journal, 86, 408 (1963).

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