Mitochondrial functions in five cases of human neuromuscular disorders

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SUMMARY We determined the respiration, respiratory control, and $P_i:O$ ratios with different substrates in mitochondria isolated from five cases of human neuromuscular disorders (two cases of central core disease, two cases of neuropathy of Dejerine-Sottas, and one case of Kugelberg-Welander's disease) and compared them with normal human muscle. In all the myopathies studied, a severe derangement of the respiratory control with variable derangement of oxidative phosphorylation was found. This supports the idea that a group of neuromyopathies shares the same biochemical lesion as the so-called mitochondrial myopathies, forming with them a group of myopathies which may be related through a similar biochemical lesion of varying degree. Alternatively, disturbance of mitochondrial functions in a number of myopathies could be considered as a non-specific finding.

Despite the frequent occurrence of morphological changes in mitochondria in many types of human neuromuscular disorders (Gruner, 1963; Aleu and Afifi, 1964; Gonatas, Perez, Shy, and Evangelista, 1965; Norris and Panner, 1966; Gonatas, 1967; Engel and Dale, 1968; Chou, 1969), several myopathies have been described in which abnormalities of this organelle were considered to be of major significance (Luft, Ikkos, Palmieri, Ernster, and Afzelius, 1962; Shy, Gonatas, Perez, 1966; Coleman, Nienhuis, Brown, Munsat, and Pearson, 1967; Van Wijngaarden, Bethlem, Meyer, Hülsmann, and Feltkamp, 1967; Price, Gordon, Munsat, and Pearson, 1967; Hülsmann, Bethlem, Meyer, Fleury, and Schellens, 1967; D'Agostino, Ziter, Rallison, and Bray, 1968; Bradley, Hudgson, Gardner-Medwin, and Walton, 1969; Spiro, Prineas, and Moore, 1970; Salomon, Esiri, and Ruderman, 1971) and for such cases, the term 'mitochondrial myopathies' has been coined (Price *et al.*, 1967).

Mitochondrial myopathies do not seem to show a uniform pattern of clinical symptoms or microscopic lesions and, therefore, are usually defined by the existence of various kinds of severe alterations of the mitochondrial structure. Five cases of mitochondrial myopathies have been studied biochemically (Luft *et al.*, 1962;

Hülsmann, *et al.*, 1967; Van Wijngaarden *et al.*, 1967; Spiro *et al.*, 1970). These studies suggest that mitochondria from mitochondrial myopathies have a characteristic functional lesion: they are loosely coupled. That is, these mitochondria lack respiratory control (ratio between respiration in the presence of ADP and respiration in the absence of ADP), while the phosphorylation (quantity of ADP phosphorylated for each molecule of oxygen consumed) remains unaltered. Since mitochondria with good phosphorylating ability are frequently referred to as 'well-coupled', the term 'loosely-coupled' is equivocal. It may be preferable to substitute the term 'uncontrolled-well-coupled' (lack of respiratory control, well-coupled phosphorylation) for 'loosely-coupled' when defining mitochondrial myopathies.

We have studied the functions of mitochondria isolated from muscle specimens of five cases of human neuromuscular disorders (none of which was considered as primarily mitochondrial) in comparison with mitochondria from normal muscle.

METHODS

The human neuromuscular diseases studied were two cases of central core disease (father and daughter)

ter), two cases of neuropathy of Dejerine-Sottas, and one case of Kugelberg-Welander's disease. The diagnosis of each was arrived at by clinical, histological, histochemical, and electron microscopy investigations. A complete description of the cases will be reported elsewhere. Normal muscle was obtained from three victims of traffic accidents and from normal rats.

Mitochondria were isolated from muscle biopsy specimens of 8 to 15 g, essentially by the method of Chappel and Perry (Ernster and Nordenbrand, 1967) with a variation in the homogenization procedure. The tissue homogenization was as follows: the excised muscle specimen, immersed in 0.15 M KCl, was blotted with filter paper, freed from fat and connective tissue, quickly weighed and immersed in an ice cold isolation medium (50 mM tris HCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP and 1 mM EDTA at pH 7.4). Then the tissue was minced with scissors, washed, and homogenized in a Sorvall omnimixer (50 ml. stainless steel chamber, rotor shaft 17165, rotor knife 1768, position 6 of speed control) for 60 seconds. The resulting homogenate was diluted 1:1 with isolation medium and further homogenized in a Teflon-glass Elvehjem-Potter homogenizer (clearance 0.15 mm. One to three strokes at 2,000 rpm, depending upon the preparation, to reach a perfect degree of homogenization). The homogenate was diluted with isolation medium and centrifugation of the mitochondria was performed by the method of Chappel and Perry (Ernster and Nordenbrand, 1967). The isolated mitochondria were washed at least three times to avoid contamination with myofibrillar ATPase.

Hülsmann, Jong, and Tol (1968) reported that homogenization with a loose-fitting homogenizer releases predominantly subsarcolemmic mitochondria with low respiratory control. Our method of homogenization gives a good yield of high quality mitochondria, as shown by our respiratory control data on normal human muscle (respiratory control from 10 to 16) and on normal rat muscle (respiratory control from 6.7 to 12) (Table 1). The rat data are included for comparison with the data of Hülsmann *et al.* (1968). The fact that the yield and the respiratory control figures found by us in normal muscle are high implies that, with our method of homogenization, a good fragmentation of the tissue was obtained, at least similar to that achieved by Hülsmann *et al.* (1968) with tight Teflon homogenizers.

Mitochondrial respiration was determined polarographically in a Clark type oxygen electrode using 0.25 M sucrose, 20 mM KCl, 7 mM $MgCl_2$, 5 mM Pi, and 10 mM tris HCl as the assay medium (pH 7.4, 22° C). The respiratory control and the Pi:O ratio

were determined as described by Estabrook (1967), by addition of a 200 micromolar aliquot of ADP to mitochondria in state 4 monitored in the oxygen electrode. The figures obtained for each parameter represent the average of three determinations. (Three aliquots of the mitochondrial suspension.) The extreme values did not surpass 7% of the average in any case.

In all cases of pathological muscle, the Pi:O ratios obtained were additionally checked by determination of Pi uptake (Slater, 1967). One aliquot of the mitochondrial suspension was incubated in the oxygen electrode for 40 minutes in the presence of 3 mM ADP. The Pi content at zero and 40 minutes was determined. Pi:O ratios determined by Pi uptake are included in Table 1 between parentheses.

Other scientists working in this field prefer, for the determination of Pi:O ratios, the assay of glucose-6-phosphate in a system in which ADP is continuously supplied by ATP, glucose, and hexokinase. Such a system usually yields low respiratory control which indicates the need for a separate determination of respiratory control adding ADP to mitochondria in state 4. Given the shortage of mitochondria imposed by human muscle biopsies, it is preferable, if respiratory control is detectable, to use a method that simultaneously yields the higher and more accurate figures of respiratory control and Pi:O ratio as in the method of Estabrook (1967). This method is able to measure the initial capabilities of mitochondria in a very short time. To illustrate the differences between the three methods of determining Pi:O ratios and respiratory control (the ADP:O ratio, Pi uptake and glucose 6 phosphate assay), the data on rat masseter shown in Table 1 were obtained with the three methods. In some preparations of rat muscle, contamination with myofibrillar ATPase cannot be avoided in spite of careful washing. Therefore, it is advisable to supplement the assay medium with EDTA when working with rat muscle. Other supplements, such as albumin or cytochrome C, are, in our opinion, unnecessary. These additions increase the respiration with NADH linked substrates but do not improve respiratory control or Pi:O ratios. Comparing the results obtained in rat masseter with the three methods used (Table 1), it can be observed that the respiratory control is lower with the glucose-6-phosphate assay method and that the figure obtained compares well with that obtained by Hülsmann *et al.* (1968) using the same method.

Mitochondrial protein was determined by the method of Gornall, Bardawill, and David (1969). The concentrations of mitochondrial substrates and other reagents in the assay medium are expressed in the legend to Table 1.

TABLE 1
COMPARATIVE CLINICAL DATA

Diagnosis	Sex, age (yr)	Clinical symptoms	Microscopic findings		Muscle biopsy mito. l g muscle (mg/g)	Glutamate plus malate as substrate				Succinate as substrate				Ascorbate plus TMPD Respiration
			Nerve	Muscle		Resp. ADP	Resp. without ADP	Resp. control (Pi:O)	ADP:O (Pi:O)	Resp. ADP	Resp. without ADP	Resp. control (Pi:O)	ADP:O (Pi:O)	
Central core (father)	M 28	Floppy baby of benign evolution		Central core Mitochondrial sub-sarcolemmal blebs	Quadriceps 2	3.91	6.57	1.68	0.41 (0.40)	4.22	9.85	1.90	0.32 (0.32)	49.00
Central core (daughter)	F 9	Floppy baby of benign evolution		Central core	Quadriceps 3-4	3.17	8.17	2.57	1.60 (1.52)	4.36	7.83	1.80	0.65 (0.74)	50.00
Dejerine-Sottas A	F 28	Peripheral neuropathy	Hypertrophic neuritis	Neurogenic atrophy Nemaline rods Mitochondrial sub-sarcolemmal blebs	Quadriceps 2-08	9.82	47.19	4.81	2.46 (2.40)	15.00	54.00	3.60	1.33 (1.30)	60.00
Dejerine-Sottas B	M 48	Peripheral neuropathy	Segmentary demyelination	Neurogenic atrophy Nemaline rods Mitochondrial sub-sarcolemmal blebs	Quadriceps 3-5	6.00	6.00	1.00	(2.60)	9.00	1.00	1.00	(1.50)	47.00
Kugelberg-Welander	M 16	Spinal atrophy pseudo-myopathic		Neurogenic atrophy Mitochondrial sub-sarcolemmal blebs	Quadriceps 3-3	3.45	4.87	1.41	2.81 (2.82)	6.00	9.42	1.57	1.77 (1.61)	48.00
Traffic accident	M 28	Normal		Normal	Gastrocnemius 6-5	2.27	22.70	10.00	2.84	3.63	32.67	9.00	1.76	43.00
Traffic accident	F 33	Normal		Normal	Sartorius 3-5	1.70	25.50	15.00	2.89	2.40	16.80	7.00	1.82	47.00
Traffic accident	M 25	Normal		Normal	Quadriceps 5-0	1.60	25.60	16.00	2.93	2.40	17.60	6.50	1.95	58.00
Rat	F 10 mth.	Normal		Normal	Massester 3-5	11.20 (a)	112.00 (a)	10.00 (a)	2.86 (2.89)	5.6 (b)	67.20 (b)	12.00 (b)	(2.80)	
						11.20 (b)	75.20 (b)	6.71 (c)	(2.80)					

Respiration is expressed in nanoatoms of oxygen per minute ad mg mitochondrial protein. The concentration of ADP was 200 μ mol per the determination of the respiratory control and the ADP:O ratios; and 3 mM per the determination of Pi uptake (P:O). The concentration of substrates was: glutamate 5 mM, malate 5 mM, succinate 6 mM, ascorbate 3 mM, and TMPD 0.2 mM. The concentration of mitochondria was 0.5 mg/ml.

(a) Data obtained with addition of 200 micromolar ADP after 3 min in state 4. The assay medium was supplemented with 0.5 mM EDTA 0.75 mg bovine serum albumin per ml. and 0.03 mM cytochrome C.

(b) Data obtained with addition of 3 mM ADP. The incubation time was 15 min in state 4 and 15 min in state 3. Pi:O ratio was obtained by Pi uptake (Slater, 1967). The medium was supplemented with 0.5 mM EDTA.

(c) Data obtained with a system of continuous production of ADP. Respiration in state 4 was measured during 15 min in the presence of glucose (25 mM) and ATP (0.5 mM). Respiration in state 3 was measured during 15 min after the addition of 1.4 u. hexokinase per ml. The Pi:O ratio was determined by the assay of glucose-6-phosphate (Slater, 1967). The medium was supplemented with 0.5 mM EDTA, 0.75 mg bovine serum albumin, and 0.03 mM cytochrome C.

RESULTS

It has been reported that a marked extent of fibrosis and fatty replacement are associated with disturbances of mitochondrial function (Peter, Stempel, and Armstrong, 1970). We observed, in our cases, only slight or discrete fibrosis and fatty replacement of exclusively perimysial localization.

SUMMARY OF HISTOLOGICAL FINDINGS IN EACH CASE

Case 1 Central core (father) Quadriceps muscle. There was slight hyperplasia of the perimysial connective tissue with an increase in fatty tissue in some areas, generally perimysial. There was an increase in endomysial fat and connective tissue in only one fascicle. Muscular structure was generally well conserved; the fibres showed diameters of 30 to 80 μ with a few isolated fibres of smaller calibre. There were fibre splitting phenomena, some isolated basophilic fibres, and some isolated signs of nuclear centring. Using histochemical techniques (ATPase), a differentiation in fibre types was seen with type 1 fibres clearly predominating (75%). Approximately half of the type 1 fibres showed, with oxidative staining (L.D.H., DPNH, Tr, and SDH), areas lacking oxidative activity; generally single and central, but occasionally eccentric and multiple. Their borders were sharp and surrounded by a halo of greater corpuscular type enzymatic activity. This same lesion appeared with conventional staining, especially in unfixed specimens. With H and E, homogeneous eosinophilic masses appeared, limited by a slight basophilic halo; with the modified Gomori method (K. Engel), they stained as homogeneous masses surrounded by an increase in the red reticulum. In longitudinal sections, these cores could be seen along the entire muscle fibre. The neuromuscular spindles were normal and did not show cores. The intramuscular nerves were normal. With trichromatic staining, many fibres showed perinuclear and subsarcolemmal accumulations of red granulation that stained strongly with oxidative stains. The core structures were confirmed by electron microscopy.

Case 2 Central core (daughter) Quadriceps muscle. There was discrete increase in perimysial connective tissue with very slight endomysial

hyperplasia. Scant quantity of adipose tissue was present in the perimysial septa and only isolated deposits endomysially. Muscle fibres of 30 to 80 μ in diameter were found with isolated atrophic fibres of 10 μ . Isolated central nuclei were present in some basophilic fibres and there were isolated signs of myofibrillar splitting. Histochemical techniques did not differentiate fibre types; all pertained to type 1. In the majority of the fibres (70%), images of central core similar to the previous cases were seen.

Case 3 Dejerine-Sottas neuropathy—A Quadriceps muscle. Discrete perimysial connective tissue and fatty proliferation without any endomysial changes were found. There was a typical picture of neurogenic atrophy, sometimes fascicular, with groups of fibres 10 μ in diameter. The non-atrophic fibres showed diameters of 30 to 60 μ . Nuclei were mostly normal with a few that were vesicular and showed prominent nucleoli. With PTAH and trichromatic methods, perinuclear and subsarcolemmal deposits of granular material that stained heavily for oxidative enzymes were seen. In the subsarcolemmal and perinuclear zones, typical nemaline rods were frequently observed. They stained intensely red with Masson and intensely blue with PTAH, sometimes forming rows and at other times displaying a random placement. With histochemical techniques, atrophy predominated in type II fibres. With oxidative stains, the majority of the fibres showed positive staining. Abundant target fibres were observed. Electron microscopy demonstrated mitochondria of normal size with crystalline inclusions, increase in intrafibrillar lipid drops (generally in contact with the mitochondria), and myofibrillar fragmentation. A biopsy of the nerve showed a typical 'onion bulb' picture.

Case 4 Dejerine-Sottas neuropathy—B Three muscle biopsies were performed on this patient: in the peroneus longus, quadriceps, and deltoid muscles. They showed a varying degree of neurogenic atrophy without significant hyperplasia of connective or adipose tissue. Perinuclear and subsarcolemmal granular accumulations, strongly positive for oxidative enzymes, were seen. They were less marked, but similar to those described in the previous case. Nemaline rods were also seen. There was a good differentiation between

type I and type II fibres. Electron microscopy confirmed the existence of accumulations of zetoide material, myofibrillar fragmentation, and alterations in the number and the morphology of the mitochondria. A biopsy of the sural nerve showed signs of segmental demyelination.

Case 5 Kugelberg-Welander syndrome Quadriceps muscle. There was slight increase in perimysial connective and adipose tissue, without endomysial invasion. A typical picture of neurogenic atrophy was present. There were isolated images of increased muscle fibre volume, with a tendency towards centring of the nuclei. There were subsarcolemmal and perinuclear granular blebs that were occasionally voluminous and stained with trichromic and PTAH stains. They were strongly positive for oxidative stains. With ATPase there was a good differentiation between type I and type II fibres. The atrophy affected both fibre types, but with a predominance of the latter. The intramuscular nerves and spindles were normal.

Electron microscopy showed an increase in the number and volume of the mitochondria, with myofibrillar derangement that started with the A band.

MITOCHONDRIAL FUNCTIONS Table 1 shows the respiration in state 4 (absence of ADP), respiration in state 3 (presence of ADP), the respiratory control, and the Pi:O ratios found in the mitochondria of the five pathological cases and in normal muscle with glutamate plus malate or succinate as substrates. This Table also includes the respiration with ascorbate plus N,N,N',N'-tetramethyl-P-phenylene diamine (TMPD), the source of the muscle, the yield of mitochondria per gram of muscle, and the age, sex, clinical, and microscopic findings in each case.

The normal muscle showed a respiratory control of 10-16 (glutamate plus malate) or 6.5-9 (succinate) and a Pi:O of 2.84-2.94 (glutamate plus malate) or 1.76-1.95 (succinate). These figures approximate to the ideal values for respiratory control and oxidative phosphorylation obtainable with isolated mitochondria. In comparison with these control values, the first case of central core disease (the father) showed a low respiration in state 3 and a relatively high respiration in state 4 with glutamate plus malate or

succinate as substrates. Consequently, the respiratory control was very low, and since in this case the phosphorylation was also very low, almost uncoupled, these mitochondria can be defined as 'uncontrolled-uncoupled' (lack of respiratory control-lack of phosphorylation). The second case of central core disease (the daughter) also showed a marked alteration of the respiratory control, but the phosphorylation was less affected than in the first case, especially the phosphorylation with glutamate and malate used as substrates. These mitochondria may be defined as 'uncontrolled-partially coupled'. Case A of neuropathy of Dejerine-Sottas showed higher respiration than the other cases; moderate derangement of respiratory control and the phosphorylation was slightly affected. Case B of Dejerine-Sottas showed a complete lack of respiratory control and a very moderate derangement of the Pi:O ratios. The fifth case, a Kugelberg-Welander's disease, showed a very low respiratory control (uncontrolled) and phosphorylation was completely conserved. These two last cases, case B of Dejerine-Sottas and the Kugelberg-Welander, present, therefore, a big chemical pattern completely similar to that designated as 'loosely coupled' in mitochondrial myopathies. (Luft *et al.*, 1962; Hülsmann *et al.*, 1963; Van Wijngaarden *et al.*, 1967; Spiro *et al.* (1970).

There was no significant difference between the controls and the myopathies in regard to the respiration with ascorbate and TMPD (Table 1).

TABLE 2
SUMMARY OF DATA IN EIGHT MYOPATHIES

Diagnosis	Respiratory control*	Pi:O*	Authors
Mitochondrial myopathy	1.25	2.6	Luft <i>et al.</i> (1962)
Mitochondrial myopathy	1.10	2.3	Wijngaarden <i>et al.</i> (1967)
Mitochondrial myopathy	3.00	2.2	Wijngaarden <i>et al.</i> (1967)
Mitochondrial myopathy	1.00	1.9	Hulsman <i>et al.</i> (1967)
Mitochondrial myopathy	2.30	—	Spiro <i>et al.</i> (1970)
Central core (Case A)	3.40	2.2	Bethlem <i>et al.</i> (1966)
Central core (Case B)	2.30	2.9	Bethlem <i>et al.</i> (1966)
Myotonic dystrophy	5.00	2.3	Wijngaarden <i>et al.</i> (1967)

* NADH linked substrates.

DISCUSSION

For comparison, Table 2 summarizes the data on respiratory control and the Pi:O ratios with NADH linked substrates in eight myopathies which have been studied biochemically in the world literature. These are: five mitochondrial myopathies, two central core diseases, and one myotonic dystrophy. The case described by Luft *et al.* (1962) shows uncontrolled mitochondria with reasonably high phosphorylation. The cases described by Hülsmann *et al.* (1967) and Van Wijngaarden *et al.* (1967) show moderate derangement of phosphorylation accompanied by lack of respiratory control, less intense in the second case of Wijngaarden. The case described by Spiro *et al.* (1970) also shows very low respiratory control. The two cases of central core disease of Bethlem, van Gool, Hülsmann, and Meijer (1966) show low respiratory control and moderately affected or normal phosphorylation. Finally, the case of myotonic dystrophy of Van Wijngaarden *et al.* (1967) has moderately affected respiratory control and phosphorylation.

Comparing Table 1 and Table 2, it can be concluded that alteration of the respiratory control is common to all myopathies and neuromyopathies studied biochemically so far. This alteration is moderate in our case A of Dejerine-Sottas neuropathy and intense in all cases of central core disease, in cases of mitochondrial myopathy, and in our case B of Dejerine-Sottas neuropathy, as well as in our case of Kugelberg-Welander's disease. The derangement of phosphorylation, however, did not occur in all these neuromuscular disorders. Our case of Kugelberg-Welander disease, our case B of Dejerine-Sottas neuropathy, the mitochondrial myopathy reported by Luft *et al.* (1962), and one central core disease of Bethlem *et al.* (1966) show practically no alteration of phosphorylation. The rest of the mitochondrial myopathies, one central core disease of Bethlem *et al.* (1966), our case of central core disease (daughter), and our case A of Dejerine-Sottas neuropathy show moderately affected phosphorylation. Finally, our central core disease (father) shows an almost complete lack of phosphorylation.

As all myopathies studied biochemically have altered respiratory control with variable alteration of oxidative phosphorylation and as our

case of Kugelberg-Welander's disease and case B of Dejerine-Sottas neuropathy show a pattern of 'loosely coupled' mitochondria (lack of respiratory control-well conserved phosphorylation) all similar to the most typical mitochondrial myopathy, there is support for the idea that there are neuromyopathies not included in the group of mitochondrial myopathies which show essentially similar biochemical lesions. This could imply that the mitochondrial myopathies along with Kugelberg-Welander's disease, central core disease, neuropathy of Dejerine-Sottas, and possibly myotonic dystrophy, form a group presenting different clinical and histological appearance but related through similar biochemical lesion of varying degree. An alternative possibility could be that the disturbance of mitochondrial function is a relatively non-specific finding seen in a number of muscle diseases. Max (1972) has reported loss of functional activity of mitochondria in experimental diffuse atrophy.

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