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Comparison of the intermediary metabolism of fatty acids in denervated and dystrophic murine skeletal muscle

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SYNOPSIS Certain aspects of lipid metabolism have been examined in denervated muscle from normal mice and in dystrophic muscle from mice of the Bar Harbor strain 129. A number of parameters show no change or similar changes. For example, the utilization of palmitate-[1-^{14}C] and palmitylcarnitine by mitochondria from denervated and dystrophic hind leg skeletal muscle showed parallel decreases in the oxidation of palmitate (30–42%) and palmitylcarnitine (37–66%). A comparable study with acetylcarnitine showed a striking difference with no change evident in mitochondria from denervated muscle and 80–85% decrease in dystrophic muscle. The study of succinate dehydrogenase and the enzymes of β-oxidation in the above mitochondrial preparation showed similar findings except for acyl CoA dehydrogenase activity (an enzyme with a regulatory role in β-oxidation) which was significantly diminished (29%) in denervated muscle, whereas no change was observed in dystrophic muscle. The findings show a close parallel in a number of parameters but distinct differences were observed in denervated as compared with dystrophic muscles. It is unlikely that the muscular disorder in murine muscular dystrophy can be explained solely on the basis of denervation or the loss of a neural trophic factor.

A basic problem in the pathogenesis of muscular dystrophy is whether the disease has a myopathic or a neuropathic origin. Because of the striking pathological alterations in dystrophic skeletal muscle and the absence of any significant change or reduction of anterior horn cells, the disorder might readily be regarded as myogenic (Papapetropoulos and Bradley, 1972). However, a number of studies have demonstrated a major role of nerve in the regulation of the metabolism and differentiation of muscle (Engel and Karpati, 1968; Guth *et al.*, 1968) and, possibly, in the causation of muscular dystrophy (cf *Nature*, 1971). The most recent evidence in support of the latter possibility derives from the electrophysiological investigations of McComas and his colleagues (McComas *et al.*, 1970; Sica and McComas, 1971; McComas *et al.*, 1971a, b). These authors provide a substantial basis for functional denervation and, in a 'neurogenic hypothesis' for muscular dystrophy, they propose a lack of a neural trophic factor, required for the survival and maintenance of muscle fibres, as the cause.

In this laboratory during the past several years a number of metabolic parameters, particularly relating to lipid metabolism, have been studied in muscular dystrophy of the mouse (Lin *et al.*, 1969, 1970; Jato-Rodriguez *et al.*, 1972a, 1974). Defects in the oxidation of palmitate (30–42% inhibition) (Lin *et al.*, 1969, 1970) and of the acetyl group of acetylcarnitine (85% inhibition) (Jato-Rodriguez *et al.*, 1972a) were demonstrated in homogenates and mitochondria isolated from dystrophic mouse. The findings that coenzyme A levels were reduced 60% in these mitochondria strongly suggested that these defects in oxidation are the result of a coenzyme

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A deficiency (Jato-Rodriguez *et al.*, 1972a) and not related to any deficiency in the enzymes of the Krebs cycle and the electron transport chain which were found to be normal (Jato-Rodriguez *et al.*, 1972b). Preliminary studies indicate that mitochondria from dystrophic muscle may be more fragile and permeable than those from normal muscle (Liang *et al.*, 1972). Should this be the case, it is possible that coenzyme A has 'leaked out' or been lost from mitochondria because of this abnormality. In experiments reported here attempts have been made to overcome the oxidation defects through the addition of coenzyme A.

In view of the evidence in favour of a 'neurogenic' hypothesis, it was decided to carry out the above type of study on denervated muscle of the hind legs of the mouse. It was reasoned that denervated muscle might in many ways act as a model for the dystrophic muscle, particularly if the prime defect is of neurogenic origin. In the experiments reported here a number of properties of denervated muscle, in particular relating to acetyl and fatty acyl oxidation, have been measured. These are compared with data previously obtained with dystrophic muscle and include measurement of (a) the enzymes monoaminoxidase, lipase and succinate dehydrogenase in whole muscle homogenates, (b) the oxidation of acetylcarnitine, palmitylcarnitine and palmitate in mitochondria and (c) the enzymes succinate dehydrogenase, cytochrome oxidase and of β-oxidation present in mitochondria.

METHODS

ANIMAL AND TISSUE PREPARATIONS Normal and dystrophic mice were of the same strain (129 ReJ) as used in the previous studies on dystrophic muscle (Lin *et al.*, 1970; Jato-Rodriguez *et al.*, 1972a). Both sciatic and femoral nerves were sectioned, in a bloodless operation under ether anaesthesia. At fixed times after the operation mice were decapitated and the muscles of the hind leg were removed, freed of connective tissue, and weighed. The isolated muscle was homogenized in 0.25 M sucrose containing 10 mM; Tris-HCl, 0.5 mM EDTA at pH 7.4. Portions of this homogenate were used for the determination of lipase, monoaminoxidase, cytochrome oxidase, and succinate dehydrogenase. Mitochondria were isolated as described by Lin *et al.* (1969, 1970).

ENZYME DETERMINATIONS Lipase was determined by the manometric method of Martin and Peers (1953) using tributyrin as substrate. Monoaminoxidase was estimated by the isotopic method of Wurtman and Axelrod (1963) and cytochrome oxidase by the manometric method described by Humoller *et al.* (1952) using ascorbate as substrate. Succinate dehydrogenase was determined as described by Alvarado-Rigault and Blanchaer (1970) using phenazine methosulphate and 2,6-dichlorophenolindophenol.

For the determination of the enzymes of β-oxidation, isolated mitochondria were pretreated with sodium deoxycholate (final concentration 1%) and then used in the assay. However, it was found that deoxycholate-treated mitochondria are not very active in the assay for acyl CoA dehydrogenase. For estimation of this enzyme the mitochondria were frozen and then thawed before being used. The different assays used and adapted to give maximal activities were as follows:

1. Acyl CoA dehydrogenase was determined as described by Ward and Fairbairn (1970) with the exception that palmityl-CoA was used as substrate (100 nmol) and the incubation was carried out *in vacuo* for one hour. The formazan formed was extracted and determined as reported by Green *et al.* (1954).
2. Hydroxy-fatty acyl CoA dehydrogenase was estimated by the method of Wakil *et al.* (1954) as modified by Weeks *et al.* (1969).
3. Enoyl CoA hydrase was assayed spectrophotometrically by the method of Stern *et al.* (1956).
4. Thiolase was assayed as described by Stern (1955).

OXIDATION OF SUBSTRATES AND RESPIRATORY CONTROL RATE (RCR) The oxidation of 1-[^{14}C]palmitate and 1-[^{14}C]acetylcarnitine were carried out in mitochondria as described by Lin *et al.* (1970) and Jato-Rodriguez *et al.* (1972a) with the exception that 2 mmol of 1-[^{14}C]acetyl-*l*-carnitine was used. The oxidation of acetylcarnitine and palmitylcarnitine and the respiratory control ratio (RCR) were carried out using the oxygen electrode. Oxygen consumption was measured polarographically using an Interscience oxygen analyser (Interscience WXB-3 fitted with oxygen electrode, Interscience YSI Model 5331 fitted to a Varicord Linear/Log recorder, Model 43, Photovolt Corp. New York). The reaction cell, volume of 1.0 ml, was maintained at 30°C using a circulating water bath (Haake, West Germany). For each measurement the following substances were added in sequence: buffer solution (KCl, 0.15 M: K_2HPO_4, 0.03 M; Tris-HCl, 0.025 M: EDTA, 0.002 M; sucrose, 0.045 M; and $MgCl_2$, 0.005 M

all adjusted to pH 7.4), substrate; mitochondria (0.3–0.5 mg) protein and ADP (0.245 μmol) added at intervals. With each estimation, after the respiration had returned to a steady state (state 4), a known amount of ADP was added to initiate state 3 respiration. The oxygen uptake, ADP:O ratio, and RCR were estimated as described by Chance and Williams (1956).

ESTIMATION OF PROTEIN Protein was estimated by the method of Lowry *et al.* (1951).

MATERIALS 1-[^{14}C] palmitate (50 mCi/μmol) was obtained from Amersham/Searle (Arlington Heights, Ill.) and prepared for utilization as described elsewhere (Lin *et al.*, 1970). 1-[^{14}C]acetyl-*l*-carnitine was prepared as detailed in a previous publication (Jato-Rodriguez *et al.*, 1972a). Acetyl-*l*-carnitine and palmityl-*l*-carnitine were kindly provided by Otsuka Pharmaceuticals (Japan). All other chemicals used were of the highest purity available commercially.

RESULTS

In this investigation, some extension has been made of earlier studies relating to acetyl- and palmityl- group oxidation by mitochondria from normal and dystrophic muscle. The data in Table 1 confirm the severe impairment observed for acetylcarnitine oxidation (80% inhibited in Table 1 compared with 85% reported by Jato-Rodriguez *et al.*, 1972a). The addition of coenzyme A (55 μM) to mitochondria isolated from dystrophic muscle resulted in a partial restoration (16% increase with five animals) of their ability to oxidize the acetyl group of acetylcarnitine. In comparison with the effect on mitochondria from normal muscle (where a 6% inhibition is observed), the stimulation of dystrophic mitochondria by CoA is highly significant ($P < 0.01$). Measurement of the oxidation of palmitylcarnitine using the oxygen

TABLE 1

EFFECT OF ADDITION OF COENZYME A (COA) ON OXIDATION OF 1-[^{14}C]-ACETYLCARNITINE BY MITOCHONDRIA OF SKELETAL MUSCLE FROM NORMAL AND DYSTROPHIC MICE

Type of animal	*Oxidation of 1-^{14}C-acetylcarnitine**		
	No CoA (nmol/mg protein)	*CoA (55 μM)*	
		(nmol/mg protein)	*Percent†*
Dystrophic			
1	242.0	282.0	116.5
2	48.2	54.1	112.2
3	85.4	90.6	106.1
4	69.6	80.1	115.1
5	97.4	126.1	129.5
		Average	115.9 ± 3.78†
Normal			
1	562.0	535.0	95.2
2	652.0	570.7	89.1
3	513.5	475.0	92.5
4	461.6	393.6	85.3
5	330.1	359.6	108.9
		Average	94.2 ± 4.05†

* Incubation conditions as described in the Methods section with 20 000 dpm 1-[^{14}C]-acetylcarnitine added to each flask.
† Average percentages relative to sample with no CoA added ± SEM. The probability $P < 0.01$ that the added CoA is equally affecting the two mitochondrial preparations.

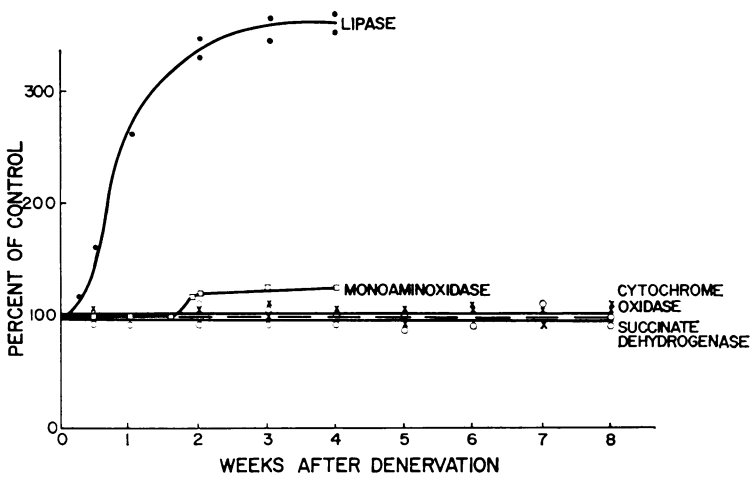

FIG. 1 *Changes in lipase, monoaminoxidase, succinate dehydrogenase, and cytochrome oxidase of homogenates of hind leg muscle of mice undergoing atrophy after denervation.*

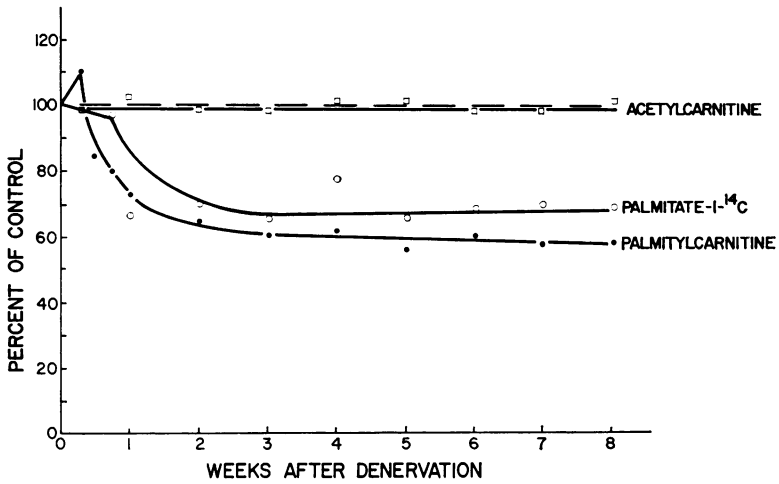

FIG. 2 *Changes in the utilization of acetylcarnitine, palmitylcarnitine, palmitate-1-[^{14}C] by mitochondria isolated from the hind leg muscles of mice at various times after denervation.*

electrode has confirmed that mitochondria from dystrophic muscle are impaired in their ability to oxidize palmityl groups (for 0.3 mmol palmityl-*l*-carnitine values were obtained of 15.67 ± 2.97 for four normal animals and 5.07 ± 1.13 for three dystrophic animals expressed as nmol O_2 utilized per min per mg mitochondrial protein; $P < 0.05$). This agrees with our earlier studies on palmitate-*l*-[^{14}C] oxidation where a highly significant inhibition of oxidation (42%) was observed in mitochondria from dystrophic muscle (Lin *et al.*, 1970).

The weight of leg muscle after denervation fell to 60% of the control value by the third week and showed no further change by the eighth week. Figure 1 shows the activities of four enzymes in homogenates of denervated muscle as compared with the contralateral control. A large increase in activity is noted for lipase which occurs quite rapidly over the first two weeks. By two weeks the level of increase begins to plateau at 250%. The increase observed with monoaminoxidase is small (25%) and becomes manifest only after two weeks of denervation. Succinate dehydrogenase and cytochrome oxidase do not show any change in the muscle for up to eight weeks after denervation. Neither the respiratory control ratio (RCR) nor the succinate dehydrogenase activity altered significantly over eight weeks of denervation. Since no changes were observed in succinate dehydrogenase (homogenate or mitochondria), it can be

TABLE 2

ACTIVITIES OF ENZYMES OF β-OXIDATION IN MITOCHONDRIA FROM NORMAL, DENERVATED, AND DYSTROPHIC MUSCLE

Enzyme	*Enzymatic activity*			
	Normal	*Dystrophic*	*Control*	*Denervated*
Acyl CoA dehydrogenase*	4.38 ± 0.50 (9) $P < 1.0$	4.63 ± 0.50	4.58 ± 0.22 (9) $P < 0.005$	3.23 ± 0.20
Hydroxyacyl CoA dehydrogenase†	561 ± 42 (4) $P < 1.0$	481 ± 47	532 ± 31 (7) $P < 1.0$	443 ± 31
Enoyl CoA hydrolase†	601 ± 51 (4) $P < 1.0$	470 ± 38	600 ± 48 (5) $P < 1.0$	433 ± 26
Thiolase†	98 ± 9 (4) $P < 1.0$	101 ± 11	109 ± 7 (8) $P < 1.0$	95 ± 7

* Activity expressed in nmol triphenyltetrazolium reduced to formazan/min/mg protein.
† Activity expressed in nmol substrate metabolized/min/mg protein.

concluded that the mitochondrial content of muscle undergoing atrophy after denervation does not change when expressed as mg mitochondrial protein per gram muscle—that is, as the muscle atrophies, there is a parallel decrease in mitochondria.

The effect of denervation on the ability of mitochondria to oxidize acetylcarnitine, palmitylcarnitine, and 1-[^{14}C]palmitate is shown in Fig. 2. No alteration is observed in respect of acetylcarnitine oxidation, which contrasts with the observation for dystrophic muscle where there is a highly significant reduction (80–85%) based on release of $^{14}CO_2$ from 1-[^{14}C]-acetylcarnitine and 36% using the oxygen electrode. With both 1-[^{14}C]palmitate and palmitylcarnitine, fairly rapid decreases in oxidation are observed for two weeks after denervation that plateau at levels of 30% and 37%, respectively, below the normal values.

An explanation for the decreases in palmitate or palmitylcarnitine oxidation observed in both dystrophic and denervated muscle was sought in an examination of the enzymes of β-oxidation, since the enzymes of the Krebs' cycle and the electron transport chain were normal (Jato-Rodriguez *et al.*, 1972b). The three enzymes, hydroxyacyl CoA dehydrogenase, enoyl CoA hydrolase, and thiolase, showed no alteration in activity in mitochondria from either denervated or dystrophic muscle (Table 2). For acyl CoA dehydrogenase, the rate limiting enzyme, it was observed that enzyme activity is reduced 29% two weeks after denervation, whereas there is no change in the dystrophic muscle. This decrease is highly significant.

DISCUSSION

This investigation extends our earlier work on lipid metabolism in dystrophic muscle to a consideration of what these changes have in common with denervated muscle. Should the changes in denervated muscle parallel those in muscular dystrophy, then substantial support would be provided for a neurogenic origin for the disease. Summarized in Table 3 are the results described in this paper compared with those previously obtained from dystrophic muscle. Many similarities were observed which include a number of activities showing no change —for example, succinate dehydrogenase, cytochrome oxidase, and three of the four enzyme activities of β-oxidation—and a number of activities showing either increases—for example, lipase and monoaminoxidase—or decreases—palmitate and palmitylcarnitine oxidation. However, examination of the defect in palmityl group

TABLE 3

COMPARISON OF SOME PROPERTIES OF DENERVATED MUSCLE (THIS PAPER) WITH DYSTROPHIC MUSCLE

Property measured	*Denervated (after 2 weeks) (% change)*	*Dystrophic (% change)*	*Reference*
Enzyme activities in homogenates			
Lipase	+250	+125	Jato-Rodriguez *et al.* (1974)
Monoaminoxidase	+25	+50	This paper
Succinate dehydrogenase	NC	NC	Jato-Rodriguez *et al.* (1972b)
Cytochrome oxidase	NC	NC	Jato-Rodriguez *et al.* (1972b)
Utilization of substrates by isolated mitochondria			
Acetylcarnitine	NC	−85	Jato-Rodriguez *et al.* (1972a)
Palmitylcarnitine	−37	−66	This paper
Palmitate-1-[^{14}C]	−30	−42	Lin *et al.* (1970)
Enzyme activities in mitochondria			
Succinate dehydrogenase	NC	NC	Jato-Rodriguez *et al.* (1974)
Enzymes of β-oxidation			
Acyl CoA dehydrogenase	−29	NC	This paper
Hydroxy acyl CoA dehydrogenase	NC	NC	This paper
Enoyl CoA hydrolase	NC	NC	This paper
Thiolase	NC	NC	This paper

* A plus denotes an increase, a minus denotes a decrease, and NC denotes no change.

—that is, palmitate and palmitylcarnitine—oxidation and acetylcarnitine oxidation in mitochondria revealed two striking differences between denervated and dystrophic muscle. These were (1) a reduction in the activity of acyl CoA dehydrogenase (a regulatory enzyme of β-oxidation) in denervated as opposed to no change for dystrophic muscle mitochondria and (2) no change in acetylcarnitine oxidation in mitochondria isolated from denervated muscle as compared with a large reduction (up to 80%) in dystrophic muscle.

Since acyl CoA dehydrogenase exhibited a much lower activity than the other three enzymes of β-oxidation (Table 2), it is considered to be the rate limiting step for β-oxidation and any reduction in activity in this enzyme could be expected to have a direct effect on the overall oxidation of acyl groups. On this basis it is reasonable to propose that the significant reduction in acyl CoA dehydrogenase activity observed in mitochondria from denervated muscle is sufficient to account for the observed impairment in oxidation of palmitate and palmitylcarnitine.

Evidence from our earlier studies (Jato-Rodriguez *et al.*, 1972a) strongly supports the view that the impairments in oxidation of acetylcarnitine, palmitate, and palmitylcarnitine observed in mitochondria from dystrophic muscle may have a common explanation which is related to the availability of coenzyme A. In a study primarily concerned with an examination of the nature of the defect in oxidation of acetylcarnitine in mitochondria from dystrophic muscle, it was found that the coenzyme A content of these mitochondria was reduced by 60%. On the assumption that the levels of coenzyme A are critical and may profoundly influence acetyl group oxidation, the above reduction appeared to be just adequate to account for the impairments noted in the oxidation of acetyl and palmityl groups. Further support for this explanation and for the possibility that the coenzyme A deficiency observed may be due to abnormal permeabilities of the mitochondria is provided by the experiments in which coenzyme A was added to mitochondria isolated from normal and dystrophic muscle (Table 1). The net stimulation of 22%—that is, a 16% stimulation for mitochondria from dystrophic muscle combined with a 6% inhibition for mitochondria from normal muscle—by coenzyme A observed for mitochondria from dystrophic muscle compared with normal muscle strongly suggests an increased permeability to coenzyme A which, if present in mitochondria *in situ*, might be great enough to account for the 60% loss in coenzyme A content.

In conclusion, it is evident that there are quite a number of biochemical similarities between dystrophic and denervated muscle and such changes are consistent with the hypothesis that murine muscular dystrophy has a neurogenic origin. However, at least two and possibly three of the properties studied show differences between dystrophic and denervated muscle and, therefore, it is unlikely that the dystrophic process can be explained solely on a basis of denervation or the loss of a neural trophic factor.

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