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IMPAIRED SUBSTRATE UTILIZATION IN MITOCHONDRIA FROM STRAIN 129 DYSTROPHIC MICE

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Summary

Mitochondria from skeletal muscle, heart and liver of strain 129/ReJ-dy dystrophic mice and their littermate controls were characterized with respect to their respiratory and phosphorylating activities. Skeletal muscle mitochondria from dystrophic mice showed significantly lower state 3 respiratory rates than controls with both pyruvate + malate and succinate as substrates ($P < 0.01$). ADP/O and Ca^{2+}/O ratios were found to be normal. A decreased rate of NADH oxidation ($0.01 < P < 0.05$) by sonicated mitochondrial suspensions from dystrophic mice was also seen. High respiratory rates with ascorbate + phenazine methosulfate as substrates indicated that cytochrome oxidase was not rate limiting in the oxidation of either pyruvate + malate or succinate. Skeletal muscle mitochondria from dystrophic mice showed no deficiency in any of the cytochromes or coenzyme Q. Mg^{2+}-stimulated ATPase activity was higher in dystrophic muscle mitochondria than in controls, but basal and oligomycin-insensitive activities were virtually identical to those of controls. A significant reduction in the intramitochondrial NAD^+ content ($0.01 < P < 0.02$) was seen in dystrophic skeletal muscle as compared to controls. Heart mitochondria from dystrophic mice showed similar, though less extensive abnormalities while liver mitochondria were essentially normal. We concluded from these results that skeletal muscle mitochondria from strain 129 dystrophic mice possess impairments in substrate utilization which may result from (1) an abnormality in the transfer of electrons on the substrate side of coenzyme Q in the case of succinate oxidation; (2) a defect on the path of electron flow from NADH to cytochrome *c*, and (3) a deficiency of NAD^+ in the case of NAD^+-linked substrates.

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

Introduction

The hereditary dystrophy of the Bar Harbor strain 129 mouse has been the subject of numerous studies since it was first described in 1955 [1]. Reports of deficiencies in the ability of skeletal muscle mitochondria from strain 129 dystrophic mice to oxidize both long and short-chain fatty acids, acyl carnitine esters, and pyruvate [2—6] have suggested an impairment in energy metabolism in these mice. This is supported mainly by a report of reduced levels of ATP in muscle homogenates from strain 129 dystrophic mice [7]. On the other hand, results in direct contrast to those mentioned above have been reported by Wrogeman and Blanchaer [8] who showed normal respiratory rates in mitochondria from strain 129 dystrophic mice using pyruvate + malate as substrates, and by Farrell and Olson [9] who observed normal levels of ATP in the muscle of these mice. There appears to be no deficiency in either the enzymes of the Krebs cycle [10] or in the supply of intermediates [11]. Glycolysis, the major pathway for anaerobic muscle metabolism also appears to be normal in these mice [12—14].

In order to determine the extent to which mitochondrial functions may or may not be impaired in this animal model, and to better characterize the nature of such impairments, we have begun a systematic study of the oxidative and energy transfer functions of isolated mitochondria from three tissues of strain 129 mice: skeletal muscle, heart and liver. In this paper we describe the results of such studies from which we conclude that skeletal muscle mitochondria from dystrophic mice possess defects in substrate utilization which occur at both the substrate and respiratory chain levels. Preliminary reports of some of these findings have already been briefly presented [15—17].

Materials and Methods

Animals. Strain 129/ReJ-dy dystrophic mice and their littermate controls were purchased from Jackson Laboratories, Bar Harbor, ME. All mice were from 10 to 16 weeks of age at the time of the experiments. Each assay was made with pooled specimens from two to six mice.

Preparation of mitochondria. Skeletal muscle mitochondria were isolated by the modification of the procedure of Makinen and Lee [18] previously described for myodystrophic mice [19]. In order to determine whether the optimal conditions for preparation of skeletal muscle mitochondria from strain 129 dystrophic mice are the same as those determined for myodystrophic mice, a study was made of the effects of proteinase concentration on the yield of mitochondrial protein, the state 3 respiratory rate, and the respiratory control index (state 3 rate/state 4 rate). As Fig. 1 shows, a proteinase concentration of 2—3 mg/g tissue with an incubation time of 5 min is the optimal condition. Dystrophic mitochondria appear to be slightly more susceptible to digestion by proteinase than mitochondria from controls as exhibited by the more rapid decrease in protein recovery at increasing levels of proteinase. All subsequent experiments were performed with mitochondria prepared using 3 mg proteinase/g of muscle.

Heart muscle mitochondria were isolated by the method of Tyler and Gonze

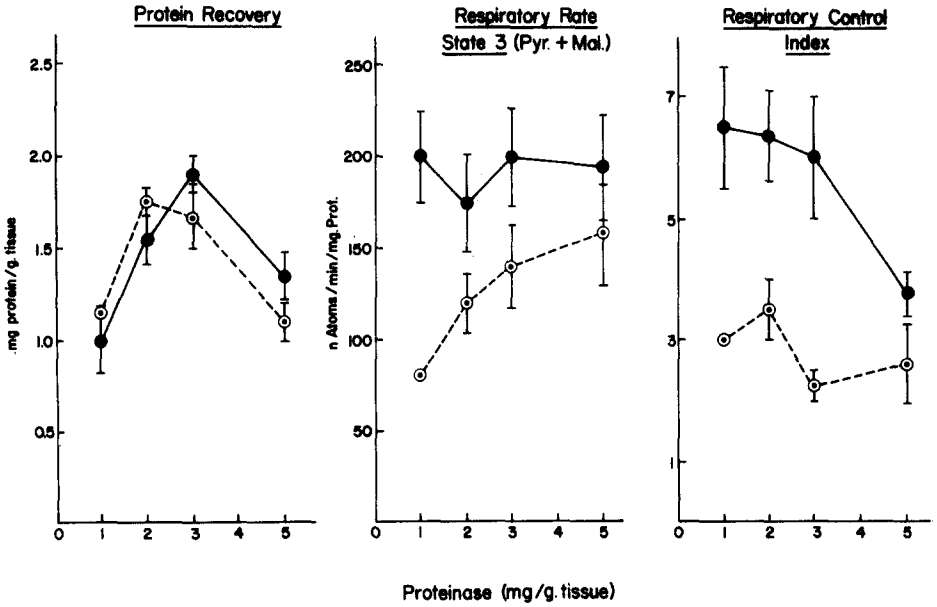

Fig. 1. Effect of proteinase on skeletal muscle mitochondrial preparations isolated from strain 129 mice. Experimental conditions are as described in the legend to Table I. Respiratory control index is the ratio of the state 3 to state 4 respiratory rates. ●———●, control; ⊙- - - - -⊙, dystrophic.

[20] using 5 mg proteinase/g of heart tissue.

Liver mitochondria were isolated by the modification of the method of Johnson and Lardy [21] as previously described [19].

Assays. Substrate oxidation rates and ADP/O ratios were determined polarographically [22] by means of a Clark oxygen electrode fitted into a thermostatted plexiglass chamber with a capacity of 1.0 ml.

NADH oxidase activity was determined spectrophotometrically at 340 nm in sonicated mitochondrial suspensions prepared as previously described [19].

ATPase activities were determined by the methods of Lindberg and Ernster [23].

The mitochondrial content of the respiratory pigments was calculated from reduced minus oxidized difference spectra recorded at room temperature using an Aminco DW-2 spectrophotometer. Concentrations were calculated using the following millimolar extinction coefficients: cytochrome *a* (605—630 nm), 24.0 [24]; cytochrome a_3 (445—455 nm), 80.0 [24]; cytochrome *b* (562—575 nm), 20.0 [25]; cytochrome $c + c_1$ (551—540 nm), 19.1 [26]; and flavoprotein (465—510 nm), 11.0 [26]. Coenzyme Q was determined as described by Redfearn [27] using paired mitochondrial suspensions from dystrophic mice and their littermate controls in each set of determinations.

The pyridine nucleotides were extracted from mitochondrial suspensions and the contents of NAD^+ and $NADP^+$ were assayed fluorimetrically by the methods of Williamson and Corkey [28] using a Perkin-Elmer MPF-2A fluorescence spectrophotometer. The excitation and emission wavelengths were 340 nm and 460 nm, respectively, and the sensitivity was adjusted so that full scale was equivalent to approximately 16 μM NADH.

Protein content was determined by the method of Lowry et al. [29] using crystalline bovine serum albumin as standard.

Statistical differences were assessed using Student's *t*-test.

Reagents. Crystalline *Bacillus subtilis* proteinase (Nagarse) was a product of Teikoku Chemical Co., Ltd., Osaka, Japan. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) was a gift from Dr. Peter Hytler, E.I. DuPont de Nemours and Co., Wilmington, DE. All other chemicals of the purest grades available were obtained commercially. Glass-redistilled water was used throughout the present investigation.

Results

Respiratory and phosphorylating activities. Table I shows the respiratory rates, the respiratory control indeces and ADP/O ratios for skeletal muscle mitochondria from both control and dystrophic mice with pyruvate + malate and succinate as substrates. As can be seen, tightly coupled mitochondria can be isolated from both control and dystrophic skeletal muscle using the procedures and incubation conditions described. These mitochondria are dependent on the presence of either ADP, Ca^{2+}, or uncoupler for maximum respiratory rates to be seen. ADP-induced stimulation is sensitive to inhibition by oligomycin and subsequent release by uncoupler. Skeletal muscle mitochondria from control and dystrophic mice do not oxidize externally added NADH under these conditions.

As can be seen in Table I, the oxidation of substrates by skeletal muscle mitochondria from dystrophic mice is characterized by significant decreases in respiratory control with both pyruvate + malate and succinate as substrates. These decreases result from reduced state 3 rates of substrate oxidation which are 43% for pyruvate + malate and 36% for succinate. There is also a 35% increase in the state 4 rate of pyruvate + malate oxidation which contributes to

TABLE I

RESPIRATORY AND PHOSPHORYLATIVE ACTIVITIES IN ISOLATED SKELETAL MUSCLE MITOCHONDRIA FROM STRAIN 129 MICE

The reaction mixture consisted of 150 mM sucrose, 25 mM Tris-HCl and 10 mM potassium phosphate, pH 7.5. Substrates and inhibitors were added at the following concentrations: 5 mM pyruvate; 2.5 mM malate; 5 mM succinate; 2.5 μM rotenone; 300 μM ADP; 5 μg/ml oligomycin; 5 μM FCCP; 300 μM $CaCl_2$. The final volume was 1.0 ml, and the assay temperature was 30°C. Values are mean ± S.E. The number of experiments is given in parentheses.

Substrates		Respiratory rates (natoms O/min per mg protein)		Respiratory control index	ADP/O
		State 3	State 4		
Pyruvate + malate	Control (66)	218 ± 8	49 ± 2	5.5 ± 0.6	3.0 ± 0.1
	Dystrophic (36)	124 ± 10 *	66 ± 6	2.1 ± 0.1 *	2.7 ± 0.1
Succinate + rotenone	Control (17)	232 ± 12	101 ± 7	2.4 ± 0.2	1.7 ± 0.1
	Dystrophic (11)	149 ± 15 *	109 ± 14	1.6 ± 0.3 *	1.7 ± 0.2

* Statistical difference from controls: $P < 0.01$.

the especially large decrease in respiratory control with these substrates. The cause of this increase is not immediately evident, since there is no change in the state 4 rate of succinate oxidation. When ascorbate + phenazine methosulfate were used as substrates, the respiratory rates were at least 50% faster than the corresponding rates with either pyruvate + malate or succinate as substrates in mitochondria from both control and dystrophic skeletal muscle. Normal ADP/O ratios, normal Ca^{2+}/O ratios (5.3 ± 0.2 for controls vs. 5.1 ± 0.2 for dystrophic), and the maintenance of oligomycin sensitivity indicate that skeletal muscle mitochondria from dystrophic mice are not deficient in the ability to couple respiration to either phosphorylation or ion transport.

The results of comparative studies on heart and liver mitochondria are shown in Tables II and III, respectively. Heart mitochondria from dystrophic mice are less severely impaired than those from skeletal muscle, as expected since a serious defect in the oxidative metabolism of the heart would very likely be fatal. Liver mitochondria from dystrophic mice appear to be normal. The slight decrease in succinate oxidation by liver mitochondria is similar to that reported by Howland and Challberg [30], but it is not statistically different from that seen in controls.

NADH oxidase activity. The rates of oxidation of externally added NADH by skeletal muscle mitochondrial suspensions which have been sonically disrupted are shown in Table IV. A 27% decrease in the NADH oxidase activity can be seen when preparations from dystrophic muscle are compared to controls.

Respiratory pigment content. From the difference spectrum (reduced minus oxidized) shown in Fig. 2, it can be seen that skeletal muscle mitochondria from dystrophic mice possess the full complement of cytochromes with both the α and γ bands of cytochromes *a*, *b*, and *c* being clearly in evidence. Table V shows the contents of these respiratory pigments in dystrophic and control mitochondrial preparations from skeletal muscle and heart tissues. The contents of these pigments in mitochondria from dystrophic mice are comparable

TABLE II

RESPIRATORY AND PHOSPHORYLATIVE ACTIVITIES IN ISOLATED HEART MITOCHONDRIA FROM STRAIN 129 MICE

Experimental conditions are as described in the legend to Table I. Values are mean ± S.E. The number of experiments is given in parentheses.

Substrates		Respiratory rates (natoms O/min per mg protein)		Respiratory control index	ADP/O
		State 3	State 4		
Pyruvate + malate	Control (30)	332 ± 16	71 ± 4	4.9 ± 0.2	2.9 ± 0.1
	Dystrophic (24)	289 ± 19 *	73 ± 7	4.6 ± 0.4	3.0 ± 0.1
Succinate + rotenone	Control (12)	414 ± 20	197 ± 11	2.1 ± 0.1	1.7 ± 0.1
	Dystrophic (11)	320 ± 20 **	190 ± 14	1.7 ± 0.1 **	1.5 ± 0.1

* Statistical difference from controls: $0.05 < P < 0.10$.

** Statistical difference from controls: $P < 0.01$.

TABLE III

RESPIRATORY AND PHOSPHORYLATIVE ACTIVITIES IN ISOLATED LIVER MITOCHONDRIA FROM STRAIN 129 MICE

Experimental conditions are as described in the legend to Table I. Values are mean ± S.E. The number of experiments is given in parentheses.

Substrates		Respiratory rates (natoms O/min per mg protein)		Respiratory control index	ADP/O
		State 3	State 4		
Pyruvate + malate	Control (18)	48 ± 6	21 ± 3	2.4 ± 0.1	2.7 ± 0.1
	Dystrophic (13)	49 ± 7	25 ± 4	2.1 ± 0.2	2.3 ± 0.2
Succinate + rotenone	Control (16)	191 ± 22	65 ± 5	3.0 ± 0.2	1.9 ± 0.1
	Dystrophic (10)	161 ± 30	54 ± 12	3.2 ± 0.2	1.8 ± 0.1

to those of controls with the exception of a 25% elevation in the levels of coenzyme Q.

ATPase activities. Table VI shows the ATPase activities of skeletal muscle, heart and liver mitochondria under various conditions. There is no difference in either basal or oligomycin-insensitive activities between mitochondria from control and dystrophic mice. Both heart and skeletal muscle mitochondria from dystrophic mice show higher rates in the presence of Mg^{2+} when compared to controls and lower rates in the presence of dinitrophenol. These differences can be abolished by prior disruption of the mitochondria (data not shown), suggesting possible alterations in the accessibility of the ATPase rather than in the structure of the enzyme itself. Liver mitochondria show normal ATPase activities.

Pyridine nucleotide contents. As Table VII shows, the total pyridine nucleotide content in skeletal muscle mitochondria from dystrophic mice is reduced significantly when compared to controls. This reduction is due entirely to a 32% decrease in the intramitochondrial content of NAD^+. Mouse skeletal muscle mitochondria contain very low levels of $NADP^+$ (less than 10% of the total pyridine nucleotide content). These levels do not differ between mitochondria from control and dystrophic mice. For comparison, we also measured

TABLE IV

NADH OXIDASE ACTIVITY OF DISRUPTED MITOCHONDRIAL SUSPENSIONS OF SKELETAL MUSCLE OF STRAIN 129 DYSTROPHIC AND CONTROL MICE

The assay medium consisted of 150 mM sucrose, 25 mM Tris-HCl, 10 mM phosphate buffer, pH 7.5, and 0.2—0.3 mg mitochondrial protein in a final volume of 3.0 ml. The reaction was carried out at 25°C. 200 μM NADH was used to initiate the reaction. Experimental conditions are as given in Materials and Methods. Values are mean ± S.E. The number of experiments is given in parentheses.

Mice	NADH oxidase activity (nmol/min per mg protein)
Control (27)	268 ± 22
Dystrophic (16)	195 ± 26 *

* Statistical difference from controls: $0.01 < P < 0.05$.

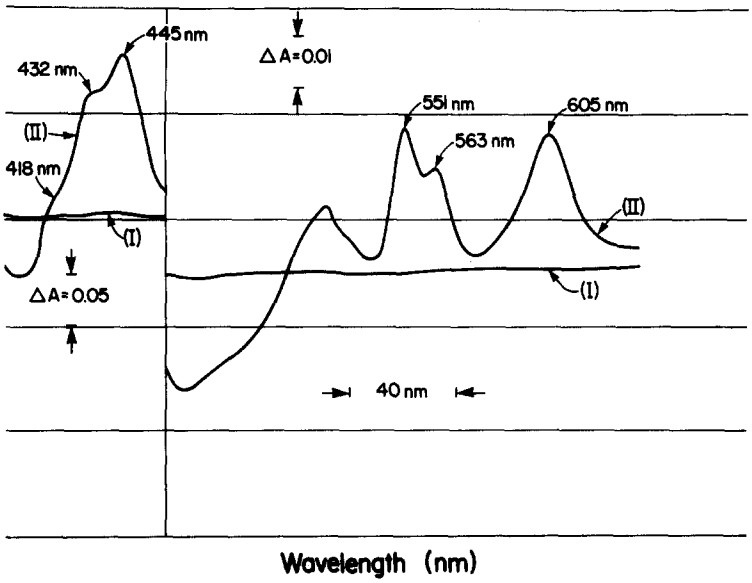

Fig. 2. Reduced minus oxidized difference spectrum of strain 129 dystrophic skeletal muscle mitochondria. Mitochondria were suspended to a protein concentration of 1.5 mg/ml in a medium consisting of 150 mM sucrose, 20 mM Tris-HCl, 10 mM potassium phosphate, pH 7.5, and 1.7 mM ADP. The mitochondrial suspension was placed in reference and sample cuvettes and the oxidized minus oxidized difference spectrum (I) was recorded to obtain the baseline. After adding 5 mM pyruvate, 2.5 mM malate, and 5 mM succinate to the sample cuvette, the reaction mixture was incubated at room temperature until cytochrome reduction was complete (approx. 2—3 min). The reduced minus oxidized spectrum (II) was then recorded.

the pyridine nucleotide content in skeletal muscle mitochondria from myodystrophic mice, for which we have reported a defect in the NADH—coenzyme Q region of the respiratory chain [19]. As expected, there was no difference in the content of either NAD^+ or $NADP^+$ in skeletal muscle mitochondria from control and myodystrophic mice.

TABLE V

RESPIRATORY PIGMENTS IN MITOCHONDRIAL PREPARATIONS ISOLATED FROM HEART AND SKELETAL MUSCLE OF STRAIN 129 MICE

Experimental conditions are as described in the legend to Fig. 2. Values are expressed as nmol/mg protein (mean ± S.E.). $N = 4$ for coenzyme Q.

	Skeletal muscle		Heart muscle	
	Control (4)	Dystrophic (3)	Control (4)	Dystrophic (3)
Cytochrome				
a	0.42 ± 0.02	0.40 ± 0.04	0.54 ± 0.03	0.53 ± 0.03
b	0.43 ± 0.04	0.35 ± 0.05	0.33 ± 0.03	0.33 ± 0.02
c	0.69 ± 0.05	0.61 ± 0.06	0.79 ± 0.05	0.81 ± 0.07
a_3	0.52 ± 0.05	0.53 ± 0.06	0.63 ± 0.07	0.62 ± 0.11
Flavoproteins	1.17 ± 0.08	1.02 ± 0.12	1.31 ± 0.13	1.35 ± 0.12
Coenzyme Q	2.41 ± 0.18	2.97 ± 0.21 *	2.77 ± 0.26	3.46 ± 0.16 *

* Statistical difference from controls: $0.05 < P < 0.10$.

TABLE VI

ATPase ACTIVITIES IN MITOCHONDRIA FROM STRAIN 129 MICE

The reaction mixture consisted of 150 mM sucrose, 50 mM Tris-HCl, pH 7.5, and 0.1—0.4 mg mitochondrial protein. The reaction was initiated by addition of 5 mM ATP. When indicated, 4 mM $MgSO_4$, 0.6 mM dinitrophenol (DNP), and/or 2 μg oligomycin were also present. Samples were incubated for 10 min at 37°C in a final volume of 1.0 ml. Values are expressed as mean ± S.E. The number of experiments is given in parentheses.

		ATPase activity (nmol/min per mg protein)				
		Basal	+ Mg^{2+}	+ DNP	+ Mg^{2+} + DNP	+ Oligomycin
Skeletal muscle	Control (13)	240 ± 30	730 ± 70	720 ± 70	790 ± 80	130 ± 20
	Dystrophic (10)	260 ± 40	900 ± 110	600 ± 50	920 ± 100	170 ± 30
Heart	Control (11)	220 ± 20	540 ± 60	700 ± 60	750 ± 60	20 ± 10
	Dystrophic (10)	220 ± 30	830 ± 100 **	530 ± 50 *	960 ± 120	50 ± 10
Liver	Control (9)	70 ± 20	160 ± 50	230 ± 50	260 ± 50	10 ± 5
	Dystrophic (10)	80 ± 10	170 ± 30	200 ± 30	240 ± 30	20 ± 5

* Statistical difference from controls: $0.05 < P < 0.10$.
** Statistical difference from controls: $0.01 < P < 0.05$.

Discussion

From the results reported here it is evident that skeletal muscle mitochondria from dystrophic mice possess an impaired ability to oxidize both pyruvate + malate and succinate. That this impairment in substrate oxidation is partly due to a functional defect in the respiratory chain is indicated by the decreased NADH oxidase activity in sonicated mitochondrial suspensions from these mice. Our results indicate that this defect is not a result of deficiencies in either phosphorylation or in the contents of the electron transport carriers. The slight elevation in the levels of coenzyme Q reported here and by Godinez

TABLE VII

PYRIDINE NUCLEOTIDE CONTENTS OF MITOCHONDRIA ISOLATED FROM SKELETAL MUSCLE OF STRAIN 129 AND MYODYSTROPHIC MICE

The assay mixture contained 0.1 M Tris-HCl, pH 8.0, 2 mM semicarbazide, 0.5 mM glucose 6-phosphate, 15 μl absolute ethanol, and the extract from 1.8 to 3.6 mg mitochondrial protein in a total volume of 1.0 ml. The change in fluorescence was measured upon addition of either alcohol dehydrogenase (NAD^+) or glucose-6-phosphate dehydrogenase ($NADP^+$). Values are mean ± S.E. The number of experiments is given in parentheses.

	Pyridine nucleotide content (nmol/mg protein)		
	NAD^+	$NADP^+$	$NAD^+ + NADP^+$
Strain 129			
Control (16)	5.08 ± 0.43	0.34 ± 0.02	5.62 ± 0.47
Dystrophic (9)	3.43 ± 0.31 *	0.36 ± 0.05	3.79 ± 0.32 *
Myodystrophic			
Control (17)	5.65 ± 0.22	0.43 ± 0.04	6.19 ± 0.24
Myodystrophic (15)	5.45 ± 0.22	0.40 ± 0.05	5.87 ± 0.22

* Statistical difference from controls: $0.01 < P < 0.02$.

et al. [31] indicates that there is not the deficiency of this carrier that has been proposed by Folkers and his coworkers [32—35] who observed a physical improvement in strain 129 dystrophic mice after treatment with ubiquinones [32—34].

Because both succinate and NADH oxidation are impaired in mitochondria from dystrophic mice, one may conclude that the defect lies in the common portion of the respiratory chain. On the other hand, succinate is oxidized more slowly than NADH in mitochondria from both control and dystrophic mice, indicating that the rate-limiting step in succinate oxidation is on the substrate side of coenzyme Q. In addition, succinate oxidation is more severely impaired than that of NADH. These two lines of evidence indicate that there is an impairment which lies in either succinate dehydrogenase itself, in its relationship to coenzyme Q, or possibly in the transport of succinate.

Our data show that in skeletal muscle mitochondria from both control and dystrophic mice, the respiratory rates with ascorbate + phenazine methosulfate as substrates are at least 50% faster than those with either pyruvate + malate or succinate as substrates, indicating that the cytochrome oxidase segment of the respiratory chain is not rate limiting. This suggests that the impairment in NADH oxidation lies on the substrate side of cytochrome *c*. If the impairment is located between coenzyme Q and cytochrome *c*, any possible effect on succinate oxidation would be masked, since this portion of the respiratory chain is not rate limiting for succinate oxidation. On the other hand, the impairment may occur in the NADH dehydrogenase region.

Jato-Rodriguez and coworkers [6,10] have measured the NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, and cytochrome oxidase activities of skeletal muscle mitochondria from strain 129 mice and have concluded that there are no differences between control and dystrophic samples. However, the rates reported were at least 50% lower than is necessary to accomodate the respiratory rates measured by either Wrogeman and Blanchaer [8] or ourselves (this paper) with any of the substrates tested.

The observed 27% decrease in the NADH oxidase activity in sonicated mitochondrial suspensions from dystrophic mice (cf. Table IV) cannot fully account for the 43% decrease in the state 3 respiratory rate with pyruvate + malate as substrates seen in intact mitochondria from dystrophic mice (cf. Table I). Skeletal muscle mitochondria must, therefore, possess additional defect(s) which lie on the substrate side of NAD^+ along the respiratory chain. Quantitative results show that these mitochondria contain 32% less NAD^+ than mitochondria from control mice (cf. Table VII). Such a deficiency in NAD^+ content could have a widespread effect on the metabolism of the muscle cell by altering respiratory rates with any of the NAD^+-linked substrates. Impairments in the oxidation rates with palmitate, and the palmityl and acetyl carnitine esters as substrates have been shown in skeletal muscle mitochondria from dystrophic mice by Lin and coworkers [3—7] and recently by us [15,16]. Of course, the results presented here do not preclude the possibility that there are other impairments which affect these oxidations. Deficiencies in carnitine acetyltransferase activities [5,6] and coenzyme A content [6] have been suggested in skeletal muscle mitochondria from strain 129 dystrophic mice. However, recent studies in our laboratory [16,17] indicate that neither the

carnitine acetyltransferase activities nor the coenzyme A contents are statistically different between mitochondria from control and strain 129 dystrophic mice. A detailed account of these studies will be presented in a subsequent paper.

Because the impairments in substrate oxidation by mitochondria from strain 129 dystrophic mice are primarily confined to muscle tissues, with heart muscle being less severely affected than skeletal muscle, it is unlikely that the primary genetic lesion lies in a gene coding for one of these enzymes. However, regardless of whether these are primary or secondary defects, an impairment in energy metabolism of the magnitude reported here could contribute significantly to the severe muscular degeneration and fatty buildup which are characteristic of the disease in these animals.

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