Sarcoplasmic Reticulum. VI. Microsomal Ca^{2+} Transport in Genetic Muscular Dystrophy of Mice* (32813)

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Of the vast and frequently controversial information on the elusive etiology of genetic muscular dystrophy, increasing attention is directed to possible alterations of cellular membranes with their potential effects on muscle metabolism and contractility.

The affected muscles possess a diminished ability to retain and accumulate K^+ (2,3), aldolase (3), and other proteins. This explains the elevated plasma level of aldolase (4), creatine kinase (5), and other sarcoplasmic enzymes in cases of human or animal muscular dystrophy, and provides a selective and sensitive tests for the diagnosis of the disease (6).

The delayed relaxation of dystrophic muscles (7), and the enhanced tension generated in the presence of NO_3 ions (8) were taken to indicate an increase in the Ca^{2+} permeability of intracellular membranes of dystrophic muscles leading to the intensification of the active state.

Motivated by these observations we studied the Ca^{2+} transport and the adenosine triphosphatase activity of sarcoplasmic reticulum fragments isolated from the leg muscles of normal and genetically dystrophic mice *in vitro*.

The Ca^{2+} transport capacity of microsomes isolated from dystrophic mouse muscle was considerably reduced in comparison with that of normal animals of the same strain. The adenosine triphosphatase activity of dystrophic microsomes showed little sensitivity to oxalate or EGTA, which are potent inhibitors of the adenosine triphosphatase activity of normal muscle microsomes.

Experimental Procedures. Normal and muscular dystrophic mice of the 129 strain were purchased from Jackson Memorial Laboratories, Bar Harbor, Maine.

Preparation of particulate fractions. The hind leg muscles of two animals were homogenized in 20 ml of 0.1 *M* KCl, 10 m*M* histidine (free base) solution in the semimicro attachment of a Waring blendor for 2 min at 4°C. The myofibrils were removed by centrifugation at 1000*g* for 30 min. The mitochondrial sediment was suspended in 2–4 ml of 0.1 *M* KCl, 10 m*M* histidine. The supernatant fluid containing the microsomes was centrifuged first at 28,000*g* for 1 hour to obtain the Grana I (Gr I) fraction, and then at 100,000*g* for 1 hour to separate the Gr II fraction from the particle-free supernatant. The Gr I fraction was extracted with 24–30 ml of 0.6 *M* KCl for 30 min in an ice bath and after centrifugation at 28,000*g* for 60 min was resuspended in 0.1 *M* KCl, 5–10 m*M* histidine to a protein concentration of 2–4 mg/ml. Particulate and supernatant fractions were stored in ice and all experiments were completed within 2 days after the animals were killed.

The fresh weight of the leg muscles ranged between 1.2–1.9 gm for normal and 0.5–0.8 gm for dystrophic animals. The mean age of dystrophic animals was 54 ± 10 days, while control animals were killed on the average 28 days after weaning.[1] The Ca^{2+} uptake, adenosine triphosphatase activity, and protein concentration were measured as described earlier (9, 10).

The experiments were carried out in widely scattered groups between July, 1963, and August, 1966. Conditions under which experiments were performed are described in legends to the tables I–III.

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[1] For control mice only weaning dates were supplied by Jackson Memorial Laboratories. Since animals are weaned 2.5–3 weeks after birth, the age of control and dystrophic groups was similar.

TABLE I. Ca^{2+} Uptake of Normal and Dystrophic Microsomes.[a]

Additions	Ca^{2+} uptake (μmole Ca^{2+}/mg of protein)[b]		Mean difference between pairs of normal and dystrophic microsomes	Statistical significance of the difference between pairs of normal and dystrophic microsomes
	Normal (N)	Dystrophic (D)	$[S(N-D)]/n$	
None	0.062 ± 0.03 (9)	0.037 ± 0.02 (9)	0.023 ± 0.037	$p < 0.05$
K oxalate (5 m*M*)	2.050 ± 0.96 (11)	0.810 ± 0.43 (11)	1.240 ± 0.90	$p \cong 0.001$
PP_i (5 m*M*)	0.980 ± 0.43 (10)	0.310 ± 0.18 (10)	0.670 ± 0.39	$p < 0.01$
P_i (5 m*M*)	0.970 ± 0.59 (7)	0.150 ± 0.02 (7)	0.820 ± 0.61	$p < 0.01$

[a] The Ca^{2+} uptake was measured in a medium of 0.1 *M* KCl, 10 m*M* histidine, 5 m*M* $MgCl_2$, 5 m*M* ATP, 0.1 m*M* $^{45}CaCl_2$ and 0.05 mg of microsomal protein/ml at room temperature for 15 min. Additions were made as indicated in the Table. Reaction was stopped by filtration through type HA Millipore filter. The radioactivity of the microsome-free filtrate was measured by liquid scintillation counting as described by Loftfield and Eigner (13). The difference between the radioactivity of unfiltered solution and filtrate, knowing the specific activity of Ca, permits the calculation of the amount of Ca^{2+} bound to microsomes. On each preparation 4–7 assays were carried out. The statistical significance of the difference between pairs of control and dystrophic microsomes prepared on the same day was evaluated according to Mather (15).

[b] Mean ± SD. Numbers in parentheses give the number of preparations (n).

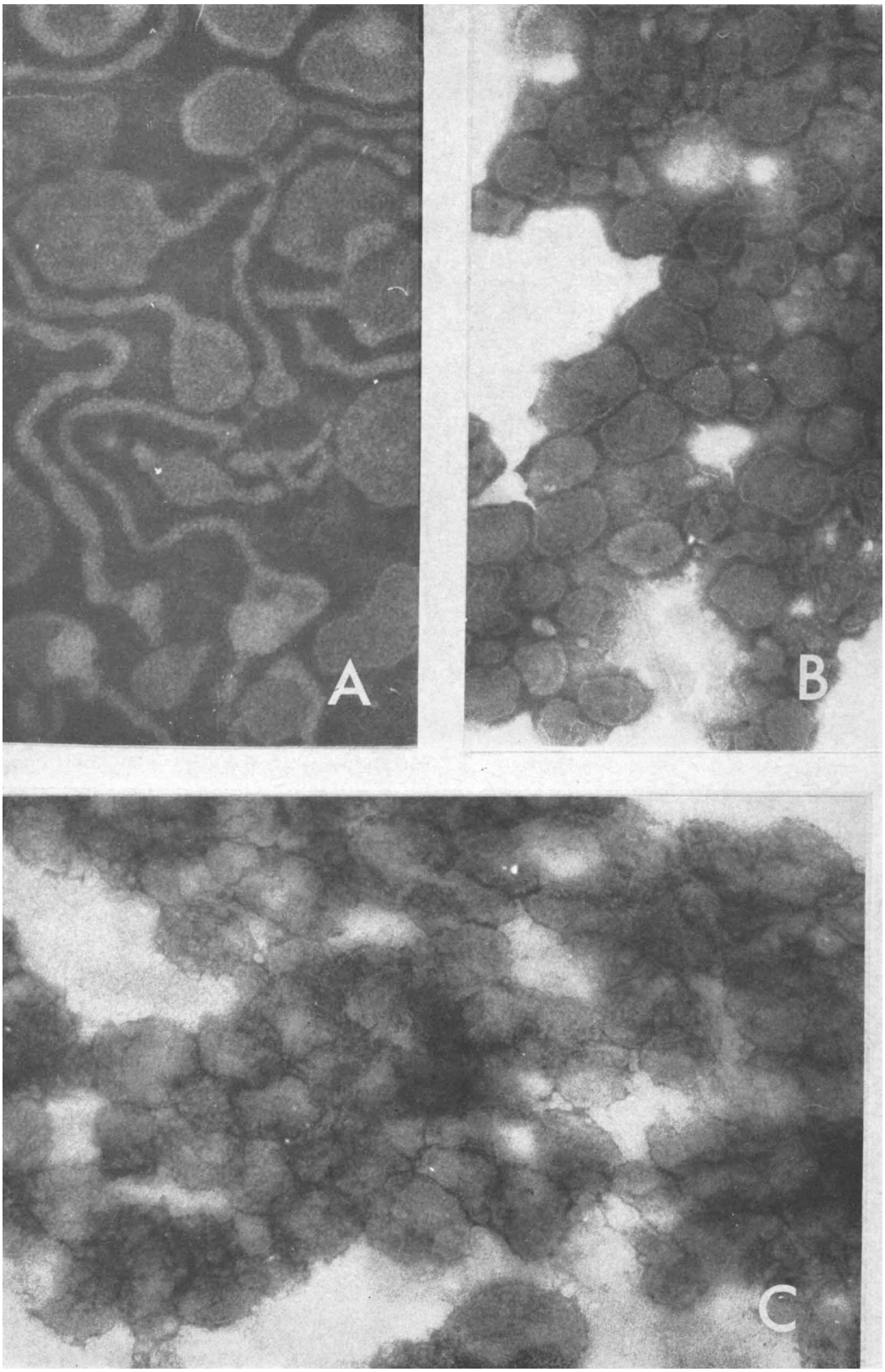
Acetylphosphate was prepared and acetylphosphatase activity was measured according to Lipman and Tuttle (11). Electron microscope analysis was carried out on a Philips EM 200 electron microscope operated at 60 kV. Microsomes were negatively stained on parlodion-coated 200 mesh grids with 0.5% K phosphotungstate, pH 7.0, and were viewed immediately under the electron microscope.

Results. The active transport of Ca^{2+} by fragments of sarcoplasmic reticulum (Gr I) isolated from leg muscles of normal and genetically dystrophic mice was studied in the presence of 5 m*M* oxalate, 5m*M* PP_i or 5 m*M* P_i as Ca-trapping agents at a series of protein concentrations. Under all experimental conditions the Ca^{2+} uptake of dystrophic microsomes was significantly lower than that of the control (Table I). These differences in Ca^{2+} uptake between pairs of control and dystrophic microsomes, prepared on the same day, were consistently observed in spite of the relatively large variation between preparations. A reduction of the Ca^{2+} transport capacity was also evident in other cell fractions of dystrophic muscle (Table II). The yield of mitochondrial and microsomal fractions did not differ significantly between normal and dystrophic animals in a limited number of experiments.

The adenosine triphosphatase activity of microsomes isolated from skeletal muscles of rabbit, rat, and normal mouse is inhibited by 0.1 m*M* EGTA, 5 m*M* K oxalate or 0.5 m*M* $CaCl_2$. Only moderate inhibition of the adenosine triphosphatase activity by EGTA and oxalate was found with microsomes isolated from dystrophic mice (Table III).

The acetylphosphatase activity of the particle-free supernatant fluid isolated from muscles of normal and dystrophic animals was similar (0.23 ± 0.08 and 0.17 ± 0.07 μmole acetylphosphate hydrolyzed/mg of protein/min, respectively). No significant acetylphosphatase activity was observed in any of the particulate fractions. These results are at variance with an earlier report (12) indicating a histochemically detectable increase of acetylphosphatase activity in dystrophic mouse muscle.

Electron microscope studies using negative staining technique did not reveal consistent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and significant differences in the surface structure of normal and dystrophic microsomes.

Fresh preparations of control mouse microsomes frequently contained tadpole-shaped particles (Fig. 1A), which were transformed during aging into the spherical elements shown in Fig. 1B. Spherical or irregularly shaped elements dominated the pictures of dystrophic microsomes (Fig. 1C). Mitochondrial fragments recognizable by their elementary particles were rarely observed and their amount in control and dystrophic microsome preparations was similar.

Discussion. The contraction–relaxation cycle of skeletal muscle is regulated by the free Ca^{2+} concentration of the sarcoplasm, which is defined primarily by the Ca^{2+} transport function of the sarcoplasmic reticulum. A biochemical lesion of these membrane systems

TABLE II. Ca^{2+} Uptake and Yield of Cell Fractions Obtained from Normal and Dystrophic Animals.[a]

Particulate fraction	Ca^{2+} uptake[b] (μmole Ca^{2+}/mg of protein)		Mean difference between pairs of normal and dystrophic preparations	Yield of particulate fractions (mg protein/gm of wet muscle)	
	Normal	Dystrophic		Normal	Dystrophic
Mitochondria	0.61 ± 0.15 (3)	0.15 ± 0.07 (3)	0.46	6.82 ± 2.08 (2)	6.15 ± 1.06 (2)
Gr I (8000–28,000 *g*)	1.78 ± 0.28 (3)	0.98 ± 0.13 (3)	0.80	2.17 ± 0.52 (9)	1.94 ± 0.63 (10)
Gr II (28,000–90,000 *g*)	0.79 ± 0.25 (3)	0.64 ± 0.21 (3)	0.15	0.63 ± 0.14 (2)	0.85 ± 0.20 (3)

[a] For details see "Experimental Procedures" and legend to Table I. The Ca uptake measurements were carried out in the presence of 5 m*M* K oxalate.

[b] Mean ± SD. Numbers in parentheses give the number of preparations (n).

TABLE III. Adenosine Triphosphatase Activity of Normal and Dystrophic Microsomes.[a]

Additions	Adenosine triphosphatase[b] (μmole P_i/mg of protein/min)		Significance of the difference between pairs of normal and dystrophic preparations
	Normal	Dystrophic	
None	0.88 ± 0.27 (11)	0.75 ± 0.36 (11)	Not significant
EGTA (0.1 m*M*)	0.39 ± 0.24 (11)	0.56 ± 0.30 (11)	$p \cong 0.05$
Oxalate (5 m*M*)	0.42 ± 0.37 (11)	0.72 ± 0.46 (11)	$p < 0.001$
$CaCl_2$ (0.5 m*M*)	0.30 ± 0.36 (2)	0.34 ± 0.30 (2)	Not significant

[a] Adenosine triphosphatase activity was measured at 34°C in a medium of 50 m*M* Tris, pH 7.0, 5 m*M* $MgCl_2$, 5 m*M* ATP, 0.01–0.05 mg of microsomal protein/ml with additions indicated in the Table. Reaction was stopped with TCA, and the liberated orthophosphate was determined according to Fiske and Subbarow (14). Evaluation of significance was based on the difference of adenosine triphosphatase activity between pairs of normal and dystrophic microsomes prepared on the same day (15).

[b] Mean ± SD. Numbers in parentheses give the number of preparations (n).

FIG. 1. Electron micrographs of negatively stained mouse microsome preparations obtained from control and dystrophic animals. A. Control microsomes stained 3 hours after killing the animal. × 210, 000. B. Control microsomes stained 3 days after killing the animal. Microsomes were stored in 0.1 *M* KCl, 10 m*M* histidine solution at pH 7.3 in ice. × 85,000. C. Dystrophic mouse microsomes stained 3.5 hours after killing the animal. × 86,000. Microsome membranes are in close contact with somewhat ill-defined contours.

in skeletal muscles of dystrophic mice is suggested by the impaired Ca^{2+} transport function of sarcoplasmic reticulum fragments isolated from dystrophic muscle.

The validity of this conclusion is dependent upon the assumption that the methods used for the isolation of cell fragments and for their assay, affect the normal and dystrophic preparations in a similar manner.

If the sensitivity of membrane structures to homogenization is greater in dystrophic than in control animals, a greater loss of Ca^{2+} uptake might result during isolation. This possibility is rendered unlikely by the fact that repeated homogenization of dystrophic muscle microsomes under conditions used for their isolation does not cause further inhibition of Ca^{2+} uptake.

Large scale contamination of dystrophic microsomes with inert particulate material might alone lead to the observed decrease in specific Ca^{2+} transport capacity. It is significant, however, that similar yields of membrane material were obtained from normal and dystrophic muscles in all particulate fractions. This finding makes extensive contamination of microsomes with mitochondria unlikely. Furthermore, electron microscope studies using negative staining did not reveal significant differences in the amount of contaminating mitochondrial fragments between normal and dystrophic microsome preparations.

On the basis of the above data the tentative conclusion is justified that in muscular dystrophy a bio-chemical defect of various phospholipid membrane systems occurs.

A unitary explanation of dystrophic changes in terms of a membrane defect must eventually be based upon some definite chemical alteration in the composition of dystrophic cellular membranes. A detailed analysis of the phospholipid and protein composition of dystrophic membrane fractions is in progress.

Summary. The Ca^{2+} transport capacity of microsomes isolated from skeletal muscles of genetically dystrophic mice was significantly less than that of the control.

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