Biochimica et Biophysica Acta, 774 (1984) 35–42
Elsevier

BBA 72108

ISOLATION AND CHARACTERIZATION OF TRANSVERSE TUBULE FROM NORMAL AND DYSTROPHIC MICE

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(Received December 2nd, 1983)

Key words: Transverse tubule; Sarcoplasmic reticulum; Fluorescence anisotropy; Muscular dystrophy; (Mouse)

I have recently reported the isolation and characterization of sarcoplasmic reticulum from normal and dystrophic mice. These sarcoplasmic reticulum fractions were similar in calcium pump function, calcium release properties, and lipid composition. In this report, I describe the isolation of mouse muscle transverse tubule membranes using a calcium phosphate-loading technique. When the relative purity of normal and dystrophic preparations was considered, transverse tubule from normal and dystrophic mice were similar in calcium-insensitive ATPase activity, cholesterol content, and membrane microviscosity (as estimated by fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene); transverse tubule yield from dystrophic muscle, however, was twice that from normal muscle, while sarcoplasmic reticulum yield from these same dystrophic muscles was only 60% that from normal muscle. This result may reflect a difference in the relative quantities of these membranes in situ.

Introduction

Recent hypotheses concerning the pathogenesis of muscular dystrophy have postulated a basic abnormality of muscle membranes [1–3]. Recently, however, Mrak and co-workers [4–6] have shown isolated sarcoplasmic reticulum from normal and dystrophic mouse muscle to be similar in lipid and protein composition, membrane microviscosity and in calcium pump function and calcium release characteristics. Thus a fundamental abnormality in dystrophic mouse sarcoplasmic reticulum appears unlikely.

In genetically dystrophic chickens, there are striking anatomic changes in the transverse tubule system, with large increases in the surface density of this organelle, while the sarcoplasmic reticulum shows more subtle changes [7]. In addition, isolated transverse tubule membranes from dystrophic chicken muscle have a greater-than-normal lipid content [8]. Similar alterations have not been described in dystrophic mouse muscle, but neither morphometric studies of muscle ultrastructure nor biochemical studies of isolated transverse tubule membranes have been available for this model. I now report the isolation and characterization of transverse tubule elements from normal and dystrophic mouse muscle.

Materials and Methods

Isolation of muscle transverse tubule membranes. Muscle transverse tubule vesicles were purified from a light membrane fraction obtained as previously described [4]. Briefly, the hind limb and lumbar musculature of normal and dystrophic ($129B6F_1/J$-*dy*) mice, aged 6 to 8 weeks, was homogenized in lots, using controlled blendor

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

speed, in a Waring Mini-Sample Blendor Accessory (Waring Products Division, Dynamics Corp. of America, New Haven, CT). Then, the homogenate was centrifuged at 7800 × *g* for 20 min, and the resulting supernatant again centrifuged at 124000 × *g* for 22 min to obtain a microsomal pellet. This pellet was incubated in 0.7 M KCl for 2 h to extract contaminating actomyosin, and then layered onto a discontinuous sucrose density gradient and centrifuged at 246000 × *g* for 90 min. Sarcoplasmic reticulum vesicles were recovered from an interface between 29.2% and 41.3% (w/w) sucrose, and a light membrane fraction recovered from an interface between 18.7% and 29.2% (w/w) sucrose [4].

The light membrane fraction, which is enriched in surface membrane enzymic 'marker' activities [4] and in cholesterol [5], was further purified using a calcium phosphate-loading technique, based on the procedure of Rosemblatt et al. [9], to remove contaminating elements of sarcoplasmic reticulum. The isolated light membrane vesicles were diluted to approx. 50 μg protein/ml with 50 mM potassium phosphate/5 mM $MgCl_2$/0.15 M KCl/0.3 mM $CaCl_2$, pH 7.35 (loading solution) at 24°C. Adenosine 5'-triphosphate (ATP; Sigma Chemical Co., St. Louis, MO) was added to a concentration of 2 mM to start the loading reaction, and 9-ml aliquots of the reaction mix were immediately underlaid with 2 ml of the loading solution, containing 2 mM ATP and 29% sucrose (w/w), in Beckman SW 41 Ti centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA). This sucrose step solution was initially at a temperature of 4°C. The centrifuge tubes were placed in Beckman SW 41 Ti rotor buckets (at 24°C) and attached to a Beckman SW 41 Ti rotor head (at 4°C). The rotor assembly was placed in a Beckman L5-65 ultracentrifuge pre-cooled to 4°C and centrifuged for 4 h at 40000 rpm (196000 × *g*). The time from initiation of the loading reaction to the start of centrifugation was approx. 15 min. Following the centrifugation, purified transverse tubule elements were recovered by Pasteur pipette as small white flecks of aggregated material floating at the interface between the loading solution and the sucrose step solution. These were homogenized in a Potter-Elvehjem glass homogenizer with a teflon pestle. The concentration of the final membrane suspension was typically 0.4–0.8 mg membrane protein per ml.

Assays. Protein was measured using a modification [10] of the method of Lowry et al. [11], with bovine serum albumin as a standard. Calcium loading was determined using a Millipore method [12]: Vesicles (20 μg protein/ml) were incubated in 0.1 M KCl/5 mM $MgCl_2$/0.1 mM $^{45}CaCl_2$ (New England Nuclear, Boston, MA)/5 mM potassium oxalate/10 mM sodium azide/10 mM Hepes (pH 6.95) at 24°C. The reaction was started by addition of ATP to a concentration of 5 mM following a 5 min equilibration period, and terminated by filtration after 16 min.

Calcium-insensitive ('basal') adenosine 5'-triphosphatase (ATPase) activity was measured by monitoring proton production at 24°C [13], using 7–15 μg membrane protein/ml in 0.1 M KCl/5 mM $MgCl_2$/5 mM potassium oxalate/1 mM EGTA (Sigma Chemical Co.)/10 mM sodium azide/5 mM ATP. The reaction was started at pH 7.35 and proton production quantitated by back titration with a KOH standard.

Calcium-stimulated phosphoenzyme formation was measured using a modification of the method of Hanna et al. [14]. Vesicles (30–50 μg protein/800 μl) were incubated at 4°C in 0.1 M KCl, 5 mM $MgCl_2$, 10 mM Hepes (pH 7.3) and either 0.1 mM $CaCl_2$ or 1.0 mM EGTA. The reaction was started by addition of [γ-^{32}P]ATP (25–50 mCi/mmol) (New England Nuclear) to a final concentration of 125 μM. After 15 s of incubation, the reaction was stopped by addition of 2 ml of ice-cold 10% trichloroacetic acid, 1 mM potassium phosphate. Aliquots of 100 μl were counted to determine the specific activity of the [^{32}P]ATP. Then, a 2.5-ml aliquot was filtered through an Amicon 0.2 μm pore-size filter (Amicon Corp., Danvers, MA), and washed with 6.0 ml of 10% trichloroacetic acid, 1 mM potassium phosphate. The filters were dissolved in 1.0 ml ethylene glycol monoethyl ether and counted in 10 ml ACS scintillant (Amersham Corp., Arlington Heights, IL). Calcium-stimulated phosphoenzyme levels are expressed as the difference between the values obtained in the presence of $CaCl_2$ and in the presence of EGTA.

Extraction and measurement of lipids. Lipid extraction was performed by the method of Bligh

and Dyer [15]. Samples were diluted with three or more volumes of water, and then extracted in (sample/water)/methanol/chloroform (0.8 : 2 : 1, v/v) [15]. Extracted lipids were back-extracted by the same procedure. This technique removed greater than 99.98% of the inorganic phosphate in the sample. Extracted lipids were spotted on Silica gel H thin-layer chromatography plates (Analtech, Newark, DE) and separated using *n*-hexane/diethyl ether/acetic acid (70 : 30 : 1, v/v). Separated spots were visualized with iodine vapor. Phospholipids were recovered at the origin, and cholesterol was identified by means of a cholesterol standard run parallel to the sample. The phospholipids were scraped into a Kjeldahl digestion tube, and total phosphorus was measured using a modification [16] of the method of Chen et al. [17]. The cholesterol spots were scraped, eluted from the silica gel with 3×1 ml of chloroform, concentrated to dryness under a nitrogen stream, and quantitated using *o*-phthalaldehyde [18].

Fluorescence anisotropy measurements. 1,6-Diphenyl-1,3,5-hexatriene was introduced into isolated membrane vesicles essentially according to the methods of Shinitzky and Barenholz [19] and Shinitzky and Inbar [20]. An aqueous dispersion of diphenylhexatriene was prepared by injecting 20 μl of 1 mM diphenylhexatriene in tetrahydrofuran, with rapid vortexing, into 100 ml of 0.1 M KCl/10 mM Hepes (pH 7.1). This mixture then was stirred at room temperature in the dark for 20 min. 1-ml aliquots of 0.1 M KCl, 10 mM Hepes (pH 7.1) containing 3.5 μg of sample lipid phosphorus, were then mixed with 1 ml of the diphenylhexatriene dispersion to give a final diphenylhexatriene concentration of 0.1 μM and a phosphorus/probe molar ratio of about 560. The samples were preincubated at 25°C for 1 h to allow partition of diphenylhexatriene into the membranes. Samples without diphenylhexatriene were prepared to determine apparent depolarization due to light scattering. Steady-state fluorescence polarization of the equilibrated samples was measured using a Perkin-Elmer MPF-44B fluorescence spectrophotometer as previously described [5,21]. Temperature studies were begun at low temperature (about 3°C). A thermocouple was used to measure sample temperature.

Electron microscopy. Samples for thin section were fixed in suspension overnight at 4°C in 0.25 M sucrose/0.1 M sodium cacodylate/2.5% glutaraldehyde/1% tannic acid (Polyscience, Warrington, PA) at pH 7.0. The solution was then filtered through a 0.22 μm pore-size Millipore filter (Millipore Corp., Bedford, MA) and the filter postfixed in buffered osmium tetroxide before dehydration, embedding, and sectioning.

Samples for freeze-fracture electron microscopy were fixed in suspension in 2.5% glutaraldehyde, 0.1 M phosphate, at pH 7.2, overnight at 4°C. Then they were sedimented and the pellet overlaid with 50% glycerol in 0.1 M phosphate, at pH 7.3 for 3 h. The pellet was quick-frozen to a gold specimen holder in liquid Freon,and fractured and shadowed as previously described [22].

Results

Normal mouse muscle yielded 35 ± 3 μg of isolated transverse tubule preparation protein per gram wet weight of muscle (Table I). This represents about 4% of the total microsomal protein recovered from the $124\,000 \times g \times 22$ min centrifugation step, and is similar to the value of 3% of microsomal protein reported for isolated rabbit muscle transverse tubule fractions (Table II). Dystrophic mouse muscle yielded approx. 60% greater quantities of transverse tubule preparation protein (Table I). This represents a 2-fold greater yield of transverse tubule elements, due to a greater purity of the dystrophic fractions (see below). In contrast to the 60% greater yield of dystrophic transverse tubule elements, the yield of dystrophic sarcoplasmic reticulum was 40% lower than normal (Table I), in agreement with a previous study [4]. As a consequence of this, the ratio of sarcoplasmic reticulum recovered to transverse tubule recovered from dystrophic mouse muscle was less than 40% of the comparable figure for normal mouse muscle (Table I).

Thin section electron micrographs of the filtered transverse tubule suspensions showed prominent large vesicles (up to 0.5 μm diameter) containing little or no stainable lumenal material (Fig. 1), as well as smaller vesicles (50–200 nm diameter), some of which showed the enhanced membrane staining characteristic of sarcoplasmic reticulum fragments prepared for electron microscopy using

TABLE I

YIELD OF MUSCLE MEMBRANE FRACTIONS FROM NORMAL AND DYSTROPHIC MICE

Fraction	Numbers of preparations	Yield (μg protein/g muscle) [a]		P
		Normal	Dystrophic	
Sarcoplasmic reticulum (SR)	13	695 ±24	413 ±14	< 0.001
Transverse tubule (TT)	13	35 ± 3	57 ± 7	< 0.001
SR/TT ratio	13	21.3± 1.7	8.2± 0.8	< 0.001

[a] Values are given as mean ± S.E.

tannic acid [23]. Such vesicles with enhanced staining were more frequent in transverse tubule fractions from normal mice than those from dystrophic mice.

Freeze-fracture replicas of the isolated transverse tubule fractions also showed a mixture of large and small vesicles (Fig. 2). Small vesicles with densely-packed 8–9 nm particles on concave fracture faces were present in fair numbers in normal membrane fractions, but were less common in dystrophic fractions. These fracture faces are typical of isolated sarcoplasmic reticulum fragments [24]. The remaining fracture faces, both concave and convex, were generally either particle-free or had infrequent particles. Large (11–13 nm) particles occurred on convex fracture faces, particularly on smaller vesicles. Occasionally these particles were found in large aggregated clumps (Fig. 2).

Calcium-insensitive ('basal') ATPase was present in both normal and dystrophic transverse tubule fractions (Table II). This enzymic activity is found in preparations of purified rabbit muscle transverse tubules [9] at levels comparable to those found in my material. Isolated transverse tubules from dystrophic mice had significantly higher levels of calcium-insensitive ATPase activity than did isolated transverse tubules from normal mice. Calcium-stimulated phosphoenzyme formation was found in fractions from normal mice, but not in fractions from dystrophic mice (Table II). This activity is characteristic of the calcium pump of sarcoplasmic reticulum, and the levels found in my normal mouse transverse tubule fractions are ap-

TABLE II

CHARACTERISTICS OF TRANSVERSE TUBULE MEMBRANE FRACTIONS ISOLATED FROM NORMAL AND DYSTROPHIC MICE

Characteristic	Numbers of preparations	Mouse transverse tubule [a]		Rabbit transverse tubule [b]	Sarcoplasmic reticulum [c]
		Normal	Dystrophic		
Yield (% of microsomal protein)		4	6	3	–
Cholesterol/lipid phosphorus (mol/mol)	5	0.33 ± 0.04	0.51 ± 0.06 *	0.55	0.06
Calcium-insensitive ('basal') ATPase (μmol/min per mg protein)	6	3.1 ±0.3	3.8 ±0.4 **	3.5 [d]	0.2
Calcium loading (μmol/mg protein)	4	< 0.2	< 0.2	0.3	5.6
Phosphoenzyme (nmol/mg protein)	6	1.9 ±0.8	−0.1 ±0.7	–	6.4 [e]

* Value is significantly different from normal from normal value: * $P < 0.05$, ** $P < 0.01$.

[a] Values are given as mean ± S.E.

[b] Values given by Rosemblatt et al. [9]

[c] Values given by Mrak and Fleischer [4,5] for normal mouse sarcoplasmic reticulum.

[d] Converted from a value measured at 32 °C, assuming an activation energy of 10 kcal/mol for calcium-insensitive ATPase (Mrak, R.E., unpublished observations, 1980).

[e] Value given by Meissner et al. [25] for rabbit sarcoplasmic reticulum.

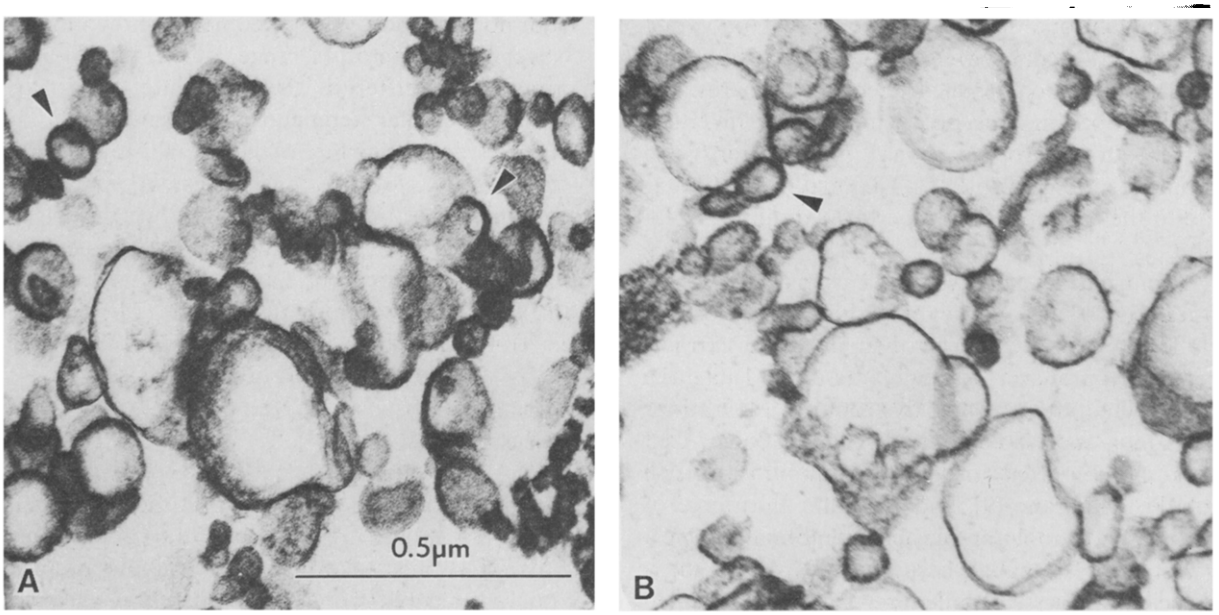

Fig. 1. Tannic acid-enhanced thin sections of isolated transverse tubule membranes. There are large empty-appearing vesicles as well as smaller vesicles. Some of the smaller vesicles show enhanced membrane staining characteristic of sarcoplasmic reticulum elements (arrowheads). These were more common in normal preparations. (A) Normal preparation. (B) Dystrophic preparation. A and B have the same magnification.

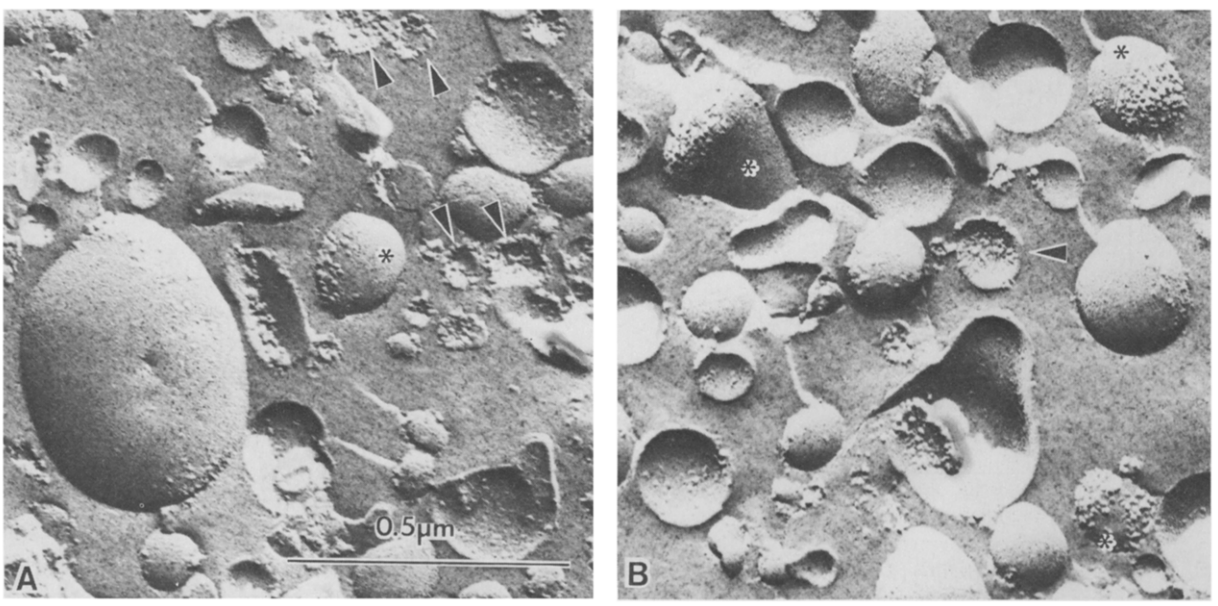

Fig. 2. Freeze-fracture replicas of isolated transverse tubule membranes. Most of the fracture faces are relatively particle-free. Some small concave fracture faces show densely-packed 8–9 nm particles characteristic of sarcoplasmic reticulum elements (arrowheads). These were more common in normal preparations. Aggregated clumps of large (11–13 nm) particles are seen on some concave fracture faces (*). (A) Normal preparation. (B) Dystrophic preparation. A and B have the same magnification.

proximately one-third the value of 6.4 nmol/mg protein reported by Meissner et al. [25] for purified rabbit sarcoplasmic reticulum preparations. Calcium loading was not demonstrable in either normal or dystrophic mouse muscle transverse tubule fractions (Table II). This may be attributable to the calcium loading performed during isolation.

Lipid extracts of dystrophic transverse tubule fractions had significantly higher ratios of cholesterol to phosphorus than did lipid extracts of normal transverse tubule fractions (Table II). The value obtained for dystrophic preparations (0.51 mol/mol) is comparable to the value of 0.55 mol/mol reported for purified rabbit transverse tubule membrane [9], and is within the range of 0.4–1.0 mol/mol for plasma membranes from a variety of mammalian tissues [26]. Normal mouse muscle transverse tubule preparations contained approximately one-third less cholesterol relative to phospholipid than did dystrophic mouse muscle transverse tubule preparations (Table II).

Fluorescence anisotropy of diphenylhexatriene in isolated muscle transverse tubule preparations from normal and dystrophic mice is shown in Fig. 3 over the temperature range 3 to 27°C. The two preparations differ in their absolute anisotropy, but have similar temperature dependences. The 0.026 lesser anisotropy of the normal preparation (0.213 vs. 0.239 at 20°C) is consistent with a 26% contamination (on a lipid basis, by weight) of the normal transverse tubule fraction by fragments of sarcoplasmic reticulum having an anisotropy of 0.139 [5] at this temperature: 0.239 (74%) + 0.139 (26%) = 0.213, since the observed anisotropy is a weight average of all lipid domains present [27].

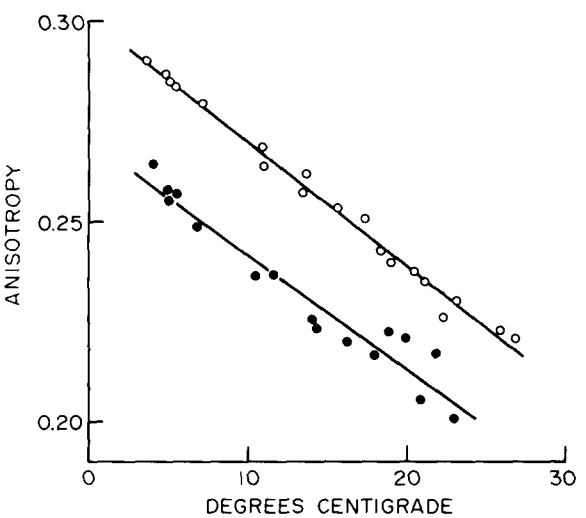

Fig. 3. Fluorescence polarization of diphenylhexatriene in isolated transverse tubule membranes from normal and dystrophic mice. Normal preparation (●). Dystrophic preparation (○). The lines are least-squares linear regression fits. The difference between normal and dystrophic preparations is consistent with a contamination of 26% (on a lipid basis) of the normal preparation by elements of sarcoplasmic reticulum.

Discussion

This study is the first report on the isolation of transverse tubule membranes from normal and dystrophic mice. The transverse tubule preparations are derived from a light membrane fraction obtained by sucrose density centrifugation during the isolation of sarcoplasmic reticulum [4]. These light membrane fractions are enriched in enzymic markers for surface membranes [4] and in cholesterol [5]. However, these light fractions also contain elements of light sarcoplasmic reticulum, and these sarcoplasmic reticulum elements can be largely removed using a calcium phosphate-loading technique based on the procedure of Rosemblatt et al. [9].

My calcium phosphate-loading technique differs somewhat from that of Rosemblatt et al. [9]. These authors incubated their rabbit light membrane fractions in loading solution for 20 min, and then collected the membranes by centrifugation prior to separation on a sucrose density gradient. The desired fraction was collected from the gradient, diluted, and then again centrifuged to a pellet. My technique involves centrifuging the loaded membrane suspensions directly against a single sucrose step solution, and thus eliminates the two pelletings. In my experience, such pelletings result in loss of up to 50% of membrane protein, and result in lower specific activities in the recovered fractions. These problems are of greater importance in small-scale preparations from mouse muscle than in large-scale rabbit preparations.

The identification of the isolated membrane fractions as transverse tubule membrane is supported by enzymic, compositional and ultrastruct-

ural data. Isolated mouse muscle transverse tubule membranes contain high levels of calcium-insensitive ATPase activity and high levels of cholesterol, which represent purifications of 2–3-fold over the normal and dystrophic light membrane fractions [4,5]. Calcium-insensitive ATPase is found in isolated rabbit transverse tubule membranes [9] at levels comparable to those found here for mouse transverse tubule membranes (Table II). Cholesterol is known to be a component of mammalian surface membranes in general [26], and the cholesterol-to-phospholipid ratios of dystrophic mouse transverse tubule fractions are similar to those of isolated rabbit transverse tubule (Table II). Thin section and freeze-fracture electron microscopy of isolated mouse muscle transverse tubule fractions show large vesicles with little stainable lumenal material and relatively particle-free fracture faces, as well as smaller vesicles with particle-free concave fracture faces and convex fracture faces showing occasional clumps of 11–13 nm particles. These 11–13 nm particles are characteristic of mouse muscle transverse tubule membranes in situ [22] and of isolated rabbit muscle transverse tubule in vitro [28]. The lack of uniform distribution of these latter particles, and the relatively particle-free appearance of the large vesicles, may be attributable to aggregation of intramembranous particles within the lipid bilayer during the isolation process (Saito, A., Seiler, S. and Fleischer, S., manuscript in preparation).

Preparations of dystrophic mouse muscle transverse tubule have significantly greater specific activities of calcium-insensitive ATPase and significantly greater quantities of cholesterol (relative to phospholipid) than do preparations of normal mouse transverse tubule membranes. This suggests greater purity in the dystrophic fractions. This suggestion is supported by the presence of calcium-stimulated phosphoenzyme formation (referable to sarcoplasmic reticulum fragments) in normal preparations, and by freeze-fracture replicas of normal preparations, which show some small vesicles densely packed with 8–9 nm particles on concave fracture faces (again referable to sarcoplasmic reticulum fragments). The phosphoenzyme levels and the relative cholesterol content suggest that approximately one-third of the material in normal preparations represents contaminating sarcoplasmic reticulum fragments which contain little or no cholesterol [5] and little or no calcium-insensitive ATPase activity [4]. In contrast, dystrophic preparations have enzymic and compositional profiles similar to isolated rabbit transverse tubule membranes (Table II), and show no calcium-stimulated phosphoenzyme formation and few fracture faces referable to sarcoplasmic reticulum on freeze-fracture replicas. This suggests that the dystrophic transverse tubule preparations are essentially free of sarcoplasmic reticulum elements. Fluorescence anisotropy measurements of diphenylhexatriene in isolated transverse tubule preparations are consistent with a contamination of approximately one-fourth (on a lipid basis) of normal preparations by sarcoplasmic reticulum elements (see Fig. 3).

The greater yield, and greater purity, of dystrophic transverse tubule elements is in contrast to the lesser yield, and lesser purity, of dystrophic sarcoplasmic reticulum preparations obtained using the same homogenization technique and the same initial centrifugation steps (Table I and Ref. 4). This suggests a difference in the relative quantities of these membrane systems in situ in the dystrophic muscle, in comparison with normal muscle. In addition, the greater purity of dystrophic mouse transverse tubule preparations suggests lesser content of light (or longitudinal) sarcoplasmic reticulum elements in dystrophic mouse muscle. Such differences might result in altered kinetics of the calcium transient, and in altered peak calcium levels during muscle activation. Physiological studies of dystrophic mouse muscle mechanics [29] and of active state kinetics [30] have suggested an alteration in excitation-contraction coupling in dystrophic mouse muscle, and the present results suggest that this alteration may be attributable to a 'microanatomic' change in the muscle membrane systems in dystrophic mouse muscle, rather than a biochemical change in membrane composition and function. The present results also suggest that reports of increased cholesterol content in dystrophic mouse muscle [31–33] may reflect an ultrastructural change in relative membrane content, rather than a biochemical change in the specific cholesterol content of dystrophic mouse muscle membranes. Ultrastructural alterations in the quantity and form of trans-

verse tubule and sarcoplasmic reticulum membranes have been described in dystrophic chicken muscle [7]. In dystrophic mice, which show greater similarities to human non-myotonic muscular dystrophies [34], ultrastructural studies have not shown abnormalities of the transverse tubule system [35], but morphometric studies quantitating membrane content in dystrophic mouse muscle are not available and small changes in relative membrane content may not be apparent on casual inspection of electron micrographs.

In summary, isolated transverse tubule fragments from normal and dystrophic mouse muscle appear to be similar (within experimental error) in calcium-insensitive ATPase activity, cholesterol content, and membrane microviscosity (as estimated by fluorescence polarization of diphenylhexatriene), when consideration is made of the relative purity of normal and dystrophic preparations. However, the yield of dystrophic transverse tubule is more than twice that of normal transverse tubule, when consideration is made of relative purity of isolated fractions. This greater yield of dystrophic transverse tubule is in contrast to a smaller yield of dystrophic sarcoplasmic reticulum elements, and may indicate a difference in the relative quantity of these membrane systems in situ.

Acknowledgements

This work was supported in part by the Muscular Dystrophy Association of America. The author thanks Ms. Carolyn Petrone for valuable technical assistance.

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