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Skeletal-Muscle Sarcolemma from Normal and Dystrophic Mice

ISOLATION, CHARACTERIZATION AND LIPID COMPOSITION

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1. Mouse skeletal-muscle sarcolemma was isolated, and the preparations obtained from normal mouse muscle and from muscle of mice with hereditary muscular dystrophy were characterized with respect to appearance under the optical and electron microscopes, distribution of marker enzymes, histochemical properties and biochemical composition. 2. The sarcolemmal membranes from normal and dystrophic muscle were subjected to detailed lipid analysis. Total lipid content was shown to increase in sarcolemma from dystrophic mice as a result of a large increase in neutral lipid and a smaller increase in total phospholipids. Further analysis of the neutral-lipid fraction showed that total acylglycerols increased 6-fold, non-esterified fatty acid 4-fold and cholesterol esters 2-fold, whereas the amount of free cholesterol remained unchanged in sarcolemma from dystrophic muscle. Significant increases were found in lysophosphatidylcholine, phosphatidylcholine and phosphatidylethanolamine in dystrophic-muscle sarcolemma; however, the relative composition of the phospholipid fraction remained essentially the same as in the normal case. 3. The overall result of alterations in the lipid composition of the sarcolemma in mouse muscular dystrophy was an increase in neutral lipid compared with total phospholipid, and a 4-fold decrease in the relative amount of free cholesterol in the membrane. The possible impact of these changes on membrane function is discussed.

Over the past few years increasing interest has been shown in the properties of the outer membrane of mammalian muscle fibres. This stems not only from a physiological interest in the function of muscle plasma membranes, but also from evidence that these membranes may be defective in certain neuromuscular diseases such as muscular dystrophy (Peter *et al.*, 1974). Isolation of skeletal-muscle sarcolemma has therefore become of increasing importance, because to probe the biochemical basis for the altered membrane properties observed in the muscular dystrophies, detailed compositional analysis is essential (Rowland, 1976).

Several authors have reported methods for the isolation of skeletal-muscle plasma membranes (Kidwai *et al.*, 1973; Shapira *et al.*, 1974) and sarcolemma (comprising the plasma membrane and its associated basement membrane) (Rosenthal *et al.*, 1965; Peter, 1970; Madeira & Antunes-Madeira, 1973) from various mammalian species, under normal and pathological conditions. The main criticism that can be levelled at many plasma-membrane-isolation procedures is the use of techniques, such as exposure to high salt concentrations, that may alter the chemical composition of the membrane, thus casting doubt on the relationship of the isolated fraction to the native membrane (De Pierre & Karnovsky, 1973; Wallach & Lin, 1973; Neville, 1975). With respect to the isolation of mammalian skeletal-muscle sarcolemma, the method of Rosenthal *et al.* (1965), as modified by Peter (1970), was found to satisfy as closely as practicable the above reservations. This method was therefore adapted for use in the isolation of sarcolemma from an inbred strain of mice that displays a form of hereditary muscular dystrophy, similar in many respects to Duchenne muscular dystrophy in humans.

The major difficulties encountered in comparative biochemical analysis of any subcellular membrane fraction are the lack of detailed knowledge of the normal case and the possibility that the various fractionation procedures may have differential effects on particular membrane properties. Peter *et al.* (1974) have stressed the importance of using multiple parameters when assessing membrane preparations, in order to overcome the objection that normal and pathological membranes may not undergo a given fractionation procedure in an identical fashion. Although the method of Peter (1970) has been widely used in recent years, in investigations into sarcolemmal composition in experimental and hereditary muscular dystrophies in rats and humans (Fiehn

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et al., 1971; Peter *et al.*, 1974), the isolation procedure has nevertheless not been characterized.

We have adapted the method of Peter (1970) to isolate the skeletal-muscle sarcolemma from normal and dystrophic mice, and have characterized the final preparation with regard to microscopical, histochemical, biochemical and enzymic properties. Detailed lipid analysis of the sarcolemmal preparations obtained have demonstrated a distinct and drastic alteration in lipid composition in sarcolemma from dystrophic mice.

Experimental

Preparation of sarcolemma

Sarcolemma was prepared by an adaptation of the method of Rosenthal *et al.* (1965), as modified by Peter (1970). The pH of all the solutions used in the preparation was measured at 20°C. Normal (+/+) or dystrophic (dy/dy) adult mice (8–24 weeks old) of the Bar Harbor 129 ReJ strain were killed by ether anaesthesia, the hind-limb muscles removed, washed in ice-cold 0.154 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.4, dissected free of fat and connective tissue, moulded into small blocks, and frozen on a liquid N_2/isopentane slurry. These blocks were stored at −70°C for not more than 4 weeks. Approx. 5 g of muscle was sliced without thawing with a Mickle tissue slicer, into slices 260 μm thick. The sections were immediately immersed in 25 ml of ice-cold 50 mM-$CaCl_2$/0.5 mM-Tris/HCl, pH 7.5. All subsequent steps were carried out at 4°C unless otherwise stated. The muscle slices were disrupted into separate myofibres by homogenization in the above solution, in a glass/Teflon homogenizer (approx. volume 30 ml; clearance 0.99 mm) at 2500 rev./min for 7 min. The suspension was then filtered through surgical gauze to remove connective tissue, diluted to 125 ml with 50 mM-$CaCl_2$ solution (fraction I), and centrifuged at 100*g* for 5 min in a Sorvall RC2-B refrigerated centrifuge. The pellet was washed twice in a buffer containing 45 mM-KCl, 30 mM-$KHCO_3$, 2.5 mM-histidine and 2.5 mM-Tris, pH 7.8. The pellet was then resuspended to a volume of 180 ml in this buffer (fraction II) and incubated with shaking at 37°C for 30 min. The suspension was centrifuged at 100*g* for 5 min and the pellet washed twice with 0.02 mM-EGTA/Tris/HCl, pH 7.5. The pellet was resuspended in 240 ml of the EGTA/Tris solution and stirred at room temperature (20°C) for 10 min. The suspension was then centrifuged at 1000*g* for 5 min. These last two steps were repeated five times to yield a pellet (fraction III), which was resuspended in 240 ml of water, pH 6.8, and centrifuged at 1000*g* for 5 min. The pellets from this centrifugation were pooled, resuspended to a volume of 30 ml in water and centrifuged at 2500*g* for 20 min. This final pellet was designated the sarcolemmal fraction (fraction IV).

Microscopy

Wet preparations of all four fractions were viewed under phase-contrast conditions with a Leitz Dialux microscope.

For histochemical staining for optical microscopy, small amounts of fraction IV were smeared on glass slides, fixed in 95% (v/v) ethanol for 1 h at 4°C and then air-dried. Sections, 8 μm thick, of whole mouse muscle were also fixed and air-dried as above. These slides were then subjected to routine haematoxylin/eosin staining, selective staining for sialic acid as described by Weber *et al.* (1975), and Karnovsky's stain for acetylcholinesterase (Karnovsky & Roots, 1964).

For electron-microscopic examination, samples of fraction IV were fixed in 2.5% (w/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4, post-fixed in 1.33% OsO_4, dehydrated and embedded in Spurr's resin. Ultrathin silver–gold sections were cut on an LKB ultramicrotome and viewed in a Hitachi HU-IIA electron microscope. In some cases, 0.1% Ruthenium Red was included in the fixation and post-fixation. Acetylcholinesterase staining was performed as described by Somogyi *et al.* (1975).

Marker enzymes

(Na^+/K^+)-stimulated Mg^{2+}-dependent ouabain-sensitive ATPase* [(Na^+/K^+)-ATPase; EC 3.6.1.3], 5′-nucleotidase (EC 3.1.3.5) and acetylcholinesterase (EC 3.1.1.7) were used as enzyme markers for plasma membranes. (Na^+/K^+)-ATPase activity was determined at 37°C in a medium containing 50 mM-imidazole, 5 mM-EGTA, 100 mM-NaCl, 20 mM-KCl, 5 mM-$MgCl_2$ and 5 mM-Tris/ATP, pH 7.4; an incubation period of 30 min was used. The amount of total activity inhibited by 1 mM-ouabain was attributed to (Na^+/K^+)-ATPase. 5′-Nucleotidase activity was determined by the method of Emmelot *et al.* (1964), and acetylcholinesterase activity was assayed by the method of Ellman *et al.* (1961). Kynurenine hydroxylase (EC 1.14.13.9), used as a marker enzyme for mitochondrial outer membranes, was determined by the method of Okamoto (1970). Acid phosphatase (EC 3.1.3.2) activity, for lysosomal contamination, and lactate dehydrogenase (EC 1.1.1.27) activity, for cytoplasmic contamination, were determined as described by Cotman & Matthews (1971) and Neilands (1955) respectively.

Lipid analysis

Lipids were extracted from samples of freeze-dried sarcolemma (1–4 mg dry wt.) by the method of

* Abbreviation: ATPase, adenosine triphosphatase.

Folch *et al.* (1957). Neutral lipids were separated by one-dimensional t.l.c. (silica gel G, Merck, Darmstadt, W. Germany; thickness 0.25mm) with *n*-hexane/diethyl ether/acetic acid (70:30:1, by vol.) as solvent. Phospholipids were separated similarly (silica gel H, Merck; thickness 0.25mm) by using chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as solvent. The separated lipids were detected by exposure to iodine vapour, and the spots were removed from the plate by suction directly into columns fitted with fine glass sinters. The neutral lipids were eluted with 5ml of chloroform/methanol (2:1, v/v). Phospholipids were eluted with 2.5ml of chloroform/methanol (2:1, v/v) followed by 1ml of methanol/acetic acid/water (94:1:5, by vol.) and 2ml of methanol. Total lipid was determined by the method of Fringo & Dunn (1970) and phospholipid by the method of Hess & Derr (1975). Cholesterol and cholesterol esters were assayed as described by Bondjers & Björkerud (1971), and non-esterified fatty acids were determined by the method of Smith (1975). Neutral lipid was determined as the difference between total lipid and total phospholipid. Total acylglycerols were determined as the difference between neutral lipid and the sum of free cholesterol, cholesterol esters and non-esterified fatty acids.

Other assays

Protein was determined by the method of Lowry *et al.* (1951), *N*-acetylneuraminic acid (sialic acid) by the method of Hammond & Papermaster (1976), and RNA as described by Tsanev & Markov (1960).

Materials

All reagents used were of the highest grade commercially available and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Calbiochem (Los Angeles, CA, U.S.A.), Mallinckrodt (St. Louis, MO, U.S.A.), BDH (Poole, Dorset, U.K.), Merck and Ajax Chemical Co. (Melbourne, Vic., Australia). Standards used in lipid analyses were sphingomyelin (Calbiochem), phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, cholesterol, cholesterol palmitate, tripalmitin and linoleic acid (all from Sigma) and olive oil (BDH). Bovine serum albumin (Cohn fraction V; Commonwealth Serum Laboratories, Melbourne, Vic., Australia) was used as the standard for protein assays. *N*-Acetylneuraminic acid, purchased from Sigma, was used as standard for the sialic acid assay.

Results and Discussion

Isolation of sarcolemma

The method of Rosenthal *et al.* (1965), as modified by Peter (1970), for preparation of rat skeletal-muscle sarcolemma, satisfied the basic requirement of an isolation procedure that did not involve high-concentration salt solutions, excessively long washes during which the membrane was in contact with intracellular components, or other steps that could damage the integrity of the membrane. It was consequently adapted for use with mouse muscle. This primarily involved an increase in concentration of the EGTA wash solution from 0.01mM to 0.02mM, and extending the time of stirring in EGTA solution at room temperature from 5min to 10min. These changes were necessary in order to wash the evacuated mouse sarcolemmal tubes completely free of intracellular components. After the eighth EGTA wash, an opaque white pellet of sarcolemma and a clear supernatant were obtained. Liberation of actomyosin did not prove to be a problem. It is proposed that the extensive washing in dilute EGTA is sufficient to wash out the actomyosin, as the pellet is swollen to two-thirds the volume by the third EGTA wash and subsequently decreases in size until the seventh wash, after which the size of the pellet is not altered by further washing in EGTA. In the method of Peter (1970), the sarcolemmal preparation was treated at this stage with Tris/ATP to remove residual actomyosin; under the conditions of preparation that we used, this step was found to be unnecessary. Washing with water at a pH of 6.4–7.0 was sufficient to remove any remaining contaminants without decreasing the yield of sarcolemma. Removal of the EGTA by this procedure resulted in swelling of the pellet, the final preparation consisting of white flocculent sarcolemma.

Phase-contrast micrographs of fractions taken at various stages in the above procedure illustrate the sequence of events in the evacuation and consequent washing of the sarcolemma (Plate 1). Although these micrographs are essentially similar to those published by Rosenthal *et al.* (1965), from preparations of rat skeletal muscle, our adapted procedure, when applied to mouse muscle, yields predominantly sarcolemmal sheets (Plate 1*c* and 1*d*), although some sarcolemmal tubes are also present. In Plate 1(*d*), folds and ridges are apparent in the membrane sheet. It is proposed that these arise from a relaxing of the sarcolemma after removal of intracellular contents and the opening of the sarcolemmal tubes to form sheets.

Electron microscopy has shown that the sarcolemma comprises four distinct regions (Rosenthal *et al.*, 1965; Zacks *et al.*, 1973*a*). From inside to out, these are the plasma membrane, an amorphous layer (ectolemma) of varying thickness (up to 50nm), and a layer of striated collagen fibrils overlayed by a meshwork of fine filaments. In sarcolemma prepared by the present procedure, the first three of these layers were detected. Large and small vesicular membranes, probably produced by sectioning across folds and invaginations in the membrane, were seen; these were

surrounded by material of the amorphous layer (Pate 2*a*). No evidence of contamination with intracellular structures was found. Similar electron micrographs of isolated sarcolemma have been published by Zacks *et al.* (1973*a*,*b*) and Beringer & Koenig (1975). No differences were detected between sarcolemma preparations from normal and dystrophic muscle at the electron-microscopic level.

Histochemical properties of isolated sarcolemma

Acetylcholinesterase staining was used at both the optical- and electron-microscope levels. At the former level, eosin was used as a counter-stain to eliminate the difficulty encountered in determining whether a dark spot on the isolated sarcolemmal membrane viewed under phase contrast was actually a specific stain or simply a fold in the membrane. For comparison, sections of whole mouse muscle were stained for acetylcholinesterase at the same time. End-plate regions in the muscle sections were stained, as were areas of similar dimensions on the sarcolemmal sheets (Plates 3*a* and 3*b*). Control slides stained with eosin alone showed no background staining. At the ultrastructural level, acetylcholinesterase staining was localized in areas of the ectolemma immediately adjacent to the plasma membrane (i.e. on the outer surface of the plasma membrane) (Plate 2*b*). Similar acetylcholinesterase staining of isolated rat sarcolemma has been reported by Zacks *et al.* (1973*a*).

Sections of whole mouse muscle and sarcolemma were also stained for sialic acid. As shown in Plates 3(*c*) and 3(*d*), the stain appeared to be localized in the areas between muscle fibres, whereas the sarcolemmal sheets stained strongly. It is probable that the sialic acid detected by this staining procedure is an integral part of the glycoprotein of the ectolemma, which has been described by Zacks *et al.* (1973*a*,*b*) as having properties attributable to highly acidic glycoproteins. Consequently, Ruthenium Red was used as a stain for cell-surface carbohydrates at the electron-microscope level. The entire sarcolemma appeared to stain intensely with Ruthenium Red, the stain being localized in the plasma membrane as well as throughout the ectolemma (Plate 2*c*). Similar results for isolated rat sarcolemma have been reported by Zacks *et al.* (1973*a*). No overt differences between sarcolemmal preparations from normal and dystrophic muscle were seen with either acetylcholinesterase staining at the optical- and electron-microscope levels or Ruthenium Red staining at the electron-microscope level. Staining for sialic acid was not performed on sarcolemmal preparations from dystrophic mice.

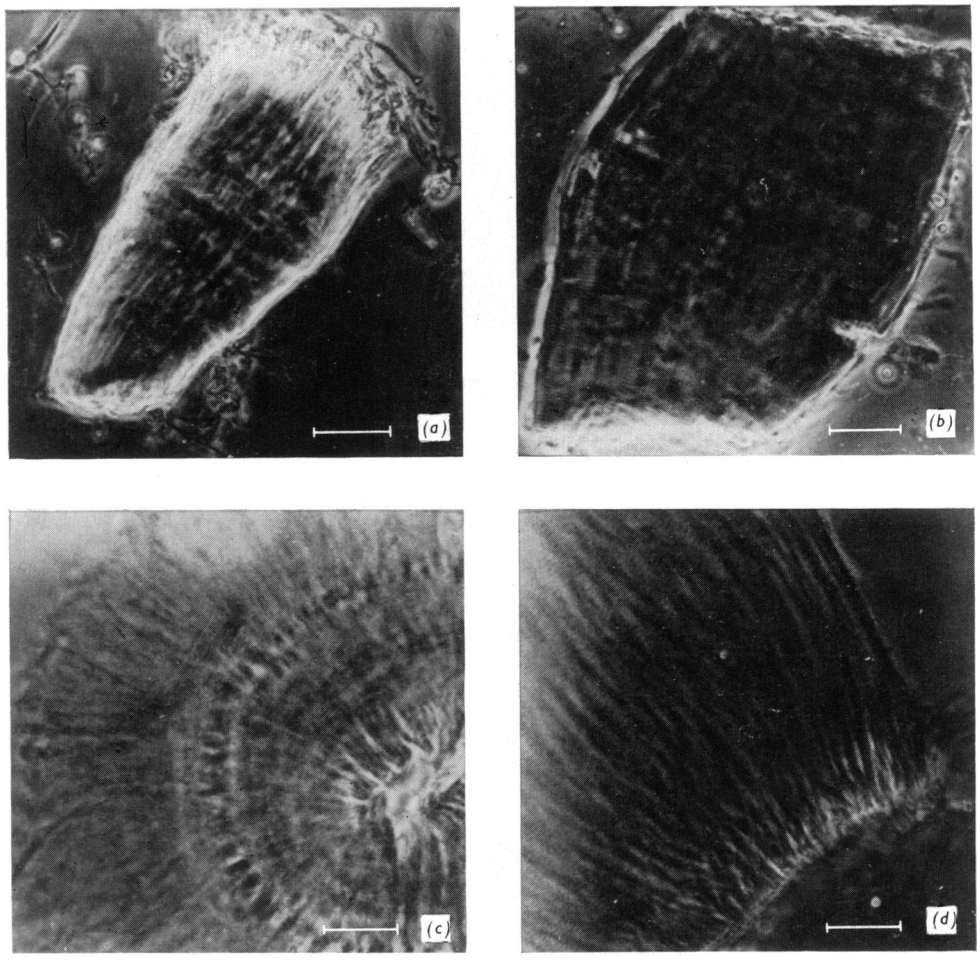
Distribution of marker enzymes

The activities of the various marker enzymes in fractions I and IV are shown in Table 1. The activities of these enzymes in fractions II and III followed the pattern expected of fractions from a plasma-membrane-isolation procedure, that is a graded increase in activities of plasma-membrane-associated enzymes and graded decrease in activities of enzymes associated with other intracellular organelles. As shown in Table 1, all three plasma-membrane-associated enzymes show an increase in specific activity of approx. 5-fold in the sarcolemmal fraction as compared with the homogenate. Conversely, all the enzymes associated with intracellular structures show a decrease in specific activity when the final preparation is compared with the homo-

Table 1. *Distribution of marker enzymes in the first and final fractions of the sarcolemmal-preparation procedure*

The following enzyme activities were determined in the first (I) and final (IV) fractions in the sarcolemmal preparation. The point in the procedure at which the fractions were taken, and all methods used, are given in the text. Specific activity is given in nmol/min per mg of protein ± S.D. A minimum of three sarcolemmal preparations was assayed for each enzyme. Numbers in parentheses indicate the number of separate samples assayed. The percentage of total activity originally found in the homogenate which remains in fraction IV is given as the percentage recovery. Difficulty was encountered in obtaining measurable activity for kynurenine hydroxylase and lactate dehydrogenase in the sarcolemmal preparations under the conditions used, hence the maximum possible activity, as calculated from the known limits of the assay, is given. Similarly, owing to large variations in lactate dehydrogenase activity in different homogenates, the minimum observed enzyme activity is given.

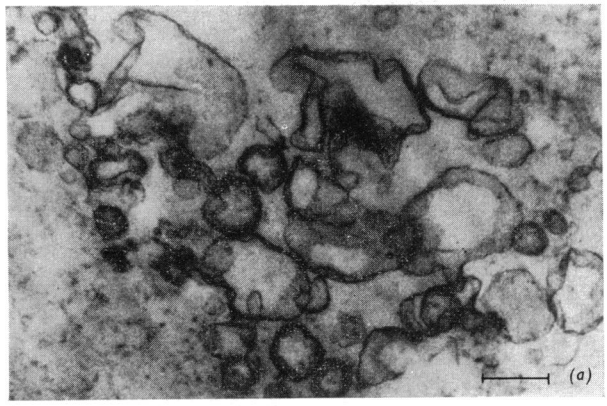
Enzyme Fraction ...	Specific activity I	Specific activity IV	Recovery in fraction IV (%)
(Na^+/K^+)-ATPase	10.4 ± 4.3 (14)	54.3 ± 6.9 (8)	16.4 ± 1.0 (8)
5′-Nucleotidase	0.33 ± 0.16 (8)	1.50 ± 0.63 (8)	22.7 ± 7.5 (8)
Acetylcholinesterase	0.14 ± 0.06 (6)	0.53 ± 0.29 (6)	13.5 ± 3.3 (6)
Kynurenine hydroxylase	6.3 ± 3.6 (12)	<2.0 (12)	<7.5 (9)
Acid phosphatase	8.3 ± 1.1 (8)	4.1 ± 1.7 (12)	5.1 ± 2.3 (12)
Lactate dehydrogenase	>80 (10)	<5 (10)	<0.4 (10)
Protein	—	—	7.6 ± 4.4 (32)

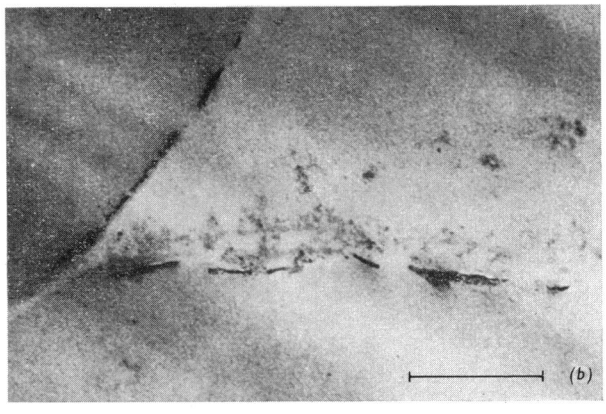


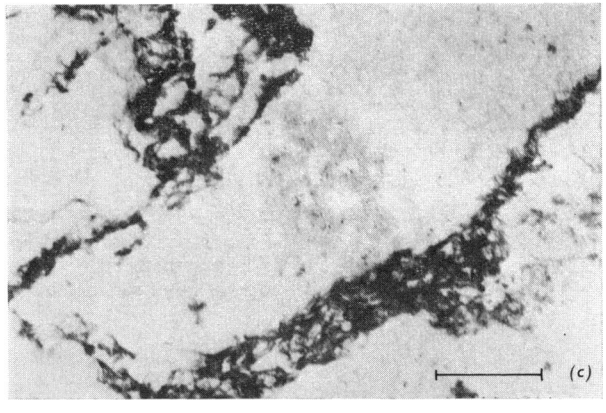
EXPLANATION OF PLATE 1

Phase-contrast micrographs of fractions I, II and IV

(*a*) Fraction I: segment of muscle fibre. Note the striations and presence of released fibrils. (*b*) Fraction II: segment of muscle fibre partially evacuated. (*c*) Fraction IV: large sarcolemmal sheet. Note the loss of bifringence in comparison with (*a*) and (*b*). (*d*) Fraction IV: view of edge of large sarcolemmal sheet. Note the ridges and folds in the membrane. Scale line represents 10μm.







EXPLANATION OF PLATE 2

Electron micrographs of isolated sarcolemma

(*a*) Sarcolemma, fraction IV, stained with OsO_4. Note the cross-sections of sheets and folds of sarcolemma. The ectolemma does not stain well with OsO_4; however, amorphous material of this layer is visible throughout the section. (*b*) Sarcolemma stained for acetylcholinesterase. This shows an area in which the plane of section passes through the sarcolemmal sheet. The acetylcholinesterase stain is located at the edge of this cross-sectional area, which is the only point where the outer layer of the plasma membrane is visible. The ectolemma does not stain well under the procedure used; certain elements of the ectolemma are just visible along the edge of the membrane. (*c*) Sarcolemma stained with Ruthenium Red and OsO_4. The reaction with Ruthenium Red appears to be localized primarily in the ectolemma, which stains intensely, although the plasma membrane also appears to stain. The ectolemma clumps together with the plasma membrane because of the binding properties of Ruthenium Red, and consequently is less evident than in (*a*). The scale line represents 0.2 μm.

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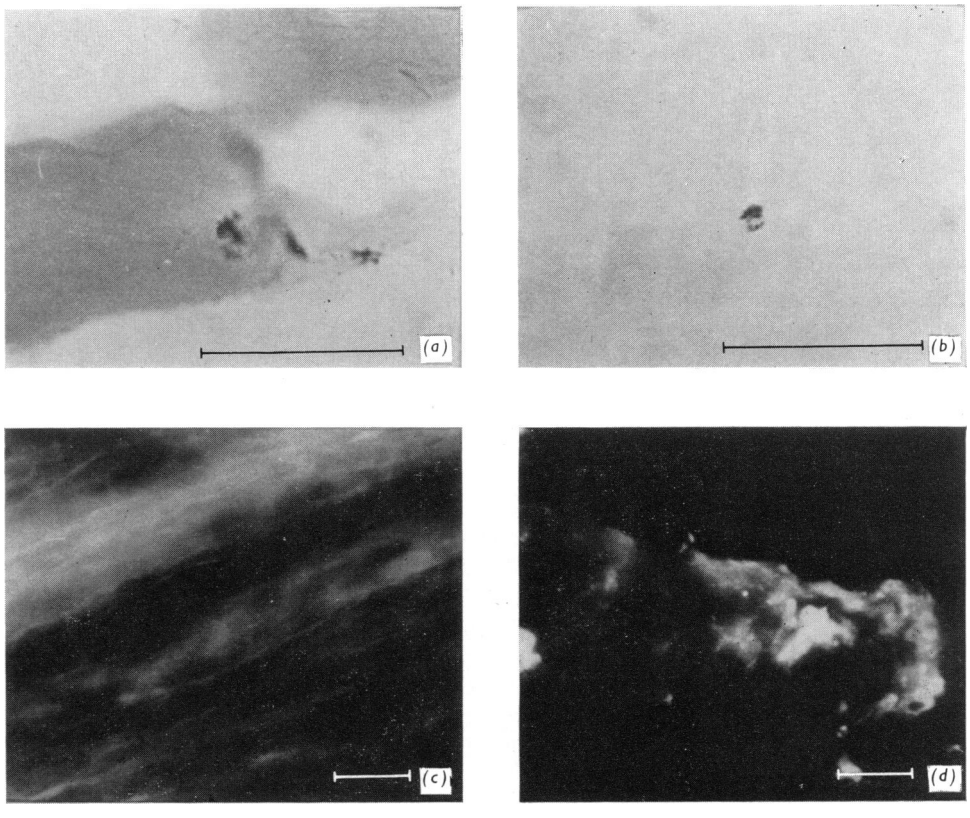

EXPLANATION OF PLATE 3

Histochemical properties of sarcolemma under the optical microscope

(*a*) End-plate region of mouse muscle fibre stained with acetylcholinesterase stain, counter-stained with eosin. (*b*) Portion of sarcolemmal sheet stained with acetylcholinestarase stain, counter-stained with eosin. Note the similar size of the acetylcholinesterase-stained region compared with those in (*a*). (*c*) Longitudinal section of whole mouse muscle stained for sialic acid. Note that, where the plane of section passes through a muscle fibre, the intracellular contents do not stain. (*d*) Sarcolemmal sheet stained for sialic acid. The entire sarcolemmal sheet stains; areas where the sheet is folded appear more intense. Scale line represents 40 μm.

genate. It is noteworthy that the specific activity of (Na^+/K^+)-ATPase in normal mouse sarcolemma prepared by the present method (Table 1) is higher than that of rat sarcolemma isolated by the method of Peter (1970) (45nmol/min per mg of protein; Peter *et al.*, 1974). As the only difference in the two isolation procedures is our substitution of water for Tris/ATP as the final wash solution, it is concluded that this change does not detrimentally affect the final preparation.

The percentage of total activity originally found in the homogenate, which remained in the final preparation, was calculated for each enzyme (Table 1). The percentage recovery of protein in the final preparation is included for comparison. The plasma-membrane-associated enzymes had percentage recoveries 2–3-fold higher than that of total protein, whereas the percentage recoveries of marker enzymes for outer mitochondrial membranes, lysosomes and cytoplasmic components were decreased with respect to that of total protein. These results suggest that the procedure used does in fact selectively isolate sarcolemmal membranes from other intracellular organelles.

No marker enzyme or property for sarcoplasmic reticulum has been included in this study, primarily because a suitable marker has yet to be found. Ca^{2+}-stimulated Mg^{2+}-dependent ATPase was once regarded as a reliable marker enzyme for sarcoplasmic reticulum, but the demonstration of a similar enzyme of high affinity in sarcolemma (Sulakhe *et al.*, 1973; Kosterin & Kursky, 1975; Gimmel'reikh & Kravets, 1976) has made its use as a marker to distinguish between sarcoplasmic reticulum and sarcolemma no longer valid. It is argued that the isolation procedure used would obviate any gross contamination by sarcoplasmic reticulum, as the numerous washing steps are punctuated by centrifugation at a speed sufficiently low to make the pelleting of membranes or membrane vesicles smaller than the very large sarcolemmal sheets unlikely. This assertion is supported by the lack of mitochondrial or lysosomal contamination, as evidenced by electron microscopy and marker enzymes.

Sarcolemmal preparations from normal and dystrophic muscle were virtually identical with respect to the distribution of marker enzymes, and no consistent difference in amounts of specific enzymes was detected, except for (Na^+/K^+)-ATPase. Results from preparations from normal and dystrophic muscle have been combined in Table 1.

Attempts to measure the (Na^+/K^+)-ATPase in sarcolemma prepared from dystrophic muscle were frustrated by the anomalous behaviour of this enzyme preparation in the presence of ouabain. Instead of the expected inhibition of activity in the presence of ouabain, as displayed by sarcolemma from normal muscle, addition of ouabain to sarcolemma from dystrophic muscle consistently resulted in an increased activity. Stimulation of (Na^+/K^+)-ATPase by oubain in tissues from dystrophic animals has been previously reported (Brown *et al.*, 1967; Rodan *et al.*, 1974).

General composition of the sarcolemma

The biochemical composition of the sarcolemma with respect to protein, lipids, RNA and sialic acid is shown in Table 2. The percentage dry wt. of the sarcolemma attributed to protein is higher in our preparation than has peen reported for normal rabbit and rat sarcolemma (Madeira & Antunes-Madeira, 1973; Vandenberg *et al.*, 1974). This may be due to species differences or to differences in the amount of protein of the ectolemma retained by the different procedures. Because of the higher protein content of sarcolemma prepared by the present method, the percentage of sarcolemmal dry wt.

Table 2. *Biochemical composition of sarcolemma from normal and dystrophic mice*

The amounts of the following components were determined in freeze-dried sarcolemma from normal and dystrophic mice, by the methods given in the text. Results are given as means ± S.D. for the numbers of separate samples assayed given in parentheses. Significance was determined by Student's *t* test: n.s., not significant ($P > 0.05$).

	Content (μg/mg of protein)			Content (% dry wt. of sarcolemma)		
Component	Normal	Dystrophic	*P*	Normal	Dystrophic	*P*
Protein				87.9 ± 5.6 (29)	85.3 ± 5.2 (27)	n.s.
Lipid	44.1 ± 7.0 (10)	86.3 ± 13.3 (12)	<0.00005	3.9	7.4	
RNA	5.31 ± 0.72 (8)	4.61 ± 0.58 (6)	n.s.	0.46	0.39	
Sialic acid	0.44 ± 0.12 (11)	0.52 ± 0.20 (12)	n.s.	0.04	0.04	
Unaccounted				7.7	6.9	

attributed to other components is decreased compared with that reported by the above authors.

Lipid composition

The gross lipid composition of normal mouse sarcolemma is shown in Table 3. The percentage composition and amounts of lipid per mg of protein differ from those reported for rat and rabbit sarcolemma (Fiehn *et al.*, 1971; Madeira & Antunes-Madeira, 1973), possibly owing to differences in species, methods of lipid determination and the amount of sarcolemmal protein retained, as discussed above.

Table 3 shows that there is a large increase in the lipid content of sarcolemma from dystrophic mice and that this increase is predominantly due to an increase in neutral-lipid content. The percentage composition of total lipid reflects this change.

The results from fractionation of sarcolemmal neutral lipids are shown in Table 4. The percentage composition of the neutral lipid of normal mouse sarcolemma is similar to that reported for rat sarcolemma (Fiehn *et al.*, 1971), except for a lower content of cholesterol esters.

The increase in total neutral lipids in dystrophic mouse sarcolemma is due to a large increase in the non-esterified fatty acid (4-fold increase) and total acylglycerol (6-fold increase) components. The amount of cholesterol esters was also increased, but the amount of free cholesterol remained unchanged. It is noteworthy that when considering the percentage composition of sarcolemmal neutral lipids, only the free cholesterol component is dramatically altered in dystrophy.

As total phospholipid was also increased in dystrophic mouse sarcolemma (1.4-fold increase), this fraction was also further separated, and the results are

Table 3. *Gross lipid composition of sarcolemma from normal and dystrophic muscle*

The following lipid classes were determined in freeze-dried sarcolemma as described in the text. Values given are means±S.D. for the numbers of separate samples assayed indicated in parentheses. Significance was determined by Student's *t* test. The cholesterol/phospholipid molar ratio (C/PL) is also given.

	Content (μg/mg of protein)			Content (% of total lipid)	
Component	Normal	Dystrophic	*P*	Normal	Dystrophic
Total lipid	44.1±7.0 (10)	86.3±13.3 (12)	<0.00005	100	100
Phospholipid	27.3±6.0 (12)	33.5±6.7 (11)	<0.05	62	39
Neutral lipid	15.4±5.7 (10)	53.7±15.5 (11)	<0.00005	35	62
C/PL ratio	0.37±0.13 (12)	0.30±0.03 (9)	n.s.	—	—

Table 4. *Neutral-lipid composition of sarcolemma from normal and dystrophic mice*

The lipid fractions of normal and dystrophic-muscle sarcolemma were further fractionated into the following neutral-lipid classes as described in the text. Values given are means±S.D. for the numbers of separate samples assayed indicated in parentheses. Significance was determined by Student's *t* test.

	Content (μg/mg of protein)			Content (% of neutral lipid)		
Component	Normal	Dystrophic	*P*	Normal	Dystrophic	*P*
Total neutral lipid	15.4±4.7 (10)	53.7±15.5 (19)	<0.00005	100	100	
Free cholesterol	3.48±1.74 (13)	3.17±0.56 (9)	n.s.	23.2±12.1 (13)	5.58±1.15 (9)	<0.0005
Cholesterol esters	0.24±0.06 (11)	0.51±0.24 9)	<0.002	1.72±0.72 (12)	0.92±0.48 (9)	<0.01
Non-esterified fatty acids	5.02±2.81 (11)	21.2±12.7 (9)	<0.001	32.4±18.9 (11)	32.3±19.1 (8)	n.s.
Total acylglycerols	6.3 ±4.0 (9)	36.9±15.1 (8)	<0.00005	43.6±22.3 (8)	61.5±19.3 (8)	n.s.

Table 5. *Phospholipid composition of sarcolemma from normal and dystrophic mice*
Lipid fractions from sarcolemma of normal and dystrophic mice were fractionated into the following phospholipid classes as described in the text. Values are means±S.D. for the numbers of separate samples assayed given in parentheses. Significance was determined by Student's *t* test.

	Content (nmol/mg of protein)			Content (% of total phospholipid)		
Component	Normal	Dystrophic	*P*	Normal	Dystrophic	*P*
Total phospholipids	32.5±8.6 (13)	46.6±11.4 (10)	<0.005	100	100	
Lysophosphatidylcholine	1.43±0.47 (10)	2.15±0.90 (10)	<0.05	4.55±1.34 (9)	4.77±2.31 (10)	n.s.
Sphingomyelin	2.84±0.81 (11)	3.22±0.80 (11)	n.s.	9.96±3.02 (12)	7.53±2.93 (10)	n.s.
Phosphatidylcholine	17.1±4.98 (13)	26.3±8.1 (10)	<0.005	54.6±5.34 (12)	56.0±5.49 (10)	n.s.
Phosphatidylserine +phosphatidylinositol	3.29±1.34 (13)	3.82±1.48 (10)	n.s.	11.9±2.78 (10)	8.78±4.03 (10)	n.s.
Phosphatidylethanolamine	6.11±1.92 (13)	9.76±3.13 (9)	<0.005	18.7±3.29 (13)	22.9±4.53 (10)	<0.02

shown in Table 5. The phospholipid classes shown account for almost all of the phospholipid found in sarcolemma. The percentage composition of phospholipids of normal mouse sarcolemma is similar to that reported for rat sarcolemma (Fiehn *et al.*, 1971). The increase in total phospholipids in dystrophic mouse sarcolemma is attributable to increases in the amounts of phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylcholine; absolute amounts of sphingomyelin and phosphatidylserine plus phosphatidylinositol do not change. However, the percentage composition of phospholipids from dystrophic mouse sarcolemma is not greatly different from that of normal sarcolemma.

Before further discussing the lipid changes in the sarcolemma of dystrophic mice, it is appropriate to consider the query raised by the work of Takagi *et al.* (1973); they reported contamination of sarcotubular-membrane preparations with membranes from fat and connective tissue, and suggested that the abnormalities in lipid composition found in sarcotubular membranes from dystrophic human muscle may thus be attributable to the increased amount of fat and connective tissue associated with this muscle. Rowland (1976) further suggested that this complication would also be applicable to isolation of sarcolemma from dystrophic muscle. In answer to this, we again draw attention to the numerous low-speed centrifugations involved in the present procedure; as discussed above, these appear to disallow major contamination by small membrane fragments or vesicles. Furthermore, if contamination by membranes other than sarcolemma was sufficiently high to influence lipid analysis of sarcolemma from dystrophic muscle, then an alteration in the percentage composition of both neutral lipids and phospholipids would be expected, as different membranes have different lipid compositions (Fiehn *et al.*, 1971; McMurray, 1973). We therefore believe that the changes in lipid composition reported here do in fact represent changes inherent in the sarcolemma of dystrophic muscle.

The alterations in lipid composition of dystrophic mouse sarcolemma are attributable to an increase in all components containing fatty acids. These alterations result in a perturbation of lipid composition as evidenced by the changes in relative lipid composition, which show an increase in neutral lipids and a corresponding decrease in total phospholipids. This will undoubtedly have an effect on the fluidity and function of the membrane. Of equal importance is the decrease in the amount of free cholesterol relative to other lipid components of the membrane (4-fold decrease), which would imply a major change in the rigidity of the lipid phase and possibly alterations in lipid–protein interactions (Demel & de Kruyff, 1976), thus affecting the structural and functional integrity of the membrane.

Others have reported similar alterations in the fatty acid-containing components of muscle membranes in dystrophy (Kunze *et al.*, 1971, 1973, 1975; Chio *et al.*, 1972; Peter & Fiehn, 1972), as well as alterations in fatty acid metabolism in dystrophic muscle (Lin *et al.*, 1969; Chio *et al.*, 1972; King & Emery, 1973; Jato-Rodriguez *et al.*, 1974; Kar & Pearson, 1975; Kwok *et al.*, 1976). Evidence to date favours increased fatty acid synthesis, possibly selective, as the primary cause of the increased deposition of fat in dystrophic tissues.

The effect of the reported changes in sarcolemmal lipid composition on membrane and cellular function is reflected in alterations of enzyme activities assoc-

iated with the sarcolemma, such as adenylate cyclase and cyclic nucleotide phosphodiesterase (Mawatari *et al.*, 1973; Susheela *et al.*, 1975; Lin *et al.*, 1976), which are recognized as playing major roles in hormone–cell interactions. That the lipid abnormality may extend to plasma membranes of other tissues is suggested by the findings of similar enzyme changes in erythrocytes from dystrophic patients (Chattopadhyay & Brown, 1972; Mawatari *et al.*, 1976). Altered hormonal responses in dystrophic patients have been reported (Beckmann, 1974; Takamori, 1975; Malarkey & Mendell, 1976).

In conclusion, a major perturbation of lipid composition in isolated sarcolemma from skeletal muscle of dystrophic mice is reported. This change consists of an increase in all components containing fatty acids, in particular total acylglycerols and non-esterified fatty acids. An abnormality of fatty acid synthesis or incorporation into membrane components is therefore implicated. It is suggested that in the alteration of lipid composition lies the explanation for the observed changes in plasma-membrane-associated properties observed in muscular dystrophy. Research into the role of lipid components in membrane structure and function, their incorporation into the membrane and the control mechanisms and restrictions to which this process is subjected, would greatly aid our present understanding of diseases in which perturbation of membrane lipid composition is implicated.

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