Macromolecular Characterization of Muscle Membranes

I. PROTEINS AND SIALIC ACID OF NORMAL AND DENERVATED MUSCLE*

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SUMMARY

Two major membrane fractions have been isolated from denervated and contralateral limb control rat muscle by a subcellular fractionation technique involving lithium bromide and potassium chlorodic extraction and differential and density gradient centrifugation. Membranes isolated from the initial nuclear pellet (Fraction I) contained Na\(^{+}\)K\(^{+}\)(Mg\(^{++}\))ATPase (20 \(\mu\) moles of P\(_{i}\) per hour per mg), sialic acid (24 nmoles per mg), and Ca\(^{++}\)/Mg\(^{++}\)ATPase (19 \(\mu\) moles of P\(_{i}\) per hour per mg). With subfractionation of Fraction I the membranes enriched in Na\(^{+}\)K\(^{+}\)(Mg\(^{++}\))ATPase and sialic acid (IM\(_{L}\)) also had the highest specific activity of membrane protein iodination using extracellular lactoperoxidase. These membrane subfractions were separated from ones containing Ca\(^{++}\)/Mg\(^{++}\)ATPase activity (IM\(_{R}\)).

Membranes isolated from the post mitochondrial supernatant (Fraction II) contained Ca\(^{++}\)/Mg\(^{++}\)ATPase (22 \(\mu\) moles of P\(_{i}\) per hour per mg), Na\(^{+}\)K\(^{+}\)(Mg\(^{++}\))ATPase (5 \(\mu\) moles of P\(_{i}\) per hour per mg), and sialic acid (10 nmoles per mg). With further subfractionation the Ca\(^{++}\)/Mg\(^{++}\)ATPase containing membranes (IM\(_{M}\) and IIM\(_{M}\)) were separated from those most accessible to iodination by extracellular lactoperoxidase and containing Na\(^{+}\)K\(^{+}\)(Mg\(^{++}\))ATPase and sialic acid (IM\(_{L}\) and IIL). Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed different protein profiles for Fractions I and II with polypeptide molecular weights ranging from 20,000 to 200,000. Both subfractions IM\(_{L}\) and IIM\(_{L}\) contained a single major glycoprotein species with an apparent molecular weight of approximately 110,000.

These data would suggest that both lighter subfractions (IM\(_{L}\) and IIM\(_{L}\)) derived from the nuclear pellet (Fraction I) and the postmitochondrial supernatant (Fraction II), respectively, were enriched in surface membranes. The heavier subfraction (IIM\(_{R}\)) from the postmitochondrial supernatant was enriched in membranes derived from sarcoplasmic reticulum.

Following 1 week of denervation no change was observed in either electron micrographs or sodium dodecyl sulfate polyacrylamide gels of the membrane fractions. The ATPase and sialic acid of Fraction I remained unaltered while a 2-fold increase was noted in the Ca\(^{++}\)/Mg\(^{++}\)ATPase (51 \(\mu\) moles of P\(_{i}\) per hour per mg) of heavier membranes of Fraction II and sialic acid (20 nmoles per mg) of lighter membranes of Fraction II.

Membranes of skeletal muscle represent an important model for studying membrane function in excitable tissue and for characterizing the effect of a nerve on its postsynaptic structure. Many of the physiological and morphological consequences of innervation and denervation have been characterized, yet their biochemical and molecular properties are incompletely understood. The advantages of muscle, namely its ample supply, relative cellular homogeneity compared to brain, and its accessibility, are offset by the difficulty of isolating purified membrane preparations and identifying their origin. Recently, several subcellular fractionation schemes have been presented which yield enriched muscle plasma membrane fractions (1–5). As a marker for surface membrane, sodium and potassium-stimulated, magnesium-dependent adenosine triphosphatase specific activity has been used while calcium- or magnesium-dependent ATPase specific activity has been employed as a marker for sarcoplasmic reticulum (6–10).

Our interest in muscle membrane isolation was prompted by our recent demonstration of increased glycoprotein sialic acid in homogenates of rabbit gastrocnemius muscle following denervation (11). All cellular glycoproteins described to date have been identified either as endogenous membrane proteins or as secretory products localized within membranous structures (12). Since one of the more prominent anatomic alterations in denervated muscle is the proliferation of intracellular membranes and tubules (13, 14), the question was raised whether the sialic acid increase might be associated with either membrane fractions enriched in Na\(^{+}\)K\(^{+}\)(Mg\(^{++}\))ATPase or Ca\(^{++}\)/Mg\(^{++}\)ATPase.

A modification of a subcellular fractionation technique devised for frog muscle (1) was utilized to obtain two major fractions of membranous vesicles from denervated and contralateral limb control rat muscle. Fraction I was derived from the initial nuclear pellet, while Fraction II was isolated from the postmitochon-
drial supernatant. Both fractions were subfractionated on sucrose density gradients and characterized by electron microscopy, ATPase activity, sodium dodecyl sulfate polyacrylamide gel electrophoresis, sialic acid content, and accessibility to iodination in intact tissue by extracellular lactoperoxidase.

MATERIALS AND METHODS

Denervation—Adult female (150 to 250 g) Wistar rats, following ether anesthesia, were unilaterally denervated by removal of a 2-cm segment of sciatic nerve from mid-thigh.

Isolation of Fraction I—The method utilized for isolation of Fraction I was modified from Boegman et al. (1). Rats were killed by decapitation and three muscles of mixed fiber type (extensor digitorum longus, anterior tibialis, and gastrocnemius) were dissected and placed in ice sucrose buffer (sucrose, 0.25 M; Na₂EDTA, 0.2 mM; Tris-HCl, 0.1 M; pH 7.6). All subsequent procedures were conducted at 4°C. The muscles were weighed, minced with scissors, suspended in 5 volumes (milliliters per g of original muscle weight) of sucrose buffer and homogenized with a Brinkmann Polytron homogenizer model PT10/30T for 2 min at a speed of 2,000 rpm. The homogenate was centrifuged at 1,000 x g for 10 min, and the supernatant (S₁) was retained and centrifuged at 105,000 x g for 30 min. The resulting pellet was washed twice with 1 volume (original muscle weight) of Medium B (KCl, 0.6 M; Tris-ATP, 10 to 20 μg of membrane protein per ml, 100 mM NaCl, 20 mM potassium chloride and/or 5 mM Mg or CaCl₂). ATPase specific activities were calculated on the basis of the amount of inorganic phosphate released during the 15-min incubation in the presence of the appropriate stimulating ions less the amount of Pi present following the same incubation without those ions and converted to terms of net micromoles of Pi released per hour per mg of protein.

Sialic Acid Determination—Membrane subfractions were hydrolyzed in 0.1 M H₂SO₄ for 60 min at 80°C. The hydrolysate was diluted, applied to Dowex 1 X 8 columns, and eluted with 0.4 N formic acid (18). The N-acetylaneuraminic acid content of the eluate was determined by the thiobarbituric acid modification of Aminoff (19).

SDS-Polyacrylamide Gel Electrophoresis—A modification of the method of Weber and Osborn (20) was utilized for polyacrylamide gel electrophoresis of membrane proteins solubilized by SDS and mercaptoethanol. The gels were composed of 0.1 M sodium phosphate buffer, pH 7.2, 0.1% SDS (recrystallized, Sigma Chemical Co., St. Louis, MO.), 0.10% N,N,N',N'-tetramethylenediamine (Eastman Organic Chemicals, Rochester, N. Y.), 7.5% acrylamide, 0.3% N,N',N,N'-tetramethylenediamine (both recrystallized from Eastman), 5% glycerol, 0.5 M urea, and 0.4% ammonium persulfate (both freshly prepared). Membrane protein (100 μg) was freeze-thawed three times, sonicated at setting 6 for 15 s on a Branson Sonifier, and lyophilized to dryness. The residue was suspended in 100 μl of 0.1 M sodium phosphate buffer, pH 7.2, containing 1% β-mercaptoethanol and 1% SDS and placed in a boiling water bath for 30 s, resulting in complete membrane solubilization. The gels (6 mm x 14 cm) were subjected to electrophoresis at 4 volts (7ma) per gel in 0.1% SDS, 0.1 M sodium phosphate, pH 7.2, for 15 hours at room temperature in a Buchler Polyanalyt disc electrophoresis apparatus. After electrophoresis, gels were fixed in 10% trichloroacetic acid, stained with 0.5% Coomassie brilliant blue in 50% methanol for 4 hours, and destained with 7% acetic acid.

Gels were calibrated with six polypeptides of known molecular weight: β-galactosidase, 115,000 (Worthington); phosphorylase a, 93,000 (Sigma); transferrin, 77,000 (Pentex); catalase, 60,000 (Sigma); ovalbumin, 43,000 (Sigma); and chymotrypsinogen A, 26,000 (Sigma). Gel scans were performed with a Gildford model 2140-S linear transport.

For glycoprotein staining of the acrylamide gels a modification of the fuchsin sulfitel staining procedure of Zacharius et al. (21) was utilized. Following the deionized water washes, additional destaining in 50% methanol further reduced the background and eliminated the nonspecific protein staining.

Lactoperoxidase Iodination—A modification of the lactoperoxidase method of Phillips and Morrison (22-24) was utilized to iodinate membrane proteins exposed to the extracellular space prior to subcellular fractionation. Twenty-five extensor digitorum longus muscles were carefully excised tendon-to-tendon and placed in ice sucrose buffer prior to iodination. The reaction was conducted on the intact muscle at room temperature in

1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Na⁺K⁺(Mg⁺⁺)ATPase specific activity was 0.10 to 0.30 mg per g wet weight. Four similar subfractions obtained from the density gradient centrifugation of Fraction II derived from postmitochondrial supernatant (Fig. 1B): a pellet (P) and high density subfraction (H), the middle density shoulder (M) at 24% sucrose; and a light density subfraction (L) at the 0/15% sucrose interface. Final yield of the middle density membrane subfraction with the highest Ca⁺⁺/Mg⁺⁺ATPase specific activity was 0.20 to 0.30 mg per g wet weight.

These membrane subfractions were examined 7 days after unilateral denervation and compared to their contralateral limb controls. During this time of denervation the muscle underwent a steady atrophy, losing 90% of its wet weight. Several changes in the continuous density gradient optical density profile of Fraction I were observed following denervation (Fig. 1A). There was a significant loss of both pellet and high density protein. The middle density subfraction also underwent a consistent protein loss and exhibited a slight shift to a lighter density. The light density subfraction alone failed to decrease significantly in its 280 nm absorbance following denervation. There was a corresponding redistribution of the Fraction II continuous density gradient optical density profile following denervation (Fig. 1D). Both the pellet and the superimposed high density protein were decreased and a new peak of protein became apparent in the high density region. The middle density subfraction shifted to lighter densities following denervation. Only the light density subfraction increased following denervation.

### Ionic stimulation of membrane Fraction I ATPase

<table>
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<tr>
<th>Stimulating ions</th>
<th>ATPase specific activity pmol Pi/hr/mg</th>
</tr>
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<tbody>
<tr>
<td>Ca⁺⁺</td>
<td>27.7 ± 1.7</td>
</tr>
<tr>
<td>Mg⁺⁺</td>
<td>21.5 ± 1.0</td>
</tr>
<tr>
<td>Mg⁺⁺, ouabain</td>
<td>22.0 ± 0.8</td>
</tr>
<tr>
<td>Na⁺, K⁺, Mg⁺⁺, ouabain</td>
<td>29.0 ± 0.3</td>
</tr>
<tr>
<td>Na⁺, K⁺, Mg⁺⁺</td>
<td>47.7 ± 2.0</td>
</tr>
<tr>
<td>Na⁺, K⁺(net)</td>
<td>26.0 ± 1.8</td>
</tr>
</tbody>
</table>

**Table I**

**Membrane Isolation**—Four subfractions were recovered from the final sucrose gradient centrifugation of Fraction I derived from the initial nuclear pellet (Fig. 1A): a pellet (P) and high density subfraction (H), a middle density subfraction (M) peaking in 280 nm absorbance at 24% sucrose, and a light density subfraction (L) at the 0/15% sucrose interface. The final yield of the middle density membrane subfraction with the highest Na⁺K⁺(Mg⁺⁺)ATPase specific activity was 0.10 to 0.30 mg per g wet weight. Four similar subfractions obtained from the density gradient centrifugation of Fraction II derived from postmitochondrial supernatant (Fig. 1B): a pellet (P) and high density subfraction (H), the middle density shoulder (M) at 24% sucrose; and a light density subfraction (L) at the 0/15% sucrose interface. Final yield of the middle density membrane subfraction with the highest Ca⁺⁺/Mg⁺⁺ATPase specific activity was 0.20 to 0.30 mg per g wet weight.

**RESULTS**

10 ml final volume of phosphate buffer 0.106 M, pH 7.4, containing KI, 10 μM; H₂O₂ (ICN carrier-free), 2.5 mCi; lactoperoxidase (Sigma), 1 μM using a millimolar extinction coefficient of 114 at 412 nm; and H₂O₂, 7.5 μM based on an extinction coefficient of 72.4 at 230 nm. The reaction was initiated by addition of the hydrogen peroxide and continued by regular addition of aliquots of H₂O₂. A control incubation was performed under identical conditions but deleting the lactoperoxidase. After 1 hour the muscles were washed on a Buchler funnel with 1 liter of cold phosphate buffer 0.106 M, pH 7.4; KI, 1.0 mM. The experimental and control muscles were pooled separately with unincubated gastrocnemius and anterior tibialis muscle, and the standard subcellular fractionation was performed as previously described.

The resulting subfractions as well as their SDS polyacrylamide gel electrophoretic polypeptides were counted directly for γ emission on a Packard γ spectrometer.

**Material and Methods**—Membrane isolation and enzyme assay were as described under "Materials and Methods." Values represent the mean ± standard error of three preparations expressed as net micromoles of P₁, hydrolysis per hour per mg of membrane protein stimulated by the aforementioned ions less the amount hydrolyzed in the absence of any ions. Net Na⁺K⁺ value represents the specific activity in presence of Na⁺, K⁺, and Mg⁺⁺ less the amount stimulated by Mg⁺⁺ alone.
lighter subfractions. Washing the crude Fraction II pellet with

different from the optical density profile. The middle density

subfraction accounted for between 70 and 80% of the total

Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase activity with a specific activity of 20

µmoles per hour per mg. This subfraction also contained most of

the Ca\textsuperscript{++}/Mg\textsuperscript{++}ATPase with a specific activity of 19 µmoles per

hour per mg. When the middle density subfraction was sub-
divided (Fig. 1A) into three subfrations, (M\textsubscript{L} 18 to 22%, M\textsubscript{M} 22 to 26%, and M\textsubscript{H} 26 to 30% sucrose), the distribution of the

Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase activity approximated the optical density

profile with peak specific activity of this enzyme appearing in the

lighter density subfractions M\textsubscript{M} and M\textsubscript{L} (Fig. 2). The Mg\textsuperscript{++}/

Ca\textsuperscript{++}-stimulated activity was skewed toward the higher density

portions of the fraction with peak specific activity in subfraction

M\textsubscript{H}.

After 1 week of denervation there was a moderate decrease in
total and specific activity of the Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase and

Ca\textsuperscript{++}/Mg\textsuperscript{++}ATPase of Fraction I (Fig. 2, Table II). There was an
apparent shift in distribution of the Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase specific activity to a lighter density position, but no change in the
distribution of the Ca\textsuperscript{++}/Mg\textsuperscript{++}ATPase specific activity.

Fraction II isolated from the postmitochondrial supernatant
was found to contain varying amounts of ATPases depending
upon the stage of isolation. Before KCl extraction this fraction
contained only a Ca\textsuperscript{++}/Mg\textsuperscript{++}-activated ATPase. LiBr extraction
revealed a latent Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase activity in the lighter
subfractions. Washing the crude Fraction II pellet with
0.6 M KCl further increased this Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase specific activity in the final preparation.

The continuous density gradient distribution of Fraction II
ATPase specific activities contrasted with that seen for Fraction
I. The middle density subfraction contained the highest activity
of both ATPases with specific activities of 22 µmoles per hour per
mg for the Ca\textsuperscript{++}/Mg\textsuperscript{++} and 5 µmoles per hour per mg for the
Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase. Subfractionation of this middle density
fraction revealed a lighter density distribution for the Na\textsuperscript{+}K\textsuperscript{+}-

(Mg\textsuperscript{++})ATPase with peak specific activity occurring in the M\textsubscript{L}
subfraction. The Ca\textsuperscript{++}/Mg\textsuperscript{++}ATPase activity had a broad
peak of specific activity around subfraction M\textsubscript{M} (Fig. 3).

Following 1 week of denervation, Fraction II ATPase demonstrated a
greater change in ATPase activity than noted with Fraction I
(Fig. 3, Table III). Instead of the slight loss of ATPase seen in
Fraction I, the middle density subfraction of Fraction II under-

![Fig. 2 (left). Distribution of ATPase specific activities of membrane Fraction I on continuous density sucrose gradients. Fractionation and ATPase assay were as described under "Materials and Methods." ———, Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase; ——, Ca\textsuperscript{++}/ Mg\textsuperscript{++}ATPase. □, Fraction I from control muscles; ■, Fraction I from muscles denervated for 1 week. Values represent the mean of three preparations.](http://www.jbc.org/content/252/3/5159/fd/supplement)

![Fig. 3 (right). Distribution of ATPase specific activities of membrane Fraction II on continuous density sucrose gradients. Fractionation and ATPase assay were as described under "Materials and Methods." ———, Na\textsuperscript{+}K\textsuperscript{+}(Mg\textsuperscript{++})ATPase; ——, Ca\textsuperscript{++}/ Mg\textsuperscript{++}ATPase. ◻, Fraction II from control muscles; ■, Fraction II from muscles denervated for 1 week. Values represent the mean of three preparations.](http://www.jbc.org/content/252/3/5159/fd/supplement)

![Fig. 4 (left). Distribution of sialic acid concentrations of membrane Fraction I on continuous density sucrose gradients. Fractionation and sialic acid assay were as described under "Materials and Methods." ◻, Fraction I from control muscles; ■, Fraction I from muscles denervated for 1 week. Values represent the mean of three preparations.](http://www.jbc.org/content/252/3/5159/fd/supplement)

![Fig. 5 (right). Distribution of sialic acid concentrations of membrane Fraction II on continuous density sucrose gradients. Fractionation and sialic acid assay were as described under "Materials and Methods." ◻, Fraction II from control muscles; ■, Fraction II from muscles denervated for 1 week. Values represent the mean of three preparations.](http://www.jbc.org/content/252/3/5159/fd/supplement)
went a 2-fold increase in both total and specific activity of Ca\(^{2+}/\)Mg\(^{2+}\)ATPase (51 nmoles per hour per mg). The minimal Na\(^{+}K^{+}(\text{Mg}^{2+})\) ATPase activity present in Fraction I appeared to decline to even lower levels.

**Sialic Acid Distribution**—Sialic acid concentrations for the membrane fractions were 25- to 50-fold greater than previously demonstrated with whole muscle (0.7 n mole per mg). The continuous density gradient distribution of the sialic acid concentration of Fraction I (Fig. 4) paralleled the Na\(^{+}K^{+}(\text{Mg}^{2+})\)-stimulated ATPase specific activity (Fig. 2). The middle density fraction contained the highest concentration of sialic acid (24 nmoles per mg). On further subfractionation, the sialic acid concentration was found to peak in the MM subfraction.

Denervation for 1 week did not significantly alter the concentration distribution of Fraction I sialic acid (Fig. 4). However, it diminished the yield of this fraction’s sialic acid and produced a shift in the total content to a lighter density fraction, thereby paralleling the previously described membrane protein (Fig. 1A) and Na\(^{+}K^{+}(\text{Mg}^{2+})\)ATPase (Fig. 2) alterations.

Fraction II contained approximately half the concentration of sialic acid found in Fraction I (Tables II and III). The middle density subfraction of Fraction II with the highest specific activity of Ca\(^{2+}/\)Mg\(^{2+}\)ATPase also had the highest concentration of sialic acid (10 nmoles per mg). However, further subfractionation of this middle density subfraction revealed a sharp peak of sialic acid concentration with the Na\(^{+}K^{+}(\text{Mg}^{2+})\)ATPase in the MM subfraction (Fig. 5) rather than the broad peak around the MM subfraction seen with the Ca\(^{2+}/\)Mg\(^{2+}\)ATPase (Fig. 3).

Denervation for 1 week produced a significant increase in the sialic acid concentration of Fraction II with a 2-fold increase noted in the ML subfraction (Fig. 5, Table III). These changes were similar to the changes in the Ca\(^{2+}/\)Mg\(^{2+}\)ATPase activity in Fraction II following denervation and differed from the lack of quantitative change of Na\(^{+}K^{+}(\text{Mg}^{2+})\) ATPase of Fraction II or of Na\(^{+}K^{+}(\text{Mg}^{2+})\)ATPase and sialic acid of Fraction I after denervation for 1 week.

**Distribution of Membranes Iodinated by Extracellular Lactoperoxidase**—Substantial levels of membrane protein iodination were achieved following exposure of intact muscle to extracellular lactoperoxidase. Radioactivity isolated in the subfractions following control incubations without the enzyme was less than 25% of the level of incorporation with lactoperoxidase. In Fraction I the distribution of specific activity of incorporated \(^{125}\)I following continuous density gradient centrifugation paralleled that seen for both Na\(^{+}K^{+}(\text{Mg}^{2+})\) ATPase specific activity and sialic acid concentration, peaking in subfractions MM and ML (Fig. 6). The same subfraction distribution of incorporated \(^{125}\)I specific activity was observed following SDS polyacrylamide gel electrophoresis of the membranes with a more generalized incorporation into membrane proteins than previously reported for erythrocytes (23, 24).

Fraction II was also iodinated by extracellular lactoperoxidase with a distribution distinct from that of the Ca\(^{2+}/\)Mg\(^{2+}\)ATPase but once again similar to the distribution Na\(^{+}K^{+}(\text{Mg}^{2+})\)ATPase and sialic acid of the lighter density membranes, peaking in the lighter density subfractions MM and ML (Fig. 7).

**Electron Microscopy**—Electron micrographs of the muscle membrane middle density subfractions revealed rather homogeneous populations of membranes vesicles for both major membrane fractions (Fig. 8, A and B). Denervation for 1 week produced no significant change in electron microscopic appearances of either Fraction I or Fraction II.

**Gel Electrophoresis**—7.5% polyacrylamide gel electrophoresis in 0.1% SDS under reducing conditions yielded characteristic and distinct protein profiles for membrane Fractions I and II (Fig. 9). The most prominent proteins in fractions MM and ML were those with molecular weights of 93,000 and 73,000. A total of 20 polypeptides was present ranging in molecular weight from

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**TABLE III**

<table>
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<tr>
<th>Membrane Fraction II denervation alterations</th>
<th>Na(^{+}K^{+}(\text{Mg}^{2+}))ATPase</th>
<th>Ca(^{2+}/)Mg(^{2+})ATPase</th>
<th>Sialic acid</th>
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<td>Muscle</td>
<td>Specific activity</td>
<td>Total activity</td>
<td>Specific activity</td>
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<td>-------------------</td>
<td>----------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Percent activity</td>
<td>nmoles hr/mg</td>
<td>nmoles hr/mg</td>
<td>nmoles hr/mg</td>
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<td>3.2</td>
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<td>Denervated...</td>
<td>1.5</td>
<td>7.1</td>
<td>51.3</td>
</tr>
</tbody>
</table>

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**Fig. 6 (left).** Distribution of specific activity of iodination by lactoperoxidase of membrane Fraction I on continuous density sucrose gradients. Iodination and fractionation were as described under "Materials and Methods." ---, control without lactoperoxidase.

**Fig. 7 (right).** Distribution of specific activity of iodination by lactoperoxidase of membrane Fraction II on continuous density sucrose gradients. Iodination and fractionation were as described under "Materials and Methods." ---, Fraction II isolated from intact muscle labeled with extracellular lactoperoxidase; ---, control without lactoperoxidase.
A polypeptide in subfraction IM$_L$ migrating with an apparent molecular weight of 109,000 was the only moiety identified as a glycoprotein on the basis of its specific fuchsin sulfite staining characteristics.

A total of 14 polypeptides was present in Fraction II ranging in molecular weight from 30,000 to 140,000. The profiles of IIM$_L$ and IIM$_H$ were readily distinguishable from the profiles of IML and IMH by the presence in the subfractions of II of a predominant polypeptide of molecular weight 93,000. Membrane subfraction IIM$_L$ also contained a single glycoprotein of apparent molecular weight of 113,000.

Although the major differences of polypeptide profiles were between Fractions I and II there were definite differences between the subfractions of each component. In IM$_H$ the 93,000 component represented a larger proportion of the total membrane protein than noted in IIM$_L$. There were also minor components of molecular weights 110,000 to 140,000 present in IIM$_L$ which did not appear in IM$_H$. Of considerable interest was the fact that polypeptides of 110,000 to 140,000 molecular weight were also quite prominent in Fraction I. The major reproducible differences between IML and IMH were the prominence of the 109,000 dalton component and the appearance of a double peak at 93,000 in the IM$_L$ subfraction.

Denervation for 1 week produced no significant change of protein profile of either muscle membrane Fraction I or Fraction II.

**DISCUSSION**

Muscle has been chosen as a model system for the biochemical analysis of the excitable membrane because of the tissue's cellular homogeneity, accessibility and the availability of various membrane isolation techniques. The major obstacles of muscle mem-
brane isolation have been the difficulty of solubilization and extraction of the tissue's high content of fibrillar contractile proteins without membrane disruption, the heterogeneous array of morphologically defined membranes present in muscle, and the difficulty in defining the origin of the resulting membranes.

As in numerous studies (1–3, 25–29) we initially used the membrane-bound ATPase as a means of identifying the cellular origin of the particular membrane fraction. Sodium and potassium-stimulated, magnesium-dependent, and ouabain-sensitive ATPase has been correlated with the ATP-dependent, ouabain-sensitive K⁺ influx and Na⁺ influx and consequently utilized as an enzymatic marker for muscle plasma membrane or sarcoplasmic reticulum (5–50). Ca++/Mg++-dependent ATPase has been correlated with the active transport of Ca++ into sarcoplasmic reticulum cisternae and both phenomena have served as biochemical markers for these cytoplasmic membranes (6–10). Other criteria of purity have been applied to further characterization of muscle membranes, including lipid composition (34, 35) and SDS polyacrylamide gel electrophoresis (36), but all are performed on membranes initially identified on the basis of their ATPase specific activities. The muscle membrane ATPase specific activities reported here (25 to 50 μmoles of P₁ per mg per hour) compare favorably to those achieved by other methods of muscle subcellular fractionation (5 to 10 μmoles of P₁ per mg per hour) (2, 4).

Protein and lipid-bound sialic acid represents another potential marker for surface membranes of muscle. Carbohydrate-containing macromolecules are significant components of biologic membranes as demonstrated by histochemical, immunologic, and electrophoretic studies on intact cells (37–39), and quantitative carbohydrate analyses of their isolated cell membranes (40, 41). Sialic acid is frequently the terminal sugar of membrane macromolecules and has been shown to be preferentially localized in the surface membranes of various cells (42–44). The levels of muscle membrane-bound sialic acid were reported here (19 to 25 μmoles per mg) are of the same order described for liver cell membrane (27 nmoles per mg) (43). Sialic acid is frequently the terminal sugar of membrane macromolecules and has been shown to be preferentially localized in the surface membranes of various cells (42–44). The levels of muscle membrane-bound sialic acid were reported here (19 to 25 μmoles per mg) are of the same order described for liver cell membrane (27 nmoles per mg) (43).

The use of lactoperoxidase for the catalysis of the specific iodination of surface membrane proteins of intact tissues has been well described elsewhere (22–24) and provides an additional method for the differentiation of subsequent subfractionation muscle surface membrane and sarcoplasmic reticulum. The method is dependent on the inability of the high molecular weight enzyme (78,000) to diffuse through the plasma membrane, with the iodination consequently restricted to extracellularly exposed membranes or, in the case of muscle, specifically to sarcolemma and transverse tubule membrane proteins.

With our present muscle membrane fractionation procedure, both Fractions I and II were found to be heterogeneous with respect to the enzymic markers currently accepted as originating in either sarcotubules or sarcoplasmic reticulum. Ca++/Mg++-ATPase as well as Na⁺K⁺(Mg++)ATPase, sialic acid, and lactoperoxidase iodination were found to be present in both Fractions I and II. Only subfractionation by continuous density gradient centrifugation permitted a separation of the membranes in contact with the extracellular space as defined by these markers from those derived from intracellular structures.

Since techniques for cell disruption and subsequent centrifugation do not permit a discrete separation of these enzyme activities, we must rely on increasing the specific activity and thus enriching for a particular fraction rather than completely purifying it. The total Fraction I derived from the original nuclear pellet can thus not be designated sarcolemma nor can the total Fraction II derived from the postmitochondrial supernatant be designated sarcoplasmic reticulum. The M₄ subfractions from both Fractions I and II were found to possess the peak specific activities of the surface membrane marker Na⁺K⁺(Mg++)ATPase and sialic acid although the ratio of these markers was different in I₄M and II₄M. Furthermore, these lighter membrane subfractions of both Fractions I and II were most highly labeled by extracellular lactoperoxidase iodination of intact muscle. These data would confirm that the lighter subfractions of both Fractions I and II are enriched in surface membranes and would suggest that membranes of the postmitochondrial supernatant, ordinarily identified as crude sarcoplasmic reticulum, also possessed membranes in contact with the extracellular space, such as sarcolemma, transverse tubular systems, or the specialized muscle membrane of the neuromuscular junction. These results are not unexpected considering our vigorous initial homogenization. Furthermore, knowledge that surface membranes may be distributed in the postmitochondrial supernatant as well as in the nuclear pellet may help explain some of the confusion in muscle membrane enzyme localization such as adenylate cyclase (45–48).

Despite the similarities of specific activity of iodination of I₄M and IIM₄ and their sialic acid content, the SDS gel electrophoretic polypeptide profiles of the two fractions were sufficiently different to indicate that they had derived from different parts of the continuous membranes in contact with the extracellular space. Furthermore, in response to denervation the sialic acid of the IIM₄ subfraction increased 100% while no change was observed in 1₄M sialic acid. The precise morphologic correlate of the membrane component in contact with the extracellular space whose sialic acid content increased with denervation is presently unknown. However, it is of interest that following denervation the sialic acid concentration of 1₄M reached levels normally present in IIM₄.

The significance of the enhanced sialic acid concentration is also unknown. Our previous report described an increased sialic acid in whole muscle homogenates following denervation, but the appropriate interpretation of the data depended upon an understanding of the fraction in which sialic acid was increasing (11).

Our present finding that the sialic acid increased in membranes with Na⁺K⁺(Mg++)ATPase activity and in contact with the extracellular space makes it unlikely that the primary effect of denervation is on the sarcoplasmic reticulum. The enhanced sialic acid may relate to the known increase in acetylcholine receptors, the ability to receive new neural connections, or the altered surface membrane properties associated with denervation. No available data can determine which of these possibilities, if any, would best explain the enhancement.

The IIM₄ and IIM₄ subfractions derived from the postmitochondrial supernatant appear to correspond to sarcoplasmic reticulum. The membranes were found to be enriched in Ca⁺⁺/Mg⁺⁺ATPase which increased 100% following denervation as had been previously reported (40, 50). Furthermore, our SDS polyacrylamide gel electrophoretic profile of the membrane polypeptides of IIM₄ was similar to that reported from other laboratories for sarcoplasmic reticulum (36). The major polypeptide comprised over 90% of the total protein with a molecular weight of 93,000 and appears to represent the polypeptide identified by guest on July 10, 2017 http://www.jbc.org/ Downloaded from
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