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# THE PREPARATION OF A SARCOLEMMAL FRACTION FROM EVACUATED MUSCLE SLICES

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## Summary

A novel procedure is described for preparing a plasma membrane fraction from skeletal muscle (i.e., sarcolemma). The procedure entails evacuating the myoplasm from muscle slices as a preliminary step to homogenization and fractionation. The evacuated muscle slices are composed of a stroma-containing sarcolemma, which is then homogenized and fractionated, utilizing a sequence of differential and discontinuous sucrose density gradient centrifugations. On the basis of electron microscopy, selective enzyme markers and α-bungarotoxin binding in innervated and denervated muscles, the fraction most enriched with sarcolemma is recovered from the 0.5/0.7 M interface of a discontinuous sucrose gradient.

## Introduction

An essential prerequisite for biochemical studies of the muscle plasmalemma (i.e., sarcolemma) is a plasma membrane fraction of high purity. A major problem in obtaining such a membrane fraction is contamination by cytoplasmic constituents of the muscle fiber, mainly membranes derived from cytoplasmic organelles such as mitochondria and sarcoplasmic reticulum, in addition to structural components derived from myofibrils (e.g., actomyosin). Fractionation techniques presently in general use for the preparation of sarco-

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Abbreviation: Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

lemma fractions are not effective in eliminating cytomembrane contaminents. If cytomembranes could be removed before homogenization, then a significant enrichment of sarcolemma could be achieved.

A procedure is described for evacuating muscle slices which is improved over that developed originally for myofiber bundles by Beringer and Koenig [1], the latter of which was based on the method of McCollester [2]. The evacuated muscle slice technique virtually eliminates all of the cytoplasmic contents of myofibers as a first step for the preparation of sarcolemma. When the stroma of the evacuated muscle slice homogenized and subjected to a sequence of differential and discontinuous sucrose gradient centrifugations, muscle plasma membrane fractions of high purity are obtained as indicated by biochemical and electron microscopic analysis.

## Methods

*Experimental preparations*

In all experiments, the extensor digitorum longus muscles of male Sprague Dawley rats (350—500 g) were used. Individual muscles weighed 100—300 mg (wet weight).

In some experiments the muscles were denervated bilaterally. Rats, anesthetized with 35 mg/kg, sodium pentabarbital, were denervated by removing 1 cm of the common peroneal nerve, underlying the biceps femoris muscle. After denervation, the separated biceps femoris was resutured with 3-0 surgical silk, and the wound was closed with surgical staples. After denervation, animals were allowed to survive for varying time intervals.

Innervated muscles were dissected bilaterally from normal, unoperated rats, and, in one case, from a bilaterally sham-operated rat. In the latter case, the rat underwent the same surgical procedure as denervated rats except that the common peroneal nerve was not disturbed.

*Preparation of evacuated muscle slices*

The intact muscles were removed bilaterally from two animals under anesthesia by cutting the tendons near their origin and insertion. The excised muscles were submerged in a dish of cold double-distilled water on ice for about 15 min. Each muscle was divided transversely through the belly of the muscle using small surgical scissors; each half muscle was placed on a Teflon disk and cut into 1—2-mm thick slices with a McIlwain tissue chopper. The muscle slices were removed from the disk and suspended in 50 ml of ice-cold double-distilled water.

The flask containing muscle slices was placed in ice for 0.5 h, after which the fluid was decanted and the slices were rinsed in approx. 25 ml of ice-cold buffered $CaCl_2$ (10 mM $CaCl_2$ buffered with 2 mM imidazole, titrated to pH 7.2—7.5 with 1 M HCl). The $CaCl_2$ was decanted and the muscle slices were transferred to a flask containing 100 ml of fresh cold buffered $CaCl_2$, the latter of which was refrigerated for 2 h. After 2 h in $CaCl_2$, the fluid was decanted and the muscle slices were washed with 50 ml of cold buffered KCl (30 mM KCl buffered with 2 mM Tris-HCl, 1 mM Tricine (pH 8.2)). The muscle slices were transferred into 200 ml of cold buffered KCl and

refrigerated overnight at 4°C. The KCl was decanted and the muscle slices were suspended in 1 l of 0.09% triethanolamine (pH 8.7—9.0; 1 g muscle/l triethanolamine) at room temperature. The muscle slices remained at room temperature for 0.5 h, at which time the fluid was removed and the slices were transferred into a flask containing 1 l of fresh triethanolamine. This flask was placed in the cold (4°C) for an additional 1.5 h and the muscle slices were vigorously swirled in the flask and poured into a beaker on ice.

The treatment described above results in the spontaneous extrusion of the cytoplasmic contents out of the cut ends of the myofibers, and generally culminated in near complete evacuation, based on microscopic inspection. The evacuated preparation is referred to as evacuated muscle slices. The evacuated muscle slices were plucked individually from the beaker and screened for completeness of evacuation under a dissection microscope.

Myofiber segments containing residual cytoplasmic material, termed 'plugs', were in most cases dissected away with a fine forceps and surgical scissors. Large pieces of connective tissue and tendon were also excised. In some cases after evacuation, muscle slices containing a significant number of plugs were discarded. After screening, the evacuated slices were blotted on bibulous paper and placed in a glass homogenized in ice.

*Isolation of muscle membrane fractions from evacuated muscle slices*

Evacuated muscle slices were homogenized in a Duall No. 22 glass-glass conical tissue grinder (Kontes Glass), containing 3 ml of ice-cold 0.32 M sucrose, using a Tri-R Model K43 homogenizer at a setting of 2 for approx. 2 min (70 pestle strokes). After rinsing the tissue grinder with 0.32 M sucrose, a total of 8 ml of homogenate was centrifuged in the cold at $1020 \times g$ (2500 rev./min) for 2.5 min (Sorvall S-22 swinging bucket rotor). The supernatant, $S_1$, was retained while the pellet, $P_1$, was discarded or saved for analysis. $S_1$ was divided between two 5-ml cellulose nitrate tubes, placed in a SW50.1 rotor (Beckman) and centrifuged at 45 000 rev./min ($189\,000 \times g_{av}$) at 4°C for 15 min. This step yielded two $P_2$ pellets which were retained. These pellets were homogenized in 0.8 ml of 0.32 M sucrose with 30 pestle strokes of a Duall No. 21 glass-glass tissue grinder. The pestle was rinsed and the $P_2$ homogenate (1 ml) was layered onto a three-step discontinuous sucrose gradient, composed of 1.7 ml of 0.5 M, 1.7 ml of 0.7 M, and 0.8 ml of 0.85 M sucrose. The gradient was centrifuged at $189\,000 \times g_{av}$ for 1 h at 4°C, yielding three interface fractions denoted L, M, H, and a pellet fraction $P_3$. Each interface fraction was recovered by aspiration with a syringe and needle, diluted and mixed with double-distilled water and placed in ice.

The $P_3$ pellet was resuspended in 0.5 ml of 0.32 M sucrose and transferred to a conical Duall No. 21 tissue grinder. Another 0.5 ml of 0.32 M sucrose was used to recover residual $P_3$ material. $P_3$ was homogenized using the same parameters as for $P_2$ (see above).

The $P_3$ homogenate was applied to a 2 step discontinuous sucrose gradient, consisting of 1 ml of 1.5 M and 2 ml of 1.0 M sucrose. An additional 0.5 ml of 0.32 M sucrose used to rinse the tissue grinder tube was also added to the gradient. This gradient was centrifuged in the same manner as the three-step gradient described above. After ultracentrifugation of the gradient, two more

interface fractions, J. and S, and another pellet, $P_4$, were obtained. The five interface fractions were sedimented in the ultracentrifuge (189 000 $\times g_{av}$ 15 min, 4°C). The pellets were generally stored in ice overnight or frozen.

*Enzyme assays*

5'-Nucleotidase (EC 3.1.3.5) activity was measured in a medium slightly modified from that used by Emmelot and Bos [3], i.e., 75 $\mu$l sample in 2 ml of 100 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.4), 5 mM sodium adenosine monophosphate (NaAMP) and 10 mM $K^+/Na^+$ tartrate. After incubation for 1 h (37°C), 1 ml of 25% trichloroacetic acid was added to stop the reaction. Inorganic phosphate released during incubation was determined according to Lowry and Lopez [4]. Protein was measured by the method of Lowry et al. [5], using bovine serum albumin as a standard. All samples were assayed in duplicate.

Acetylcholinesterase was assayed using acetylthiocholine as substrate [6]. The total incubation volume of 1 ml included 50 mM phosphate buffer, (pH 7.0) $1 \cdot 10^{-4}$ M dithiobisnitrobenzoate (DTNB, Aldrich Chem.), $1.0 \cdot 10^{-3}$ M acetylthiocholine iodide (Eastman), and 0.1 ml of sample. The samples were shaken for 1 h in a water bath (37°C). The reaction was stopped by adding 0.1 ml $1.0 \cdot 10^{-3}$ M neostigmine bromide (Sigma). The relative distribution of acetylcholinesterase activity in the various fractions was expressed as the $\Delta A$ of each sample divided by the total $\Delta A$ of all the samples, multiplied by 100. Each fraction was assayed in duplicate.

*Assay of acetylcholine receptors*

Acetylcholine receptors were assayed by binding with $^{125}$I-labelled $\alpha$-bungarotoxin, according to the procedure of Kemp, et al. [7]. Membrane fractions (see above) were prepared from either four innervated or four 14-day denervated extensor digitorum longus muscles. Membrane fractions assayed for $^{125}$I-labelled $\alpha$-bungarotoxin binding were not frozen, but were stored on ice in a coldroom overnight.

Acetylcholine receptors were solubilized in each fraction by the addition of 200 $\mu$l 1% Triton-X 100 (Sigma) in 10 mM $NaH_2PO_4$ (pH 7.2). For the $P_4$ pellet, 1 ml of the detergent solution was used for extraction. The tubes were vortexed briefly, and left at room temperature for 15—20 min. All samples were then centrifuged at 8000 $\times g$ for 10 min in the cold with an Eppendorf table-top microcentrifuge. Two 75-$\mu$l aliquots were taken from the supernatant of each sample and placed into separate tubes (two 200-$\mu$l aliquots were taken for $P_4$). All tubes were brought to a final volume of 200 $\mu$l with 1% of the Triton-X sodium phosphate solution. To one of the duplicate tubes of each sample unlabelled $\alpha$-bungarotoxin was added. 5 min later $^{125}$I-labelled $\alpha$-bungarotoxin was introduced to give a final concentration of approx. $1.0 \cdot 10^{-9}$ M. In these samples the unlabelled toxin concentration was 1000-fold greater than that of the $^{125}$I-labelled $\alpha$-bungarotoxin. The other sample of each duplicate contained only 1 nM of $^{125}$I-labelled $\alpha$-bungarotoxin. All samples were incubated with the toxin at room temperature for 1.5 h.

Acetylcholine receptor/$^{125}$I-labelled $\alpha$-bungarotoxin complex was separated from free $^{125}$I-labelled $\alpha$-bungarotoxin by chromatography on Sephadex CM-50

columns (for details, see Ref. 7). Effluents were collected and counted in a Beckman gamma counter. Correction for nonspecific binding was made by subtracting the radioactivity (cpm) of the sample containing both labelled and unlabelled toxin from that containing the labelled toxin alone. The rationale for this is based on the assumption that the unlabelled toxin will rapidly saturate specific binding sites, so that binding of labelled toxin, added a short time later in a much lower concentration (1 : 1000), will be to nonspecific sites.

*Light and electron microscopy*

Electron micrographs were made of the membrane fractions and muscle slices. Membrane fractions were prepared for electron microscopy as follows. Samples, removed from sucrose gradients, were diluted with ice cold solution of 50 mM sodium cacodylate, pH 7.4 (Fisher), 4% gluteraldehyde fixative (Tousimis Res.), to which a few mg of $CaCl_2$ had been added (final concentrations, 33 mM sodium cacodylate and 2.7% gluteraldehyde). The fractions were sedimented in the fixative and were fixed for a minimum of 1—2 days in vials containing 50 mM sodium cacodylate and 4% gluteraldehyde with $CaCl_2$ (pH 7.4) at 4°C. After fixation, all samples were stained en bloc with uranyl acetate and lead citrate (Fisher) and then embedded in epon (Polysciences, Inc.).

Thin and thick sections were cut on a MT2B ultra microtome (Sorvall). Thick sections were stained on the slide with methylene- or toluidine blue while thin sections were restained on the grid with lead citrate and uranyl acetate. Thick sections were photomicrographed in a Zeiss phase-contrast microscope and thin sections were photographed with a JEM electron microscope.

## Results

*Structural evaluation of muscle slices*

Within 15 min following suspension in 0.09% triethanolamine at room temperature, considerable myoplasmic extrusion can be seen from the cut ends of the muscle slices. Continued exposure to triethanolamine solution at room temperature and at 4°C results in further extrusion of cytoplasmic material from cut surfaces. Evacuation of myoplasm goes to completion approximately 2 h after suspension in 0.09% triethanolamine. At this time the extruded cytoplasmic material remains associated at the ends of the slices, however, agitation separates the myofibrillar mass and becomes dispersed in aggregate form.

When viewed in a dissecting microscope, the evacuated muscle slices have a honeycomb appearance (Fig. 1), reflecting cross-sectional profiles of evacuated myofibers. Staining the evacuated muscle slices for acetylcholinesterase revealed circumscribed areas containing reaction product which appeared to be associated with nerve and axon branches (Fig. 2), indicating that they contained endplate regions. In addition, the binding of $^{125}$I-labelled $\alpha$-bungarotoxin to either whole muscle homogenates (non-evacuated muscle) or to homogenized evacuated muscle slices was equivalent (data not shown). These

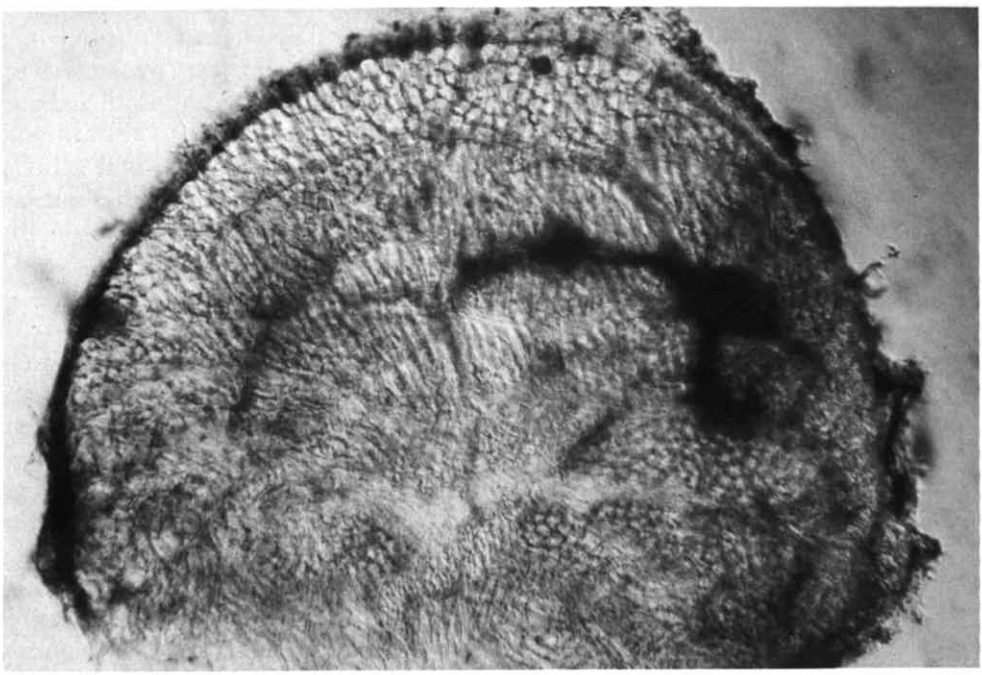

Fig. 1. A low power micrograph of an evacuated muscle slice, taken through a dissecting microscope. The honeycomb appearance (best orientation in the upper left hand field) is due to the vacant spaces left by evacuated myofibers in the endomysium. A single myofiber 'plug' can be seen at the top. Intramuscular nerve fasicles and blood vessels can be seen at the right of the micrograph (original magnification, X35).

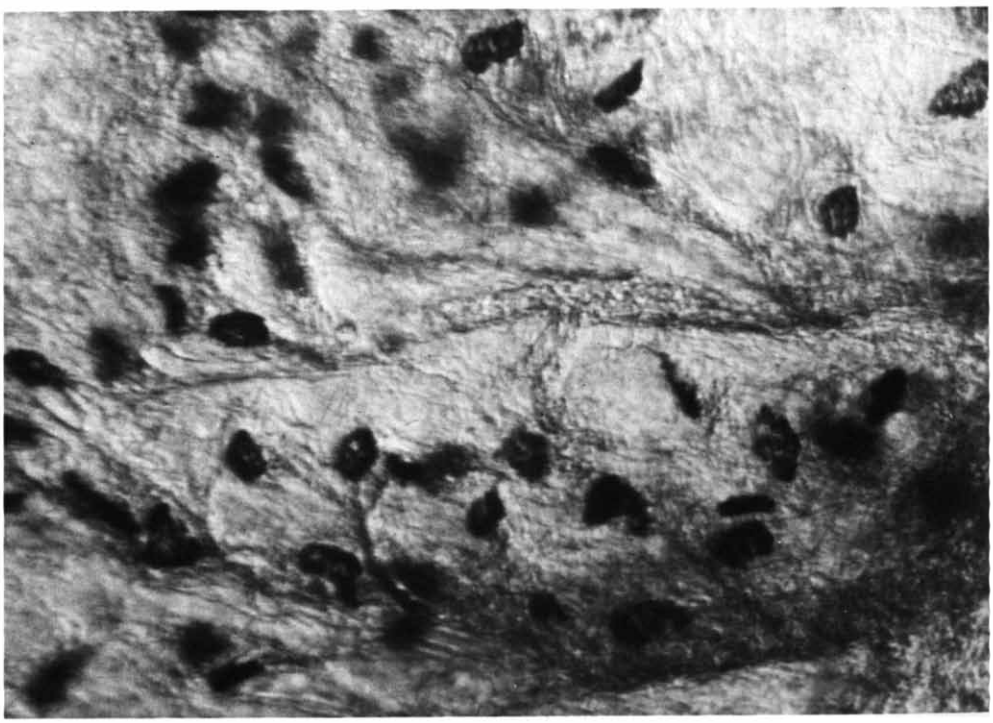

Fig. 2. An evacuated muscle slice stained for acetylcholinesterase, showing endplates. Axons terminating in some of the endplates can be discerned (original magnification, X150).

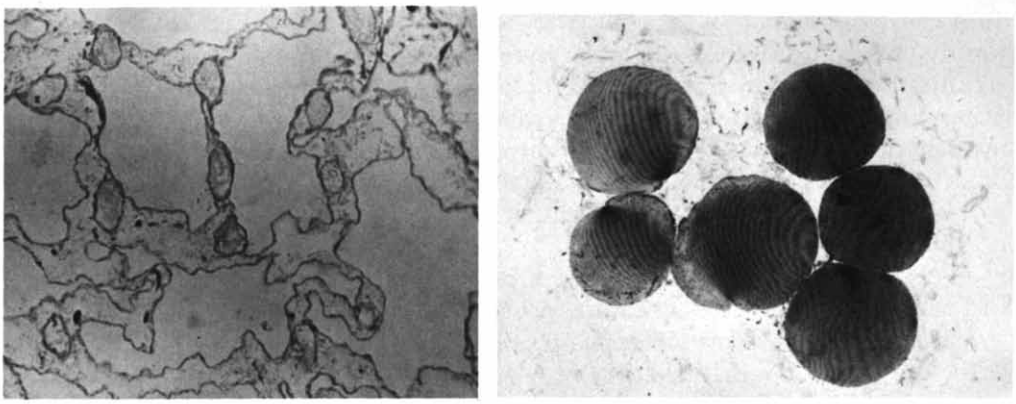

Fig. 3. High power light micrographs of (A) an evacuated muscle slice showing complete evacuation, and (B) another evacuated muscle slice showing a region containing myofiber plugs (i.e., unevacuated). Oblong profiles in A are capillaries surrounding evacuated myofibers (original magnifications, A ×400, B ×256).

results suggest that the junctional complex, including the postsynaptic membrane, is not lost during the evacuation process.

A higher magnification light micrograph provides greater detail of the evacuated muscle slice stroma (Fig. 3a). The adequacy of the evacuation proce-

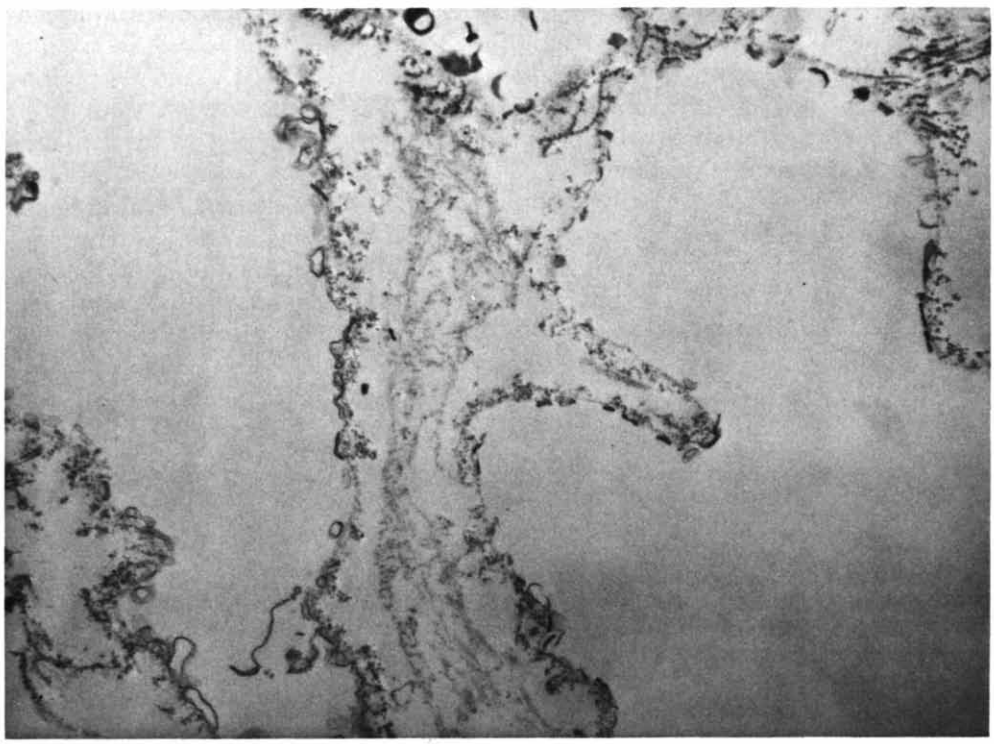

Fig. 4. An electron micrograph of an evacuated muscle slice showing two adjacent evacuated myofibers. The sarcolemma is disrupted and partially attached to the basal lamina. Connective tissue of the endomysium separates the two myofibers (magnification, ×18 100).

dure can be appreciated by comparing Fig. 3a with that of Fig. 3b, a 'plugged' myofiber region (i.e., unevacuated segment) of an evacuated muscle slice. The six intensely stained areas are myoplasmic plugs, which would represent a potential source of contaminating cytomembranes (e.g. sarcoplasmic reticulum, mitochondria) during the preparation of membrane fractions unless excised. Such plugged segments, however, occur with only occasional frequency and would represent considerably less than 1% of the myofiber content of a well evacuated muscle.

Fig. 4 is an electron micrograph of an evacuated muscle slice stroma, showing incomplete cross-sectional profiles of two evacuated myofibers. They reveal that while the myofiber plasma membrane generally remains associated with the basal lamina, the membrane undergoes disruption and tends to separate from the basal lamina during the evacuation process. These observations are similar to those made earlier on evacuated myofiber bundles [1]. The lumina of evacuated myofibers, on the other hand, are largely devoid of structural elements, but some contain variable amounts of residual myofibrillar material.

*Characterization of membrane fractions prepared from evacuated muscle slices*

*Distribution of selective plasma membrane markers.* The specific enzymic activities in each of the fractions obtained after discontinuous sucrose density gradient centrifugation are shown in Table I. 5′-Nucleotidase, a nonspecific plasma membrane marker, was used to identify fractions enriched with plasmalemma. The M and H fractions exhibit the highest 5′-nucleotidase specific activities recovered from the sucrose density gradient. The L, J and S fractions show little, if any, specific enzymatic activity. The 5′-nucleotidase activity in $P_4$ is probably a consequence of plasma membranes entrapped in the collagen and basal lamina of this fraction.

Acetylcholinesterase activity was used to profile the distribution of the end-plate regions of the evacuated muscle slices in the fractions, as well as of axonal membranes of intramuscular nerve fasicles. Almost all of the acetylcholinesterase activity was found in the $P_4$ pellet (Table I). The relatively low proportion of acetylcholinesterase activity in the M and H fraction may represent a small contribution by axonal membranes.

TABLE I

DISTRIBUTION OF KEY ENZYMIC MARKERS IN FRACTIONS PREPARED FROM EVACUATED MUSCLE SLICES

Specific activity is expressed as $P_i$/mg protein per h ± S.E. for three experiments. % total *A* = ($\Delta A$ individual fraction/$\Delta A$ total of all fractions) × 100 ± S.E. for three experiments.

| Fraction | 5′-Nucleotidase Spec. Act. | Actylcholinesterase % total *A* of fractions |
|---|---|---|
| L | 9.06 ± 9.06 | 0.60 ± 0.31 |
| M | 88.62 ± 11.14 | 3.33 ± 1.67 |
| H | 80.23 ± 21.22 | 2.67 ± 1.20 |
| J | 0 | 0 |
| S | 3.77 ± 3.77 | 0.47 ± 0.47 |
| $P_4$ | 7.28 ± 1.52 | 93.00 ± 2.65 |

TABLE II

RELATIVE DISTRIBUTION * OF $^{125}$I-LABELLED α-BUNGAROTOXIN BINDING TO STROMAL MEMBRANE FRACTIONS FROM EVACUATED INNERVATED AND DENERVATED MUSCLES

| Membrane source | Membrane fractions | | | | |
|---|---|---|---|---|---|
| | L | M | H | J | S |
| Innervated Muscle | 15.0 ± 7.3 | 3.0 ± 3.0 | 11.8 ± 4.9 | 10.4 ± 4.3 | 63.6 ± 12.3 |
| Denervated Muscle | 7.5 ± 2.0 | 31.3 ± 5.0 | 27.3 ± 5.4 | 19.0 ± 3.9 | 15.3 ± 4.0 |

* Figures are percentages ± S.D.

To further profile the distribution of sarcolemma prepared from evacuated muscle slices, $^{125}$I-labelled α-bungarotoxin binding assays were performed on fractionated material from innervated and denervated extensor digitorum longus muscles. The specificity and the relative irreversibility of α-bungarotoxin binding can be used to determine the postjunctional membrane in innervated myofibers. As shown in Table II, the S fraction from innervated muscle contained approximately 2/3 of the recoverable $^{125}$I-labelled α-bungarotoxin bound in the interface fractions, exclusive of the binding in $P_4$. $P_4$ contained 17% and 21% of the total $^{125}$I-labelled α-bungarotoxin bound to all the fractions from innervated and denervated muscle, respectively. The binding in $P_4$ was irretrievable and probably indicated the loss of some sarcolemma during fractionation. The smallest amount of specific $^{125}$I-labelled α-bungarotoxin binding from innervated muscle is seen in the M fraction (Table II); indeed, in only one of five experiments did M exhibit any binding at all.

Following 14 days of denervation, the distribution of $^{125}$I-labelled α-bungarotoxin binding among the general fractions was quite different (Table II). Of particular interest was the shift in the proportion of binding of the M fraction from one showing the lowest specific binding to one showing the highest. The results of the $^{125}$I-labelled α-bungarotoxin binding assays provided evidence that the S fraction was enriched with postjunctional sarcolemma, while the M and the H fractions were enriched with extra-junctional sarcolemma.

The apparent discrepancy between the localization of the junctional region using acetylcholinesterase as a marker (i.e., in $P_4$), and using $^{125}$I-labelled α-bungarotoxin binding in innervated muscle as a marker (i.e., in the S fraction), is probably based on a difference in the regional disposition of these junctional molecules. Recent morphological and biochemical results have demonstrated that acetylcholinesterase appears to be located in the basal lamina (i.e., ectolemma), not the sarcolemma of the junctional region (Ref. 8, and unpublished observations). Furthermore, electron microscopy of evacuated muscle slices in cross-section reveals that the sarcolemma separates from the ectolemma [1]. Thus, following evacuation and homogenization of the muscle slices, the ectolemma does not co-distribute with the plasma membrane.

*Electron microscopy of the membrane fractions.* Based on the results of the 5′-nucleotidase assays and the $^{125}$I-labelled α-bungarotoxin binding assays, muscle plasmalemma appears to be distributed among M, H and S fractions. These fractions, recovered from the gradients, yielded 14.13 ± 2.16; 12.41 ±

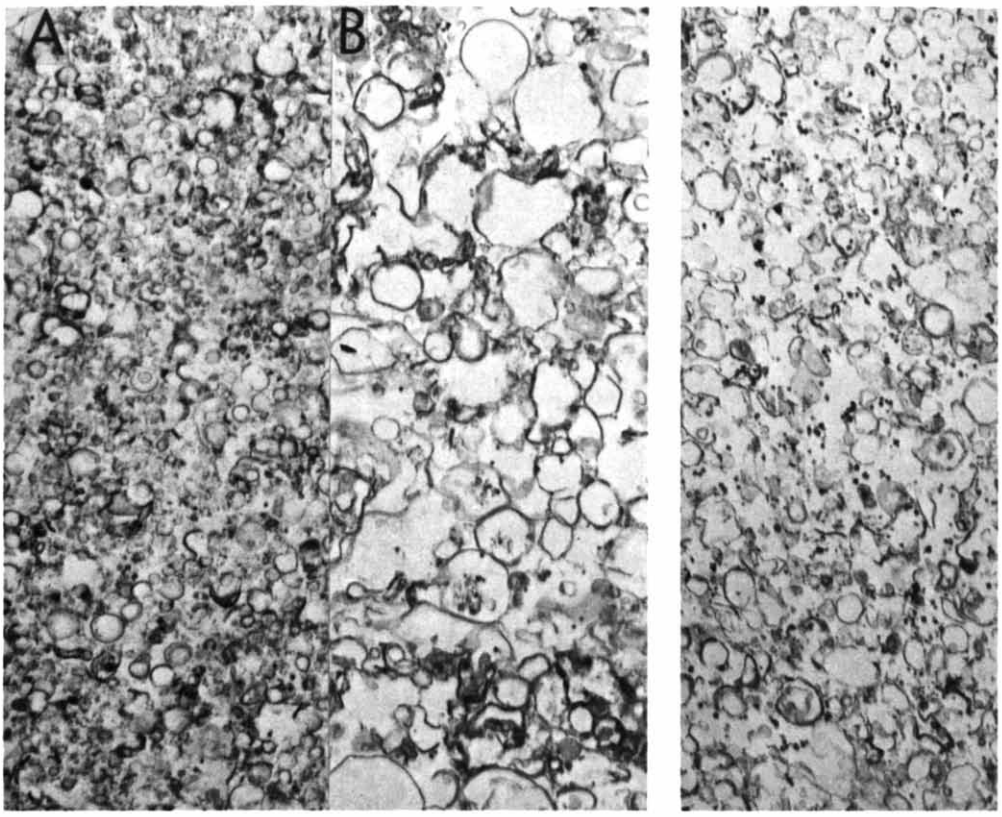


Fig. 5. Electronmicrographs of the M membrane fraction showing a discontinuous distribution of (A) small vesicles, and (B) large vesicles (magnification, X12 600).

Fig. 6. Electronmicrograph of the H membrane fraction. Electron-dense particles are probably an artifact of preparation (magnification, X12 600).

1.45; and 6.87 ± 0.59 $\mu$g protein for M, H and S respectively (data based on three experiments). Each of these membrane fractions was examined in the electron microscope. Fig. 5 is a low magnification electron micrograph of a region from the M fraction. Numerous membrane vesicles of varying sizes were a prominent feature of this fraction, and there was little evidence of non-membranous structures. However, the vesicles of the M fraction appeared to be composed of two sub-populations based on size (Figs. 5a and 5b). The separation of the two sub-populations suggested a stratification of the M fraction pellet.

A representative electron micrograph of the H fraction is provided in Fig. 6. While membrane vesicles were prevalent in this fraction also, there were numerous electron-dense particles and aggregates of particles. The dense particles were of unknown origin and the possibility of preparative artifact was not ruled out.

The S fraction was the most heterogeneous of the three membrane fractions, and included mitochondria, which may have derived from cells associated

with the stroma, such as blood vessels. Nevertheless, the S fraction did contain abundant membrane vesicles (data not shown).

## Discussion

The evacuated muscle slice procedure is a modified and improved version of the Beringer and Koenig technique [1]. The procedure allows bulk evacuation of transverse muscle slices, which is more reliable and provides sufficient yield to permit subfractionation and preparation of enriched plasma membrane fractions.

The mechanisms involved in evacuation are not known, however, $Ca^{2+}$ is necessary for successful evacuation [1,2,9]. Furthermore, Beringer and Koenig [1] observed that myofibers incubated in 1 mM $CaCl_2$ underwent 'extraction' of Z-band material, and the absence of electron-dense Z-band material was correlated with successful emptying. Considering that a $Ca^{2+}$-activated protease, which degrades Z-disk material, has been purified from porcine muscle [10], evacuation of muscle slices may be dependent on the $Ca^{2+}$-induced activation of such an endogenous protease. With the removal of transverse structural restraints (i.e., Z-disks), exposure to a low ionic strength, alkaline medium, very likely generates local repulsive forces, which probably provide the motive force for the en masse myoplasmic extrusion out the open ends of truncated myofibers. In view of the bulk extrusion of myoplasm it seems likely that the transverse tubules are sheared off and carried out as well.

Previous methods of preparing muscle membrane fractions were strictly dependent on differential and density gradient centrifugation for the isolation of sarcolemma from other muscle cytomembranes [11—17]. This approach increases the likelihood of significant contamination by cytomembranes, especially sarcoplasmic reticulum, because buoyant density differences are insufficient for complete separation. The major advantage of the evacuated muscle slice technique, therefore, is the elimination of cytoplasmic organelles such as sarcoplasmic reticulum and mitochondria prior to homogenization. This results in a significant enrichment of stromal constituents, which include muscle plasmalemma and endothelial elements. The structurally intact evacuated muscle slice stroma can be screened in addition, using a dissecting microscope to assess adequacy of evacuation. Occasionally, plugged myofiber segments can be excised, or a slice can be rejected if the incidence of plugged segments is unacceptably high. In this manner more than 99% of the myoplasmic contents of the muscle can be eliminated (see Fig. 3a and b). The stroma can then serve as starting material for homogenization and preparation of membrane fractions, using standard differential and sucrose density centrifugation.

The fractions obtained by differential and discontinuous sucrose gradient centrifugation of homogenized evacuated muscle slice stroma have been characterized by selected plasma membrane markers and fine structural examination. Using 5′-nucleotidase (5′-AMPase) as a general enzyme marker for plasma membrane [18—21], the M and H fractions appear to be most enriched with regards to plasma membrane. More specifically, 5′-AMPase activity has also been used by Kidwai, et al. [13], Shapira, et al. [15], Agapito and Cabezas

[16], and Del Molino and Cabezas [22] as one criterion for the identification of plasma membrane fractions obtained from homogenized muscle. The highest activity for 5′-AMPase reported for a purified rat muscle plasmalemma fraction was 5.8 $\mu$mol $P_i$/mg protein per h [13], which was about 15-times less than the specific activity found in either the M or H fractions in this study. However, extraordinarily high 5′-AMPase specific activity was reported by Del Molino and Cabezas [22] for hamster and chicken sarcolemmal membrane fractions; indeed, even their crude muscle homogenate had a higher specific activity than that reported here for purified membrane fractions. Reasons for the inordinately high values in the latter study are not readily apparent.

Further characterization of the muscle plasmalemma using $^{125}$I-labelled $\alpha$-bungarotoxin binding assays and acetylcholinesterase assays revealed their peak activities in fractions S and $P_4$, respectively. Similar results were obtained by Festoff and Engel [14] who found that their plasma membrane marker ($Na^+ + K^+$)-ATPase, $^{125}$I-labelled $\alpha$-bungarotoxin binding and acetylcholinesterase activities were located at different interfaces of the discontinuous gradient that they employed, the buoyant density distribution of which was similar to that characterizing M, H, S and $P_4$ for the corresponding markers.

On the basis of the nonspecific plasma membrane marker, 5′-AMPase, the M and H fractions are essentially equivalent. However, the presence of plasma membrane from cells other than from myofibers in evacuated muscle slice stroma (e.g. endothelial cells) could contribute significantly to 5′-AMPase activity in the M and/or H fractions. In order to distinguish which contained a greater enrichment of sarcolemma membrane, the distribution of $^{125}$I-labelled $\alpha$-bungarotoxin binding was determined in the fractions following 2 weeks of denervation, a time when spread of acetylcholine receptor to extrajunctional sarcolemma should have been maximal [23]. The binding assay showed that the M fraction bound the largest percentage of $^{125}$I-labelled $\alpha$-bungarotoxin of all the fractions. Nonetheless, the H fraction also bound almost as much $^{125}$-labelled $\alpha$-bungarotoxin as M, allowing no clear distinction to be drawn. The S fraction on the other hand, showed an apparent decrease in the percent binding of $^{125}$-labelled $\alpha$-bungarotoxin. Since the S fraction is thought to contain most of the junctional membrane, based on toxin binding of innervated muscle, and considering that the number of acetylcholine receptors in the endplate does not change appreciably following short-term denervation [24,25], the decrease in the percentage of $^{125}$I-labelled $\alpha$-bungarotoxin bound by the S fraction probably represents a relative, and not an absolute, decrease in acetylcholine receptors.

As to the question of whether the M or the H fraction is most enriched with extrajunctional sarcolemma, additional information is required. Several observations, however, suggest that the M fraction is the more enriched one. Firstly, the M fraction lacked significant binding of $^{125}$I-labelled $\alpha$-bungarotoxin when prepared from innervated extensor digitorum longus muscle. Secondly, from a morphological standpoint, the fraction showed less evidence of contamination by extraneous non-membrane material. Finally, electrophoretic analysis of the M, H and S fractions from innervated and denervated extensor digitorum longus muscle has indicated that the polypeptide composition of the M fraction is most sensitive to denervation while H is very much less so and S is least [26].

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