

ISOLATION AND CHARACTERIZATION OF THE SARCOPLASMIC RETICULUM OF SKELETAL MUSCLE

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ABSTRACT

The sarcoplasmic reticulum (SR) of rabbit skeletal muscle was studied after isolation of a vesicle fraction and of vesicular subfractions by means of differential and density gradient centrifugations. The different fractions were examined electron microscopically by negative and positive staining; their content in protein and phospholipid and their ability to bind Ca^{++} were determined. After homogenization, differential centrifugation yielded a "sarco-vesicular fraction" (SVF) which was mainly composed of numerous vesicles of different types mixed with fibrous proteins and mitochondrial fragments. This SVF contained 2% of the protein and 25% of the phospholipid of the initial tissue extract. It had a high Ca^{++} binding activity that was preserved for several days by storage in the presence of oxalate. After centrifugations of the SVF on sucrose density gradients, two vesicular subfractions were obtained which were characterized by different sedimentation rates, isopycnic banding, morphology, and composition in protein and phospholipid. (a) The low-density subfraction (ρ 1.10–1.12) contained a heterogeneous population of membranous structures: thick- and thin-walled vesicles, tubular formations, triads, and plasma membranes. Its content in protein and phospholipid was very low. (b) The high-density subfraction (ρ 1.13–1.17) was a very pure subfraction composed only of thin-walled vesicles. Its content in phospholipid was high and the ratio of phospholipid-phosphorus to protein was about 20. The calcium-binding activity found in the total SVF was recovered only in this latter homogeneous subfraction. The origin of these two subfractions from the SR is discussed.

INTRODUCTION

The sarcoplasmic reticulum (SR) of skeletal muscle is composed of two distinct membrane-bounded systems of vesicles and tubules: the longitudinal cisternae which are considered to be a cell organelle derived from the endoplasmic reticulum (46), and the transverse system, the T system, which is now proven to consist of tubular invaginations of the sarcolemmal membrane: the T tubules (11, 16, 18, 27, 43, 44). At the places where the terminal cisternae of the longitudinal system face the transverse tubules, triads are

formed, which consist of complex differentiations of the membranes (46).

Correlative physiological and morphological studies have shown that the T tubule of the triad is involved in transporting the depolarization of the plasma membrane into the depth of the muscle tissue (28, 19, 44, 45, 20). This causes the release of Ca^{++} ions which, in turn, causes muscle contraction by activating the sliding filament mechanism (42, 6, 53). The liberation of Ca^{++} ions from the SR may occur at the site of the terminal cisternae

(5, 53). Inversely, during muscle relaxation, the longitudinal SR may reaccumulate the calcium released from actomyosin (10, 21).

The major findings concerning the function of the SR, in releasing and in binding Ca^{++} ions during the contraction-relaxation processes, have been obtained by *in vitro* studies on microsomal fractions (relaxing factor preparations) isolated from the SR by differential centrifugation of homogenized muscle tissue (8, 9, 39, 10, 23-25).

Several solutions have been used as media for homogenization of skeletal muscle. These include bicarbonate (10, 32, 22), histidine, phosphate or Tris buffer with or without KCl (41, 25, 37, 50, 30), and sucrose (39, 50, 12, 54). Sucrose density gradient centrifugation methods have been used by some authors to obtain relatively pure SR vesicles (25, 33, 50, 49, 54).

Ultrastructural descriptions of fragmented SR have been provided by several investigators, using sectioned or negatively stained material (39, 10, 50, 12, 22, 36, 30). In these studies the fractions and subfractions include a mixture of morphologically distinct vesicles. In addition to vesicles, "tadpole" configurations were found in negatively stained preparations of SR (12, 36, 30), whereas in sectioned material, spherical vesicles and tubular fragments were observed (39, 10, 50). However, no correlation has been found between these different structures and the two parts of the SR, the T system and longitudinal system. Ikemoto et al. (29, 30) have speculated that the globular portion of the tadpole structures represents the terminal cisternae and that the tail-like portion corresponds to the longitudinal reticulum.

More recently, Damer and Baskin (7) examined fragmented SR by negative staining and freeze-etch techniques. They concluded that the most probable configuration of the SR vesicles is spherical and that the tadpole or tubular structures may be artifactual.

The purpose of our investigation was to further characterize fractions and subfractions isolated from the SR. Our data show that the two subfractions obtained correspond to the two different membrane-bounded systems, the T tubules and the longitudinal SR.

METHODS

Animals and Tissue Preparation

Adult rabbits, 4-5 months of age and weighing between 2-3 kg, were used as the source of SR.

The animals were anaesthetized by intravenous injection of Nembutal and carefully bled by sectioning the neck vessels.

The muscles of the hind legs were excised and rapidly cleaned of fat and connective tissue. Those predominantly composed of red fibers were discarded, and the selected white muscles were chilled and packed in the cooled sucrose solution prepared for homogenization. Older animals were not used because the differentiation into white and red muscle was less marked and also because of fatty infiltration of the muscle fibers.

Preparation of Sarcovesicular Fraction (SVF) and Its Subfractionation

All the steps summarized in Fig. 1 were carried out at 5°C.

To preserve the structure and the characteristic features of the differentiated membranes, the homogenization was executed in three successive steps.

Homogenization was carried out in two different media: (1) 0.25 M sucrose (saccharose high purity; E. Merck AG, Darmstadt, West Germany) or (2) 0.18 M sucrose in 0.1 M phosphate buffer (pH 7.2). The latter molarity was selected because it gave the most definite separation of the different cell fractions. The differences observed during the centrifugation experiments will be described in the Results section. Tissue was homogenized in 5 vol of either medium 1 or medium 2.

A preliminary low-speed centrifugation was used to pellet cell debris. The supernate, called "extract," was filtered through four layers of gauze to adsorb part of the lipid micelles and was then centrifuged at 15,000 *g* for 20 min in a No. 21 Spinco rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting pellet contained mainly mitochondria and variable amounts of fragmented myofibrils. Mitochondrial and postmitochondrial fractions were normally contaminated with protein which sedimented as a gelled precipitate. In order to keep the fibrous proteins in solution, potassium chloride was added to the extract at a final concentration of 0.25 M (Fig. 1).

The supernate of the second centrifugation was then centrifuged at 123,000 *g* for 60 min in a No. 50.1 Spinco rotor (Beckman). The resulting compact pellet was composed of vesicles, derived from the SR, and also of a small amount of fibrous protein. This fraction was called the SVF. The SVF pellets were gently re-suspended in a 0.25 M sucrose solution containing 0.05 M KCl and diluted to a final protein concentration of 10-15 mg/ml. Lowering the KCl concentration at this step allowed the fibrous proteins to agglutinate. The agglutinated fibrous material was easily precipitated by a low-speed 15 min centrifugation. This technique yielded a very pure sarcovesicular supernate, al-

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|---------------------------------|---|
| Homogenization of muscle tissue | With meat-grinder, mortar (6 min), Potter homogenizer (3 min); In 0.18 M Sucrose-0.1 M Phosphate, pH 7.2 (w/v:1/5) or In 0.25 M Sucrose (w/v:1/5); At 4°C |
| Differential centrifugations | (a) Preliminary centrifugation to discard cell debris, 1,500 $g \times$ 5 min; Addition of KCl (final molarity, 0.25 M) (b) Sedimentation of myofibrils and mitochondria, 15,000 $g \times$ 20 min (c) Sedimentation of the SVF as a pellet or on a sucrose cushion, 123,000 $g \times$ 57 min (d) Washings in 0.25 M Sucrose-0.05 M KCl |
| Gradient centrifugations | Continuous sucrose density gradients, 0.74 M-2.05 M; In SW 39 tubes, 39,000 rpm \times 3 hr or In SW 25 tubes, 25,000 rpm \times 15 hr or In a zonal rotor, Anderson type, 39,000 rpm \times 2 hr |

FIGURE 1 Homogenization and centrifugation procedures used to obtain the SV fraction and subfractions.

though there was some loss of vesicles which sedimented with the contaminating fibrous protein.

Further separation of subfractions was achieved by centrifugation of the SVF on linear sucrose density gradients prepared either for swinging-bucket rotors or for rotors of the Anderson type (Beckman). The centrifugations in the cylindrical rotor of Anderson, type L 4, was carried out with the assistance of Dr. E. Schram, Laboratory of Biochemistry, University of Brussels.

Chemical Analysis

The protein determinations on individual fractions were made either according to the method of Lowry et al. (34) or automatically with a Technicon auto-analyser unit (Technicon Corp., Ardsley, N. Y.).

Phospholipids were extracted following the technique of Folch et al. (15), and their phosphorus content was determined according to Ames and Dusen (1) or to Berenblum and Chain (3).

The cytochrome oxidase activity was determined by the measurements of the oxidation of a reduced cytochrome *c* (Sigma Chemical Co., St. Louis, Mo., type III, horse heart) (4).

Determination of Calcium-Binding Activities of the SVF and Its Subfractions

Ca⁺⁺ uptake by the different preparations of vesicular material was measured by the Millipore filtration technique (10, 37).

The different vesicular fractions were incubated at 22°-25°C in a medium composed of histidine (5 mM), MgCl₂ (5 mM), K-oxalate (5 mM), ATP (5 mM), ⁴⁵Ca Cl₂ (0.1 mM) (pH 7.2). Vesicles in suspension were

added at a concentration of 0.1-2 mg protein/ml, and the final total volume was uniformly adjusted to 2 ml.

The reaction was terminated by passing the mixture through Millipore filters, type Ha (0.45 μ) (Millipore Corp., Bedford, Mass.) with negative pressure. The radioactivity in 0.5-1.0 ml samples of the filtrates was measured in a Packard liquid scintillation counter¹ (Packard Instrument Co., Downers Grove, Ill.), using the scintillation cocktail recommended by Nadarajah et al. (40).

The amount of membrane-bound calcium was expressed as the difference in the radioactivity between the filtrates of the vesicle-free incubation medium and the filtrates of the incubated vesicle-containing samples.

Electron Microscopy

SECTIONED MATERIAL: 200- μ l samples of SV fraction and SV subfractions in 0.25 M sucrose were centrifuged at 15,000 rpm for 4 min, in microtest tubes of a Beckman microfuge, model A. The very small pellets (1-2 mm thick) were fixed with 2.5% distilled glutaraldehyde (13) in 0.18 M sucrose-0.1 M phosphate buffer at pH 7.2, for 3 hr, or in 2% osmium tetroxide buffered with 0.1 M phosphate, for 2 hr (38). The fixations were performed at room temperature or at 4°C. The glutaraldehyde fixation was followed by several washings in phosphate-buffered sucrose (pH 7.2) and then by postfixation for 1 hr in a 1% OsO₄-0.18 M sucrose-0.1 M phosphate buffer. The osmium tetroxide fixation was followed by rapidly washing in phosphate-

¹ Measurements were carried out in the Laboratory of Nuclear Medicine, University of Brussels.

buffered sucrose or in 0.35 M Veronal-acetate buffer. The latter procedure was used when tissue blocks were to be stained with uranyl acetate 0.5%. Uranyl acetate staining was performed in Veronal-acetate buffer at pH 5.3 (14). The blocks were then dehydrated in a graded ethanol-water series up to 94% ethanol. They were then removed from the tubes and transferred to 100% ethanol. The blocks were embedded in Epon 812 (35) and sectioned on an LKB ultratome (Laboratorie och Kemikalista Produkter, Stockholm), then double-stained with uranyl acetate and lead citrate (48).

NEGATIVELY STAINED MATERIAL: 200-mesh grids were coated with a Formvar film and covered by a layer of evaporated carbon. Negative staining was carried out in a solution of 3% phosphotungstic acid (PTA) brought to pH 7.2 with potassium hydroxide. Dilutions of the suspended material were made with the PTA solution. The mixtures were applied to the grids for 5 min and then removed. The grids were dried on filter paper, and a second very thin layer of carbon was evaporated on the grid.

Electron micrographs were taken with a Siemens Elmiskop 1 at 20,000 times magnification, using a 50 μ diameter diaphragm in the objective lens and very low illumination of the specimen.

RESULTS

Preparation and Chemical Analysis of the Fractions

THE SVF: On gross examination, the SVF appeared as a distinctly heterogeneous pellet: the superficial layer was opalescent and viscous, while the deeper regions were dense and less translucent.

The mean protein content of the SVF was 0.7 mg/g wet weight of muscle tissue. This represented about 2% of the total protein content of the extract (Table I). The latter was the same whether homogenization was carried out in buffered or unbuffered sucrose.

In contrast, the total phospholipid-phosphorus of the extract was higher when prepared in buffered than in unbuffered sucrose. Nevertheless, in both cases, 25% (Table I) of the total was recovered in the SVF (9.5 μ g and 5.9 μ g phospholipid-phosphorus/g of muscle tissue, respectively).

THE SV SUBFRACTIONS: Two subfractions were separable from the SVF by isopycnic banding in linear sucrose gradients varying from 0.74 M to 2.05 M. Fig. 2 represents a SW 25 swinging-bucket tube containing a sucrose density gradient in which two subfractions (1 and 2) are clearly separated. In some cases, a third layer (*c*)

TABLE I
Per Cent Recovery of Proteins, Phospholipids, and Cytochrome-Oxidase Activity of the SVF and Subfractions

| Fractions and subfractions | Protein (%) recovery | Phospholipid-phosphorus (%) recovery | Cytochrome oxidase (% content) |
|----------------------------|----------------------|--------------------------------------|--------------------------------|
| Extract | 100 | 100 | 100 |
| Mitochondrial fraction | 5 | 42 | <50 |
| SVF after washings | 2 | 25 | 3 |
| Low-density subfraction | 0.2 | 1.7 | <0.01 |
| High-density subfraction | 0.5 | 13 | <0.1 |
| Contaminations | 1 | 7 | 2 |

of coarse material accumulated in variable amounts, immediately below subfraction 2.

Subfraction 1, banding near the top of the gradient between densities 1.10 and 1.12, consisted of a thin layer of blue opalescent material which was very unstable. Subfraction 2, banding between densities 1.13 to 1.17, was weakly opalescent and very stable with time, independent of the isolation medium used. The lower part of this subfraction sometimes displayed an increased opalescence caused by overlapping with the upper part of band *c*.

Occasionally, during centrifugation, subfraction 1 separated into two bands in close proximity; however, the variability of this separation and the closeness of the two bands did not allow isolation and characterization of these new subfractions.

The presence of subcellular components in the gradients was also detected by automated protein analysis. Fig. 3 shows a recording of the protein analysis of 30 1-ml fractions collected from a SW 25 gradient tube.

The zonal rotors B-IV and B-XIV also separated two subfractions. These subfractions were located in the gradients by measuring the absorbance of gradient fractions at 280 m μ and by determining the protein content, as shown in Fig. 4. The material remaining at the starting level, after the time of centrifugation, is called the "sample zone fraction." Peak 1, ranging in density between 1.10 and 1.12, was not always distinct from the sample zone fraction. Peak 2, extending from fractions 30 to 50, corresponded to sucrose

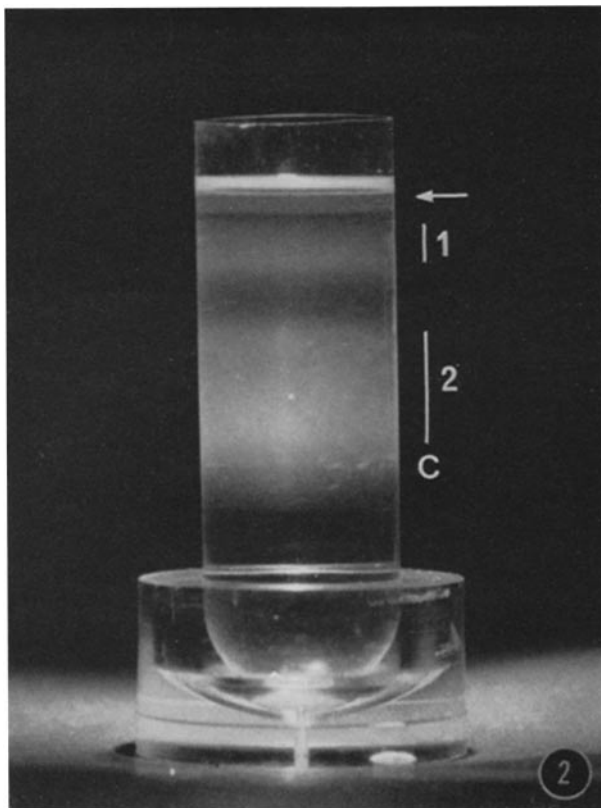


FIGURE 2 SV subfractions banded in a centrifuge tube which was spun in a swinging-bucket at 25,000 rpm for 15 hr: the SVF was layered on top of the linear sucrose gradient (arrow); subfractions (1) (densities 1.10–1.12) and (2) (densities 1.13–1.17) are clearly distinguished. C, contaminating band.

densities ranging from 1.12 to 1.16. The presence of one or two less important peaks in the denser end of the gradient, immediately after peak 2, varied from one experiment to another.

The two subfractions obtained after centrifugation in zonal rotors, corresponding to the protein and UV absorption peaks of Fig. 4, were concentrated and layered on top of sucrose gradients prepared in SW 25 tubes. After centrifugation, peaks 1 and 2 were found to band at the level of the low-density and high-density subfractions, respectively. Therefore, the subfractions separated by zonal centrifugation corresponded to those separated by isopycnic banding in swinging-bucket rotors.

THE LOW-DENSITY SUBFRACTION: This subfraction showed a marked variability in protein and phospholipid content and a certain instability in the course of time. The variability in protein content depended mainly upon the homogeniza-

tion technique, since high protein values in the original extract generally coincided with high values in the corresponding low-density subfractions. Therefore, it is more meaningful to express protein content of the low-density subfraction as per cent of the protein in the original extract (Table I). It was repeatedly observed that a delay of 48 hr or more from the time of preparation of the SVF to the time of gradient centrifugation resulted in a significant reduction in the amount of material present in the light-density subfraction.

Similar considerations apply to variability in the phospholipid content; higher values were obtained after fractionation in phosphate-buffered extracts compared to unbuffered preparations (Table II). When considering the average values, the ratio of phospholipid-phosphorus to protein ranged from 3.1 to 7.5 under the different conditions of preparation, the highest ratios corresponding to the phosphate-buffered preparations.

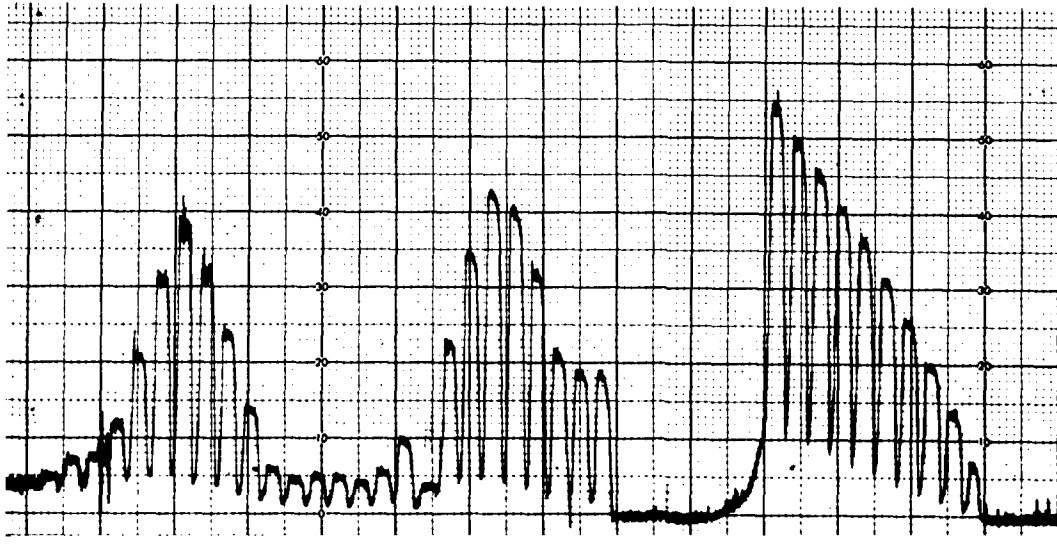


FIGURE 3 A recording of the automated protein analysis of 30 1-ml fractions, collected from a SW 25 tube (to be read from right to left). At the right of the graph are the different values of the standard protein concentrations (albumin, fraction 5) ranging from 10 γ to 100 γ . The first two fractions from the top of the gradient represent the protein content of the remaining SVF. Fractions 3-8 represent the protein content of subfraction 1; fractions 17-23, the protein content of subfraction 2.

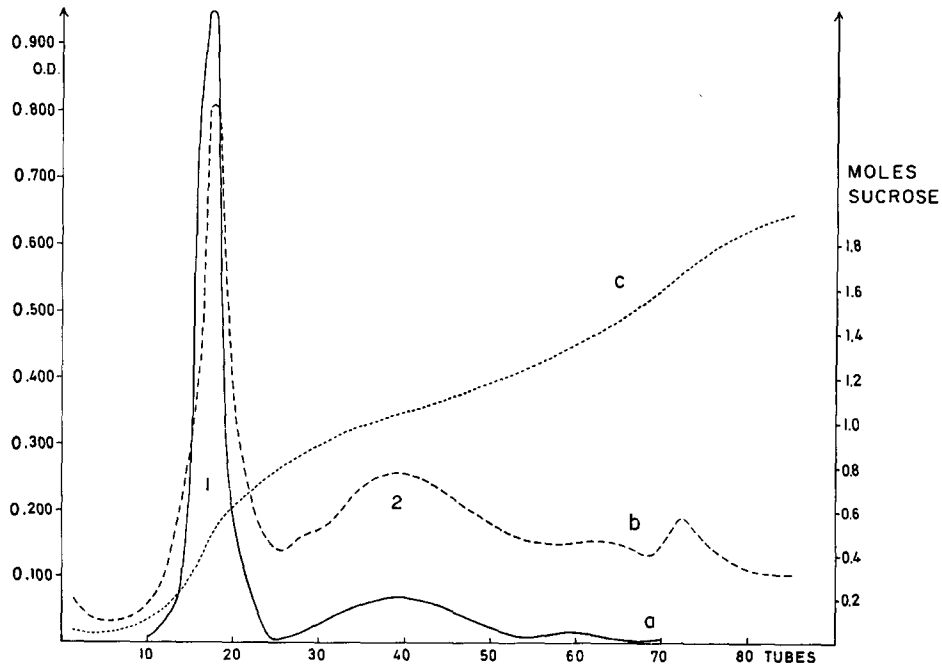


FIGURE 4 Distribution of proteins (a) and optical densities (b) measured at 280 $m\mu$ (O.D.) of samples collected from the B-IV zonal rotor; (c) molar concentration of the sucrose gradient. The two peaks 1 and 2 corresponding to the SV subfractions were obtained after centrifugation of the SV at 39,000 rpm for 2 hr.

TABLE II
Protein and Phospholipid Content of the SV
Subfractions

| Subfractions of the SVF | Protein (mg/g tissue) | Phospholipid-Phosphorus (PL-P) ($\mu\text{g/g}$ tissue) | Ratio PL-P ($\mu\text{g/mg}$ protein) |
|---|-----------------------|--|--|
| Fractionation in phosphate-buffered sucrose | | | |
| Low-density subfraction | 0.05-0.08 (0.07)* | 0.25-0.89 (0.53) | 7.5 |
| High-density subfraction | 0.15-0.37 (0.21) | 2.93-4.94 (3.92) | 18.6 |
| Fractionation in sucrose without buffer | | | |
| Low-density subfraction | 0.05-0.12 (0.09) | 0.15-0.44 (0.28) | 3.1 |
| High-density subfraction | 0.11-0.18 (0.13) | 2.54-3.62 (3.05) | 23.4 |

* Numbers in parenthesis are average values.

Cytochrome oxidase determinations performed on this subfraction did not reveal any activity (Table I).

THE HIGH-DENSITY SUBFRACTION: This subfraction was a much easier and substantial one to manipulate; it represented 0.5% of the protein and 13% of the phospholipid found in the extract. One-half the phospholipid content of the SVF was recovered in this subfraction. The phospholipid-phosphorus to protein ratio, obtained after using buffered or unbuffered sucrose for homogenization, ranged around 20 (18.6 and 23.4, respectively).

Very small mitochondrial contamination traced by the cytochrome oxidase determinations was found in this subfraction. This represented less than 0.1% of that present in the starting extract. When observed, this activity was always located in the lower region of the subfraction, close to the coarse material banded at the *c* level. This contaminating material contained up to 70% of the cytochrome oxidase activity present in the SVF. The protein content of this *c* layer was variable, but always higher than that of the low- and high-density subfractions.

DISTRIBUTION OF Ca^{++} UPTAKE IN THE VARIOUS FRACTIONS: The SVF bound Ca^{++} in the absence or in the presence of oxalate. After

10 min incubation at 37°C, the preparations bound 0.2-0.5 μM Ca^{++} /mg of protein. Binding activity was maintained at a constant level in the presence of oxalate for at least 6 days. When they were stored in oxalate-free media, gradual decrease in Ca^{++} uptake was observed. Studies of Ca^{++} uptake in the two subfractions 1 and 2 clearly indicate that only the high-density subfraction 2 bound the divalent ions. Subfraction 1 was completely devoid of this property (Fig. 5).

Morphology

THE SVF: Fig. 6 represents a rabbit adductor magnus from which the SVF was extracted (see legend).

The general aspect, at low magnification, of a SVF negatively stained with phosphotungstate is represented in Fig. 7. It is composed of numerous vesicles of different structures. This fraction appears contaminated with many well-defined fibrous proteins, the bulk of organelles appearing mixed with a granular amorphous material in the background.

Fig. 8 shows, at higher magnification, details of the elements contained in the SVF. Two types of vesicular structures are apparent: thick-walled vesicles (v_1), which are often prolonged by a dense

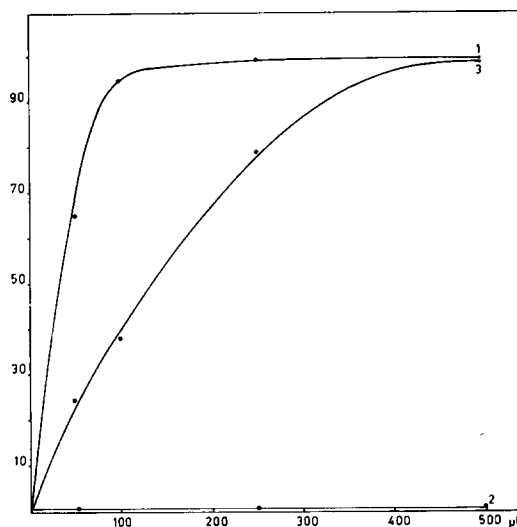


FIGURE 5 Per cent Ca^{++} uptake by vesicles of the SVF (1), 1.75 mg protein/ml; of the low-density subfraction (2), 0.25 mg protein/ml; and of the high-density subfraction (3), 0.15 mg protein/ml.

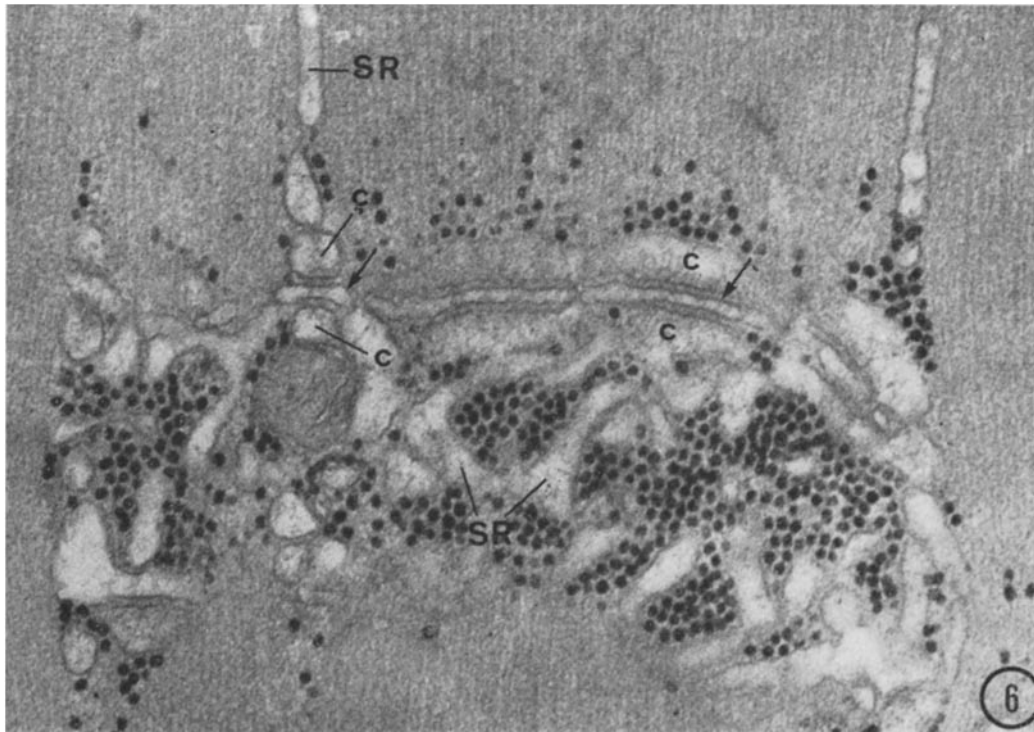


FIGURE 6 I band and part of the adjacent A bands of rabbit adductor magnus in longitudinal section. The triad located at the I-A limit is composed of a transverse T tubule (arrow) 300 A thick and, at each side, of terminal cisternae (*c*) which are connected to the longitudinal cisternae of the SR. The T tubule and, likewise, the lateral cisternae of the SR adjacent to the intermediate tubule are bounded by membranes of high opacity. These joined membranes exhibit an average thickness of about 500 A and are separated by a space of 110 A which is occupied by an electron-opaque material; β -glycogen particles are accumulated in the irregular spaces which are delineated by the reticulum; others, more or less aligned, infiltrate the myofilaments. $\times 48,000$.

tubular structure, and thin-walled vesicles (v_2) of round or oval shape.

The diameter of the thin-walled vesicles (v_2) ranges from 50 to 100 $m\mu$. The membrane limiting them has a constant thickness of 100 A.

The thick-walled vesicles (v_1), which sometimes appear in tadpole-like configuration, appear more complex structurally. The vesicular part has a diameter varying from 80 to 150 $m\mu$. The limiting membrane reaches 300 A in thickness. The tail-like structure consists of a tubule 100–250 $m\mu$ in length, with a thickness varying from 250 to 500 A.

The fibrous proteins which contaminate this fraction are either reconstituted actomyosin filaments (*am*) or fragments of collagen fibers (*co*). The latter, which are identified by their characteristic periodicity, are not often found. The reconstituted actomyosin filaments are very similar to the

arrowhead structures (Acto-HMM filaments) described by Huxley (26) and Ikemoto et al. (29, 30).

THE LOW-DENSITY SUBFRACTION: This subfraction examined in negative contrast contains a mixture of the two types of vesicles described above for the total SVF, but appears enriched in thick-walled vesicles and tadpole-like structures. The dimensions of the vesicles and of the tubular structures remain similar to those measured in the whole SVF. Occasionally, disrupted thin-walled vesicles reach diameters of 200 $m\mu$.

The thick-walled vesicles, an example of which has been selected for Fig. 9, is prolonged with a tubular formation 250 $m\mu$ in length and 50 $m\mu$ thick. On its surface, particles of 85 A diameter are densely packed and regularly arranged (arrow).

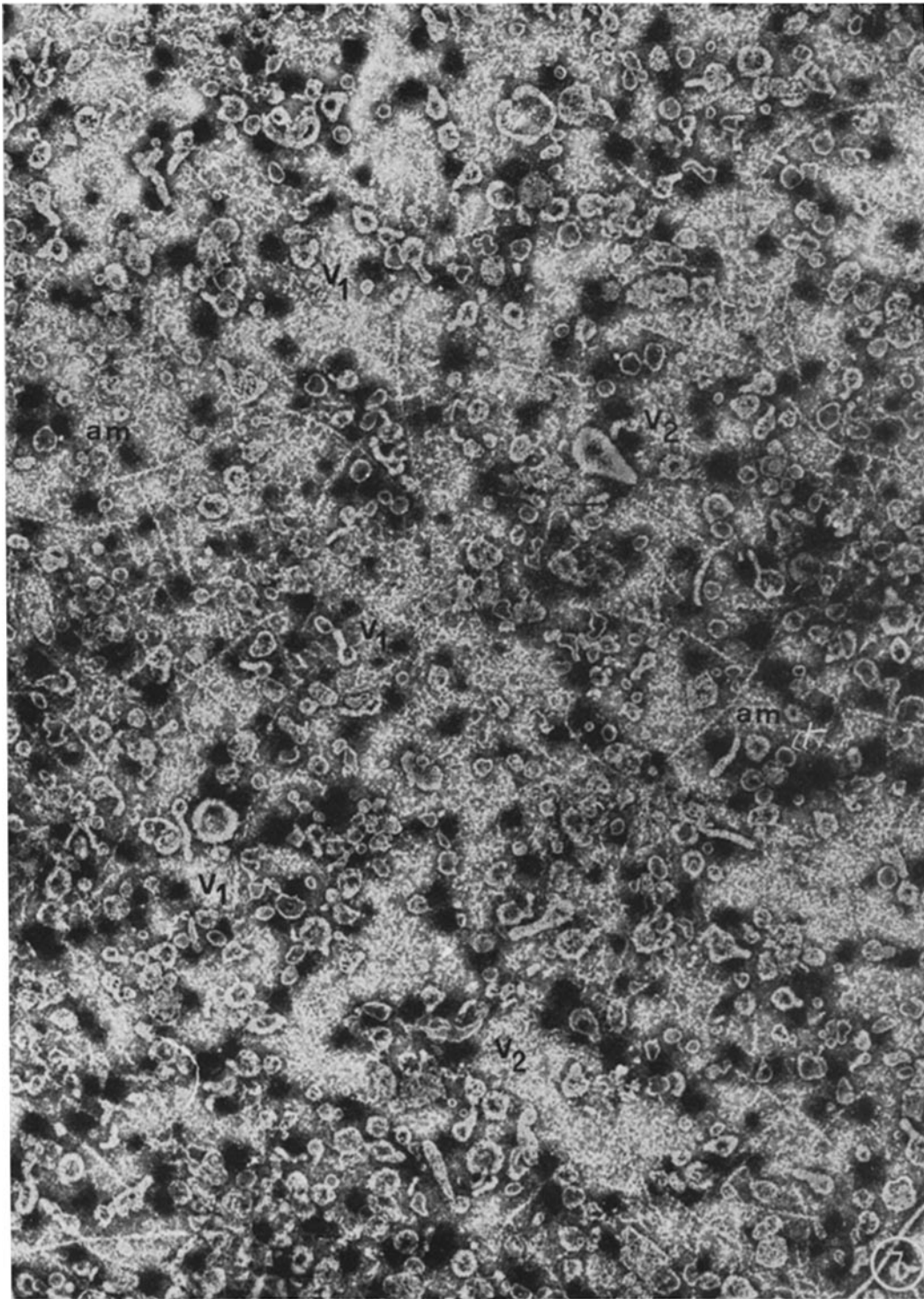


FIGURE 7 Negative staining of the SVF. This is a general view which shows the variety of components present: vesicles of two types, the thin (v_1), and the thick-walled vesicles (v_2), the latter often being elongated with a tubule (tadpole-like) structure; arrowhead structure of the actomyosin type (am), and a granular substance in the background. $\times 48,000$.

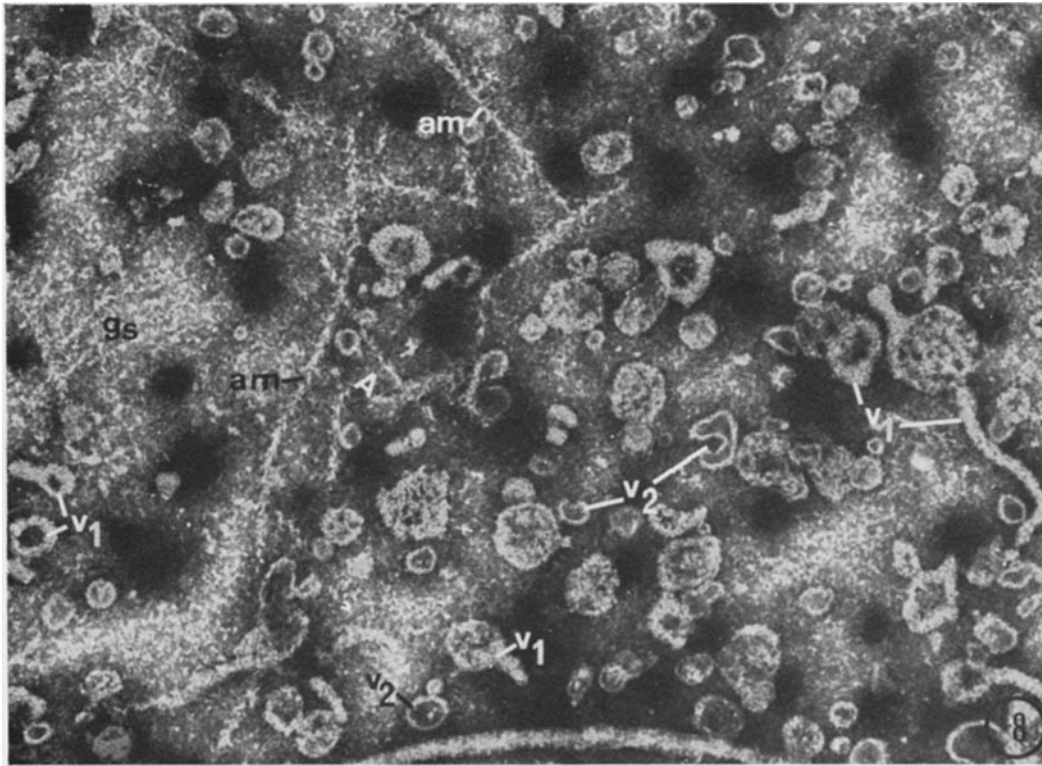


FIGURE 8 This micrograph represents a negative staining which shows more details of the various elements which compose the SVF. The thick-walled vesicles (v_1) contrast with the thin-walled vesicles (v_2). The arrowheaded filaments (am) measuring up to 1μ in length are mixed with a granular substance (gs) in which short, irregularly shaped and branched filamentous structures emerge. At the lower part of the picture, a collagen filament can be identified. $\times 75,000$.

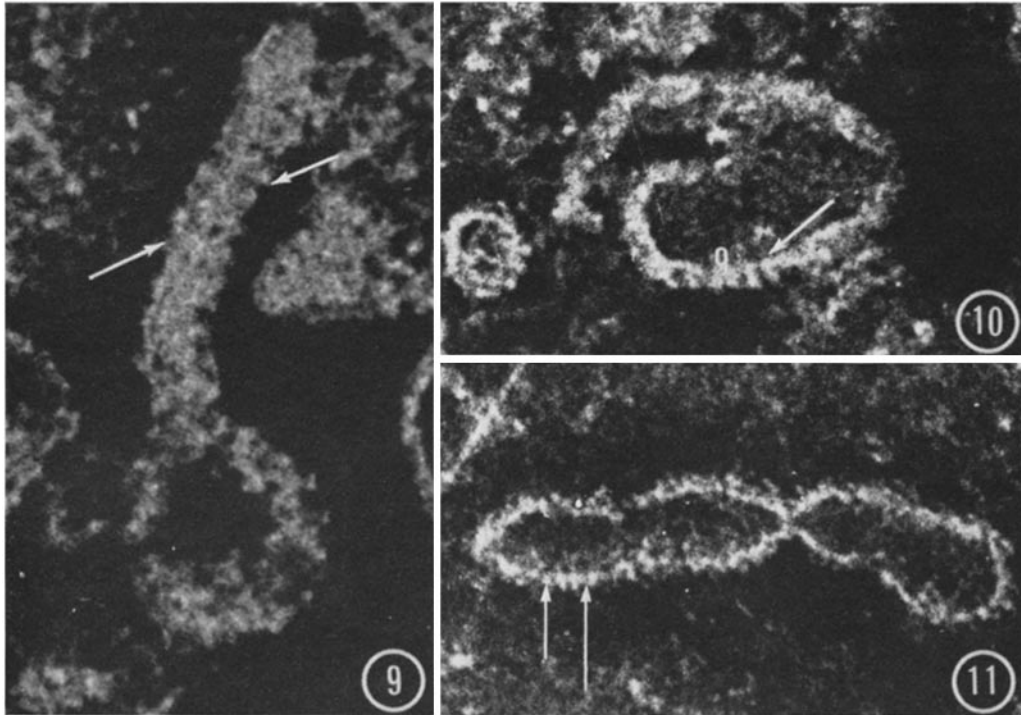
The membrane which delineates the vesicular head seems to be composed of the same round, closely packed particles.

The vesicular and tubular structures observed in the negatively stained specimens were also observed in sections of pelleted low-density material. Moreover, other sarcoplasmic elements could be identified in these pellets such as associations of vesicles and tubules, suggesting isolated triads, and also large and folded membranes of sarcolemmal origin.

The low magnifications (Figs. 12 and 13) show the heterogeneity of this material and the irregularity in size and shape of the vesicular structures. It was considered important to determine whether any of these vesicles could be related to the tubular and tadpole-like structures considered to be artifacts of negative staining. Despite the difficulty due to the plane of the section, frequent tubular

but well-differentiated structures occur (arrows). Some resemble triads of intact muscle (T), as shown in Fig. 13. A high magnification of that particular structure (Fig. 14) illustrates an example of two oval vesicles separated by a tubule-like element 150 A thick. The localized increase in density of the membranes that characterizes, *in situ*, the opposed membranes of the terminal cisternae and the tubular element is also present. Dense material appears in the space 100 A in width which is interposed between vesicles and tubule.

Fig. 15 is another example of a tubular element measuring 250 A in thickness with a lumen 100 A in width. The limiting membrane is a triple-layered structure with an average thickness of 80 A, each leaflet measuring 25 A. Dense, minute material can be detected in the lumen of the tubule.



FIGURES 9-11 Details on membrane structure of three vesicles selected in negatively stained material. $\times 224,000$.

FIGURE 9 This tadpole-like structure consists of an elongated vesicle with a tubular segment from the surface of which projects particles 80 Å in diameter (arrows).

FIGURE 10 The membrane of this thin-walled vesicle is composed of small particles (arrow), each being composed of two subunits: the inner base piece and the outer globular element. The base pieces of these particles are regularly aligned and form a more or less continuous inner leaflet.

FIGURE 11 Fig. 11 illustrates contiguous base pieces within the membrane of a thin-walled vesicle (arrows).

Fig. 16 represents a third selected tubular element in which four apposed membranes are heavily osmiophilic and densely packed. This picture strongly resembles the configuration of the tadpole-like vesicles (thick-walled vesicles) which appeared in negative staining.

Typical sarcolemmal membranes were also found in this subfraction as shown in Fig. 17. This picture represents a typical plasma membrane with its associated vesicular elements. A slightly granular material, resembling a stain precipitate, is also found in this low-density subfraction (Fig. 17, arrows). This material is interpreted as being fibrous proteins in single form, not reconstructed into filaments.

THE HIGH-DENSITY SUBFRACTION: Fig 18 is a representative sample of negatively stained, high-density material. It appears very homogeneous, composed only of thin-walled vesicles. The thick-walled vesicles were never observed. These thin-walled vesicles have a homogeneous substructure and present a somewhat variable size in the order of $120 \text{ m}\mu$. This diameter is slightly larger than that of similar vesicles in the low-density subfraction. The membrane thickness can be estimated to be about 80 Å. A fine granular material can be detected within some vesicles, although the majority appear empty.

The fine structure of membranes in negatively stained preparations of high-density subfractions

(Fig. 18) is less discernible than that of membranes of similar vesicles examined in the SVF (Fig. 8). Repeated centrifugation of these vesicles appears to damage the membranes. In membranes of thin-walled vesicles, particles which are regularly arranged close to one another are found. Two subunits may be distinguished within each particle: a base piece which forms with the contiguous elements a continuous inner leaflet (Fig. 10, arrow), and a globular subunit which constitutes the outer leaflet.

In sections of the fixed and embedded high-density subfractions, the homogeneity of the population of vesicular elements was obvious (Fig. 19). The majority of the vesicles were round or oval in shape, with a diameter of about 90 m μ . The vesicles of larger size are disrupted. At higher magnification (Fig. 20), the membrane which enclosed each vesicle appeared triple-layered with an over-all thickness of about 75 A. It consisted of two opaque leaflets, 25 A thick, separated by a light-density layer. In some cases, the membrane appeared to be composed of minute, closely packed spots (arrows), measuring about 25 A.

DISCUSSION

The results indicate that there are two major types of membranous structures that can be isolated from the SR of skeletal muscle. These can be distinguished from one another not only by their morphologic appearance but also by their sedimentation and biochemical properties. The SVF is morphologically identical to that isolated by others from several types of skeletal muscles, using similar techniques (39, 50, 12, 54). The SVF is composed mainly of two distinct types of vesicles. There is minor contamination by fibrous proteins

reminiscent of actomyosin (26, 30) and by mitochondrial fragments. One type of vesicle is bounded by a thick membrane. These vesicles are tubular or tadpole-like structures. The other vesicles are bounded by thinner membranes and represent the spherical or elliptical vesicles already described in the literature (23, 39, 10, 50, 12, 37, 30).

The protein content for SVF described here is lower than the values obtained by Yu et al. (54) and Seraydarian and Mommaerts (50). The lower values are due to the paucity of contaminating fibrous proteins in our preparations where KCl was used during the centrifugation and, at a lower concentration, during the successive washing. The phospholipid content is high, representing 25% of the total phospholipid in the initial extract. The high phospholipid content fits with the morphological observation of a large amount of vesicular membranes. Mitochondrial contamination is very low as estimated by cytochrome oxidase activity. Extreme care during homogenization is important in eliminating mitochondrial contamination.

SVF is very active in binding Ca⁺⁺, but the possibility cannot be excluded that, in addition to the vesicles, other components such as the contractile proteins also bind Ca⁺⁺. However, before Ca⁺⁺ binding assays were performed, all SVF were resuspended in 0.25 M sucrose, 0.05 M KCl, and most of the contractile proteins were precipitated in this solution.

"The low-density subfraction" is rather complex and has a weak opalescence that is unstable with time. The particles in this subfraction have a complex morphology that is of considerable interest. The two types of vesicles present in SVF are found in this fraction, but the thick-walled

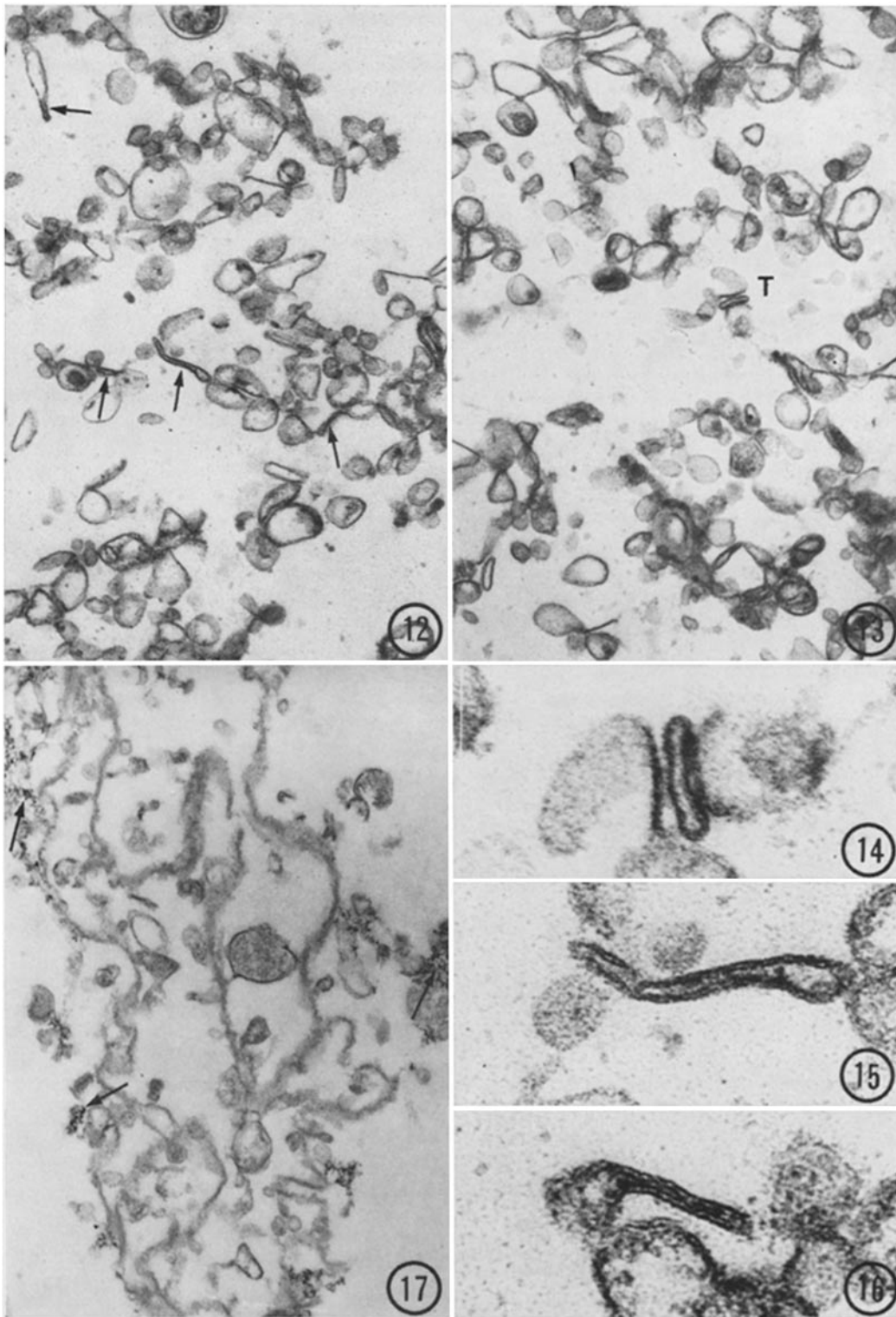
FIGURES 12-17 General views and details of selected areas of sections of the low-density subfraction.

FIGURES 12 and 13 Two general views of the pellet of low-density material which show the heterogeneity of the vesicular structures. Frequent tubular, tail-like structures (arrows) and triads (*T*) reminiscent of those described in tissue sections occur. $\times 37,000$.

FIGURE 14 Fig. 14 represents a high magnification of an area selected in Fig. 13. This triadic structure is characterized by the thickness and electron density of the membranes of the T tubule and of the terminal cisternae. $\times 150,000$.

FIGURES 15 and 16 Figs. 15 and 16 illustrate the tubular elements which are strongly suggestive of the configuration of the thick-walled vesicles observed in negative staining. $\times 150,000$.

FIGURE 17 These typical folded sarcolemmal membranes were found in the low-density subfraction. The granular material (arrows) may correspond to fragmented fibrous proteins. $\times 37,000$.



structures are much more numerous. The general morphology of the vesicles resembles that described by Seraydarian and Mommaerts (50) in the subfraction obtained from their "large microsomal material." In addition to these vesicles, we also observed a large number of contorted tubular fragments which Seraydarian and Mommaerts never mentioned. This difference could be accounted for by the fact that their material banded with a density of 1.15 while ours banded at 1.11.

Negative staining shows numerous tadpole-like structures identical to those described by Engel and Tice (12), Ikemoto et al. (29, 30), and Martonosi (36). These authors did not observe 85 Å subunits in the membranes of the tails and vesicles as described here. Similar membrane subunits were indeed observed by Deamer and Baskin (7) by the technique of freeze-etching, but valid comparison with our results is impossible in view of the different methods used. The 85 Å subunits have the same size as the "elementary particles" of the internal mitochondrial membranes. However, that the structures we observed are not mitochondrial membranes is shown by the total absence of cytochrome oxidase activity. It is thus suggested that these subunits might constitute the fine structure of some of the SR membranes.

The sectioning technique also confirms the complex morphology of the low-density subfraction. An important feature is the presence of typical triadic structures. Such triads have been observed by other authors in the total SVF (10, 22). We were not successful in separating these triadic structures from numerous tubular elements also located only in this subfraction. Morphologically, the tubular structures represent T tubules with a swollen terminus. An alternative hypothesis is that these tubules are composed of the lateral membrane of the terminal cisternae and the apposed membrane of the T tubule. The tiny spots in the lumen of these tubules could then represent the

bridges recently described between the two structures (17). On the other hand, the resemblance between these tubular structures and tadpole-like vesicles observed in the negatively stained preparations provides evidence that the tadpole-like structures are not artifacts, as proposed by Deamer and Baskin (7), but represent a true configuration of part of the membranes of the SR.

In addition to these structures, the low-density subfraction contains plasma membrane fragments which are structurally similar to the liver plasma membranes (2). The simultaneous presence of typical plasma membranes, of numerous tubular elements, and also of triadic configurations strongly suggests that this subfraction is part of a system connected with the sarcolemma and may be the transverse system. In agreement with Hasselbach and Elfvin (22), it would appear that after homogenization, the T system does not always maintain its complete triad structure, but is present mainly in the form of three tubular elements. This is probably the reason why we did not observe large amounts of intact triads.

The absence of any binding of Ca^{++} by the low-density subfraction separated from the active total SVF suggests that the T tubules, the main constituent of this fraction, do not contribute to the Ca^{++} uptake. If our interpretation is correct that the lateral vesicles observed in negative and positive staining correspond to the terminal cisternae described *in situ*, then we may further conclude that the entire triadic system is not involved in calcium uptake. This does not exclude, however, the possibility that the terminal cisternae, being in continuity with the active resorbing longitudinal system, play a role in the storage and the outflow of the Ca^{++} (5).

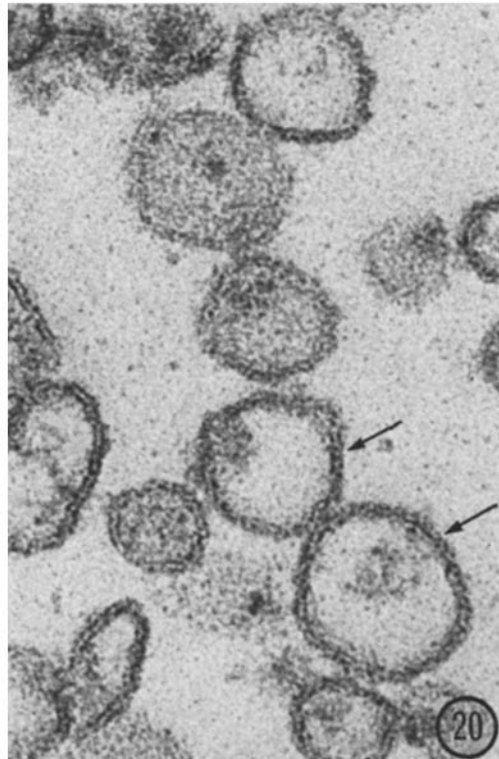
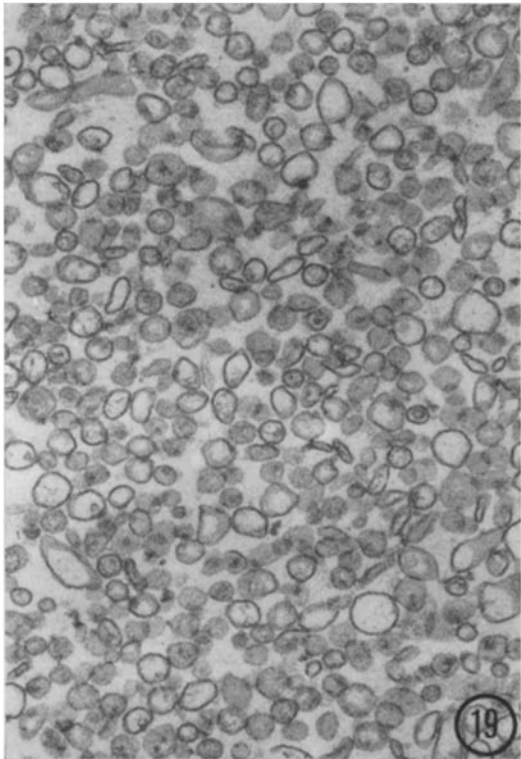
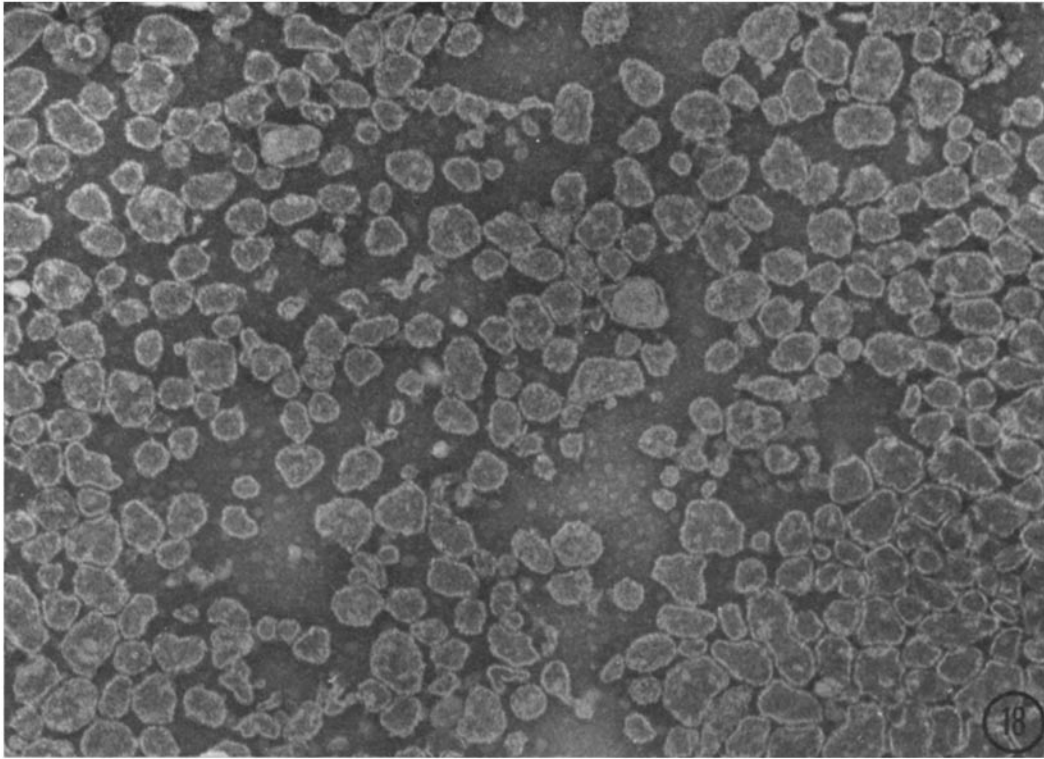
"The high-density subfraction" has a very low protein and high phospholipid content (high PL-P: protein ratio). The relative amounts of protein and phospholipid are very similar to those ob-

FIGURE 18 Fig. 18 is a representative area of the homogeneous population of thin-walled vesicles which comprise the high-density subfraction. Negative staining. $\times 37,000$.

FIGURES 19 and 20 Figs. 19 and 20 illustrate the high-density subfraction examined in sections.

FIGURE 19 This general view shows clearly the homogeneity of the population of the vesicles, which are round or oval in shape. $\times 38,000$.

FIGURE 20 At higher magnification, the membranes appear triple-layered (arrows). Sometimes, minute spots are observed. $\times 200,000$.



tained for microsomes isolated from other tissues such as the liver (see Reid (47), for review). The buoyant density of the high-density subfraction is also similar to that of microsomes.

The most significant finding is the high degree of purity and homogeneity of the structures present in this subfraction. In this purified state, these vesicles maintain a high Ca^{++} binding capacity. They are the only vesicles which exhibit such a high uptake. These are new arguments in favor of the hypothesis that these vesicles are derived from the longitudinal SR which is supposed to accumulate calcium during the relaxation process (10, 21).

The 40 A particles which appear to compose the membranes of the thin-walled vesicles are reminiscent of those observed by Ikemoto et al. (29, 30), Inesi and Asai (31), Martonosi (36), and Deamer and Baskin (7).

According to Stoekenius and Engelman (51), the membranes of the SR are composed of a lipid-protein bilayer.

The interpretation of our data that are most consistent with this hypothesis is that the 40 A and 85 A subunits constitute the protein moiety component of the membrane bilayer of the thin-walled and thick-walled vesicles, respectively.

In our experiments, we have thus separated two types of membranes each composed of different subunits: the 85 A particles forming the thick-walled vesicles in the low-density subfraction and the 40 A particles of the thin-walled vesicles in the high-density subfraction.

Moreover, the membranes of the thin-walled vesicles are very similar to those of microsomes. This morphological feature represents a third analogy between the high-density subfraction and a microsomal subfraction.

In the sectioned material, the morphology appears quite similar. The subfraction is composed of round vesicles surrounded by a membrane with a triple-layered structure closely resembling that of microsomes. We never observed the asymmetric structure described by Hasselbach and Elfvin (22), but the techniques used were quite different. We also observed minute spots, 25 A thick, in the membranes, similar to those described by Hasselbach and Elfvin. Weinstein (52) suggests that a substantial amount of lipid and protein are extracted during the fixation procedures for electron microscopy and that many alterations may occur that make these techniques unsuitable for determining the structure of membranes. These

minute spots could then be artifacts of the electron microscopic techniques.

In conclusion, it is suggested, from the morphologic and biochemical data presented, that we successfully separated the two systems which compose the SR in vivo. According to this interpretation, the longitudinal systems, considered as representing the smooth endoplasmic reticulum of the muscle cells, are isolated as thin-walled vesicles in the high-density subfraction. The transverse system, composed in vivo of triads and invaginated tubules of the plasma membrane, is recovered in the low-density subfraction.

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