Biochimica et Biophysica Acta, 389 (1975) 51–68

BBA 76923

ISOLATION AND CHARACTERIZATION OF TWO TYPES OF SARCOPLASMIC RETICULUM VESICLES

GERHARD MEISSNER*

Department of Molecular Biology, Vanderbilt University, Nashville, Tenn. (U.S.A.)

(Received October 11th, 1974)

SUMMARY

A purified preparation of sarcoplasmic reticulum from rabbit skeletal muscle has been found to consist of a heterogeneous population of vesicles. Isopycnic centrifugation was used to obtain "light" and "heavy" vesicles from the upper and lower ends of a 25 to 45 % (w/w) linear sucrose gradient. Each fraction accounted for about 10 to 15 % of the total vesicles. The remainder of the vesicles were of intermediate density and banded between the light and heavy fraction. Light vesicles were composed of about equal amounts of phospholipid and Ca^{2+} pump protein which contained approx. 90 % of the protein. Heavy vesicles contained in addition to the Ca^{2+} pump protein (55–65 % of the protein) two other major protein components, the Ca^{2+} binding and M_{55} proteins which accounted for 20–25 and 5–7 % of the protein of these vesicles, respectively. The sarcoplasmic reticulum subfractions had ^{32}P-labelled phosphoenzyme levels proportional to their Ca^{2+} pump protein content and contained similar Ca^{2+}-stimulated ATPase activities. They were capable of accumulating Ca^{2+} in the presence of ATP and of releasing the accumulated Ca^{2+} when placed into a medium with a low Ca^{2+} concentration. The vesicles differed significantly in that heavy vesicles had a greater number of non-specific Ca^{2+} binding sites than light vesicles (approx. 220 vs 75 nmol of bound Ca^{2+} per mg protein), in accordance with their high content of Ca^{2+} binding protein.

Electron dense material could be seen within the compartment of heavy but not light vesicles. Removal of Ca^{2+} binding and M_{55} proteins from heavy vesicles resulted in empty membranous structures consisting mainly of Ca^{2+} pump protein and phospholipid. Electron micrographs of sections of muscle showed dense material in terminal cisternae but not in longitudinal sections of sarcoplasmic reticulum. These experiments are consistent with the interpretation that (1) the electron dense material inside heavy vesicles may be referable to Ca^{2+} binding and/or M_{55} proteins, and that (2) light and heavy vesicles may be derived from the longitudinal sections and terminal cisternae of sarcoplasmic reticulum, respectively.

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* Present address: Departments of Biochemistry and Physiology, School of Medicine, University of North Carolina, Chapel Hill, N. C. 27514, U.S.A.

INTRODUCTION

Within skeletal muscle cells, sarcoplasmic reticulum forms an extensive membranous network consisting of longitudinal sections and terminal cisternae which surround the myofibrils in a sleevelike fashion [1, 2]. This network is responsible for regulating the Ca^{2+} concentration of the sarcoplasm [3, 4]. During homogenization, sarcoplasmic reticulum is disrupted to form membrane vesicles which can be isolated by differential and sucrose gradient centrifugation [5–10]. In this study, it is shown that purified sarcoplasmic reticulum can further be fractionated into vesicles of differing buoyant density. Two types of sarcoplasmic reticulum vesicles, "light" and "heavy" vesicles, have been isolated from the upper and lower ends of a sucrose gradient and their composition and functional properties have been characterized. A preliminary report of this work has appeared [11].

MATERIALS AND METHODS

Materials

"Ultrapure" grade sucrose from Schwartz/Mann (Orangeburg, N.Y.) was used throughout the experiments. All sucrose concentrations are given as percentage sucrose (w/w) and were determined at 25 °C with the use of a Bausch and Lomb refractometer. The ionophore X537A was kindly donated by Dr Julius Berger (Roche Institute of Molecular Biology, Nutley, N. J.). [γ-^{32}P]ATP was prepared according to Post and Sen [12] and was a generous gift of Dr Robert Post (Department of Physiology, Vanderbilt University).

Assays

Protein was determined by the procedure of Lowry et al. [13] using bovine serum as a standard. Total phosphorus was measured as an estimate of lipid phosphorus [14].

Formation of ^{32}P-labelled phosphoenzyme, Ca^{2+}-stimulated and basic ATPase activities, Ca^{2+} loading (+ oxalate) and Ca^{2+} uptake (− oxalate) capacities were determined similarly as previously described [15]. Ca^{2+}-stimulated ATPase activity was estimated as the difference between total and basic ATPase activity.

Total ATPase activity was measured at 23 °C in a medium containing 20–40 μg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 5 mM Mg^{2+}, 5 mM ATP, 50 μM Ca^{2+}, 40 μM EGTA (ethyleneglycol-bis(β-aminoethylether)-*N*,*N*′-tetraacetic acid), and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.1. The enzyme concentrations used resulted in less than 10 % hydrolysis of ATP. Specific activities were estimated from ATP hydrolysis obtained after 2.5, 5 and 10 min. Basic ATPase activity was measured in the same medium but containing 1 mM EGTA and no added Ca^{2+}. Ca^{2+} loading was carried out for 8 min at 23 °C in a medium containing 5–20 μg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 5 mM Mg^{2+}, 5 mM ATP, 100 μM $^{45}Ca^{2+}$, 5 mM oxalate and 10 mM HEPES (pH 7.1). Ca^{2+} uptake was measured by Millipore filtration [15] or in a dual-wavelength spectrophotometer with murexide as an indicator for free Ca^{2+} [16]. The time resolution of these two methods was 15 and 2 s, respectively. When the Millipore filtration technique was used, the amount of accumulated Ca^{2+} was determined by measurement of

the Ca^{2+} concentration of the complete medium and of the Millipore filtrate by atomic absorption spectroscopy [9]. In both assays, the Ca^{2+} uptake medium contained 200 μg sarcoplasmic reticulum protein per ml, 0.1 M KCl, 5 mM Mg^{2+}, 5 mM ATP, 50 μM $^{45}Ca^{2+}$ and 10 mM HEPES, pH 7.1.

Contamination with other cell organelles was monitored using succinate-cytochrome *c* reductase [17] and monoamine oxidase [18] activities for mitochondrial inner and outer membrane, respectively, glucose-6-phosphatase activity [17] for endoplasmic reticulum, rotenone-insensitive NADH-cytochrome *c* reductase activity [17] for endoplasmic reticulum and mitochondrial outer membrane [19], 5'-nucleotidase activity [20] for sarcolemma, and arylsulfatase activity [21] for lysosomes.

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were purified by zonal centrifugation as previously described [9, 10] except that the homogenization buffer and the sucrose gradient solutions were not supplemented with HEPES buffer. Briefly, 2000 g of ground rabbit muscle (white muscle of leg) was homogenized in 0.3 M sucrose for 45 s in a Waring Blendor. Cell debris, nuclei, mitochondria and myofibrils were removed by centrifugation for 13 min at 8000 rev./min in a Beckman JA 10 rotor (7 020 $\times g$ at R_{av} = 9.8 cm). Purified vesicles were then obtained from the supernatant fraction by two successive sucrose gradient centrifugations using Beckman Ti 15 zonal rotors.

RESULTS

When sarcoplasmic reticulum from rabbit skeletal muscle was purified by rate separation in a Beckman Ti 15 zonal rotor, most of the vesicles were recovered between 25 and 33 % sucrose [9, 10]. Isopycnic centrifugation of these vesicles resulted in a broad band ranging from 25 to 45 % sucrose suggesting that purified sarcoplasmic reticulum consists of a heterogeneous population of vesicles. The total purified sarcoplasmic reticulum present between 25 and 33 % sucrose (w/w) [9, 10] was collected and diluted with water to give about 750 ml of a 23 % sucrose solution. It was placed into a Beckman Ti 15 zonal rotor and a linear sucrose gradient was established as the rotor spun at 4000 rev./min. The gradient had a volume of 900 ml and ranged from 26 to 45 % sucrose. After centrifugation at 28 000 rev./min for 20 h, the sample was unloaded through the center of the rotor by pumping in 55 % sucrose. The first 600–700 ml were discarded. Aliquots of 25 ml were then collected and pooled to form four fractions: up to 28 %, 28–32 % (light sarcoplasmic reticulum vesicles), 32–39 % (intermediate sarcoplasmic reticulum vesicles), and 39–43 % sucrose (heavy sarcoplasmic reticulum vesicles). The fractions were diluted with an equal volume of 1.2 M KCl in 5 mM HEPES, pH 7.4, and kept in ice for about 2 h to remove small amounts of extraneous muscle protein from the vesicles. They were centrifuged at 30 000 rev./min for 90 min in a Beckman 30 rotor. The pellets were resuspended in 0.3 M sucrose/1 mM HEPES, pH 7.4, quick-frozen using liquid N_2 and stored at − 70 °C.

Starting with 2000 g of rabbit skeletal muscle, about 125 to 150 mg protein of both light and heavy vesicles were obtained (Table I). The remainder of the vesicles, representing about 70 % of the total material recovered, was present at a density

TABLE I

YIELD AND PHOSPHOLIPID CONTENT OF SARCOPLASMIC RETICULUM VESICLES

	Buoyant density (% sucrose)	Bound phosphorus* (μmol P/mg protein)	Yield** (mg protein/ 2000 g muscle)
Crude sarcoplasmic reticulum vesicles[a]	(25–45)	–	4000±1000
Purified sarcoplasmic reticulum vesicles[b]	(25–45)	0.8	1500± 300
Light sarcoplasmic reticulum vesicles[c]	28–32	1.15–0.95	125± 50
Intermediate sarcoplasmic reticulum vesicles[c]	32–39	0.95–0.65	750± 200
Heavy sarcoplasmic reticulum vesicles[c]	39–43	0.65–0.45	150± 50

[a–c] Sarcoplasmic reticulum fractions were obtained from first, second and third zonal centrifugation, respectively.

* Practically all bound phosphorus is present in phospholipid [14].

** Mean of four preparations ±S.E.

intermediate between that of light and heavy vesicles. The material present at 24–28 % sucrose represented about 1 % of the vesicles. It was slightly contaminated with other cell organelles and was therefore generally discarded. In order to determine whether equilibrium was achieved during centrifugation, each of the KCl-washed vesicle fractions was placed on a 25 to 45 % linear sucrose gradient and recentrifuged for 12 and 24 h at 38 000 rev./min in a Beckman SW 41 rotor. The sedimentation pattern of the vesicles corresponded to the one obtained using the zonal rotor indicating that the vesicles had indeed different buoyant densities. The differences in buoyant densities seemed to reflect differences in the phospholipid to protein ratio of the vesicles (Table I). From the amount of bound phosphorus [14], it can be calculated that

TABLE II

"MARKER ENZYME" ANALYSIS OF SARCOPLASMIC RETICULUM SUBFRACTIONS

Activities are expressed as μmol substrate hydrolyzed or reduced/mg protein per min. The material sedimenting in the layer of 24–28 % (w/w) sucrose represents about 1 % of the total protein of the gradient and is generally discarded because it is slightly contaminated with other cell organelles such as plasma membrane and endoplasmic reticulum. The data are the average of four determinations ±S.E.

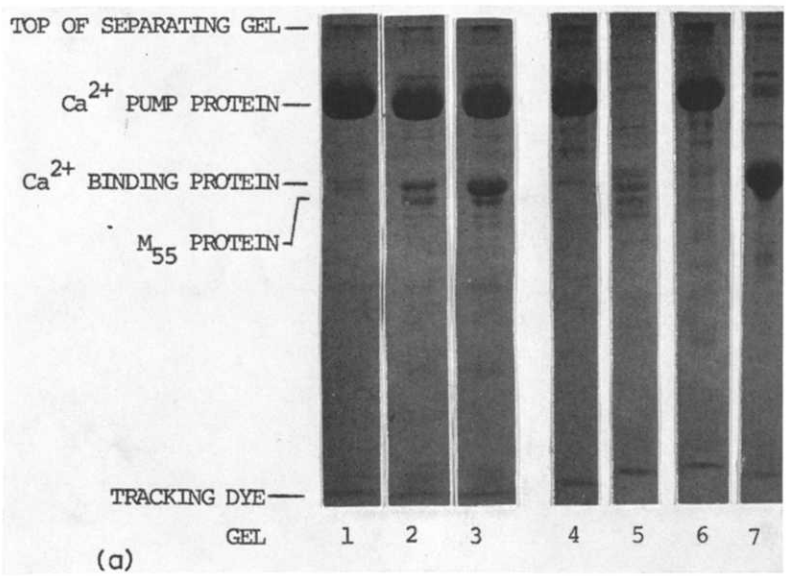
Fraction	Buoyant density (% sucrose)	^{32}P-labelled phosphoenzyme (nmol ^{32}P/mg protein)	Glucose-6-phosphatase	5′-nucleotidase	Succinate-cytochrome *c* reductase
	24–28	6.4	0.007±0.003	0.015±0.004	0.003±0.002
Light sarcoplasmic reticulum vesicles	28–32	7.4	0.005±0.002	0.002±0.001	<0.001
Intermediate sarcoplasmic reticulum vesicles	32–39	6.5	0.005±0.002	0.001±0.001	<0.001
Heavy sarcoplasmic reticulum vesicles	39–43	5.0	0.005±0.002	0.001±0.001	0.001±0.001

phospholipid accounted for about 45 and 30 % of the weight of light and heavy vesicles, respectively. The different buoyant densities do not appear to be caused by the presence of Ca^{2+} or Mg^{2+}, since these cations accounted for less than 0.2 and 0.5 % of the total mass of light and heavy vesicles, respectively, as isolated from the zonal gradient. KCl washing lowered the amounts of Ca^{2+} and Mg^{2+} by a factor of approx. 2.

The three sarcoplasmic reticulum subfractions had low specific activities of marker enzymes for endoplasmic reticulum, sarcolemma, and inner mitochondrial membrane (Table II). Arylsulfatase (lysosomes) and monoamine oxidase (outer mitochondrial membrane) activities were less than 0.001 μmol/mg protein per min (not shown). Electron microscopy studies indicated that the original and derived three sarcoplasmic reticulum subfractions were practically free of mitochondria, lysosomes and sarcolemma (cf. Fig. 5). When the sucrose gradient solutions contained 5 mM HEPES (pH 7.4), the phosphoenzyme level of the material banding between 24 and 27 % sucrose was lowered from 6.4 to 3.5 nmol ^{32}P/mg protein. At the same time, noticeable amounts of endoplasmic reticulum and sarcolemma were found at the upper end of the gradient. Glucose-6-phosphatase and rotenone-insensitive NADH-cytochrome *c* reductase activities were 0.03 and 0.1 μmol/mg protein per min, respectively, and 5'-nucleotidase activity was 0.06 μmol/mg protein per min. Because of the lack of availability of purified cell organelles from muscle, their presence in sarcoplasmic reticulum can be assessed only qualitatively by the variations in the specific activities of the marker enzymes.

As previously reported [9], only three major protein bands are discernible on acid and sodium dodecylsulfate/polyacrylamide gels of purified sarcoplasmic reticulum vesicles which have been prepared by zonal centrifugation and have been washed with KCl. The preparation was largely free of extraneous muscle proteins such as myosin (mol wt ≈ 225 000), actin (mol wt ≈ 45 000) and phosphorylase (mol wt ≈ 95 000) as indicated by the absence of major protein bands on the gels which correspond to the molecular weights of these compounds. The protein composition of the three vesicle subfractions differed with respect to the distribution of the three major proteins of sarcoplasmic reticulum as seen on sodium dodecylsulfate/polyacrylamide gels (Fig. 1). Light vesicles (Gel 1) contained only one major protein, the Ca^{2+} pump protein (mol wt ≈ 105 000), which accounted for approx. 90 % of the total protein as judged from the densitometry tracing in Fig. 1. The gels of intermediate and heavy vesicles showed two additional major bands, labelled Ca^{2+} binding protein (mol wt ≈ 65 000) and M_{55} protein (mol wt ≈ 55 000) (Gels 2 and 3). In heavy vesicles, Ca^{2+} pump, Ca^{2+} binding and M_{55} proteins accounted for about 55–65, 20–25 and 5–7 % of the area of the densitometry tracing, respectively.

Several of the characteristic enzymatic properties of sarcoplasmic reticulum are summarized in Table III. The three sarcoplasmic reticulum subfractions contained a Ca^{2+}-stimulated ATPase and were capable of accumulating Ca^{2+} in the presence of ATP. It may be noted that the Ca^{2+}-stimulated ATPase activities were not optimal due to partial inhibition by the accumulated Ca^{2+}. The presence of detergents [14] resulted in a several-fold increase in activity (not shown). The basic ATPase activities contributed only little to the total ATPase activities of the three fractions. It was found that Ca^{2+} uptake reached maximal levels within 2 s in both light and heavy vesicles when measured spectroscopically with the use of a dual-wavelength



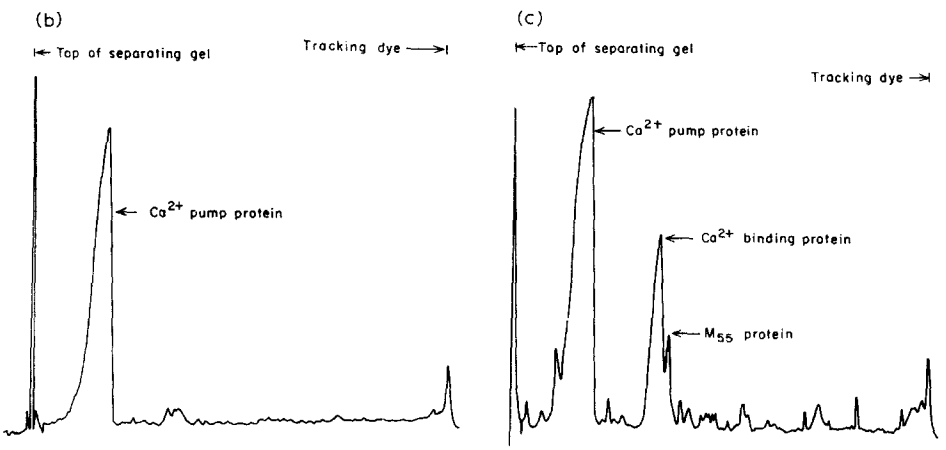


Fig. 1. Separation of sarcoplasmic reticulum proteins by sodium dodecylsulfate/polyacrylamide gel electrophoresis. Gels containing 11 and 5 % acrylamide in the separating and stacking gel, respectively, were prepared and run as described by Laemmli [22] except that gels contained 4 M urea and 1 mM EDTA and the electrophoresis buffer had a glycine concentration of 0.096 M. Samples (40 μg protein in Gels 1–4, 6, 7; 10 μg protein in Gel 5) were reduced with 2.5 % 2-mercaptoethanol by heating for 4 min at 100 °C prior to gel electrophoresis. Gels were stained with 1 % Amido Schwartz [9]. Ca^{2+} pump and Ca^{2+} binding proteins were previously identified by electrophoresing them together with the two isolated proteins [9]. M_{55} protein has an apparent molecular weight of 55 000 on the sodium dodecylsulfate-polyacrylamide gels [9]. The densitometry tracings [14] of Gels 1 (left side) and 3 (right side) are also shown. Gel 1, light sarcoplasmic reticulum vesicles; Gel 2, intermediate sarcoplasmic reticulum vesicles; Gel 3, heavy sarcoplasmic reticulum vesicles; Gels 4 and 5, membranous fraction and soluble extract of light sarcoplasmic reticulum vesicles, respectively (cf. Table IV); Gels 6 and 7, membranous fraction and soluble extract of heavy sarcoplasmic reticulum vesicles, respectively (cf. Table IV).

TABLE III

PROPERTIES OF SARCOPLASMIC RETICULUM VESICLES

Values represent the mean of four determinations ±S.E.

	Sarcoplasmic reticulum vesicles		
	Light	Intermediate	Heavy
^{32}P-labelled phosphoenzyme (nmol ^{32}P/mg protein)*	7.4 ± 0.5	6.5 ± 0.5	5.0 ± 0.5
ATPase activity (μmol P_i/mg protein per min)**			
with 50 μM Ca^{2+} +40 μM EGTA	1.0 ± 0.3	0.9 ± 0.3	1.3 ± 0.3
with 1 mM EGTA	0.03 ± 0.02	0.02 ± 0.01	0.02 ± 0.01
Ca^{2+} uptake capacity (nmol Ca^{2+}/mg protein)***	90 ±15	120 ±20	120 ±25
Ca^{2+} loading capacity (μmol Ca^{2+}/mg protein)***	12 ± 2	6 ± 1.5	2.5 ± 1
Ca^{2+} loading rate			
(μmol Ca^{2+}/mg protein per min)***	3–6	–	1–2

* ^{32}P-labelled phosphoenzyme level of total muscle homogenate of rabbit (white muscle of leg) is 0.18 nmol ^{32}P/mg protein.

** Ca^{2+}-stimulated ATPase activity is the difference of total ATPase activity (measured in the presence of 50 μM Ca^{2+} +40 μM EGTA) and basic ATPase activity (measured in the presence of 1 mM EGTA) and was determined in the absence of oxalate.

*** Ca^{2+} uptake and Ca^{2+} loading were measured in the absence and presence of 5 mM oxalate, respectively.

spectrophotometer (not shown).When energized Ca^{2+} accumulation was measured in the presence of oxalate, precipitates of Calcium oxalate formed inside the vesicles resulting in the accumulation of large amounts of Ca^{2+} [23]. Light vesicles accumulated Ca^{2+} more rapidly in the presence of oxalate and sequestered three to four times as much Ca^{2+} as heavy vesicles (Table III).

Sarcoplasmic reticulum membranes form a phosphorylated intermediate during Ca^{2+} pump activity [24–26]. The highest level of this intermediate under steady-state conditions was found in light vesicles, in accordance with their high content of Ca^{2+} pump protein (Table III). Using a molecular weight of 105 000 and one phosphorylation site per molecule [9, 27], one can calculate that the Ca^{2+} pump protein accounted for about 80 and 55 % of the total protein of light and heavy vesicles, respectively. These data are in agreement with the densitometry tracings of gels which indicated a Ca^{2+} pump protein content of about 90 and 60 %, respectively (cf. Fig. 1).

Previous binding studies have shown that sarcoplasmic reticulum vesicles contain specific and non-specific Ca^{2+} binding sites [9, 28–30]. In this study the binding of Ca^{2+} by light, heavy and purified Ca^{2+} pump protein vesicles was investigated by equilibrium dialysis. The actual binding of Ca^{2+} to a binding site rather than the trapping of Ca^{2+} within a compartment was measured by omitting ATP from the medium. Otherwise, an ionic environment similar to that of the sarcoplasm was used, i.e. the dialysis buffer contained 100 mM KCl and 1 mM $MgCl_2$ at pH 6.8. Fig. 2 illustrates that at low free Ca^{2+} concentrations (approx. 10 μM), all three preparations bound 10–15 nmol of Ca^{2+} per mg protein, in agreement with a recent study which showed that there are two specific, high-affinity Ca^{2+} binding sites per phosphorylation site in sarcoplasmic reticulum [27]. Under the above conditions

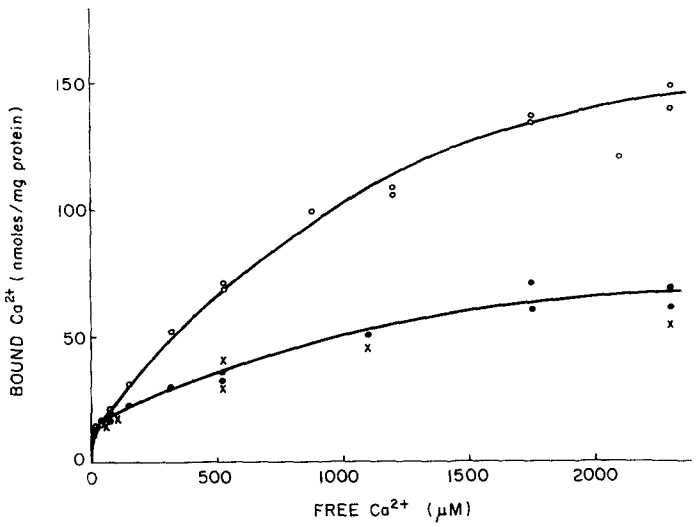


Fig. 2. Ca^{2+} binding by light and heavy sarcoplasmic reticulum vesicles and a purified Ca^{2+} pump protein preparation. The Ca^{2+} pump protein was purified by partial extraction of sarcoplasmic reticulum vesicles with deoxycholate [9] and contained 0.8 μmol bound P per mg protein. Ca^{2+} binding was measured by equilibrium dialysis as previously described [9]. Samples (2 mg protein in 0.4 ml) were dialyzed against a 2.5 mM maleate/5 mM Tris buffer (pH 6.8 at 4 °C) containing 100 mM KCl, 1 mM $MgCl_2$ and varying concentrations of Ca^{2+}. ○–○, heavy sarcoplasmic reticulum vesicles; ●–●, light sarcoplasmic reticulum vesicles; ×–×, Ca^{2+} pump protein preparation.

little non-specific Ca^{2+} binding occurred [9, 27]. However, at higher Ca^{2+} concentrations, Ca^{2+} effectively competed for the non-specific binding sites (Fig. 2). Heavy vesicles bound greater amounts of Ca^{2+} than both the light and purified Ca^{2+} pump

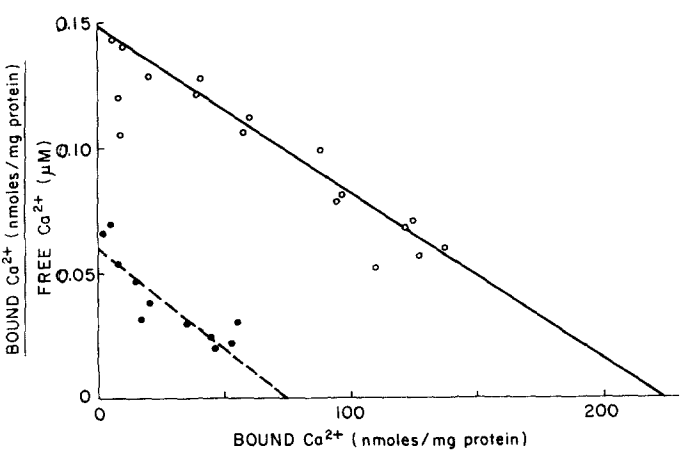


Fig. 3. Scatchard plots of non-specific Ca^{2+} binding by light and heavy sarcoplasmic reticulum vesicles. Data are obtained from Fig. 2. The amount of Ca^{2+} which binds specifically to the vesicles (15 and 11 nmol of Ca^{2+}/mg protein of light and heavy vesicles, respectively) is subtracted from the amount of total bound Ca^{2+}. The corrected data are then rearranged in the form of Scatchard plots. ○–○, heavy sarcoplasmic reticulum vesicles; ●–●, light sarcoplasmic reticulum vesicles.

protein vesicles at free Ca^{2+} concentrations of 0.25–2.5 mM. Further, it was found that an aqueous microdispersion of sarcoplasmic reticulum phospholipid (5 μmol P/ml) [31] bound less than 5 % of the Ca^{2+} present in the sample volume. This would indicate that most of the Ca^{2+} is bound by the protein and not by the phospholipid components of the vesicles.

Scatchard plot analysis of Ca^{2+} binding by the vesicles is shown in Fig. 3. The amount of Ca^{2+} which bound with high affinity and specificity to the vesicles (15 and 11 nmol of Ca^{2+} per mg protein of light and heavy vesicles, respectively) was subtracted from the total amount of bound Ca^{2+}. For heavy vesicles a straight line was obtained suggesting that only one major type of Ca^{2+} binding site remained. From the intercepts with the abscissa and the slopes, it can be calculated that heavy vesicles bound 220 nmol of Ca^{2+} per mg protein, with an apparent binding constant of approx. $0.7 \cdot 10^3$ M^{-1}. The data obtained with light vesicles are less clear. However, it appears that light vesicles contained an appreciably smaller number of non-specific binding sites than heavy vesicles. Their number has been estimated to range from 50 to 100 nmol bound Ca^{2+}/mg protein. The original mixture of purified sarcoplasmic reticulum vesicles averaged 90–100 nmol of non-specific Ca^{2+} binding sites per mg protein [9].

One of the least understood steps in muscle contraction is the mode of release of Ca^{2+} by sarcoplasmic reticulum. Light and heavy vesicles have been used to test the influence of Ca^{2+} binding and M_{55} proteins on Ca^{2+} release. Vesicles were filled with $^{45}Ca^{2+}$ using 1.5 mM acetylphosphate and approx. 50 μM $^{45}Ca^{2+}$ (Fig. 4). Release of Ca^{2+} was achieved by diluting the vesicles 30-fold into a medium of similar

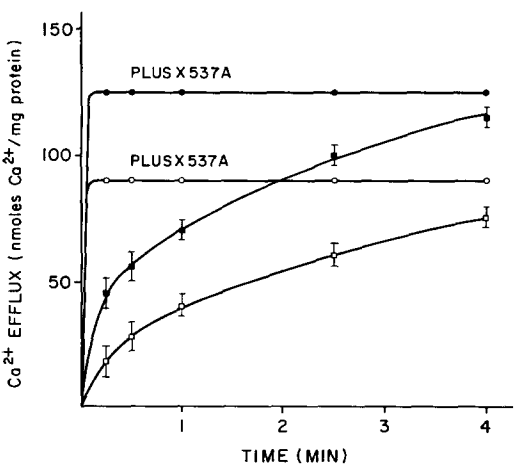
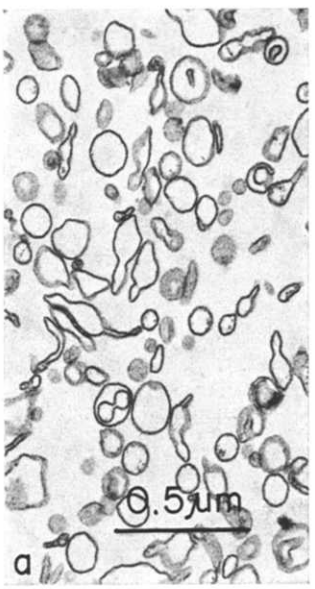
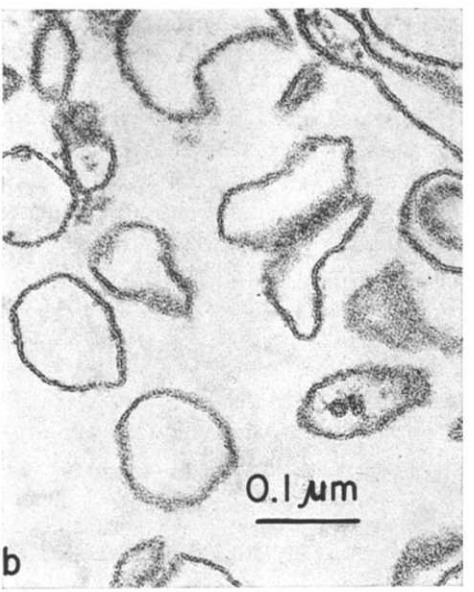
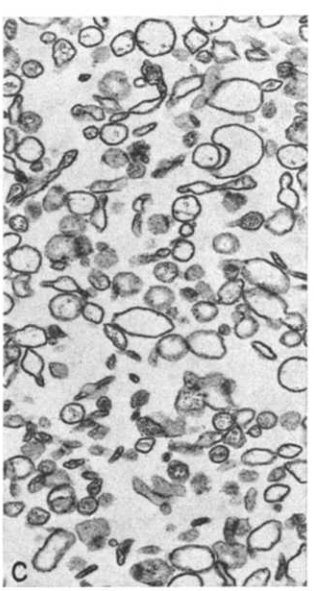


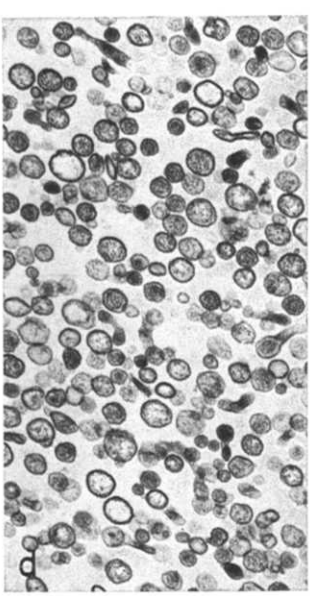
Fig. 4. Release of Ca^{2+} from light and heavy sarcoplasmic reticulum vesicles. The vesicles were filled with $^{45}Ca^{2+}$ at 23 °C in a 30 mM maleate/60 mM Tris buffer (pH 6.95) containing 400–500 μg sarcoplasmic reticulum protein, 50 μM $^{45}Ca^{2+}$, 150 mM KCl, 2 mM $MgCl_2$ and 1.5 mM acetylphosphate. The efflux of the accumulated $^{45}Ca^{2+}$ was measured by diluting the samples with 30 vol. of 30 mM maleate/60 mM Tris (pH 6.9), 150 mM KCl, 5 mM $MgCl_2$, and 3 mM EGTA. In controls the ionophore X537A (30 μg/ml) was added to the release medium. The amount of accumulated and released $^{45}Ca^{2+}$ was followed by Millipore filtration. Total radioactivity and radioactivity of the Millipore filtrates were measured. □–□ and ○–○, light vesicles without and with X537A in release medium, respectively; ■–■ and ●–●, heavy vesicles without and with X537A in release medium, respectively.

composition but with EGTA and no acetylphosphate. As indicated in Fig. 4, both types of vesicles released the accumulated Ca^{2+} rather slowly when compared with the Ca^{2+} uptake and Ca^{2+} loading rates of the vesicles. Heavy vesicles released a greater amount of Ca^{2+} somewhat more rapidly than light vesicles. In 4 min, more than 80 % of the Ca^{2+} was released in both types of vesicles. Practically all of the accumulated $^{45}Ca^{2+}$ was available for rapid efflux since $^{45}Ca^{2+}$ was fully released within 10–15 s when the release medium contained the ionophore X537A (30 μg/ml). Addition of 1 mM ATP or 10 mM caffeine to the release medium did not appreciably









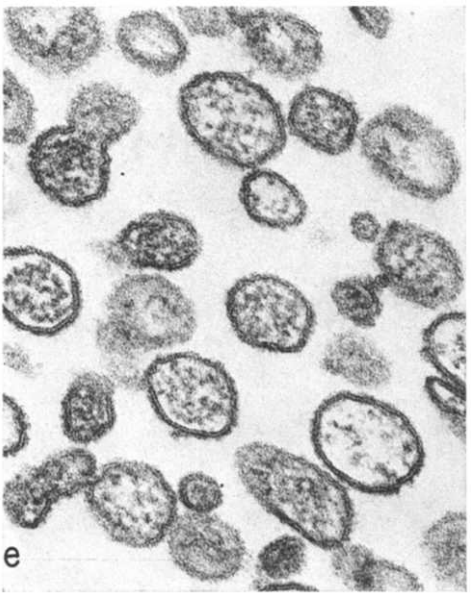


Fig. 5. Electron micrographs of sarcoplasmic reticulum vesicles. Samples were first fixed for 2 h at 0 °C in a solution of 2.5 % glutaraldehyde (0.25 M sucrose, 0.1 M cacodylate, pH 7.2) followed by a second fixation in a solution of 1 % OsO_4 (2.4 mM $CaCl_2$, 120 mM NaCl, 28 mM veronal-acetate, pH 7.2). They were then further processed for electron microscopy as previously described [14]. Representive electron micrographs are shown. Pellets were sectioned throughout and found to be homogeneous. Dense material may be noted within the compartment of heavy vesicles. (a) Light sarcoplasmic reticulum vesicles (×35 000). (b) Light sarcoplasmic reticulum vesicles (×140 000). (c) Intermediate sarcoplasmic reticulum vesicles (×35 000). (d) Heavy sarcoplasmic reticulum vesicles (×35 000). (e) Heavy sacroplasmic reticulum vesicles (×140 000).

change the Ca^{2+} efflux rates of light and heavy vesicles. Caffeine has been found to cause muscle contraction [32] and to release Ca^{2+} from a sarcoplasmic reticulum fraction [33].

Typical electron micrographs of the three sarcoplasmic reticulum subfractions are shown in Fig. 5. In all three cases closed membranous vesicles were found to be present. Light vesicles have a somewhat larger average size than heavy vesicles. More important, light vesicles appear as empty sacs while heavy vesicles are filled with electron dense material. The fraction which banded in the middle of the gradient (32–39 % sucrose) appears to be composed of light and heavy vesicles as well as vesicles which contain intermediate amounts of electron dense material (Fig. 5c). In an attempt to define the nature of the electron dense material inside heavy vesicles, sarcoplasmic reticulum vesicles were repeatedly extracted with a 10 mM Tris · HCl buffer, pH 8.5, containing 1 mM EDTA [34]. About 90 % of the material of light and heavy vesicles could be recovered in a particulate and soluble fraction (Table IV). Considerably larger amounts could be extracted from heavy vesicles. Electron microscopy studies indicated that the two particulate fractions contained membranous structures which were free of electron dense material (Fig. 6). Gel electrophoresis (Fig. 1) and chemical analysis showed that these membranes were composed mainly of Ca^{2+} pump protein and phospholipid. Interestingly, membranes derived from

TABLE IV

FRACTIONATION OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES INTO A SOLUBLE AND MEMBRANOUS FRACTION

Light and heavy vesicles (20 mg protein each) were extracted four times with 15 ml of a 10 mM Tris · HCl buffer, pH 8.5, containing 1 mM EDTA. The suspensions were centrifuged in a Beckman 65 rotor at 45 000 rev./min for 45 min. The final pellet was resuspended in the above Tris buffer at a protein concentration of 10 mg/ml. The supernatants of the four extracts were pooled and concentrated to a volume of about 1 ml using an Amicon Diaflow apparatus equipped with a PM 30 membrane. The concentrated soluble extracts, the membranous fractions and untreated light and heavy vesicles (5 mg protein each in 0.5 ml) were dialyzed for 48 h against a 0.4 mM HEPES buffer, pH 7.4. The derived fractions were then analyzed by electron microscopy (cf. Fig. 6) and gel electrophoresis (cf. Fig. 1). The Lowry et al. [13] and Biuret procedures were used to estimate protein with bovine serum albumin as a standard. Both procedures gave similar results. Prior to protein determination, samples were solubilized by heating them for 5 min at 100 °C in a small volume of 0.1 M NaOH, 2.5 % Na_2CO_3 and 0.5 % sodium dodecylsulfate. Total phosphorus was determined as an estimate of lipid phosphorus and phospholipid content [14]; it was found that there are 26 μg phospholipid per 1 μg P. The dry weights of the samples were obtained using a Cahn Electro Balance after drying in vacuo over $CaCl_2$. Some material has been lost during the handling of the samples. Recovery of about equal amounts of dry weight, protein and phospholipid suggests that no components have been lost selectively.

Fraction	Dry weight (mg)	Protein (mg)	Phospholipid (mg)	Lipid Phosphorus / Protein (μmol P/mg protein)
Light sarcoplasmic reticulum vesicles				
Untreated vesicles	48.7	20.0	17.8	1.10
Membranous fraction	43.9	17.6	15.5	1.09
Soluble extract	1.4	0.1	0.18	0.23
Heavy sarcoplasmic reticulum vesicles				
Untreated vesicles	34.0	20.0	8.4	0.52
Membranous fraction	25.1	12.9	7.2	0.69
Soluble extract	7.2	5.1	0.1	0.02

light vesicles had a higher phospholipid content than those obtained from heavy vesicles. Ca^{2+} binding and M_{55} proteins were found to be the major protein constituents of the soluble extract of heavy vesicles. These two proteins accounted for approx. 25 % of the protein of these vesicles (Table IV). These results suggest that the electron dense material inside heavy vesicles is likely referable to Ca^{2+} binding and/or M_{55} proteins.

Electron micrographs of intact rabbit skeletal muscle have been taken in an attempt to correlate the morphology of light and heavy vesicles with specific sections of the sarcoplasmic reticulum structure (Fig. 7). Longitudinal sections of sarcoplasmic reticulum and triads consisting of transverse tubules of the sarcolemma and terminal cisternae of sarcoplasmic reticulum are apparent. The terminal cisternae contain electron dense material while the longitudinal sections appear empty. These studies indicate that longitudinal sections and terminal cisternae may give rise to the light and heavy vesicles, respectively.

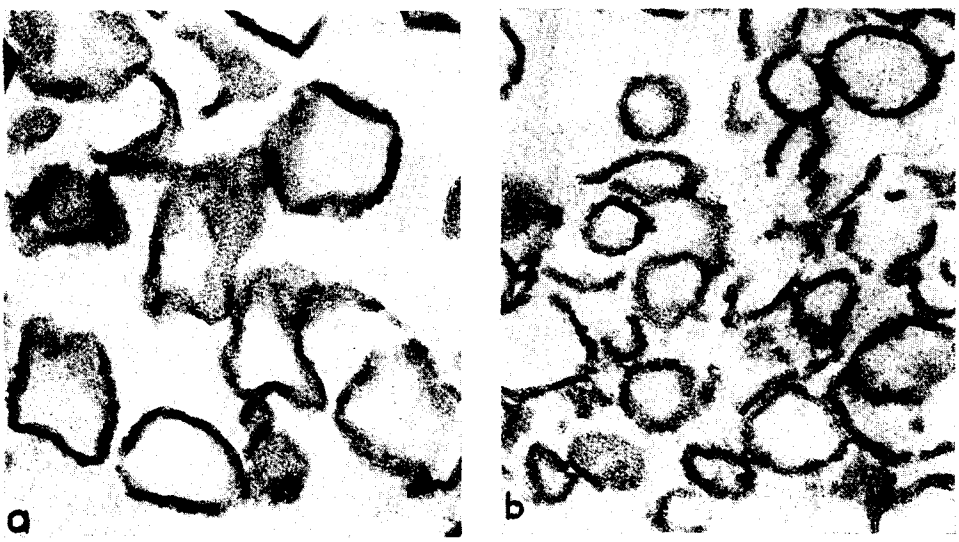


Fig. 6. Electron micrographs of membranous fraction of light and heavy sarcoplasmic reticulum vesicles. The membranous fraction of the vesicles was prepared as described in the legend of Table IV and was then processed for electron microscopy as described in the legend of Fig. 5. (a) Membranous fraction of light vesicles. (b) Membranous fraction of heavy vesicles. Magnification, 140 000×.

DISCUSSION

Earlier we described the isolation of a purified sarcoplasmic reticulum preparation [9, 10]. The vesicles were composed of about 60 % protein and 40 % lipid of which greater than 95 % was phospholipid. Three major protein components were observed, the Ca^{2+} pump, Ca^{2+} binding and M_{55} proteins which accounted for about 70 %, 5–10 % and 5–10 % of the protein, respectively.

In this study it is shown that purified sarcoplasmic reticulum can be further resolved into vesicles of differing buoyant density. Light, intermediate, and heavy vesicles have been isolated from a sucrose gradient and characterized. The three types of vesicles differ in composition, morphology and functional properties. Sodium dodecylsulfate/polyacrylamide gel electrophoresis indicates that these differences may be attributed to variable amounts of the three major proteins in sarcoplasmic reticulum. Light vesicles have a density between 1.12 and 1.14 and are mainly composed of the Ca^{2+} pump protein (approx. 90 % of the total protein) and phospholipid. The density of heavy vesicles is 1.17 to 1.20. In addition to the Ca^{2+} pump protein (approx. 60 % of the protein), heavy vesicles contain two additional major proteins, the Ca^{2+} binding and M_{55} proteins (mol. wt of 55 000) which account for 20-25 % and 5–7 % of the protein of these vesicles, respectively. Intermediate vesicles isolated from the middle of the sucrose gradient have a Ca^{2+} binding and M_{55} protein content between that of light and heavy vesicles.

Differential and sucrose gradient centrifugation has been used earlier to fractionate a "microsomal" fraction from a homogenate of skeletal muscle [5–8, 33]. However, it is not clear from the data reported whether purified sarcoplasmic reticulum subfractions were obtained. Heuson-Stiennon et al. [35] used sucrose gradient

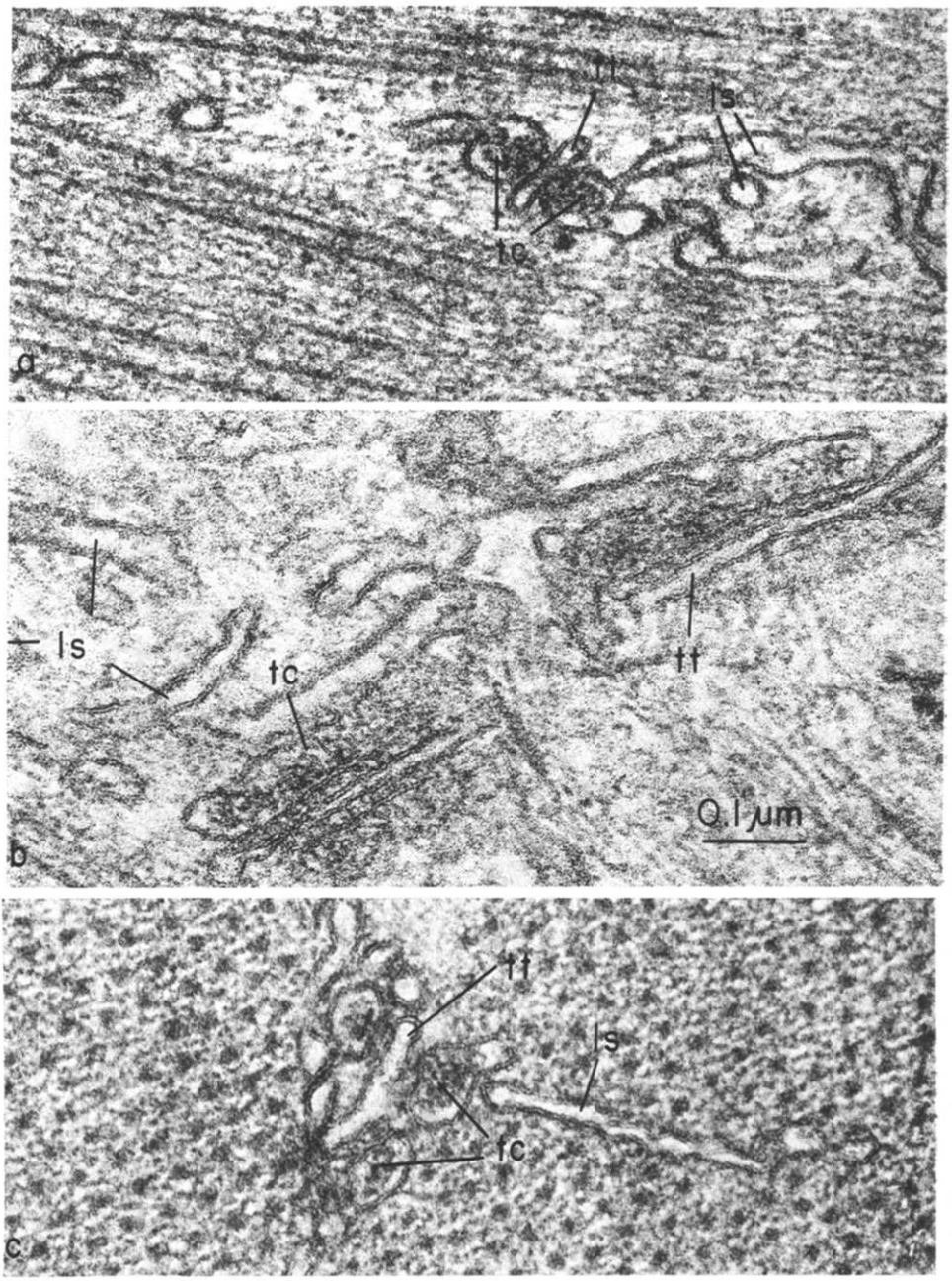


Fig. 7. Electron micrographs of white muscle of rabbit leg. Thin slices of muscle tissue were fixed overnight at 0 °C in a solution of 2.5 % glutaraldehyde (0.25 M sucrose, 0.1 M cacodylate, pH 7.2) and then further processed as described in Fig. 5. The sections have varying orientations with respect to the myofibrils. Triads consisting of two terminal cisternae of sarcoplasmic reticulum (tc) and a centrally located transverse tubule (tt) are seen. It may be noted that the terminal cisternae contain electron dense material as has been observed in heavy vesicles (cf. Fig. 5e). Longitudinal sections of sarcoplasmic reticulum (ls) appear to be devoid of electron dense material as has been found in light vesicles (cf. Fig. 5b). Magnification, 140 000 × .

centrifugation to obtain two membranous fractions from a muscle homogenate which banded at densities of 1.10 and 1.12, and 1.13 to 1.17. Only the high-density fraction was capable of energized Ca^{2+} accumulation. From their morphological data, Heuson-Stiennon et al. [35] concluded that the low-density fraction was enriched with transverse tubules and sarcolemma, while the high-density fraction represented sarcoplasmic reticulum. The zonal separation described in this study indicates subfractionation of a heterogeneous purified sarcoplasmic reticulum preparation rather than removal of contaminating membranes. The three subfractions which represented about 99 % of the recovered material of a purified sarcoplasmic reticulum preparation (cf. Table I) were capable of Ca^{2+} accumulation and storage. The accumulated Ca^{2+} could be released by placing the vesicles into a medium of low Ca^{2+} concentration.

When sarcoplasmic reticulum vesicles were isolated in the presence of 5 mM HEPES, appreciable glucose-6-phosphatase and rotenone-insensitive NADH cytochrome *c* reductase activities were detected at the upper part of the sucrose gradient. Although their localization is not well established in muscle, it is reasonable to expect that they reside in endoplasmic reticulum as they do in liver [36]. Most of the sarcoplasmic reticulum equilibrated at a higher buoyant density where little glucose-6-phosphatase and rotenone-insensitive NADH cytochrome *c* reductase activities were present. Since endoplasmic and sarcoplasmic reticulum activities could be separated on a sucrose gradient, it appears that they may form distinct membrane structures in skeletal muscle cells.

One interpretation of the morphological data (Figs 5 and 7) is that light and heavy vesicles are derived from the longitudinal sections and terminal cisternae of sarcoplasmic reticulum, respectively. Heavy vesicles and terminal cisternae contain electron dense material, while light vesicles and longitudinal sections appear empty. The proportion of vesicles obtained, however, probably does not reflect the content of longitudinal sections and terminal cisternae since only a fraction (approx. 10–20 %) of the sarcoplasmic reticulum present in muscle was recovered from the homogenate. Also, light and heavy vesicles did not form well defined bands on the sucrose gradient. Rather they were isolated from the upper and lower ends of a broad band. Further, vesicles with intermediate amounts of electron dense material appeared to be present in the middle of the gradient (Fig. 5c). This raises the possibility that part of the Ca^{2+} binding and M_{55} proteins were lost during disruption of the sarcoplasmic reticulum structure or that sarcoplasmic reticulum contains portions with gradually changing concentrations of these two proteins.

After removal of Ca^{2+} binding and M_{55} proteins, the membranous fractions of light and heavy vesicles contained different phospholipid contents (cf. Table IV). The protein was mainly referable to Ca^{2+} pump protein. It follows that the membranes of light and heavy vesicles contained approx. 110 and 70 molecules of phospholipid per Ca^{2+} pump protein molecule, respectively. This difference in phospholipid content supports that the vesicles were derived from different sections of the sarcoplasmic reticulum structure.

It has been found that Ca^{2+} binding and M_{55} proteins are released from the vesicles under conditions which largely increase the permeability of sarcoplasmic reticulum vesicles such as the use of EDTA or EGTA at elevated pH [34], the use of low concentrations of detergent [9, 37], or phospholipase treatment [31]. Also,

both proteins are water soluble, have a low tendency to rebind to the membrane [15, 38, 39], and are protected from tryptic digestion [40]. These earlier data suggest the Ca^{2+} binding and M_{55} proteins are localized inside the vesicles. Some evidence against an internal location of the Ca^{2+} binding protein has been presented by Thorley-Lawson and Green [41]. These authors found that the Ca^{2+} binding protein is iodinated by lactoperoxidase suggesting that it is at least partially located on the outer surface of the membrane. We have found that the amorphous material seen inside intermediate and heavy vesicles (cf. Figs 5c–5e) corresponds to the Ca^{2+} binding and M_{55} proteins. Further, it appears that these proteins are not an integral part of the membrane, but are rather trapped inside the vesicles.

One approach used in the past to decipher the function of the individual components in sarcoplasmic reticulum involved their solubilization, purification and reconstitution into functional vesicles [15, 39, 42] or liposomes [38, 43, 44]. Reconstituted vesicles prepared from a purified Ca^{2+} pump protein preparation have a composition practically identical to that of light vesicle but do not accumulate Ca^{2+} as efficiently in the presence of ATP. The availability of light and heavy vesicles opens another avenue to the study of the role of the three major proteins in sarcoplasmic reticulum function. The two types of vesicles may be obtained with relative ease in reasonable amounts while avoiding detergent treatments. A comparison of the enzymatic data shows that under the experimental conditions used, Ca^{2+} transport and release were largely independent of Ca^{2+} binding and M_{55} proteins. It is difficult at present to evaluate the significance of the somewhat higher ATPase activity. Ca^{2+} uptake capacity and Ca^{2+} release rate of heavy vesicles. An increased ATPase activity and Ca^{2+} release rate generally point to a "leaky" membrane [15, 34, 45]. A more permeable membrane would also explain why heavy vesicles had a lower Ca^{2+} uptake capacity than one would have expected from their high number of non-specific Ca^{2+} binding sites. For reasons we do not understand well at present, light vesicles had a much higher Ca^{2+} loading capacity than heavy vesicles. It may be that because of the somewhat larger size of light vesicles and the absence of the Ca^{2+} binding and M_{55} proteins, there is more space available inside these vesicles. The vesicles differed significantly in that heavy vesicles had a greater number of non-specific Ca^{2+} binding sites than light vesicles. Since the Ca^{2+} binding protein binds non-specifically 900–1000 nmol of Ca^{2+} per mg protein [9, 37, 46], practically all of the additional Ca^{2+} binding sites of heavy vesicles may be accounted for by this protein. The Ca^{2+} binding protein, although not essential for sarcoplasmic reticulum function, may then allow sarcoplasmic reticulum to store the amounts of Ca^{2+} necessary to initiate muscle contraction.

Such a role for the Ca^{2+} binding protein would support a suggestion of Winegrad [47] who observed that the longitudinal sections and terminal cisternae may represent the "relaxation" and "release" sites of sarcoplasmic reticulum. Accordingly during the contraction-relaxation cycle of muscle, the released Ca^{2+} would be preferentially taken up by the longitudinal sections as represented by the light vesicles. The presence of the Ca^{2+} binding protein in terminal cisternae (heavy vesicles) would then act as a sink for the Ca^{2+} accumulated in the longitudinal sections. Part of the Ca^{2+} would therefore slowly diffuse to the terminal cisternae where it would then be available again for release to initiate muscle contraction.

ACKNOWLEDGEMENTS

I wish to thank Dr Sidney Fleischer for his interest and helpful suggestions, and Mrs Connie Turvy for excellent technical assistance. The skilled electron microscopy was performed by Mr. Akitsugu Saito. This investigation was supported by grants of the American Heart Association and Muscular Dystrophy Association of America. The work was done during the tenure of an Established Investigatorship of the American Heart Association to the author.

REFERENCES

1 Peachey, L. D. (1965) J. Cell Biol. 25, 209–231
2 Porter, K. R. (1961) J. Biophys. Biochem. Cytol. 10, Suppl. 219–226
3 Martonosi, A. (1971) in Biomembranes (Manson, L. A., ed.), Vol. 1, pp. 191–256, Plenum Press, New York
4 Inesi, G. (1972) Ann. Rev. Biophys. Bioeng. 1, 191–210
5 Ebashi, S. and Lipmann, F. (1962) J. Cell Biol. 14, 389–400
6 Hasselbach, W. and Makinose, M. (1963) Biochem. Z. 339, 94–111
7 Seraydarian, K. and Mommaerts, W. F. H. M. (1965) J. Cell Biol. 26, 641–656
8 Yu, B. P., DeMartinis, F. D. and Masoro, E. J. (1968) Anal. Biochem. 24, 523–530
9 Meissner, G., Conner, G. E. and Fleischer, S. (1973) Biochim. Biophys. Acta 298, 246–269
10 Meissner, G. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds), Vol. 31, pp. 238–246, Academic Press, New York
11 Meissner, G. (1974) Fed. Proc. 33, 1283
12 Post, R. L. and Sen, A. K. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 773–776, Academic Press, New York
13 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
14 Meissner, G. and Fleischer, S. (1971) Biochim. Biophys. Acta 241, 356–378
15 Meissner, G. and Fleischer, S. (1974) J. Biol. Chem. 249, 302–309
16 Inesi, G. and Scarpa, A. (1972) Biochemistry 11, 356–359
17 Fleischer, S. and Fleischer, B. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 406–433, Academic Press, New York
18 Schnaitman, C., Erwin, V. G. and Greenawalt, J. W. (1967) J. Cell Biol. 32, 719–735
19 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) in Methods in Enzymology (Estabrook, R. W. and Pullman, M. E., eds), Vol. 10, pp. 448–463, Academic Press, New York
20 Michell, R. H. and Hawthorne, J. N. (1965) Biochem. Biophys. Res. Commun. 21, 333–338
21 Roy, A. B. (1953) Biochem. J. 53, 12–15
22 Laemmli, U. K. (1970) Nature 227, 680–685
23 Hasselbach, W. and Makinose, M. (1961) Biochem. Z. 333, 518–528
24 Yamamoto, T. and Tonomura, Y. (1968) J. Biochem. Tokyo 64, 137–145
25 Makinose, M. (1969) Eur. J. Biochem. 10, 74–82
26 Martonosi, A. (1969) J. Biol. Chem. 244, 613–620
27 Meissner, G. (1973) Biochim. Biophys. Acta 298, 906–926
28 Cohen, A. and Selinger, Z. (1969) Biochim. Biophys. Acta 183, 27–35
29 Chevallier, J. and Butow, R. A. (1971) Biochemistry 10, 2733–2737
30 Fiehn, W. and Migala, A. (1971) Eur. J. Biochem. 20, 245–248
31 Meissner, G. and Fleischer, S. (1972) Biochim. Biophys. Acta 255, 19–33
32 Endo, M., Tanaka, M. and Ogawa, Y. (1970) Nature 228, 34–36
33 Weber, A. and Herz, R. (1968) J. Gen. Physiol. 52, 750–759
34 Duggan, P. F. and Martonosi, A. (1970) J. Gen. Physiol. 56, 147–167
35 Heuson-Stiennon, J. A., Wanson, J. C. and Drochmans, P. (1972) J. Cell Biol. 55, 471–488

36 Fleischer, S. and Kervina, M. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds), Vol. 31, pp. 6–41, Academic Press, New York
37 MacLennan, D. H. and Wong, P. T. S. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1231–1235
38 MacLennan, D. H., Ostwald, T. J. and Stewart, P. S. (1974) Ann. N.Y. Acad. Sci. 227, 527–536
39 Meissner, G. and Fleischer, S. (1973) Biochem. Biophys. Res. Commun. 52, 913–920
40 Stewart P. S. and MacLennan, D. H. (1974) J. Biol. Chem. 249, 985–993
41 Thorley-Lawson, D. A. and Green, N. M. (1973) Eur. J. Biochem. 40, 403–413
42 Meissner, G. and Fleischer, S. (1974) in Calcium binding proteins (Drabikowski, W., Strzelecka-Golaszewska, H. and Carafoli, E., eds), pp. 281–313, Elsevier Publ. Co., Amsterdam, and Polish Scientific Publ., Warszawa
43 Racker, E. (1972) J. Biol. Chem. 247, 8198–8200
44 Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. and Metcalfe, J. C. (1974) Proc. Natl. Acad. Sci. U.S. 71, 622–626
45 Scarpa, A., Baldassare, J. and Inesi, G. (1972) J. Gen. Physiol. 60, 735–749
46 Ikemoto, N., Nagy, B., Bhatnagar, G. M. and Gergeley, J. (1974) J. Biol. Chem. 249, 2357–2365
47 Winegrad, S. (1970) J. Gen. Physiol. 55, 77–88