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ISOLATION OF SARCOPLASMIC RETICULUM BY ZONAL CENTRIFUGATION AND PURIFICATION OF Ca^{2+}-PUMP AND Ca^{2+}-BINDING PROTEINS

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SUMMARY

A procedure for the isolation of highly purified sarcoplasmic reticulum vesicles from rabbit skeletal muscle has been described using sucrose gradient centrifugation in zonal rotors. The yield of our purest fraction was 300 mg of sarcoplasmic reticulum protein using 1 kg muscle. The sarcoplasmic reticulum vesicles were relatively simple in composition. The Ca^{2+}-pump protein accounted for most (approx. two-thirds) of the sarcoplasmic reticulum protein. Two other protein components, a Ca^{2+}-binding protein and a M_{55} protein (approx. 55000 daltons) each accounted for about 5–10% of the protein. Enrichment in the level of phosphoenzyme by the Ca^{2+}-pump protein was regarded as an important index of the purification of sarcoplasmic reticulum vesicles. The sarcoplasmic reticulum vesicles were capable of forming 6.4 nmoles of ^{32}P-labelled phosphoenzyme per mg protein and had a high capacity of energized Ca^{2+} uptake. The Ca^{2+}-dependent formation of phosphoenzyme has been used to estimate the sarcoplasmic reticulum protein content in rabbit skeletal muscle and found to be about 2.5% of the total muscle protein.

The Ca^{2+}-pump and Ca^{2+}-binding proteins were isolated with a purity of 90% or more by treating the purified sarcoplasmic reticulum vesicles with bile acids in the presence of salt. The solubilized Ca^{2+}-pump protein reaggregated during dialysis together with phospholipid to form membranous vesicles which were capable of forming approx. 9 nmoles ^{32}P-labelled phosphoenzyme per mg protein. The Ca^{2+}-binding protein was water soluble and contained a high percentage of acidic amino acids (35% of total residues).

Ca^{2+} binding by sarcoplasmic reticulum vesicles and by the Ca^{2+}-pump and Ca^{2+}-binding proteins was studied by equilibrium dialysis. Sarcoplasmic reticulum vesicles and Ca^{2+}-pump protein contained nonspecific high-affinity Ca^{2+} binding sites with a capacity of 90–100 and 55–70 nmoles Ca^{2+} per mg protein, respectively. Both of them specifically bound 10–15 nmoles Ca^{2+} per mg protein. The binding constants for nonspecific and specific Ca^{2+} binding by both preparations were approx. 1 μM^{-1}. The Ca^{2+}-binding protein nonspecifically bound 900–1000 nmoles Ca^{2+} per mg protein with a binding constant of about 0.25 μM^{-1}.

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

INTRODUCTION

In recent years it has been found that the simplicity of sarcoplasmic reticulum function, *i.e.* Ca^{2+} release and uptake (for reviews, see refs 1 and 2), is matched with a relatively simple protein[2–6] and lipid [3,6–11] composition, thus making it a promising system to study in detail how a membrane system is capable of active transport. One important approach toward this goal involves dissociation of the sarcoplasmic reticulum membrane and purification of its membrane components, so that the properties of the individual sarcoplasmic reticulum proteins and lipid may be studied. These components would then be used for the reconstitution of a functional membrane system capable of active transport, an accomplishment not yet achieved. The availability of a highly purified preparation of sarcoplasmic reticulum greatly facilitates such studies by providing a framework for the composition and function which is to be reconstituted.

Sarcoplasmic reticulum vesicles which are capable of carrying out energized Ca^{2+} uptake can be isolated from muscle[1,2]. In the best preparations, contamination with other cell organelles is low, however, significant amounts of other muscle proteins are associated with the preparation. Salt extraction with 0.6 M KCl (ref. 2) or 0.5 M LiBr (ref. 6) has been employed to decrease the amount of these proteins.

The isolation of purified membrane proteins with some functional properties from sarcoplasmic reticulum vesicles has been attempted in only a few instances. MacLennan and co-workers[3,12,13], using deoxycholate as the solubilizing agent, succeeded in purifying two of the proteins of sarcoplasmic reticulum, the Ca^{2+}-pump protein, a membrane-forming enzyme containing phospholipid, and a water soluble, acidic protein with a molecular weight of 44000. The Ca^{2+}-pump protein was not capable of binding Ca^{2+} to any extent. The acidic protein was named "Calsequestrin" because it could bind large amounts of Ca^{2+} and other cations. Ikemoto *et al.*[5] isolated a protein with a molecular weight of 62000 from a Triton X-100 extract by precipitation with 4 mM Ca^{2+} and purified the Ca^{2+}-pump protein several-fold.

The present study describes a zonal density gradient centrifugation procedure for the large scale isolation of highly purified, functional sarcoplasmic reticulum vesicles. The procedure requires less handling than previous sarcoplasmic reticulum preparations. Further, using the purified sarcoplasmic reticulum vesicles as the starting material, we describe the purification of two of the major proteins of sarcoplasmic reticulum by procedures which are simpler than previously described[12,13]. The Ca^{2+}-pump protein (referred to also as ATPase protein or ^{32}P-labelled phosphoenzyme) and a Ca^{2+}-binding protein with a molecular weight between 50000 and 65000 were isolated with a purity of 90% and more. The Ca^{2+}-pump protein contained specific and nonspecific high-affinity Ca^{2+}-binding sites. In a subsequent paper the properties of the Ca^{2+}-pump protein will be described with regard to the initial steps of Ca^{2+} transport by sarcoplasmic reticulum[14].

MATERIALS AND METHODS

Materials

"Ultrapure" grade sucrose was obtained from Mann Research Laboratories (New York) and was used throughout the experiments. Cholic acid (Sigma, St. Louis

Mo.) and deoxycholic acid (Matheson Coleman and Bell, Norwood, Ohio) were recrystallized before use. The bile acid (100 g) was dissolved in 1 l of boiling ethanol containing charcoal (Norit A, 5 g/1000 ml) and 1 mM EDTA. The charcoal treatment was repeated till little color remained. The bile acids were then recrystallized from a cold 50% ethanol solution. [^{14}C]Deoxycholate and [^{14}C]cholate were purchased from ICN Chemical and Radioisotope Division (Irvine, California) and Mallinckrodt (St. Louis, Mo.), respectively. [γ-^{32}P]ATP was prepared according to Post and Sen[15] and was a generous gift of Dr Robert Post (Dept. of Physiology, Vanderbilt University). Non-radioactive ATP was obtained from P-L Biochemicals (Milwaukee, Wisc.).

Assays

Protein was determined by the procedure of Lowry *et al.*[16] using bovine serum albumin as a standard. Total phosphorus was measured as an estimate of lipid phosphorus[6] using a modification[17] of the method of Chen *et al.*[18]. Neutral lipids were quantitated as previously described[6].

Ca^{2+} uptake capacity (at 23 °C using 5 mM ATP and 5 mM oxalate), ^{32}P-labelled phosphoenzyme formation under steady state conditions (at 0 °C using 0.1 mM [γ-^{32}P]ATP) and total ATPase activity (at 32 °C using 5 mM ATP) were determined as previously described[6]. Each of these activities was measured in the presence of 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10 mM histidine (pH 7.4). Ca^{2+}-stimulated ATPase activity was estimated as the difference between total ATPase and "basic" ATPase activity. The latter was determined in a medium containing 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in place of 0.1 mM $CaCl_2$. In the Ca^{2+} uptake assay the protein concentration was adjusted for 30–70% Ca^{2+} uptake. The enzyme concentrations used in the ATPase assays resulted in less than 10% of ATP hydrolysis. Ca^{2+} uptake in the absence of oxalate was determined at 23 °C in 2 ml of a medium containing 0.30 mg sarcoplasmic reticulum protein, 0.1 M KCl, 5 mM $MgCl_2$, 4 mM ATP, 50–75 μM $^{45}Ca^{2+}$ and 10 mM histidine (pH 7.4). The assay was begun by addition of 40 μl of 200 mM ATP and was terminated after 1 and 2 min by pressing the solution through a 0.22 μm Millipore filter. The Ca^{2+} uptake was calculated from the decrease of radioactivity in the filtrate.

Ca^{2+} and Mg^{2+} were determined using a Perkin–Elmer Model 403 atomic absorption spectrometer. Standard Ca^{2+} and Mg^{2+} solutions containing 0.5% $LaCl_3$ and 5% trichloroacetic acid were used for calibration of the instrument[19]. The concentrations of both cations could be measured with an accuracy of about ± 20 and ± 5% at 1 and 10 μM, respectively. Protein was removed with 5% trichloroacetic acid in the presence of 0.5% $LaCl_3$ prior to analysis. All glassware was treated with 1 mM EGTA and 3 mM EDTA followed by five rinses with deionized water.

Measurement of $^{45}Ca^{2+}$ binding to various protein preparations was determined by equilibrium dialysis. Samples (0.5 mg protein in 0.5 ml) were dialyzed for 44 h against 100 ml of a solution containing various concentrations of $^{45}Ca^{2+}$, Mg^{2+} and K^+ in 1.0 or 2.0 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.4). The dialysis buffer was exchanged once after 12 h dialysis time. The first dialyzate was discarded, since it served only to remove salt and other contaminants. The second dialyzate was used for analysis of total Ca^{2+} by atomic

absorption spectroscopy and for measurement of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ in samples and dialysis buffer (100 μl each) was counted in 4 ml of a scintillation fluid containing 60 g naphthalene, 4.2 g 2,5-diphenyloxazole, 180 mg 1,4-bis-(5-phenyloxazolyl-2)-benzene and 70 ml water in 900 ml of dioxane.

Acrylamide gel electrophoresis

The protein profile of sarcoplasmic reticulum preparations was obtained by polyacrylamide gel electrophoresis using soaked-acid gels as well as two different systems of sodium dodecyl sulfate gels[20,21].

System 1. Sodium dodecyl sulfate gels contained 7.5 and 3% acrylamide in the separating and stacking gel, respectively, and were prepared and run as described by Laemmli[20]. Samples were dissolved in 4 M urea, 2% sodium dodecyl sulfate, 0.06 M Tris–HCl buffer (pH 6.8) and 0.001% bromophenol blue as a tracking dye. In some cases samples were reduced by adding 3% 2-mercaptoethanol and heating for 4 min at 100 °C. Gels were stained with 1% Amido black in 9% acetic acid and 50% methanol for 10 h. They were destained by soaking in several changes of 10% acetic acid and 20% methanol for several days.

System 2. Sodium dodecyl sulfate gels contained 6% acrylamide, 0.23% *N,N'*-methylene-bis-acrylamide, 4 M urea, 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer (pH 7.0). Protein samples were dissolved in a solution of 4 M urea, 1% sodium dodecyl sulfate and 0.01 M sodium phosphate (pH 7.0). After layering the sample (in up to 0.1 ml) on top of the gel a current of 2 mA per tube was passed through the gels for the first hour after which the current was increased to 4 mA per tube for 7 h. Gels were then stained and destained as before (*cf.* System 1).

System 3. Soaked-acid gels were prepared and run as previously described[6]. The gels were stained with 1% Amido black in 7% acetic acid and destained in several changes of 7% acetic acid as described previously[6].

Preparation of sarcoplasmic reticulum vesicles

Albino rabbits, about 3 kg each, were killed by injecting an overdose of nembutal (60 mg/kg rabbit). The leg and back muscles were excised, chilled in ice, freed of fat and connective tissue, and passed through a meat grinder. All operations were carried out at 0–4 °C. Ground muscle, 60 g, was homogenized in 170 ml of 0.3 M sucrose containing 10 mM HEPES buffer (pH 7.5), for 30 s in a Waring Blender. The homogenate of 10 blendings was centrifuged in six bottles for 20 min at 8000 rev./min in a JA 10 rotor in a Beckman Model J-21 centrifuge. The supernatant was poured through five layers of cheesecloth to remove varying amounts of floating fat particles. A Spinco Ti 15 zonal rotor equipped with a B-29 type core *plus* liner to allow loading and unloading of the gradient from the edge of the rotor was filled with 1200 ml of the filtrate. After accelerating the rotor to 4000 rev./min three sucrose solutions were pumped in at a speed of 10 ml/min with a Sage tubing pump Model 375. The gradient layers at the outer edge of the rotor consisted of 80 ml of 23%, 50 ml of 40% and 50 ml of 50% sucrose in 5 mM HEPES buffer (pH 7.4). The "percent sucrose" (w/w) was adjusted using a Bausch and Lomb refractometer at 25 °C. The rotor was then accelerated to 34000 rev./min and maintained at this speed for 30 min. For unloading, the rotor was decelerated to 3000 rev./min and distilled water was pumped into the center of the rotor (20 ml/min) to unload a crude

sarcoplasmic reticulum preparation from the edge of the rotor. Fractions of 20 ml were collected. The material sedimenting in the gradient between 38 and 23% sucrose was collected. The contents of two zonal runs, run either parallel or sequentially, were combined and diluted with 5 mM HEPES buffer (pH 7.4), to give about 250 ml of a crude sarcoplasmic reticulum preparation in 18% sucrose, and were then placed into a Spinco Ti 14 zonal rotor. For further purification a sucrose gradient was established as the rotor spun at 4000 rev./min by successively pumping in at the outer edge at a rate of 8 ml per min 70 ml each of 26.5, 29.1, 31.6, 33.9 and 37.1% sucrose containing 5 mM HEPES buffer (pH 7.4) and about 50 ml of 50% sucrose. The rotor was accelerated to and centrifuged at 43000rev./min for a total of 60 min. The rotor was then decelerated to 4000 rev./min and the sample was unloaded through the center of the rotor by pumping in a solution of 55% sucrose. The first 150 ml were discarded. The following 100 ml and then portions of 25 ml were collected, so that the material in the gradient in up to 26.5% (Fraction 1), 26.5–30.5% (Fraction 2) and 30.5–33% (Fraction 3) sucrose could be combined. Fractions 1–3 were diluted with an equal volume of 5 mM HEPES buffer (pH 7.4) and centrifuged for 90 min at 30000 rev./min in a Spinco 30 rotor. The pellets were resuspended in a solution containing 0.3 M sucrose, 1 mM HEPES buffer (pH 7.4), and 10–20 mg protein/ml, and quick-frozen using liquid N_2. These preparations were stable for many months when stored at −70 °C in a low temperature freezer with regard to energized Ca^{2+} uptake and phosphoenzyme formation.

Purification of the Ca^{2+}-pump protein

The Ca^{2+}-pump protein of sarcoplasmic reticulum was purified by three different procedures.

(*1*) *Partial extraction of sarcoplasmic reticulum vesicles with cholate.* Sarcoplasmic reticulum vesicles (2.5 mg/ml) were partially clarified with cholate (3.7 mg/ml) for 10 min at 0 °C in a medium containing 0.3 M sucrose, 0.5 M KCl, 1 mM EDTA, 1.5 mM $MgCl_2$ and 5 mM HEPES buffer (pH 7.7). The particulate matter was isolated by centrifugation in a 50.1 Spinco rotor at 42000 rev./min for 75 min. The pellet was washed to remove EDTA and excess detergent; it was resuspended to about 5 mg sarcoplasmic reticulum protein per ml in 5 mM HEPES buffer (pH 7.7), 0.3 M sucrose, 0.5 M KCl, 0.5 mM Mg^{2+} and 10^{-5} M Ca^{2+}. After centrifugation at 42000 rev./min for 75 min, the pellet which was enriched with respect to the Ca^{2+}-pump protein was finally resuspended in 0.3 M sucrose–1 mM HEPES buffer (pH 7.5) at a protein concentration of about 10 mg/ml, quick-frozen with liquid N_2, and stored at −70 °C.

(*2*) *Partial extraction of sarcoplasmic reticulum vesicles with deoxycholate.* Sarcoplasmic reticulum vesicles were extracted two times with deoxycholate. Sarcoplasmic reticulum vesicles (2.5 mg/ml) were incubated for 10 min at 0 °C in a medium containing deoxycholate (0.5 mg/ml), 0.3 M sucrose, 0.5 M KCl, 1 mM EDTA, 1.25 mM Mg^{2+}, 10^{-5} M Ca^{2+} and 10 mM Tris.HCl buffer (pH 7.9). The suspensions were centrifuged in a 50.1 Spinco rotor at 42000 rev./min for 75 min. The pellet was washed to remove EDTA and excess detergent as described under (1) above, quick-frozen, and stored at −70 °C.

(*3*) *Solubilization of sarcoplasmic reticulum vesicles with deoxycholate and reaggregation by dialysis.* Sarcoplasmic reticulum vesicles (3 mg/ml) were solubilized

with deoxycholate (2 mg/ml) in 10 mM Tris–HCl buffer (pH 8.0) containing 0.3 M sucrose, 0.5 M KCl, 20 μM Ca^{2+}, 1 mM EDTA, and 1.5 mM $MgCl_2$. Insoluble material was removed by centrifugation in a Spinco 65 rotor at 48000 rev./min for 60 min. The supernatant (5–10 ml) was dialyzed for varying times against 2 l of the above buffer (*minus* detergent) using a rocker dialyzer[22]. Removal of detergent resulted in reaggregated sarcoplasmic reticulum protein and lipid which was recovered by centrifugation in a Spino 65 rotor at 48000 rev./min for 60 min. Ca^{2+}-pump protein preparation was resuspended in 0.3 M sucrose–1 mM HEPES buffer (pH 7.5) at a protein concentration of 5 mg/ml, quick-frozen, and stored at −70 °C.

Purification of Ca^{2+}-binding protein

The protein of 65000 molecular weight, as determined by sodium dodecyl sulfate acrylamide gel electrophoresis (System 1) of sarcoplasmic reticulum vesicles can be purified from the deoxycholate extract obtained during the purification of the Ca^{2+}-pump protein (Method 2 above). The supernatant (100 ml) of the first deoxycholate extraction was dialyzed for 2 days at 4 °C against 2.5 l of 2.5 mM Tris–HCl buffer (pH 7.8) containing 5 mM KCl, 0.1 mM $MgCl_2$ and 0.075 mM EDTA using a rocker dialyzer. The dialysis buffer was changed every 12 h. Reaggregated material was removed by centrifugation in a Spinco No. 30 rotor at 30000 rev./min for 120 min. The supernatant was concentrated to a volume of 15 ml using an Amicon Diaflo apparatus equipped with a PM 30 membrane and recentrifuged to remove small amounts of reaggregated material. The soluble protein fraction was then applied to a DEAE-cellulose (Whatman DE52) column (1.5 cm × 30 cm) and eluted with 400 ml of 5 mM Tris–HCl buffer (pH 8.0) containing a linear gradient of KCl from 5 mM to 0.7 M. The Ca^{2+}-binding protein was the last band eluted (at a KCl concentration of about 0.35 M) and formed the dominant peak of the eluate at 280 nm. The fractions comprising the last band which was eluted were concentrated to a volume of 10 ml by Diaflo filtration, diluted with 25 ml of 2.5 mM Tris–HCl buffer (pH 8.0) and reconcentrated to a protein concentration of 2–4 mg/ml. The solution, containing the purified protein, was quick-frozen with N_2 and stored at −70 °C.

RESULTS

Preparation of sarcoplasmic reticulum vesicles

The procedure described under Materials and Methods, involved one differential centrifugation of the homogenate to remove most of the debris, myofibrils and mitochondria and two consecutive sucrose gradient centrifugations using zonal rotors. The purification of sarcoplasmic reticulum was monitored by the enrichment of ^{32}P-labelled phosphoenzyme. The first zonal rotor separation served both to reduce the volume of the sarcoplasmic reticulum suspension as well as to give a preliminary purification. This was achieved by introducing a steep, narrow density gradient ranging from 23 to 50% sucrose into the outer edge of the zonal rotor. It resulted in a 10-fold increase in the concentration of the sarcoplasmic reticulum vesicles and removal of most of the soluble proteins. A 20-fold purification over the total muscle homogenate was achieved at this stage (Table 1). This crude sarcoplasmic reticulum vesicle preparation was further purified in a subsequent zonal run containing a density

gradient from 26 to 37% sucrose. Several fractions containing sarcoplasmic reticulum vesicles were collected (see Materials and Methods), the purest one sedimenting in the layer of 26.5 to 30.5% sucrose (Fraction 2). Fraction 1 (18–26.5% sucrose) was contaminated mainly by muscle contractile proteins which could be partly extracted with 0.6 M KCl (ref. 2) or 0.5 M LiBr (ref. 6). About 35% of the Ca^{2+}-pump protein of the crude sarcoplasmic reticulum vesicle preparation was recovered in Fraction 1. Some of the properties of this fraction are recorded in Table I. Fraction 3 (30.5–33% sucrose) was free of contaminating muscle protein but was contaminated with up to 10% of mitochondrial fragments as judged by the succinate–cytochrome *c* reductase activity[22]. This fraction was discarded.

Fraction 2 was used in our experiments, unless otherwise specified. Approx. 40% of the Ca^{2+}-pump protein of the crude sarcoplasmic reticulum vesicle preparation was recovered in Fraction 2 (Table I). As isolated, the sarcoplasmic reticulum vesicles contained 70 and 60 nmoles/mg protein of bound Ca^{2+} and Mg^{2+}, respectively. The lower values of bound Ca^{2+} and Mg^{2+} shown for Fraction 1 were a consequence of the extraction with 0.6 M KCl. Fraction 2 accumulated Ca^{2+} in the presence or absence of oxalate, it contained a Ca^{2+}-stimulated ATPase, and it

TABLE I

PROPERTIES OF SARCOPLASMIC RETICULUM VESICLES

Crude sarcoplasmic reticulum vesicles were obtained from the first zonal rotor separation and purified vesicles from the subsequent zonal rotor separation. Fraction 1 of the second zonal rotor separation was extracted with 0.6 M KCl overnight at 0 °C by adding an equal volume of 1.2 M KCl. Fraction 2 did not contain KCl extractable proteins and was not extracted with salt. When Ca^{2+} uptake was measured in the absence of oxalate, sarcoplasmic reticulum vesicles (1.5 mg/ml) were incubated prior to analysis in 10 mM phosphate buffer (pH 7.4) containing 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM ADP and 0.1 mM EGTA to lower the amount of endogenous Ca^{2+} (ref. 24). Samples were then layered on top of 2 ml of 23% sucrose containing 5 mM HEPES buffer (pH 7.4), 100 mM KCl and 2.5 mM $MgCl_2$ and centrifuged. The pellet, which was taken up in 0.3 M sucrose–1 mM HEPES buffer (pH 7.4) contained 20–25 nmoles Ca^{2+} per mg protein. The ^{32}P-labelled phosphoenzyme was characterized by gel electrophoresis on soaked-acid gels[6]. Approx. 75 and 90% of the recovered ^{32}P radioactivity was found in the phosphoenzyme band of gels containing total muscle and purified sarcoplasmic reticulum, respectively. Each value represents the mean of 10 determinations ± S.E.

	Total muscle	*Crude sarcoplasmic reticulum*	*Purified sarcoplasmic reticulum*	
			Fraction 1	*Fraction 2*
Yield (mg protein/1000 g muscle)	≈230000	1700 ± 200	320 ± 50	300 ± 50
μg bound P/mg protein	—	—	21.0 ± 2.0	24.5 ± 0.5
Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)				
+oxalate	—	2.2 ± 0.3	—	5.0 ± 0.5
−oxalate	—	—	—	0.13 ± 0.02
[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein)	0.15 ± 0.03	3.0 ± 0.3	5.4 ± 0.4	6.4 ± 0.3
ATPase (μmoles P_i/mg protein per min)				
with 1 mM EGTA	—	—	—	0.10 ± 0.05
with 0.1 mM Ca^{2+}	—	—	—	0.95 ± 0.15
Bound Ca^{2+} (nmoles Ca^{2+}/mg protein)	—	35 ± 5	30 ± 5	70 ± 5
Bound Mg^{2+} (nmoles Mg^{2+}/mg protein)	—	65 ± 10	17 ± 4	60 ± 4

had a phosphoenzyme level of 6.4±0.3 nmoles ^{32}P/mg protein under steady-state conditions (Table I). Succinate-cytochrome *c* reductase was less than 0.005 μmoles cytochrome *c* reduced per min per mg protein indicating a contamination by mitochondria of less than 1%. There was low contamination of sarcoplasmic reticulum with other muscle protein, since a wash with 0.6 M KCl or 0.5 M LiBr extracted less than 5% of the protein. Most of the extracted protein had a molecular weight of less than 50000.

Highly purified sarcoplasmic reticulum vesicles (Fraction 2) were further characterized by electron microscopy and analysis of neutral lipid content and by gel electrophoresis. Electron microscopy revealed not only the generally observed closed membranous vesicles, but also many flattened sacs which were in close contact with others (Fig. 1a). The neutral lipid content was found to account for 3.4% of the total lipid. Cholesterol and triglycerides accounted for 1.3% each, free fatty acid and cholesterol ester for 0.4% each (all values are averages of two preperations). The protein profile of purified sarcoplasmic reticulum is shown in Fig. 2 and is compared with the crude sarcoplasmic reticulum fraction and total muscle protein. Soaked-acid gels, which have been found to resolve best the high molecular weight proteins of sarcoplasmic reticulum[6], and sodium dodecyl sulfate gels (System 1, *cf.* Materials and Methods) were used. The Ca^{2+}-pump protein was by far the dominant band in both gel systems accounting for approximately two-thirds of the area of densitometry tracings of sodium dodecyl sulfate gels (Fig. 2). Two other bands, labelled Ca^{2+}-binding and M_{55} proteins each accounting for about 5–10% of the area of the densitometry tracing, were also consistently found in purified sarcoplasmic reticulum. The apparent molecular weights of the Ca^{2+}-pump protein and the two other protein bands were previously estimated to be 125000, 63000 and 52000, respectively, as found by electrophoresis in soaked-acid gels[6]. Using sodium dodecyl sulfate gels (System 1) and reduced samples, the molecular weights of these three proteins were estimated to be 105000, 65000 and 55000. In addition, a number of bands were observed, some of which varied in intensity with the preparation, but each of them was generally less than 5% of the protein.

Purification of Ca^{2+}-pump protein

The dominant band of Gels 1–3 in Fig. 2 has been previously identified as the Ca^{2+}-pump protein[3,6,23]. Three procedures are described under Materials and Methods for the purification of the major component of sarcoplasmic reticulum membranes. Each made use of the selective solubilization of the low molecular weight components in the presence of bile acids and salt. Two preparations of Ca^{2+}-pump protein purified by partial extraction with cholate and deoxycholate, respectively, are characterized in Table II and Fig. 3. It can be seen that most of the low molecular weight proteins have been extracted (*cf.* Gels 2 and 3 with Gel 1 in Fig. 3). In addition to the Ca^{2+}-pump protein band two high molecular weight bands were present as contaminants. Both of them seemed to be referable to trace bands in the starting sarcoplasmic reticulum preparation.

Ca^{2+} uptake capacity was generally lost and basic ATPase activity (assayed without Ca^{2+} present) was decreased while Ca^{2+}-stimulated ATPase activity was increased several-fold (Table II). The preparation prepared with cholate treatment had a somewhat higher level of phosphoenzyme. In both cases the enrichment with

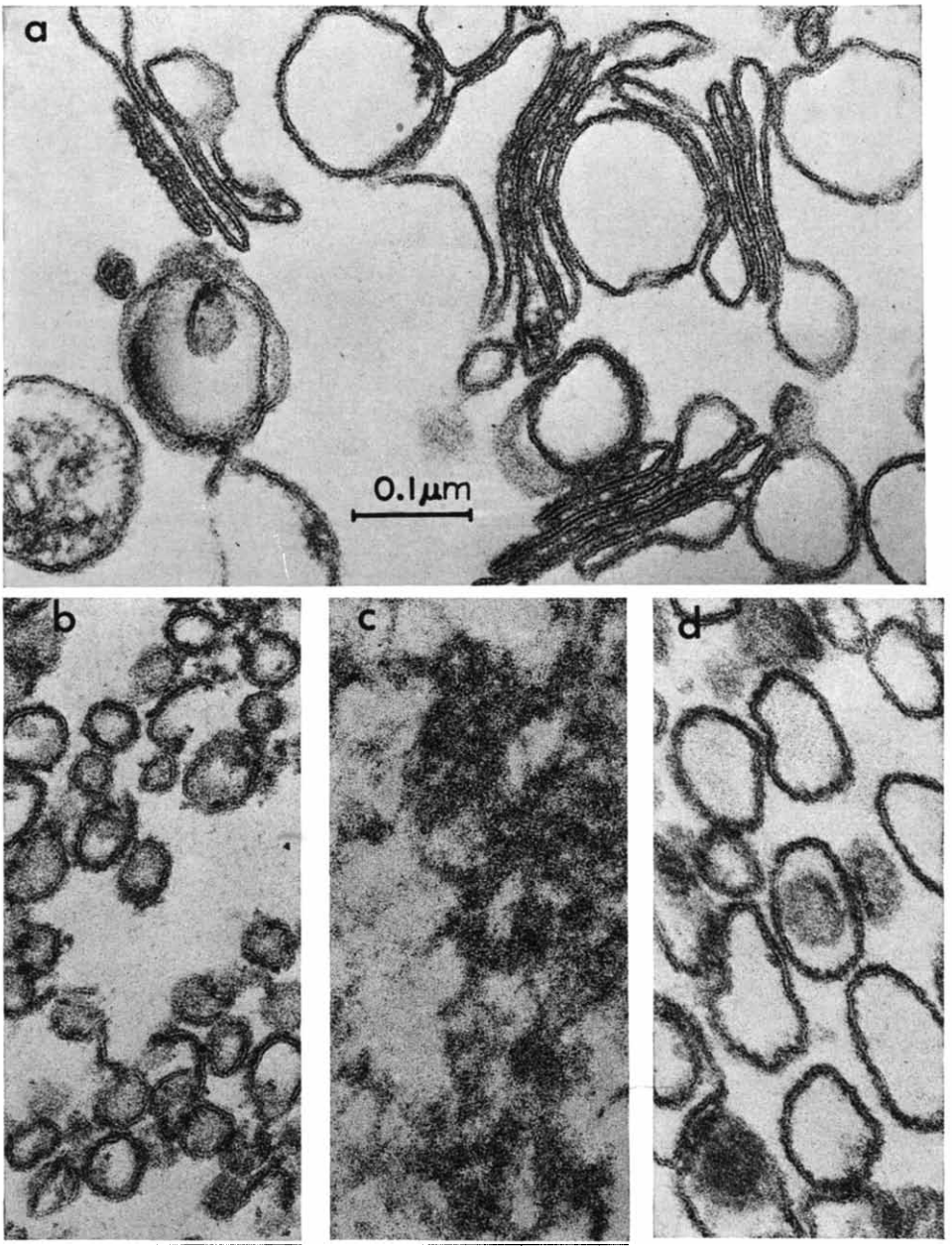
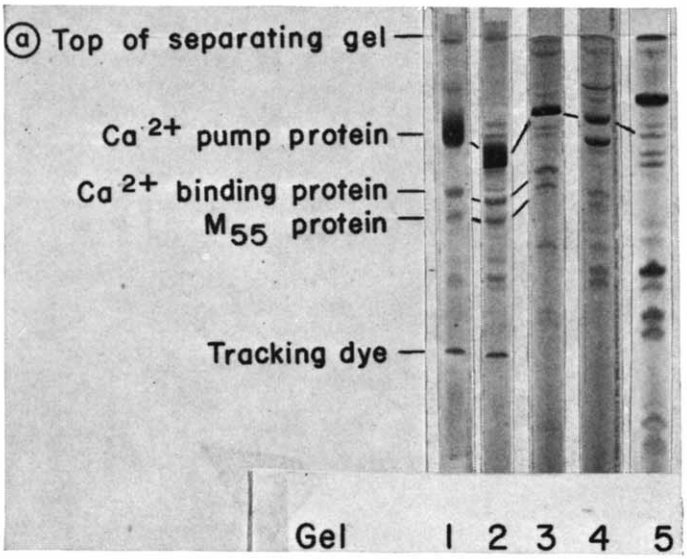


Fig. 1. Electron micrographs of native and detergent treated sarcoplasmic reticulum vesicles. Samples were fixed in 1% OsO_4, embedded and sectioned as previously described[6]. Magnification 160000×. (a) Purified sarcoplasmic reticulum vesicles (Fraction 2 of Table I). (b) Ca^{2+}-pump protein prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate (Method 1, Table II). (c) The supernatant fraction from sarcoplasmic reticulum vesicles, solubilized with deoxycholate and centrifuged to remove insoluble matter, was directly fixed with 1% OsO_4 by adding 1 vol. of 4% OsO_4 to 3 vol. of the sample after centrifugation (Method 3 for purification of Ca^{2+}-pump protein). (d) Ca^{2+}-pump protein prepared by solubilization of sarcoplasmic reticulum vesicles with deoxycholate and reaggregated by dialysis for 1 h (Method 3, Table III).



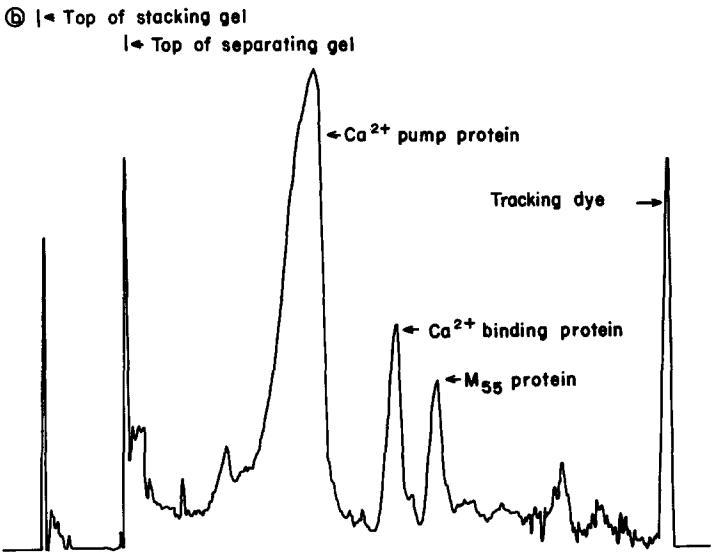


Fig. 2. Separation of sarcoplasmic reticulum proteins by gel electrophoresis. Sodium dodecyl sulfate gels (System 1) (Gels 1 and 2) containing 50 μg protein and soaked-acid gels (Gels 3–5) containing 20 μg protein were used. The densitometry tracing[6] of Gel 1 is also shown. Preparations described in Table I were used. Reduction of the sample in Gel 2 was achieved with 3% 2-mercaptoethanol and heating for 4 min at 100 °C. Sodium dodecyl sulfate gels of System 1 made use of a stacking gel. The top of the gel in all cases refers to the top of the separating gel. Gel 1, purified sarcoplasmic reticulum, Fraction 2, not reduced; Gel 2, purified sarcoplasmic reticulum, Fraction 2, reduced; Gel 3, purified sarcoplasmic reticulum, Fraction 2, not reduced; Gel 4, crude sarcoplasmic reticulum, not reduced; Gel 5, total muscle, not reduced.

respect to phosphoenzyme was small as would be expected by the high content of Ca^{2+}-pump protein already present in the original sarcoplasmic reticulum vesicles. The conditions of preparation which included the presence of 1 mM EDTA complexed

TABLE II

PROPERTIES OF PARTIALLY PURIFIED Ca^{2+}-PUMP PROTEIN

Partially purified Ca^{2+}-pump protein was obtained by partial extraction of sarcoplasmic reticulum vesicles with cholate or deoxycholate (Methods 1 and 2). The amount of bound detergent was determined using [^{14}C]cholate or [^{14}C]deoxycholate after washing as described. Ca^{2+} uptake capacity was determined in the presence of 5 mM oxalate. Standard deviation is given when values were determined for at least five preparations.

	Sarcoplasmic reticulum vesicles	*+Cholate*	*+Deoxycholate*
Yield (% protein recovered)	—	40 ±10	50 ±10
μg bound P/mg protein	24.5	11.5± 2	24 ± 3
μg detergent/mg protein	—	12	65
Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)	5.0	<0.1	<0.1
[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein)	6.4	7.6± 0.7	6.8± 1.0
ATPase (μmoles P_i/mg protein per min)			
with 1 mM EGTA	0.12	0.02	0.04
with 0.1 mM Ca^{2+}	1.0	3.5	5.5

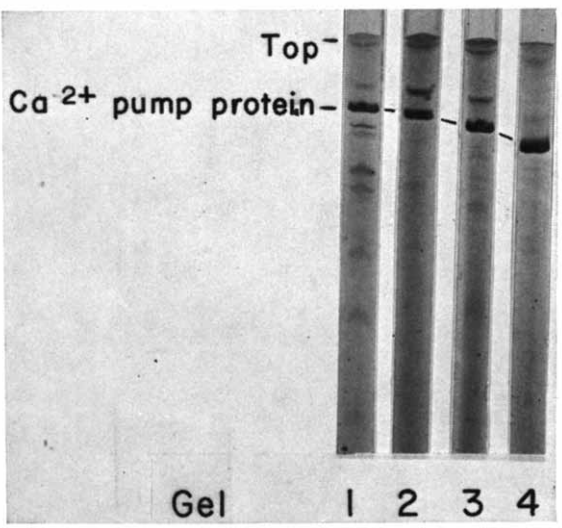


Fig. 3. Polyacrylamide gel electrophoresis of Ca^{2+}-pump protein on soaked-acid gels. Soaked-acid gels of 7.5% polyacrylamide were used. Samples (20 μg protein per gel) were prepared as described in Tables II and III and were not reduced prior to electrophoresis. Gel 1, sarcoplasmic reticulum; Gel 2, Ca^{2+}-pump protein prepared by partial extraction of sarcoplasmic reticulum vesicles with cholate (Method 1, Table II); Gel 3, Ca^{2+}-pump protein prepared by partial extraction of sarcoplasmic reticulum vesicles with deoxycholate (Method 2, Table II); Gel 4, Ca^{2+}-pump protein prepared by solubilization of sarcoplasmic reticulum vesicles with deoxycholate and reaggregation by dialysis for 1 h (Method 3, Table III).

with a small excess of Mg^{2+} (1.2–1.5 mM), the presence of both sucrose (0.25–0.5 M) and KCl (0.5–1.0 M), and a pH between 7.6 and 8.1, were important in obtaining reproducible results and optimal phosphoenzyme formation. Only small amounts of detergent were retained in these preparations (Table II). The preparations appeared mostly as closed membranous vesicles with a trilaminar arrangement of the membrane; some nonvesicular membranes were also observed (Fig. 1b).

The preparation of Ca^{2+}-pump protein, using partial extraction with cholate, had a lower lipid content (11.5 μg P/mg protein) than the starting sarcoplasmic reticulum vesicles (24.5 μg P/mg protein). There was preferential release of phospholipid when sarcoplasmic reticulum vesicles were treated with bile acids in the presence of salt, shown in Fig. 4 for varying cholate and varying salt concentrations. Phospholipid was preferentially lost as either the salt concentration was increased at constant cholate concentration (3.6 mg/ml) (Fig. 4a) or as the cholate concentration increased at constant salt concentration (0.5 M KCl) (Fig. 4b). The protein which

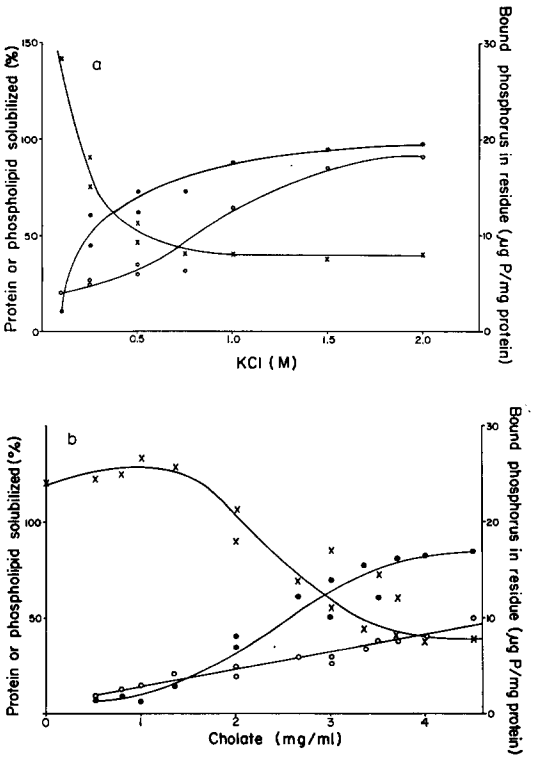


Fig. 4. The effect of ionic strength and cholate concentration on solubilization of protein and phospholipid from sarcoplasmic reticulum. Sarcoplasmic reticulum vesicles (2.5 mg protein per ml) were incubated for 20 min at 0 °C with a 10 mM Tris–HCl buffer (pH 8.0) containing 0.3 M sucrose, 1.5 mM Mg^{2+}, 1 mM EDTA and the indicated KCl and cholate concentrations. After centrifugation for 45 min at 50000 rev./min in a Spinco No. 65 rotor protein and phosphorus of the supernatant and residue fractions were measured. ○—○, % protein solubilized; ●—●, % phosphorus solubilized; ×—×, μg bound P per mg protein in residue fraction. Practically all of the phosphorus could be recovered in the phospholipid fraction[6]. (a) 3.6 mg cholate per ml at various KCl concentrations. (b) 0.5 M KCl at various cholate concentrations.

was first extracted under both of these conditions was mainly the low molecular weight components (Ca^{2+}-binding and M_{55} proteins, *cf.* Gels 1–3 of Fig. 2). The Ca^{2+}-pump protein was preferentially recovered with the membranous fraction whose bound phosphorus to protein ratio approached 8 μg P per mg protein. The partial extraction procedure with bile acid in the presence of salt gave qualitatively the same results between pH 7 to 8.5.

The preparation of the Ca^{2+}-pump protein using partial solubilization with deoxycholate (Method 2) had about the same phospholipid to protein ratio as that of the starting sarcoplasmic reticulum vesicles in contrast with the preparation described using cholate. We do not mean to indicate that there was a fundamental difference in the pattern of release of components from sarcoplasmic reticulum treated with cholate or deoxycholate. It may be noted that different concentrations of detergent were used. The ratio of phospholipid to protein of the Ca^{2+}-pump protein could be varied with the detergent and salt concentration so that at 0.5 mg deoxycholate per ml, ratios of 25–30 μg and 8–10 μg bound P per mg protein were obtained when the salt was varied from 0.1 to 1.0 M KCl, respectively (not shown). Either preparation showed high levels of phosphoenzyme formation as long as the salt concentration was at 0.5 M KCl or greater.

Method 3 for the preparation of Ca^{2+}-pump protein made use of a higher concentration of deoxycholate (2.0 mg/ml) which resulted in solubilization of most of the sarcoplasmic reticulum membrane in the presence of 0.5 M KCl. Subsequent dialysis to remove detergent caused reaggregation of sarcoplasmic reticulum protein and lipid. When the dialysis was interrupted after a short time (approx. 1–3 h), a Ca^{2+}-pump protein preparation with a phosphoenzyme content of 9 nmoles ^{32}P per mg protein was obtained. Assuming a molecular weight of 105000 and one phosphorylation site per molecule, the Ca^{2+}-pump protein had a purity of more than 90%. This was in accordance with densitometry tracings of gels (Gel 4 in Fig. 3) containing the purified preparation. If dialysis was too long, the upper bands (*cf.* Gel 2 and 3 with Gel 4, Fig. 3) were present. Thus, there is a compromise between yield and purity of the preparation. The precise conditions for the dialysis must be strictly controlled, which was achieved in part by using a rocker dialyzer. In agreement with MacLennan *et al.*[3] we found that the purified Ca^{2+}-pump protein formed membranous vesicles

TABLE III

PROPERTIES OF REAGGREGATED Ca^{2+}-PUMP PROTEIN

Sarcoplasmic reticulum vesicles (3.0 mg protein/ml) were solubilized with deoxycholate (2 mg/ml) in a 10 mM Tris–HCl buffer (pH 8.0) containing 0.3 M sucrose, 0.5 M KCl, 1 mM EDTA, 1.5 mM Mg^{2+} and 20 μM Ca^{2+} (Method 3). Reaggregation was achieved by dialysis against the same buffer except that deoxycholate was omitted.

Dialysis time (h)	*Bound phosphorus (μg P/mg protein)*	*[^{32}P]Phosphoenzyme (nmoles ^{32}P/mg protein)*
1	12.0	9.0
4	14.2	8.9
24	19.2	8.5

when the detergent was removed by dialysis (Fig. 1d). The sarcoplasmic reticulum vesicles, solubilized with deoxycholate had an amorphous appearance and were, in the main, clearly nonmembranous (Fig. 1c). The amino acid composition of the Ca^{2+}-pump protein is presented in Table IV. In agreement with two earlier analysis[2,3] it was found that the acidic amino acids with about 20% of the total residues outnumbered the basic amino acids which accounted for about 10% of the total residues. The Ca^{2+}-pump protein as isolated is not capable of appreciable Ca^{2+} uptake measured in the presence of 5 mM oxalate (<0.2 μmoles Ca^{2+} per mg protein).

TABLE IV

AMINO ACID COMPOSITION OF Ca^{2+}-PUMP AND Ca^{2+}-BINDING PROTEINS

The Ca^{2+}-pump protein was purified by reaggregation of solubilized sarcoplasmic reticulum vesicles (Method 3). The preparation was extracted with 90% aqueous acetone to remove phospholipid before acid hydrolysis of the protein. The number of residues per mole was calculated by extrapolating the results from a 20, 40 and 72 h hydrolyzate and assuming molecular weights of 110000 and 60000 for the Ca^{2+}-pump and Ca^{2+}-binding proteins, respectively. Cysteine and methionine were estimated from a sample which was oxidized with performic acid[25] before a 20-h hydrolysis.

Amino acid	*Mole %*		*Amino acid residues per mole*	
	Ca^{2+}-pump protein	*Ca^{2+}-binding protein*	*Ca^{2+}-pump protein*	*Ca^{2+}-binding protein*
Lys	5.42	5.66	54	30
His	1.27	1.97	13	10
Arg	5.08	2.24	51	12
Asp	8.72	17.85	87	96
Thr	5.90	3.21	59	17
Ser	5.00	4.00	50	21
Glu	10.60	17.70	106	93
Pro	5.50	4.73	55	25
Gly	7.03	4.33	70	23
Ala	8.48	6.30	85	33
Cys	2.33	0.16	23	1
Val	7.66	6.54	77	35
Methionine sulfone	3.39	1.82	34	10
Ile	6.18	5.21	62	27
Leu	9.75	9.00	97	47
Tyr	2.33	3.03	23	16
Phe	5.30	4.94	53	26

Purification of the Ca^{2+}-binding protein

In the course of preparing the Ca^{2+}-pump protein, it became clear that the low molecular weight proteins were preferentially released on treatment of sarcoplasmic reticulum with detergents. The first extract of deoxycholate treated vesicles was used to purify the Ca^{2+}-binding protein. The procedure to purify the Ca^{2+}-binding protein involved dialysis to remove detergent and a concentration step. The M_{55} protein largely aggregated while the Ca^{2+}-binding protein stayed in

solution and was the dominant protein species in the supernatant fraction after centrifugation (Gel 9 in Fig. 5). Using a DEAE-cellulose column, a linear KCl gradient eluted first contaminating proteins at relatively low ionic strength, while the release of the Ca^{2+}-binding protein required a 0.35 M KCl solution. The Ca^{2+}-binding protein was the last protein being eluted from the DEAE-cellulose column. It had a purity of more than 95% as judged by gel electrophoresis (Gels 3, 6 and 8 in Fig. 5). The purification of Ca^{2+}-binding protein is summarized in Table V. It contained only a small amount of bound phosphorus (expressed as μg P/mg protein) and was capable of binding large amounts of Ca^{2+}. In the course of purification and

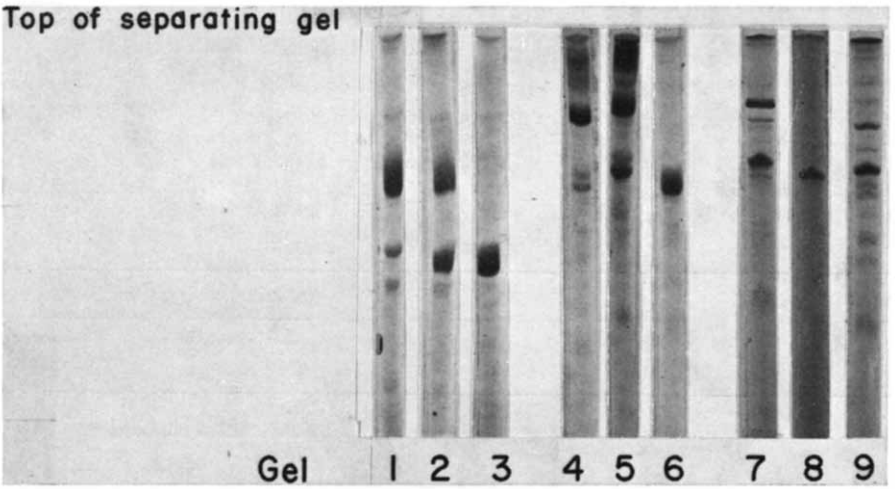


Fig. 5. Polyacrylamide gel electrophoresis of Ca^{2+}-binding protein. Sodium dodecyl sulfate gels of 7.5% (System 1, Gels 1–3) and 6% (System 2, Gels 4–6) polyacrylamide and soaked-acid gels containing 7.5% polyacrylamide (Gels 7–9) were used. 50 and 20 μg protein were run on sodium dodecyl sulfate and soaked-acid gels respectively. Samples were not reduced. Gels 1, 4, sarcoplasmic reticulum; Gels 2, 5, 7, sarcoplasmic reticulum *plus* purified Ca^{2+}-binding protein; Gels 3, 6, 8, purified Ca^{2+}-binding protein (*cf.* Table V); Gel 9, crude Ca^{2+}-binding protein (soluble deoxycholate extract of sarcoplasmic reticulum, *cf.* Table V).

TABLE V

PURIFICATION OF Ca^{2+}-BINDING PROTEIN

The soluble extract was obtained by treating sarcoplasmic reticulum vesicles with deoxycholate (0.5 mg/ml) in 0.5 M KCl, dialysis of the supernatant fraction and concentration on an Amicon filter. The purified Ca^{2+}-binding protein was then further purified from the soluble extract by DEAE-cellulose chromatography. Ca^{2+} binding was determined by equilibrium dialysis against a 2 mM HEPES buffer (pH 7.4) containing 20 μM $^{45}Ca^{2+}$.

Fraction	*Recovery (mg protein)*	*Bound phosphorus (μg P/mg protein)*	*Ca^{2+} binding (nmoles Ca^{2+}/mg protein)*
Sarcoplasmic reticulum	250	24.5	120
Soluble extract	30	6.5	245
Purified Ca^{2+}-binding protein	6	1.0	690

characterization of the Ca^{2+}-binding protein, it became increasingly clear that it was similar if not identical to "Calsequestrin" an acidic, water-soluble cation binding protein, previously described by MacLennan and Wong[13].

The Ca^{2+}-binding protein and Calsequestrin had a similar amino acid composition. Aspartic and glutamic acid were clearly the two dominant amino acids (35% of total residues) and outweighed by far the two basic amino acids arginine and lysine (8% of total residues) (Table IV), thus being responsible for the acidic character of this protein. Significant differences in cysteic acid and tyrosine content may be noted between the two preparations.

Gel electrophoresis in both sodium dodecyl sulfate and in the soaked-acid system showed that the Ca^{2+}-binding protein moved with a mobility identical to that of one of the major proteins in sarcoplasmic reticulum (Fig. 5). The apparent molecular weight of this band had been previously determined to be 50000 in sodium dodecyl sulfate gels (System 2) and 63000 in soaked-acid gels[6]. In sodium dodecyl sulfate gels of System 1 which can be also used for molecular weight determinations[20] a molecular weight of 65000 was estimated. Such large differences in apparent molecular weights using the three different gel systems were only observed for the Ca^{2+}-binding protein. Fig. 5 shows that the Ca^{2+}-binding protein had a slower mobility compared with the M_{55} protein in soaked-acid and sodium dodecyl sulfate (System 1) gels; however the order was reversed for the two protein bands in sodium dodecyl sulfate gels of System 2. The content of the Ca^{2+}-binding protein in sarcoplasmic reticulum was estimated from densitometry tracings of sodium dodecyl sulfate gels of sarcoplasmic reticulum using the purified Ca^{2+}-binding protein for calibration. Accordingly the Ca^{2+}-binding protein made up 7.0% of the total sarcoplasmic reticulum protein. Using Coomassie Blue stain MacLennan and Wong[13] had also estimated that Calsequestrin accounted for 7% of the sarcoplasmic reticulum protein.

TABLE VI

Ca^{2+} BINDING BY SARCOPLASMIC RETICULUM, Ca^{2+}-PUMP AND Ca^{2+}-BINDING PROTEINS AS A FUNCTION OF Ca^{2+}, Mg^{2+} AND K^+ CONCENTRATIONS

The Ca^{2+}-pump protein, prepared by partially extracting sarcoplasmic reticulum vesicles with cholate, contained 13 μg bound P per mg protein. Ca^{2+} binding was measured by equilibrium dialysis. Samples were dialyzed against a 2 mM HEPES buffer (pH 7.4) containing 20 μM $^{45}Ca^{2+}$ and the indicated concentrations of Mg^{2+} and K^+. All values are averages of at least two determinations.

Components added		*Ca^{2+} binding (nmoles Ca^{2+}/mg protein)*		
[Mg^{2+}] (mM)	*[K^+] (mM)*	*Sarcoplasmic reticulum*	*Ca^{2+}-pump protein*	*Ca^{2+}-binding protein*
—	—	120	90	690
1	—	23	17	70
—	100	17	14	21
1	100	15	12	16

Ca^{2+} binding by sarcoplasmic reticulum vesicles, Ca^{2+}-pump and Ca^{2+}-binding proteins

The Ca^{2+} binding by sarcoplasmic reticulum vesicles, Ca^{2+}-pump and Ca^{2+}-binding proteins was studied by equilibrium dialysis. In the absence of salt (100 mM KCl or 1 mM $MgCl_2$), the Ca^{2+}-binding protein bound 6 and 8 times greater amounts

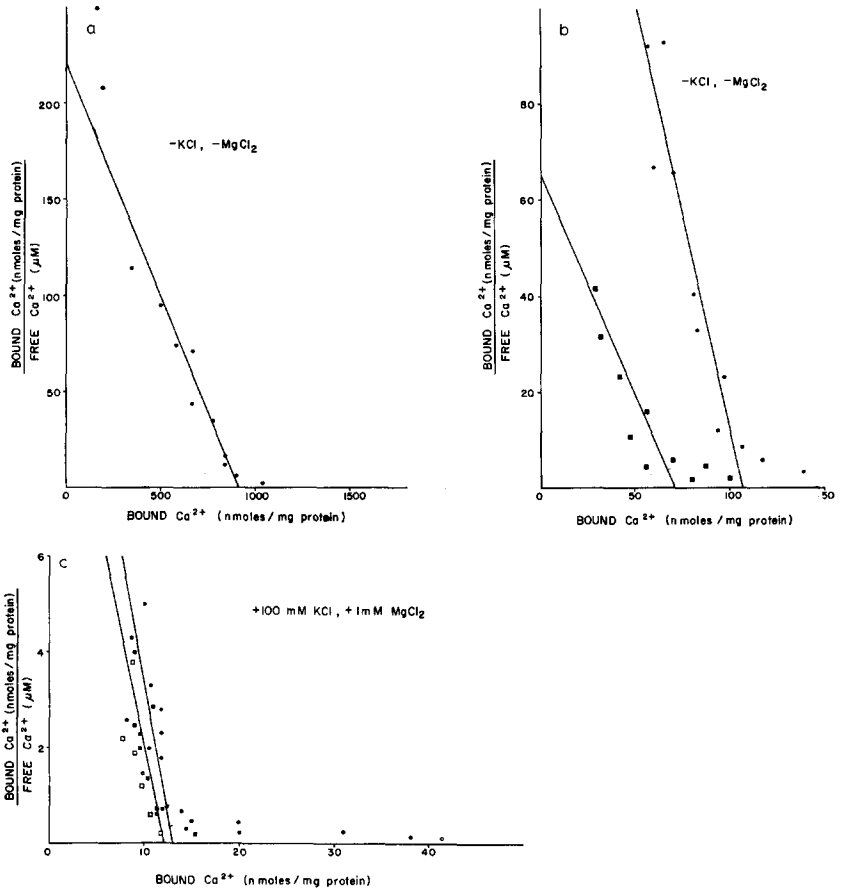


Fig. 6. Scatchard plots of Ca^{2+} binding by sarcoplasmic reticulum vesicles, Ca^{2+}-pump and Ca^{2+}-binding proteins. Ca^{2+}-pump protein, prepared by partially extracting sarcoplasmic reticulum vesicles with cholate (Method 1, *cf.* Materials and Methods), contained 12.5 μg bound P per mg protein. Total binding was determined by dialyzing the samples against 1 mM HEPES buffer (pH 7.4) containing varying concentrations of Ca^{2+}. Specific binding was measured in the presence of 2 mM HEPES buffer (pH 7.4) containing 100 mM KCl, 1 mM $MgCl_2$ and varying concentrations of Ca^{2+}. When Ca^{2+} binding by sarcoplasmic reticulum vesicles and Ca^{2+}-pump protein was studied in the presence of salt, the amount of bound Ca^{2+} determined by atomic absorption spectroscopy was somewhat greater (10–25%) than that obtained on the basis of radioactivity assuming complete equilibrium. This would indicate that there was a small amount of Ca^{2+} which was not readily exchangeable. (a) Ca^{2+}-binding protein; Ca^{2+} binding was measured in the absence of added KCl and $MgCl_2$. (b) Sarcoplasmic reticulum vesicles (●—●) and Ca^{2+}-pump protein (■—■); Ca^{2+} binding measured in the absence of added KCl and $MgCl_2$. (c) Sarcoplasmic reticulum vesicles (●—●, ○—○) and Ca^{2+}-pump protein (■—■, □—□); Ca^{2+} binding, measured in the presence of salt, was estimated by atomic absorption spectroscopy (●—●, ■—■) or by radioactivity (○—○, □—□).

of Ca^{2+} than sarcoplasmic reticulum and the Ca^{2+}-pump protein, respectively (Table VI). The presence of 100 mM KCl severely decreased the binding of Ca^{2+} in all three preparations. $MgCl_2$ at two orders of magnitude lower concentrations than KCl had a similar effect. Ca^{2+}-binding by the three preparations is presented for varying Ca^{2+} concentrations in the absence of salt in form of Scatchard plots (Fig. 6). In addition, Ca^{2+} binding by sarcoplasmic reticulum vesicles and Ca^{2+}-pump protein was studied in the presence of salt (1 mM $MgCl_2$ *plus* 100 mM KCl). The approximate binding constants, K_B, and number of binding sites, n, were calculated from the slopes and the intercepts of the Scatchard plots, respectively, and are summarized in Table VII. All three preparations bound Ca^{2+} with a similar high affinity. The Ca^{2+}-binding protein had a somewhat lower binding affinity for Ca^{2+} than the other two preparations. The number of total high affinity binding sites varied greatly in the three preparations. Sarcoplasmic reticulum vesicles, Ca^{2+}-pump and Ca^{2+}-binding proteins had a capacity of approx. 100–110, 65–80 and 900–1000 nmoles Ca^{2+} per mg protein, respectively. The addition of 1 mM $MgCl_2$ and 100 mM KCl to the dialysis buffer reduced the number of Ca^{2+}-binding sites to 10–15 nmoles per mg protein for sarcoplasmic reticulum vesicles and Ca^{2+}-pump protein without changing the binding constants appreciably. Thus it seems that both preparations contained about an equal number of specific high-affinity Ca^{2+} binding sites. At high Ca^{2+} concentrations some low-affinity Ca^{2+} binding was observed in the presence as well as absence of salt (Fig. 6). The binding constant for the low-affinity Ca^{2+} binding by sarcoplasmic reticulum vesicles in the presence of salt was about two orders of magnitude lower than the high-affinity binding.

TABLE VII

HIGH-AFFINITY Ca^{2+} BINDING BY SARCOPLASMIC RETICULUM VESICLES, Ca^{2+}-PUMP AND Ca^{2+}-BINDING PROTEINS

Data were obtained from Scatchard plots in Fig. 6.

Preparation	*Type of binding*	K_B (μM^{-1})	*Binding sites* (*nmoles* Ca^{2+}/*mg protein*)
Sarcoplasmic reticulum vesicles	Nonspecific	1.25–2.5	90–100
	Specific	0.5 –1.5	10– 15
Ca^{2+}-pump protein	Nonspecific	0.5 –1.5	55– 70
	Specific	0.5 –1.5	10– 15
Ca^{2+}-binding protein	Nonspecific	0.2 –0.3	900–1000

DISCUSSION

A large scale isolation procedure of highly purified sarcoplasmic reticulum has been described and the preparation has been characterized. The procedure made use of two successive gradient centrifugations using zonal rotors to process 1000 g of rabbit skeletal muscle. When two zonal rotors (Ti-15) were used in parallel for the first separation and one (Ti-14) for the second, it took 10–12 h to prepare 300 mg

of our purest sarcoplasmic reticulum (Fraction 2 of Table I); approximately an equal amount was also obtained in a fraction of somewhat lesser purity (Fraction 1 of Table I), which was also of good quality and was useful for many purposes. We recovered approx. 10% of the sarcoplasmic reticulum of muscle in the purified sarcoplasmic reticulum preparation (Fractions 1 and 2 together). The efficient scale-up in the preparation of sarcoplasmic reticulum using zonal rotors demanded much less effort and netted greater amounts of superior material than did our previous isolation procedure[6] as well as procedures described by others (*cf.* refs 2 and 6). This procedure has been further scaled up for 2000 g of muscle tissue using four Ti 15 zonal rotors (two times two in parallel) for the first separation and one Ti 15 zonal rotor for the second gradient separation[26].

The sarcoplasmic reticulum prepared as described had good functional characteristics. It was capable of accumulating 4–6 μmoles of Ca^{2+} per mg protein in the presence of 5 mM oxalate. The preparation as isolated did not contain appreciable amounts of contaminating muscle protein. Thus the salt extraction used previously[6] has been eliminated for our best fraction. The new preparation had a lower neutral lipid content than our older preparation, accounting for 3.4% of the total lipid; less than 2% of the lipid was cholesterol and cholesterol ester. These values were similar to those of Sanslone *et al.*[11] and were much lower than reported recently by others[3,8–10].

A phosphoenzyme content of 6–7 nmoles ^{32}P/mg protein was found which is approx. 50% greater than that in our previous preparation. The increase appears in part referable to a decrease in the content of some of the high molecular weight constituents, and seems in part to be due to the gentler handling of the sample which was made possible by the use of the zonal procedure. Accordingly the Ca^{2+}-pump protein accounts for at least 2/3 of the total sarcoplasmic reticulum protein assuming a molecular weight of 105000 and one phosphorylation site per molecule. Energized Ca^{2+} uptake and Ca^{2+}-stimulated ATPase activity are less useful criteria for the estimation of the purity in our experience. These activities reflect in part the intactness of the membrane. This is especially true for the derived preparations obtained by detergent treatment, since the ATPase activity can be increased several-fold just by addition of detergent to sarcoplasmic reticulum vesicles.

We consider that Ca^{2+}-dependent phosphoenzyme formation is also a good index of the amount of sarcoplasmic reticulum present in muscle. Of the ^{32}P recovered, 75% or more was associated with the Ca^{2+}-pump protein as observed by acrylamide gel electrophoresis under acid condition. A comparison of the phosphoenzyme levels in muscle and purified sarcoplasmic reticulum vesicles shows that the overall purification of sarcoplasmic reticulum vesicles was about 40-fold (Table I). Accordingly sarcoplasmic reticulum protein accounts for about 2.5% of the total muscle protein of rabbit skeletal muscle or 1 g wet muscle contains 5–6 mg of sarcoplasmic protein. For comparison, Peachey[27] estimated from measurements on electron micrographs that frog muscle contains a total sarcoplasmic reticulum volume of approx. 13%.

The Ca^{2+}-pump protein is clearly the dominant band as observed by acrylamide gel electrophoresis. Besides several minor bands, two other bands were consistently observed each accounting for 5–10% of the protein. One of these was the Ca^{2+}-binding protein, the other was the M_{55} protein. The purification of two of the

sarcoplasmic reticulum proteins, the Ca^{2+}-pump and the Ca^{2+}-binding proteins has been described in this study.

The Ca^{2+}-binding protein described here bears strong resemblance to Calsequestrin which was previously isolated from sarcoplasmic reticulum by MacLennan and Wong[13]. Both preparations are soluble acidic proteins, have a molecular weight of about 50000 as determined by sodium dodecyl sulfate acrylamide gel electrophoresis (System 2), have similar amino acid composition (with some noticeable differences) and have a remarkable ability to bind calcium (approx. 1 μmole/mg protein). We find significantly tighter binding, *i.e.* the binding constant of the Ca^{2+}-binding protein was 0.25 μM^{-1} *versus* 0.025 μM^{-1} for Calsequestrin. In both cases, the binding of Ca^{2+} was essentially all nonspecific as judged by the decrease in binding caused by the presence of other salts. MacLennan and Wong have suggested that Calsequestrin serves to bind and hence retain Ca^{2+} which is transported into the sarcoplasmic reticulum vesicles. Ikemoto *et al.*[5] isolated a protein with a molecular weight of 62000 from a Triton X-100 extract of sarcoplasmic reticulum in the presence of 4 mM Ca^{2+}. Since in this study sodium dodecyl sulfate gels were used which showed only one band in the 50000–60000 molecular weight region, it is not clear whether the protein with a molecular weight of 62000 was pure and was identical with Calsequestrin and the Ca^{2+}-binding protein isolated by MacLennan and Wong[13] as well as by us, respectively.

Several modifications for the purification of the Ca^{2+}-pump protein have been described in this paper. Each makes use of bile acids in the presence of salt. In Methods 1 and 2 small amounts of detergent which did not appreciably clarify the sarcoplasmic reticulum vesicle suspension were used to remove low molecular weight components including the Ca^{2+}-binding protein. The Ca^{2+}-pump protein, prepared in this manner, was membranous and was estimated to be 85% pure as judged by densitometry tracings of the gel pattern. The contaminating proteins were components of 130000 molecular weight or greater. To separate away most of the high molecular weight proteins it was necessary to use higher amounts of bile acid to first solubilize the membrane. The solubilized membrane was then dialyzed to allow reaggregation. The material which formed on reaggregation after a short dialysis interval consisted mainly of Ca^{2+}-pump protein (*cf.* Method 3). Its appearance was that of membranous vesicles as viewed by electron microscopy (Fig. 1) and its phospholipid content was approximately half of that of the starting sarcoplasmic reticulum. Most important it was enriched with respect to functional Ca^{2+}-pump protein as judged by an increase of the amount of phosphoenzyme, from 6.4 in sarcoplasmic reticulum to 9.0 nmoles/mg protein. This was a reasonable enrichment considering that approximately two-thirds of the protein of sarcoplasmic reticulum consists of the Ca^{2+}-pump protein.

MacLennan and co-workers[3,12] were the first to describe the purification of Ca^{2+}-pump protein from sarcoplasmic reticulum and to show that it could reaggregate to form membranes upon removal of detergent. Attempts to reaggregate the Ca^{2+}-pump protein with ammonium acetate according to the procedure of MacLennan[12], gave less reproducible results in our hands. Also we were not able to obtain a Ca^{2+}-pump protein preparation free of high molecular weight proteins using his procedure. The preparation of Ca^{2+}-pump protein described by us was simpler and had a somewhat higher phosphoenzyme content than the one by Mac-

Lennan *et al.*[3] (9 *vs* 7 nmoles/mg protein). The Ca^{2+}-pump protein has been also purified by Ikemoto *et al.*[5] using Triton X-100 as a solubilizing agent. A phosphoenzyme level of 14.7 nmoles/mg protein has been reported for their purest fraction suggesting that the Ca^{2+}-pump protein contains more than one phosphorylation site per molecule. We cannot explain their data; the studies of this paper as well as ATP binding studies[14] would argue against this.

The purification of the Ca^{2+}-pump and Ca^{2+}-binding proteins was greatly facilitated by the availability of highly purified sarcoplasmic reticulum vesicles as a starting material. Such a preparation further offers the powerful advantage in that it provides a frame of reference to consider functional sarcoplasmic reticulum. For example, MacLennan[12] and Ikemoto *et al.*[5] reported a several-fold purification of the Ca^{2+}-pump protein from sarcoplasmic reticulum. This clearly is not meaningful in light of the fact that the Ca^{2+}-pump protein makes up two-thirds of the protein of sarcoplasmic reticulum vesicles.

The above mentioned studies suggest that sarcoplasmic reticulum contains two groups of proteins: a group of membrane associated proteins[28] which are loosely attached to the membrane and are easily removed under relatively mild conditions (Ca^{2+}-binding and M_{55} proteins), and intrinsic membrane proteins, which make up the primary structure of the membrane together with the phospholipid, most notably the Ca^{2+}-pump protein, and which require extreme conditions for solubilization[28]. In this regard, Duggan and Martonosi[29] found that washing of sarcoplasmic reticulum with solutions at pH 8–9 containing EGTA or EDTA released two proteins, probably the Ca^{2+}-binding and M_{55} proteins. These proteins were likewise released when sarcoplasmic reticulum was treated with phospholipase A (ref. 30).

The amount of phospholipid associated with the Ca^{2+}-pump protein varied with the precise conditions used. The membranous Ca^{2+}-pump protein which was formed on reaggregation of a solubilized sample bound 12–19 μg P per mg protein. The lowest bound P to protein ratio, which was equal to about 8 μg P/mg protein, was obtained by partially extracting sarcoplasmic reticulum vesicles with cholate or deoxycholate. Attempts to lower this ratio further by varying the conditions of detergent treatment were unsuccessful indicating that about one-third of the phospholipid was bound much tighter to the Ca^{2+}-pump protein than the remaining part.

In our binding studies we found that sarcoplasmic reticulum vesicles contained specific and nonspecific high-affinity Ca^{2+}-binding sites with a capacity of 10–15 and 90–100 nmoles Ca^{2+} per mg protein, respectively and with binding constants of about 1 μM^{-1}. In the case of nonspecific binding the apparent binding constant varies with the ionic strength of the medium. The binding constants in our studies as well as those in the literature are only apparent binding constants, since they were not extrapolated to ionic strength equal to zero. Our binding data agree qualitatively with those of Cohen and Selinger[31] who report both specific (5 nmoles Ca^{2+} per mg protein) and nonspecific (45 nmoles Ca^{2+} per mg protein) Ca^{2+} binding. Their binding constants for both types of binding was about 40-fold lower than what we found. In contrast, Chevallier and Butow[32] did not find nonspecific high-affinity binding, *i.e.* two binding constants were found, equal to approx. 1 and 0.05 μM^{-1} (at pH 6.5) and accounting for 10–20 and 90–130 nmoles Ca^{2+} per mg protein, respectively. Some of their specific low-affinity binding may be referable to extraneous muscle protein, since little effort was made to obtain a highly purified and well-

characterized sarcoplasmic reticulum preparation. It is also difficult to directly correlate our binding data with those of Bertrand *et al.*[33] who studied Ca^{2+} binding to an enriched protein preparation obtained by sodium dodecyl sulfate treatment of sarcoplasmic reticulum. Carvalho and Leo[34] found that the total amount of Ca^{2+}, Mg^{2+} and K^+ in sarcoplasmic reticulum was about 350 nequiv/mg protein at neutral pH and that it remained approximately constant during energized Ca^{2+} uptake. Similarly Fiehn and Migala[35] reported that sarcoplasmic reticulum can bind 100–150 nmoles of Ca^{2+} per mg protein under nonenergized as well as energized conditions when a Ca^{2+} precipitating agent such as oxalate was absent. We found that the amount of Ca^{2+} and Mg^{2+} (130 nmoles/mg protein, Table I) isolated together with the purified sarcoplasmic reticulum vesicles was in the range of Ca^{2+} uptake (in the absence of oxalate). Ca^{2+} uptake (about 130 nmoles Ca^{2+}/mg protein, Table I) exceeded somewhat the number of specific and nonspecific high-affinity Ca^{2+}-binding sites of sarcoplasmic reticulum (100–110 nmoles/mg protein, Table VII). This difference might be accounted for by some low-affinity Ca^{2+} binding. The total (both bound and free) Ca^{2+} concentration of sarcoplasmic reticulum vesicles can be calculated to be 30–35 mM, assuming that 1 mg protein has a vesicular volume of 4 μl (ref. 29) and taking into account a Ca^{2+} uptake of 130 nmoles Ca^{2+} per mg protein. Thus, it is likely that under the conditions of the Ca^{2+} uptake assay, the Ca^{2+} accumulated was sufficiently high to compete favorably with other cations for the nonspecific high-affinity binding sites. If some nonspecific high-affinity Ca^{2+} binding sites were present on the outside of the membrane, then they would not be capable of binding Ca^{2+} under the conditions of the Ca^{2+} uptake assay and the role of low-affinity Ca^{2+} binding sites would be greater.

Partial extraction of sarcoplasmic reticulum vesicles with cholate resulted in a decrease of the number of nonspecific high-affinity Ca^{2+}-binding sites of the membranous fraction possibly because, as first postulated by MacLennan and Wong[13], a component such as Calsequestrin or the Ca^{2+}-binding protein isolated by us, was released. The Ca^{2+}-binding protein contained more than enough Ca^{2+} binding sites to account for the difference [approx. 35 nmoles Ca^{2+} per mg protein (Table VII)] between total high-affinity Ca^{2+} binding by sarcoplasmic reticulum and Ca^{2+}-pump protein. Assuming the Ca^{2+} binding protein accounted for 7% of sarcoplasmic reticulum protein and bound 900 nmoles Ca^{2+} per mg protein, it had a binding capacity of 63 nmoles Ca^{2+} per mg sarcoplasmic reticulum protein. The detergent treatment removed also part of the phospholipid from the membrane. The binding constants for Ca^{2+} by phospholipid reported in the literature vary from 0.01 to 10 μM^{-1}, the highest binding having been measured at low ionic strength[36]. It is then quite possible that some of the nonspecific high-affinity binding sites in sarcoplasmic reticulum could be also referable to phospholipid and that the decrease in the number of these binding sites in the Ca^{2+}-pump protein could be accounted for in part by the removal of phospholipid. The binding studies described indicate that it is too preliminary to assign the nonspecific Ca^{2+} binding simply to one membrane component.

The specific binding of Ca^{2+} by sarcoplasmic reticulum seems to be referable to the Ca^{2+}-pump protein (Table VII and Fig. 6). In a subsequent paper we will report that the Ca^{2+}-pump protein contained two specific high-affinity binding sites for Ca^{2+} and one for ATP per phosphorylation site[14]. Thus the Ca^{2+}-pump protein

contains many of the characteristics of the Ca^{2+} transport system. It combines with lipid and can be reaggregated to form membranous vesicles. In the presence of Ca^{2+}, ATP is hydrolyzed. A phosphoenzyme intermediate is formed which is discharged to release inorganic phosphate. Phospholipid has been shown to be required for the discharge of phosphoenzyme[30]. The membranous vesicles of the Ca^{2+}-pump protein have, however, lost their energized Ca^{2+} uptake capacity. To restore full transport capacity of Ca^{2+}, it may be necessary to add back the Ca^{2+}-binding protein or some of the other components of the sarcoplasmic reticulum membrane.

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