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CHARACTERIZATION OF SARCOPLASMIC RETICULUM FROM SKELETAL MUSCLE

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SUMMARY

Highly purified sarcoplasmic reticulum vesicles were isolated from rabbit muscle by sucrose gradient fractionation followed by a wash with 0.5 M LiBr. The purity of the vesicles was tested by loading with calcium oxalate and separating on a sucrose gradient the vesicles which accumulated more calcium oxalate. The more active fraction with regard to calcium oxalate accumulation had the same composition as that of the more poorly loaded vesicles or the entire preparation.

Phospholipids were more than 90 % of the total lipid and more than 35 % of the total membrane weight. Phosphatidylcholine (73 % of total lipid P), phosphatidylethanolamine (14 %) and phosphatidylinositol (9 %) were the major phospholipids. Triglycerides accounted for about 4 % and cholesterol and free fatty acids for less than 2 % each of the total lipid.

A protein profile was obtained by gel electrophoresis of sarcoplasmic reticulum vesicles having full Ca^{2+} uptake capacity. The molecular weights of the major proteins of sarcoplasmic reticulum vesicles were estimated to be $>$ 200000, approx. 200000, 140000, 115000, 60000 and 50000. The phosphoprotein intermediate of the Ca^{2+} pump was identified on acid gels. The steady-state concentration of the ^{32}P-labelled phosphoprotein was approx. 4.5 nmoles ^{32}P per mg protein and it had a molecular weight of about 115000. This ^{32}P-labelled phosphoprotein accounted for approx. 50 % of the total membrane protein of purified sarcoplasmic reticulum vesicles.

INTRODUCTION

The sarcoplasmic reticulum of skeletal muscle is a highly specialized membrane which regulates the Ca^{2+} distribution and thus contraction and relaxation inside muscle cells[1–3]. In the initiation of muscle contraction, Ca^{2+} is released from the sarcoplasmic reticulum. Muscle relaxation requires removal of Ca^{2+} energized by ATP through rapid and active pumping back into the sarcoplasmic reticulum. Isolated sarcoplasmic reticulum vesicles rapidly accumulate Ca^{2+} from the medium and contain a Ca^{2+} stimulated ATPase[4–6]. The splitting of ATP occurs through a covalently-linked phosphoprotein intermediate[7–9]. Since the regulation of Ca^{2+} distribution

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

appears to be the only function of sarcoplasmic reticulum, one may expect that this specialized membrane has a relatively simple lipid and protein composition. A simple membrane composition is ideal for studying the arrangement and function of the membrane components and their role in active transport.

This study characterizes a highly purified preparation from rabbit skeletal muscle of sarcoplasmic reticulum vesicles which are capable of actively transporting Ca^{2+}.

MATERIALS AND METHODS

Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared using a procedure somewhat modified from that of SERAYDARIAN AND MOMMAERTS[10]. Albino rabbits, about 3 kg each, were used. A rabbit was killed by injecting an overdose of nembutal (60 mg/kg rabbit), and the leg and back muscles were excised, chilled in ice, freed of fat, and passed through a meat grinder. All operations were carried out at 0–4°. Ground muscle, 50 g, was homogenized in 150 ml of homogenization buffer (0.3 M sucrose and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4)) for 30 sec in a Waring blendor. The homogenate from six blendings was centrifuged in six bottles for 20 min at 7000 rev./min in a GSA Rotor in a Sorvall centrifuge. The supernatant was poured through five layers of cheesecloth and a crude sarcoplasmic reticulum vesicle fraction was obtained by centrifugation for 75 min at 27000 rev./min in a Spinco 30 rotor. The supernatant was poured off and the pellets were resuspended in a total volume of 30 ml of homogenization buffer using a 50-ml glass–Teflon homogenizer. The crude fraction was placed on top of a step gradient (three tubes) containing 5 mM HEPES (pH 7.4) and different percentages of sucrose in each layer. The "percent sucrose" (w/w) was adjusted using a Bausch and Lomb refractometer at 25°. The gradient consisted of 12 ml 37.2 %, 10 ml 33.9 %, 8 ml 31.6 %, 8 ml 29.1 %, 12 ml 26.4 % sucrose. After application of a 10-ml sample, it was spun for 2.5 h at 24000 rev./min in the Spinco SW 25.2 rotor.

Vesicle fractions were taken off the gradient by pumping in heavy sucrose (50 %) from the bottom to displace the fractions through the top. The top 13 ml were discarded and fractions of 6 ml (Fraction 1), 12 ml (Fraction 2), 8 ml (Fraction 3) and 5 ml (Fraction 4) were successively collected (*cf.* Fig. 1). The fractions were diluted with 2 vol. of 5 mM HEPES (pH 7.4) added in three parts over a period of 30–45 min as a precaution to minimize osmotic shock, and then were centrifuged for 1 h at 45000 rev./min in a Spinco 50.1 rotor. The pellets were resuspended in a solution containing 0.3 M sucrose, 2.5 mM HEPES (pH 7.4) and 10–20 mg protein per ml, quick-frozen using liquid N_2 and stored at —70° in a low-temperature freezer. These preparations were stable for several months when stored in this manner.

To remove varying amounts of contaminating protein the preparation was extracted with a salt wash. Fractions 2 and 3 were thawed at 37°, combined, diluted to a protein concentration of 1 mg/ml in a solution containing 0.25 M sucrose, 0.5 M LiBr and 10 mM histidine (pH 7.3) and kept on ice for 1 h. The vesicles were recovered by sedimentation at 45000 rev./min for 1 h in a Spinco 50.1 rotor. The pellet was resuspended to a protein concentration of 10–20 mg protein per ml in 0.3 M sucrose containing 2.5 mM HEPES (pH 7.4). Aliquots of washed sarcoplasmic reticulum

vesicles were quick-frozen using liquid N_2. They were stable for several months when stored at —70°. Unless otherwise specified only these salt extracted sarcoplasmic reticulum vesicles were used in our experiments.

Enzymic assays

^{32}P-labelled phosphoprotein formation. Sarcoplasmic reticulum vesicles (1 mg protein) were incubated in 0.95 ml of a medium containing 0.105 M KCl, 5.25 mM $MgCl_2$, 0.105 mM $CaCl_2$ and 10.5 mM histidine (pH 7.3) at 0 or 32°. The reaction was started by the addition of 50 μl of 2 mM [^{32}P]ATP (10^3–10^5 counts/min per nmole ATP) with rapid stirring and was stopped after 6 sec at 0° or 3–4 sec at 32° by addition of 25 ml ice cold 4.25 % trichloroacetic acid containing 0.5 mM ATP and 1 mM P_i (ref. 11). The suspension was centrifuged at 20000 × *g* for 15 min and the pellet was washed twice with 25 ml of 2 % trichloroacetic acid and once with 10 ml of 5 % trichloroacetic acid by resuspension and centrifugation. The final pellet was resuspended in 1 ml of 0.1 M NaOH containing 2 % Na_2CO_3 and heated at 100° for 10 min. Control samples were incubated with 1 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in place of $CaCl_2$. Aliquots were taken for protein and radioactivity determination. A 0.1-ml sample was dissolved in 0.2 ml Protosol (New England Nuclear, Boston, Mass.) and then 5 ml of a toluene solution containing 4 g 2,5-diphenyloxazole and 0.3 g 1,4-bis-(2-phenyloxazolyl)benzene per l were added. Counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer, using mini vials and adapters (Nuclear Associates, Westbury, N.Y.).

ATPase assay. Sarcoplasmic reticulum vesicles (0.03–0.1 mg protein) were preincubated for 5 min at 32° in 2 ml of a mixture containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 10 mM histidine (pH 7.3). The reaction was started by addition of 50 μl of 0.1 M ATP and stopped after 5 or 10 min with 0.7 ml 1.5 M $HClO_4$. Inorganic phosphate was determined on 1 ml of the protein-free supernatant[12] using Elon as a reducing agent.

Ca^{2+} uptake capacity of sarcoplasmic reticulum vesicles (0.03–0.1 mg protein) was measured in 3 ml of a mixture containing 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $^{45}CaCl_2$, 5 mM ATP, 5 mM potassium oxalate, and 10 mM histidine (pH 7.3). The uptake reaction was carried out at 23° for 8 min and was terminated by pressing the mixture through a millipore filter (either HA 0.45 μm or GS 0.22 μm) in a syringe adapter[13]. A control mixture without added sarcoplasmic reticulum vesicles was used to determine the zero time concentration of ^{45}Ca. Samples of the filtrate (0.2 ml) were counted in 4 ml of scintillation fluid containing 60 g naphthalene, 4.2 g 2,5-diphenyloxazole, 180 mg 1,4-bis-(2-(5-phenyloxazolyl)) benzene and 70 ml water in 900 ml of dioxane.

Contamination with other organelles was monitored using succinate–cytochrome *c* reductase[14] and monoamine oxidase[15] activities for mitochondrial inner and outer membrane respectively, 5'-nucleotidase activity[16] for sarcolemma and acid phosphatase activity for lysosomes[17].

Electron microscopy

Samples for electron microscopy were prepared by fixation of a pellet with 1 % OsO_4 in 28 mM veronal–acetate buffer (pH 7.2) containing 2.4 mM $CaCl_2$ and 120 mM NaCl, overnight in the cold. The pellet was block stained with uranyl acetate, dehydrated, embedded in Araldite and treated as described previously[18]. In checking

for contamination oriented thin pellets were embedded. The section was cut across the pellet so that the whole cross-section from the top to the bottom could be observed.

Negative staining with phosphotungstic acid was carried out as described previously[18].

Chemical assays

Protein was determined by the procedure of LOWRY *et al.*[19] using bovine plasma albumin as a standard.

Total phosphorus was measured as an estimate of lipid phosphorus using a modification[20] of the method of CHEN *et al.*[21]. Two methods were used to show that this was a good approximation. When sarcoplasmic reticulum was extracted with 0.8 M $HClO_4$ at room temperature for 10 min, only 2–3 % of the total phosphorus was solubilized. Lipid was extracted from sarcoplasmic reticulum vesicles with chloroform–methanol (2:1, v/v) containing 1 μg butylated hydroxytoluene per ml and the extract was then passed through a Sephadex G-25 column[22,23] to separate phospholipid from other water-soluble phosphorus containing substances. Greater than 95 % of the total P was recovered in the phospholipid fraction (chloroform–methanol (19:1, by vol.) saturated with water.

Phospholipid analysis was carried out as described by ROUSER and co-workers[20,22–24]. The lipids of the chloroform–methanol (19:1, by vol.) phase of a Sephadex G-25 column[22] were separated by two-dimensional thin-layer chromatography[24].

Two solvent combinations were used: System (i) chloroform–methanol–28 % aq. ammonia (65:35:5, by vol.), followed by chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5, by vol.); and System (ii) chloroform–methanol–water (65:25:4, by vol.) followed by 1-butanol–acetic acid–water (60:20:20, by vol.). Lipids were visualized by spraying with conc. H_2SO_4–30 % formaldehyde (97:3, by vol.) followed by heating at 180°. The phospholipid distribution was determined by measuring the phosphorus content of the spots[24]. The phospholipids were identified by their relative migration. In addition, phosphatidylethanolamine and phosphatidylserine were confirmed by their ninhydrin-positive reaction, phosphatidylinositol and sphingomyelin by cochramotography with standards. System (i) was used routinely for phospholipid quantitation. System (ii) gave a complete separation of sphingomyelin and phosphatidylinositol and was used to obtain the phospholipid content of these two lipids.

Neutral lipids were isolated by passing the total lipid extract over a silicic acid column in chloroform (Unisil-Clarkson Chemical Co., Williamsport, Pa.). The chloroform eluate was defined as neutral lipids. The neutral lipid was analyzed by one-dimensional thin-layer chromatography using the solvent mixture *n*-hexane–diethyl ether–acetic acid (70:30:1, by vol.). The neutral lipid fraction was also used to quantitate cholesterol according to SEARCY *et al.*[25] and free fatty acid using the ^{63}Ni distribution method of Ho[26]. The weights of the neutral and total lipids dried *in vacuo* were determined using a Cahn Electro Balance.

To determine the cytochrome content, difference absorption spectra were recorded using a split-beam spectrophotometer designed at the Johnson Research Foundation (Univ. of Pennsylvania, Philadelphia). The concentration of cytochrome b_5 was calculated using a difference extinction coefficient of 21.0 $cm^{-1} \cdot mM^{-1}$ at

557–567 nm; cytochrome $a + a_3$ was calculated using a difference extinction coefficient of 16.0 $cm^{-1} \cdot mM^{-1}$ at 605—630 nm. To determine cytochrome P-450, a difference spectrum was obtained between the sample treated with CO and reduced with dithionite *versus* the sample oxidized by shaking with air[27].

Electrophoresis

The protein profile of sarcoplasmic reticulum vesicles was obtained by polyacrylamide-gel electrophoresis using the following conditions unless otherwise described:

(1) Electrophoresis in the presence of sodium dodecyl sulfate was carried out using the gel formulation of LENARD[28]. Gels containing 6 % acrylamide, 1 % sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.0) were prepared in glass tubes 5 mm in diameter and 10 cm in length. Samples were dissolved in a solution containing 4 M urea and 3 % sodium dodecyl sulfate. To reduce disulfides 2 % 2-mercaptoethanol was added and the mixture was heated for 5 min at 100°. The sample (25 μg protein in up to 0.1 ml) was layered on top of the gel and electrophoresis carried out for the first 30 min at 1.0 mA per tube. The current was then increased to 2.0 mA per tube and electrophoresis was continued for 15 h. Gels were stained and destained according to LENARD[28].

(2) Acid gels were prepared by a modification[29] of the method of TAKAYAMA *et al.*[30].

(3) Soaked, acid gels were prepared as described by MACLENNAN[31] with the following modifications: Polyacrylamide gels were prepared as described by ZAHLER *et al.*[29]. After the preelectrophoresis step the gels were removed from the glass tubes and soaked for 3 h in a solution containing 50 g phenol, 25 ml glacial acetic acid and 25 ml 8 M urea (soaking solution). The gels were sucked back into the same tubes with the aid of a water aspirator, overlayered with soaking solution and stored at room temperature for 12 h. Samples were dissolved in soaking solution and 20 μg protein in up to 0.1 ml was layered on top of the gel. In some cases samples were reduced with 2 % 2-mercaptoethanol in the presence of 4 M urea by heating for 5 min at 100° or treated with performic acid overnight at 0° (ref. 32) prior to the addition of soaking solution. Sucrose was removed from the sample by precipitation with 5 % trichloroacetic acid prior to performic oxidation. The pellet was resuspended in an excess of performic acid solution (1 mg protein/0.4 ml) and the mixture was finally diluted with 5 vol. of soaking solution. Electrophoresis was carried out at 4° with 10 % acetic acid as upper and lower chamber buffer. A current of 0.5 mA per tube was passed through the gels for the first 30 min after which the current was increased to 1 mA per tube for 5–9 h. Gels were stained with Coomassie Brilliant Blue (0.25 % in 7 % acetic acid) for 2 h and destained by soaking for several days in several changes of 7 % acetic acid. Some acid gels were stained with Amido Schwartz (1 % in 7 % acetic acid) for 2.5 h and destained in the same manner as for Coomassie Blue.

When working with the ^{32}P-labelled protein intermediate, all of the steps including solubilization of the sample, staining and destaining of the gels were carried out at 4°. For localization of radioactivity on polyacrylamide gels, the gels were sliced into approx. 2-mm segments and soaked overnight in 0.2 ml Protosol. Radioactivity was determined after the addition of 5 ml of toluene scintillation solution (see above).

Densitometry tracings of gels were obtained using a Photovolt Corp. densitometer, Model 52-C, modified according to PRIVETT *et al.*[33]. The mobility of standards and sarcoplasmic reticulum proteins relative to ovalbumin (R_M) was determined using densitometry tracings.

Materials

"Ultrapure" grade sucrose from Mann Research Laboratories (New York) was used throughout the experiments. [γ-^{32}P]ATP was prepared according to POST AND SEN[34] and was a generous gift of Dr. Robert Post (Dept. of Physiology, Vanderbilt University). Non-radioactive ATP was purchased from P-L Biochemicals, (Milwaukee, Wisc.).

RESULTS

Separation of the crude sarcoplasmic reticulum vesicle fraction on a sucrose gradient is shown in Fig. 1. Three components can be distinguished, a broad continuous band in the upper half of the gradient, a band at the 33.9/37.2 % sucrose interface and a pellet of varying size. Increasing the time of centrifugation shifts the upper broad band further down the gradient without significantly changing the overall distribution. Vesicle fractions were collected corresponding roughly to the material sedimenting in the 26.4 % sucrose layer (fraction 1), at and between interfaces 26.4/29.1 % and 29.1/31.6 % (fraction 2), at interface 31.6/33.9 % (fraction 3) and in the 33.9 % sucrose layer (fraction 4). Analysis of these fractions showed that they all accumulated calcium oxalate, had the ability to form a phosphoprotein intermediate and contained a Ca^{2+} stimulated ATPase (Table I). Fractions 2 and 3 had the highest Ca^{2+} uptake capacity and steady-state concentration of ^{32}P-labelled phosphoprotein. These fractions were pooled for use in the experiments described below, except when noted otherwise.

Markers for contaminating cell fractions reveal that Fractions 2 and 3 had negligible contamination with sarcolemma and lysosomes and slight contamination with mitochondria. Monoamine oxidase and 5'-nucleotidase[35] activities were not detectable. Acid phosphatase activity was 0.002–0.005 μmole P_i per min per mg protein, indicating negligible contamination of the fractions by lysosomes. Succinate–cytochrame *c* reductase in Fractions 2 and 3 varied from 0.005 to 0.01 μmoles cytochrome *c* reduced per min per mg protein. Assuming a rate for purified mito-

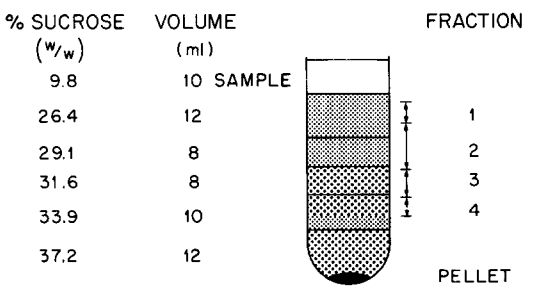

Fig. 1. Fractionation of the crude sarcoplasmic reticulum fraction on a sucrose step gradient. The relative protein concentration is indicated by the degree of stippling.

TABLE I

PROPERTIES OF SARCOPLASMIC RETICULUM VESICLES

The sarcoplasmic reticulum vesicles are obtained from the sucrose gradient in Fig. 1. Fraction I indicates sarcoplasmic reticulum vesicles of Fractions 2 and 3 combined. Fraction II indicates Fraction I washed with 0.5 M LiBr. Ca^{2+} uptake capacity, ^{32}P-labelled phosphoprotein formation at 0° and ATPase activity were measured as described in MATERIALS AND METHODS. ATPase activity was also determined in a medium which contained 1 mM EGTA instead of 0.1 mM Ca^{2+}.

Fraction (No.)	*Yield (mg protein/ 300 g muscle)*	*Total phosphorus (μg P/ mg protein)*	*Ca^{2+} uptake (μmoles Ca^{2+}/ mg protein)*	*^{32}P-labelled phosphoprotein (nmoles P/ mg protein)*	*ATPase (μmoles P_i/mg protein per min*	
					With 1 mM EGTA	*With 0.1 mM Ca^{2+}*
1	33 ± 10*	16.7 ± 4.5*	2.9 ± 1.3*	2.4 ± 0.6**	0.06 ± 0.03**	0.40 ± 0.10*
2	120 ± 40	18.8 ± 3.2	4.0 ± 0.8	3.3 ± 0.4	0.06 ± 0.03	0.50 ± 0.12
3	30 ± 12	21.2 ± 2.2	4.2 ± 0.8	3.4 ± 0.5	0.06 ± 0.03	0.55 ± 0.12
4	18 ± 5	21.0 ± 1.6	3.9 ± 1.0	3.0 ± 0.7	0.08 ± 0.04	0.60 ± 0.10
I	—	19.5 ± 3.0	4.0 ± 0.8	3.4 ± 0.5	0.06 ± 0.03	0.52 ± 0.12
II	—	24.0 ± 1.4	3.7 ± 1.0	4.1 ± 0.4	0.08 ± 0.05	0.85 ± 0.15

* Mean ± S.D. for six preparations.
** Mean ± S.D. for three preparations.

chondria of 0.5 this would indicate a contamination by mitochondria of 1–2 %. Fraction 4 was appreciably contaminated by mitochondrial fragments to the extent of 4–15 % and was therefore discarded.

Difference spectra on salt washed sarcoplasmic reticulum vesicles (Fraction II of Table I) showed that cytochromes typical of mitochondria were present confirming our enzymic data that a small amount of mitochondrial contamination is present. The cytochrome ($a+a_3$) content was 0.033 nmole/mg protein. Vesicles prepared from bovine heart mitochondria gave a value of 1.26 nmoles/mg protein. Assuming a similar value for vesicles from rabbit skeletal muscle mitochondria, the contamination by mitochondria would be 2.6 %.

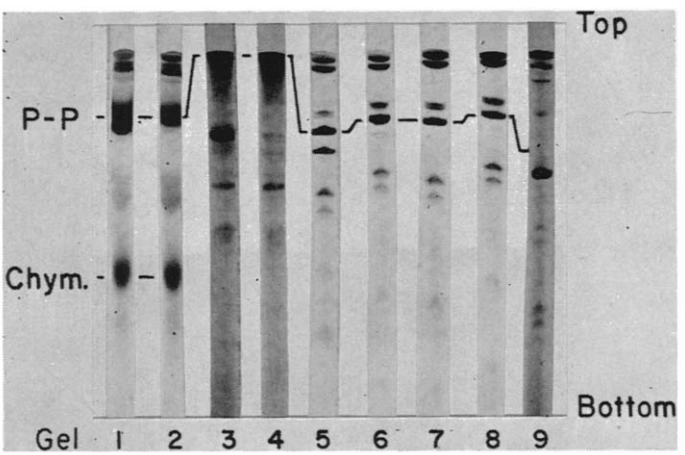

Fig. 2 a.

The concentration of cytochrome b_5 was 0.076 nmole/mg protein. This value is appreciably less than that obtained for liver microsomes, *i.e.* 1.4 nmoles/mg protein and 1.11 nmoles/mg protein from bovine and rat liver, respectively[27]. The cytochrome P-450 content was at the level of the "noise" and therefore negligible.

Typical protein profiles of isolated sarcoplasmic reticulum are shown in Fig. 2 using the three polyacrylamide gels described in MATERIALS AND METHODS. The proteins are best resolved in acid gels which were soaked in a mixture containing

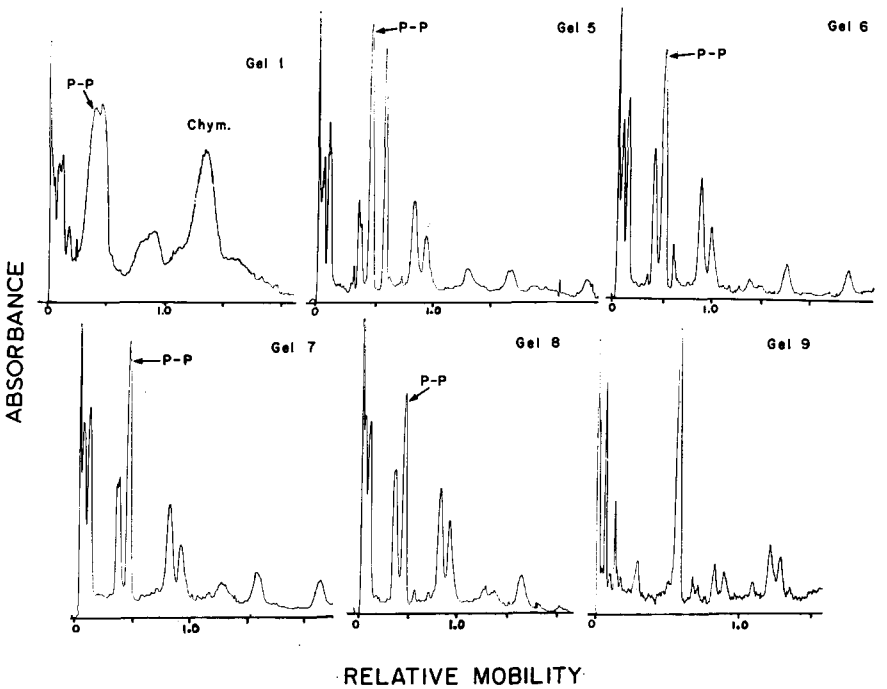

Fig. 2. Separation of sarcoplasmic reticulum proteins by gel electrophoresis. Sodium dodecyl sulfate gels contained 25 μg sarcoplasmic reticulum protein. Acid and soaked, acid gels contained 20 μg sarcoplasmic reticulum protein. Gels were stained with Coomassie Blue. The densitometer tracings of Gels 1, 5–9 are also shown. The mobility relative to ovalbumin is plotted on the abscissa in the tracings. P-P refers to ^{32}P-labelled phosphoprotein and Chym. refers to chymotrypsinogen A which is added as an internal standard to determine relative mobility. ^{32}P-labelled phosphoprotein was identified by relative mobility and/or labelling with ^{32}P. The ^{32}P-labelled phosphoprotein did not penetrate into the gel in Gels 3 and 4.

Gel	*Gel system*	*Sample*
1	Sodium dodecyl sulfate	Fraction I (*cf.* Tables I and II)
2	Sodium dodecyl sulfate	Same as in Gel A but in addition extracted with 0.5 M LiBr (Fraction II of Tables I and II)
3	Acid	Same as in Gel 1
4	Acid	Same as in Gel 2
5	Acid soaked	Same as in Gel 1
6	Acid soaked	Same as in Gel 2
7	Acid soaked	Pellet Fraction IIb of Ca^{2+} loading experiment
8	Acid soaked	Interface Fraction IIb (Table II)
9	Acid soaked	0.5 M LiBr extract of Fraction I (*cf.* Tables I and II)

phenol prior to electrophoresis (Gel 5). In agreement with earlier reports[36], an appreciable amount of protein stays on top of normal acid gels, *i.e.* those not presoaked in phenol (Gel 3). In sodium dodecyl sulfate gels (Gel 1) all of the protein enters the gel, however, this gel system gives considerably poorer resolution of the sarcoplasmic reticulum proteins than soaked, acid gels.

The lipid phosphorus to protein ratio (μg total P per mg protein) varied somewhat from preparation to preparation averaging 18–21 μg P per mg protein for Fractions 2 and 3 (*cf.* Table 1, Fraction I). Part of the protein could be removed by extraction with either 0.6 M KCl (ref. 37) or 0.5 M LiBr. We preferred 0.5 M LiBr, since the purification was more reproducible and one treatment was usually sufficient. One of the major protein bands, with a mobility relative to ovalbumin of 0.60 (mol. wt 100 000), was almost completely removed form the original vesicle protein (*cf.* Gels 3 and 4 or Gels 5 and 6) and was the predominant protein in the extract (Gel 9 of Fig. 2). The protein profile in Gel 9 and the amount of protein extracted varied somewhat from preparation to preparation. When the phosphorus to protein ratio was low, salt extraction removed more protein. The phosphorus to protein ratio of the extracted vesicles reached 23–25 μg P per mg protein (*cf.* Table I, Fraction II). Sonication in LiBr solution or increase of LiBr concentration up to 1.8 M removed additional protein, but did not selectively remove additional bands. This treatment also decreased Ca^{2+} uptake capacity and ^{32}P-labelled phosphoprotein concentration.

Electron microscopy of the salt-washed sarcoplasmic reticulum showed essentially closed membranous vesicles. The trilaminar arrangement of the membrane

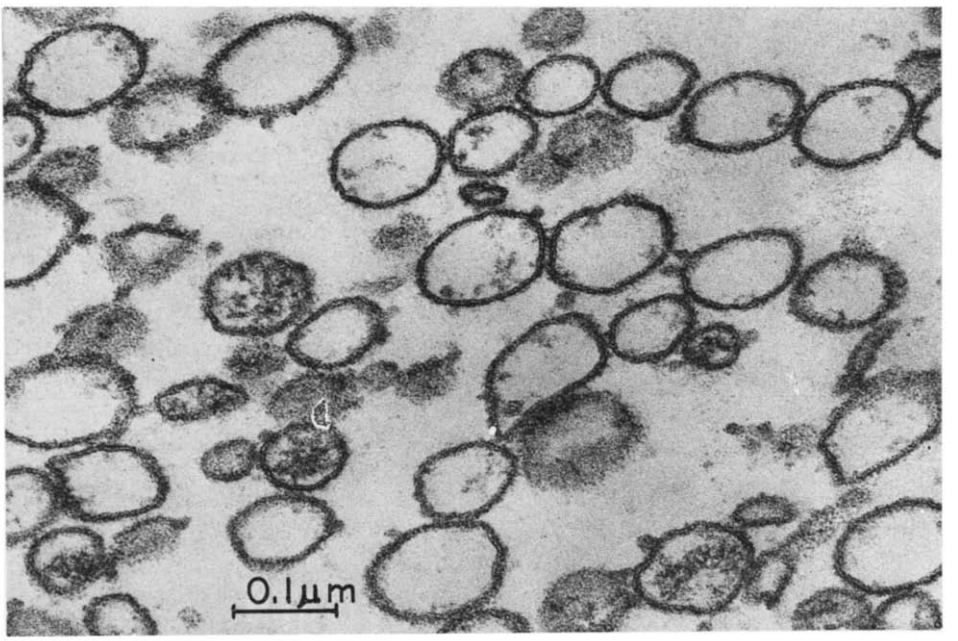

Fig. 3. Electron micrograph of sarcoplasmic reticulum vesicles isolated on a sucrose gradient followed by a wash with 0.5 M LiBr. Sample was fixed in 1 % OsO_4, embedded and sectioned. Magnification 140000 ×.

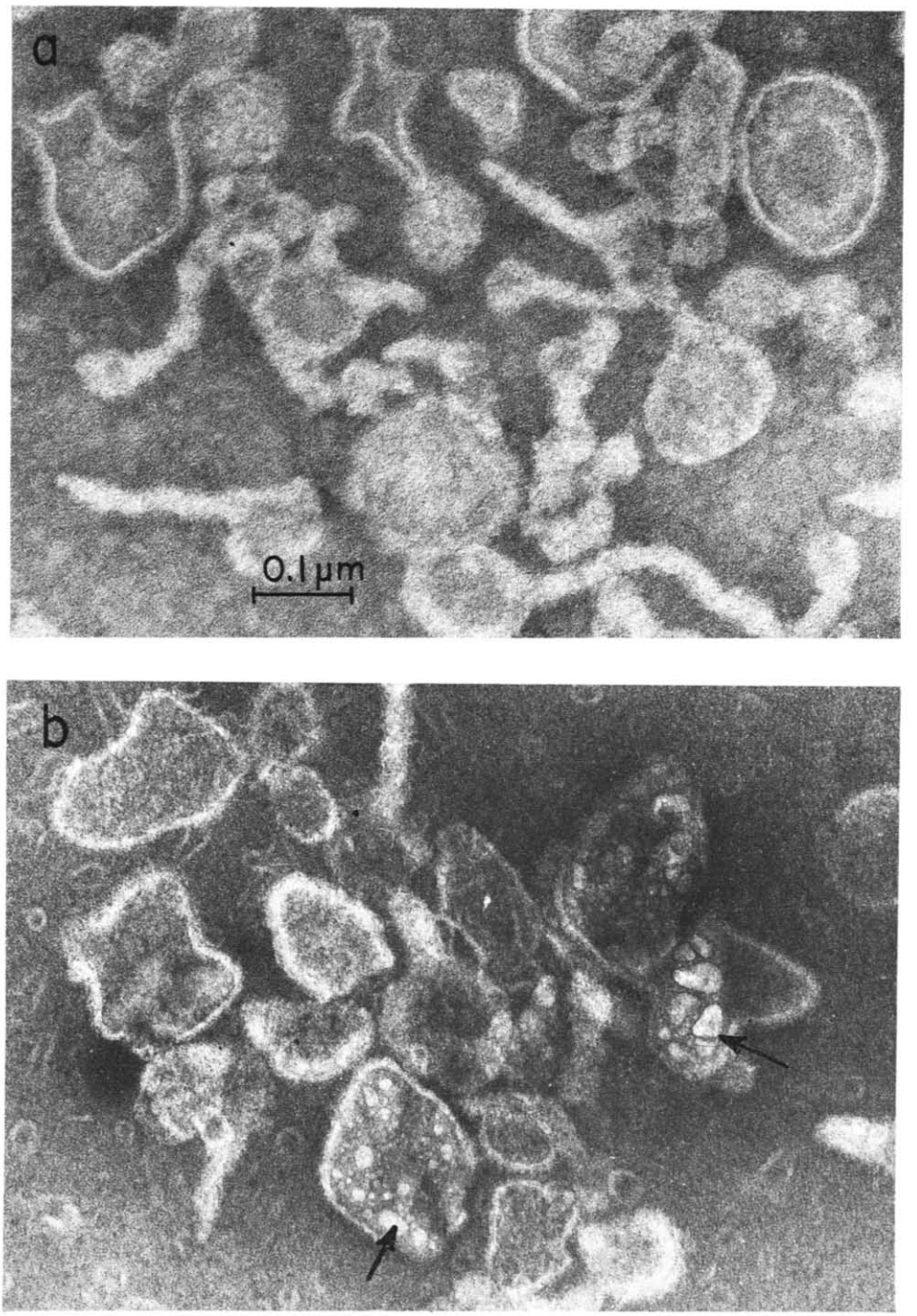

Fig. 4. Negatively-stained sarcoplasmic reticulum vesicles. a. Interface Fraction IIb. b. Pellet Fraction IIb of Ca^{2+}-loading experiment (see Table II). Arrows indicate calcium oxalate deposits in pellet fraction. Magnification 140000 ×.

is clearly visible (Fig. 3). Electron microscopy did not reveal any lysosomes or mitochondria, although 2 % mitochondrial contamination was detected chemically. Hence the contamination was probably in the form of submitochondrial vesicles.

As a further test of homogeneity, sarcoplasmic reticulum preparations were incubated with 0.25 mM Ca^{2+}, 5 mM ATP and 5 mM oxalate, which allowed accumulation of calcium oxalate by intact vesicles, and only intact vesicles. They were then placed on a sucrose gradient and centrifuged. Two fractions were obtained. One fraction had a higher density than the starting material and sedimented through

TABLE II

PROPERTIES OF SARCOPLASMIC RETICULUM VESICLES PURIFIED BY CALCIUM OXALATE ACCUMULATION

Sarcoplasmic reticulum vesicles not extracted (I) and extracted (II) with 0.5 M LiBr were used. As a precaution against extraneous dense material, the sarcoplasmic reticulum vesicles were prespun by placing 10 mg protein in 0.5 ml of 0.3 M sucrose *plus* 2.5 mM HEPES (pH 7.4) on top of 4 ml of 35 % sucrose containing 5 mM histidine (pH 7.3). The gradient was spun for 2 h at 48000 rev./min in the Spinco SW-50 rotor. A small pellet was discarded. 2 mg protein of the vesicle fraction collected in the top 3.5 ml of the sucrose gradient was incubated as usual in 50 ml Ca^{2+} uptake solution except that the Ca^{2+} concentration was 0.25 mM. About 70 % of $^{45}Ca^{2+}$ was taken up by the vesicles. 45 ml of the suspension were layered on top of sucrose layers consisting of 6 ml 28 % and 6 ml 40 % sucrose in 5 mM histidine (pH 7.3). After centrifugation in the Spinco SW-25.2 rotor for 1 h at 24000 rev./min, two fractions were obtained, one which accumulated high amounts of calcium oxalate and sedimented to the bottom of the gradient, and a second one which took up little or no calcium oxalate and banded at the 28/40 % sucrose interface. About 50 % of the protein was recovered in these experiments; 50–75 % of the recovered protein was in the pellet. Less than 2 % of the protein was recovered in the pellet when ATP was omitted. The pellet and the interface fractions contained 29–33 and 24–27 μg P/mg protein, respectively, when the incubation was carried out in the presence of ATP. The phosphorus to protein ratios shown are those obtained after acid extraction of the vesicles to remove soluble phosphate. Values are averages of two preparations.

	Total phosphorus (*μg P/mg protein*)	^{32}*P-labelled phosphoprotein* (*nmoles P/mg protein*)
I. Sarcoplasmic reticulum vesicles*	19.2	3.9
Ca^{2+} loading experiment		
(*a*) *ATP omitted*		
(1) Interface fraction	23.2	4.3
(2) Pellet	No sarcoplasmic reticulum vesicles	—
(*b*) *ATP added*		
(1) Interface fraction	23.7	4.4
(2) Pellet	24.7	4.0
II. Sarcoplasmic reticulum vesicles extracted with 0.5 M LiBr*	23.5	4.3
Ca^{2+} loading experiment		
(*a*) *ATP omitted*		
(1) Interface fraction	24.1	4.6
(2) Pellet	No sarcoplasmic reticulum vesicles	—
(*b*) *ATP added*		
(1) Interface fraction*, **	25.0	4.75
(2) Pellet*, **	26.1	3.3

* Protein profiles of these fractions are shown in Fig. 2.
** Electron micrographs of these fractions are shown in Figs. 4a and 4b.

both 28 and 40 % sucrose layers and pelleted. These were the relatively intact vesicles. The second fraction sedimented to the 28/40 % interface and therefore had a density similar to that of the starting material. This interface fraction consisted of relatively leaky vesicles and possibly other material. In the absence of ATP, all of the sarcoplasmic reticulum vesicles banded at the 28/40 % interface. Electron micrographs of both fractions showed the same appearance, namely, mainly sacs and tubules (Figs. 4a and 4b). About 30 % of the vesicles in the pellet fraction contained electron opaque granules, similar to those identified as calcium oxalate deposits by previous workers[38]. No deposits were found within the vesicles of the interface fractions.

To test the homogeneity of the original preparation, the composition and activity of the fractions were compared. Furthermore the test was done on vesicles before or after they were washed with LiBr. There was no difference between the interface fractions and the pellet fractions with respect to the ratio of total phosphorus to protein, the ratio of ^{32}P-labelled phosphoprotein to protein, or the distribution of protein into bands by gel electrophoresis (Table II and Fig. 2, Gels 7 and 8). Actually the ^{32}P -labelled phosphoprotein to protein ratio was lower in the pellet fraction. This is probably due to the damaging effects of the large amounts of calcium oxalate during handling of the loaded vesicles. Because the two fractions were basically the same, it was concluded that they differed only in the leakiness of the vesicles and that the original preparation was homogeneous in other respects. With respect to vesicles tested before washing with LiBr, the test itself purified the vesicles to the same extent as did the wash with LiBr. There was no such further purification in vesicles which had already been washed with LiBr (Table II).

Sarcoplasmic reticulum vesicles contained approx. 0.65 mg lipid per mg protein (Table III). Phospholipids accounted for 93 %, and total neutral lipids for about 7 %. The neutral lipid pattern was quite simple (Fig. 5). Triglyceride, cholesterol and free fatty acid were the main components and accounted for approx. 60, 25 % and 15 % by weight respectively. Cholesterol esters were detected only as a trace component. Coenzyme Q_{10} was not detectable in our preparation indicating its absence from sarcoplasmic reticulum vesicles. Two-dimensional thin-layer chromatograms of the phospholipids of sarcoplasmic reticulum vesicles are shown in Figs. 6a

TABLE III

LIPID COMPOSITION OF SARCOPLASMIC RETICULUM VESICLES

The lipid phosphorus to protein ratio of sarcoplasmic reticulum vesicles was 23.5 μg P/mg protein. Fatty acids and cholesterol were quantitated directly on the neutral lipid fraction. The triglyceride value is obtained by difference. The triglyceride value is somewhat inflated since a small amount of residual butylated hydroxytoluene, added during the extraction procedure, is present in the neutral lipid fraction. Values are averages of two sarcoplasmic reticulum vesicle preparations.

	% (*w*/*w*)	% *of total lipid* (*w*/*w*)
Protein	60.5	—
Lipid	39.5	—
Phospholipid	—	92.8
Total neutral lipid	—	7.2
Cholesterol	—	1.9
Fatty acids	—	1.0
Triglycerides	—	4.3

and 6b and the phospholipid composition is shown in Table IV. The major phospholipid is phosphatidylcholine which accounts for 73 % of the total lipid phosphorus. 95 % of the lipid phosphorus is in phosphatidylcholine, phosphatidylethanolamine

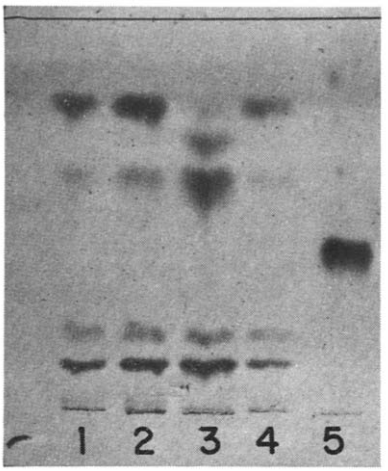

Fig. 5. Thin-layer chromatography of neutral lipids of sarcoplasmic reticulum. Neutral lipids were chromatographed in a solvent containing *n*-hexane–ether–acetic acid (70:30:1, by vol.). 65 μg lipid were spotted (Column 3) and compared with a standard containing 38 μg (Column 1), 76 μg (Column 2) and 15 μg (Column 4) total lipid. The standard mixture contained equal amounts of cholesterol, palmitic acid, tripalmitin and cholesterol palmitate in the sequence from origin to front. Column 5 contains 10 μg coenzyme Q_{10}. The sample contains five visible bands, cholesterol, fatty acids, triglycerides, butylated hydroxytoluene and a trace of cholesterol ester. The butylated hydroxytoluene is normally not present but is added during the lipid extraction to prevent lipid peroxidation. The plate was charred at 180° for 30 min after spraying with H_2SO_4–30 % formaldehyde (97:3, by vol.).

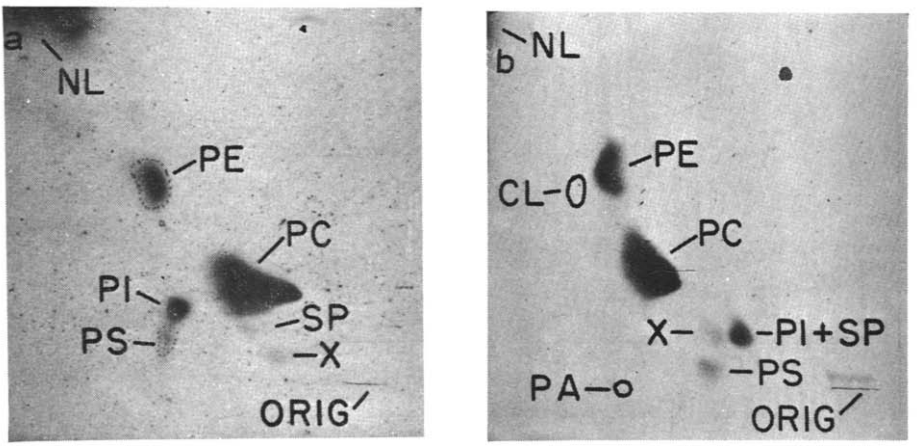

Fig. 6. Separation of sarcoplasmic reticulum phospholipid by two-dimensional thin-layer chromatography. Solvent combinations in (a): chloroform–methanol–water (65:25:4, by vol.) followed by 1-butanol–acetic acid–water (60:20:20, by vol.), in (b): chloroform–methanol–28 % aq. ammonia (65:35:5, by vol.) followed by chloroform–acetone–methanol–acetic acid–water (5:2:1:1:0.5, by vol.). 7 μg lipid P was applied to the plates. In (a) two spots are outlined by dots. This indicates the ninhydrin-positive material. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Sp, sphingomyelin; CL, cardiolipin; PA, phosphatidic acid; NL, neutral lipid; X, unidentified lipid.

or phosphatidylinositol. The data of MARTONOSI[13] are included for comparison (Table IV).

Since the Ca^{2+} pump is a major component of sarcoplasmic reticulum vesicles, optimal conditions for formation of ^{32}P-labelled phosphoprotein were studied. At 0° the amount of ^{32}P -labelled phosphoprotein was nearly independent of moderate changes in ATP, Ca^{2+} or Mg^{2+} concentration (Fig. 7). The concentrations of Ca^{2+} (0.1 mM) and Mg^{2+} (5 mM) were those in the ATPase assay mixture. At 0° the ^{32}P-labelled phosphoprotein concentration was constant between 2 sec and 2 min (Fig. 8). At room temperature, the ^{32}P-labelled phosphoprotein concentration decreased after 15 sec incubation, probably because of hydrolysis of the ATP. The addition of 1 mM EGTA in place of 0.1 mM Ca^{2+} decreased ^{32}P-labelled phosphoprotein forma-

TABLE IV

PHOSPHOLIPID COMPOSITION OF SARCOPLASMIC RETICULUM

	Percent of total lipid phosphorus	
	Mean ± S.D. of 8 thin-layer chromatography plates from two different sarcoplasmic reticulum preparations	*Values reported by* MARTONOSI[13]
Phosphatidylcholine	72.7 ± 1.4	65.0
Phosphatidylethanolamine	13.5 ± 0.9	12.3
Phosphatidylinositol	8.7 ± 0.9	—
Phosphatidylserine	1.8 ± 0.3	6.2
Sphingomyelin	1.0 ± 0.2	—
Cardiolipin	0.3 ± 0.2	—
Phosphatidic acid	0.2 ± 0.1	—
Unidentified phospholipids	1.8	16.5

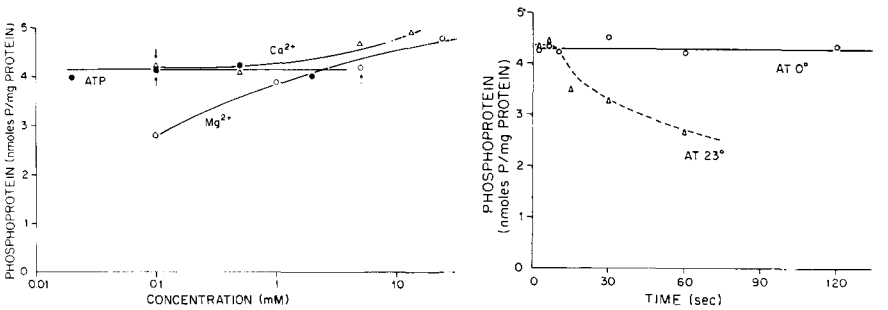

Fig. 7. Dependence of ^{32}P-labelled phosphoprotein formation on the concentration of ATP, Mg^{2+} or Ca^{2+}. Sarcoplasmic reticulum vesicles (1 mg protein per ml) were incubated at 0° in 0.95 ml of the standard medium containing 0.105 M KCl, 5.25 mM Mg^{2+}, 0.105 mM Ca^{2+}, 10.5 mM histidine (pH 7.3). The reaction was started by addition of 50 μl 2 mM [^{32}P]ATP and stopped after 6 sec by addition of 25 ml cold trichloroacetic acid solution. To obtain the three separate curves, the concentrations of ATP (●—●), Ca^{2+} (△—△) or Mg^{2+} (○—○) were individually varied from the standard conditions as shown. Standard conditions are shown by the arrows.

Fig. 8. Time course of the steady-state concentration of the ^{32}P-labelled phosphoprotein intermediate at 0° and 23°. Sarcoplasmic reticulum vesicles (1 mg/ml) were incubated in 0.95 ml of a mixture containing 0.105 M KCl, 5.25 mM Mg^{2+}, 0.105 mM Ca^{2+}, and 10.5 mM histidine (pH 7.3) and the reaction was started with the addition of 50 μl 2 mM [^{32}P]ATP. The reaction was stopped with trichloroacetic acid solution at the indicated time.

tion to less than 0.05 nmole P per mg protein, indicating that Ca^{2+} is required for formation. To compare ^{32}P-labelled phosphoprotein formation with other tests for the presence of sarcoplasmic reticulum vesicles detergents were added. Triton X-100, deoxycholate, or oleate did not affect ^{32}P-labelled phosphoprotein formation but prevented Ca^{2+} uptake and increased ATPase activity several fold (Table V). These concentrations of the detergents did not appreciably clarify the solutions. The level of ^{32}P-labelled phosphoprotein seems to be a better index of the amount of Ca^{2+}-pump enzyme in preparations of sarcoplasmic reticulum than ATPase or Ca^{2+} uptake capacity.

TABLE V

THE EFFECT OF DETERGENTS ON THE FUNCTION OF SARCOPLASMIC RETICULUM VESICLES

Samples were preincubated for 5 min at 32° in a medium containing 0.1 M KCl, 5 mM Mg^{2+}, 0.1 mM Ca^{2+} and 10 mM histidine (pH 7.3). The concentration of sarcoplasmic reticulum protein during the preincubation was 1.25 mg/ml except for the incubation with oleate where 0.4 mg/ml was used. Aliquots were then taken for determining Ca^{2+} uptake capacity, ATPase activity, and ^{32}P-labelled phosphoprotein formation at 0 and 32°.

Detergent	*Ca^{2+} uptake (μmoles Ca^{2+}/mg protein)*	*ATPase (μmoles P_i/mg protein per min)*	*^{32}P-labelled phosphoprotein (nmoles P/mg protein)*	
			At 0°	*At 32°*
—	3.4	0.90	3.75	3.6
Triton X-100 (0.15 %)	0	3.3	3.6	3.1
Deoxycholate (0.05 %)	0	3.5	3.65	3.25
Oleate (0.6 mM)	0	2.7	3.4	3.4

For the characterization of the ^{32}P-labelled phosphoprotein, the soaked, acid gel system is ideal since this labile intermediate is relatively stable under acid conditions. When trichloroacetic acid precipitates of ^{32}P-labelled membranes were applied to soaked, acid gels, the same protein profile was found as for sarcoplasmic reticulum vesicles (Fig. 2, Gel 6). By running the electrophoresis at 0–4° and slicing the gels immediately after electrophoresis, 75–90 % of the ^{32}P was recovered from the gel. After staining and destaining the gels for 1–2 days in 7 % acetic acid at 4°, 40–60 % of the ^{32}P label was still recovered. The latter procedure has the advantage that the protein bands are visible and the ^{32}P-containing bands are accurately identified. In both procedures about 90 % of the recovered ^{32}P was associated with the main protein band (R_m = 0.48 relative to ovalbumin, Gel 1 of Fig. 9), while the remaining ^{32}P stayed near the top of the gel. Sodium dodecyl sulfate gels were also used to characterize the ^{32}P-labelled phosphoprotein intermediate. 25–50 % of the ^{32}P was recovered in one band with a mobility of 0.41 relative to ovalbumin (Gel 2 of Fig. 2). The ^{32}P distribution in sodium dodecyl sulfate gels was very similar to that reported by MARTONOSI[36]. In these studies a current of 4 mA per tube was applied for 4–6 h. The gels were immediately sliced and slices were treated and analyzed as for the soaked, acid system.

The molecular weights of the sarcoplasmic reticulum proteins were estimated by comparing their mobility to the mobility of proteins with known molecular weights using both sodium dodecyl sulfate and soaked, acid gels. Separation on the basis of

molecular weight has been previously established in sodium dodecyl sulfate gels[39–41]. The usefulness of the soaked, acid gels for molecular weight determination was also investigated because of their superior resolution of the sarcoplasmic reticulum proteins and accurate identification of the ^{32}P-labelled phosphoprotein. In Figs. 10a and 10b the mobility of a number of proteins relative to ovalbumin is plotted against the logarithm of their molecular weight. As expected, the sodium dodecyl sulfate system (Fig. 10a) yielded approximately a straight line over the molecular weight range from 17000 to 140000. In the soaked, acid system (Fig. 10b) the plot was reasonably linear from 30000 to 160000, with more variation in the points than for the sodium dodecyl sulfate system. Table VI presents the molecular weights of the major sarcoplasmic reticulum proteins as estimated from the plots in Figs. 10a and 10b. The molecular weight of the ^{32}P-labelled phosphoprotein was estimated to be 115000. A second major protein with a molecular weight of approximately 100000 was found in preparations not washed with LiBr. Treatment with LiBr removed this band, as already mentioned. The three major bands migrating between the ^{32}P-labelled phosphoprotein band and the top of soaked, acid gels showed inhomogenities indicating that all of them are composed of more than one protein. The band above the ^{32}P-labelled phosphoprotein ($R_m = 0.37$) was sometimes clearly resolved into a doublet. Reduction with 2-mercaptoethanol or performic acid oxidation dramatically changed the profile of the large molecular weight group (mol. wt. $>$ 130000) by completely removing the doublet with a molecular weight of 140000 and by significantly decreasing the intensity of the bands with a molecular weight of approx. 200000 and larger. Two proteins with a molecular weight of about 50000 and 60000 stood out among several proteins

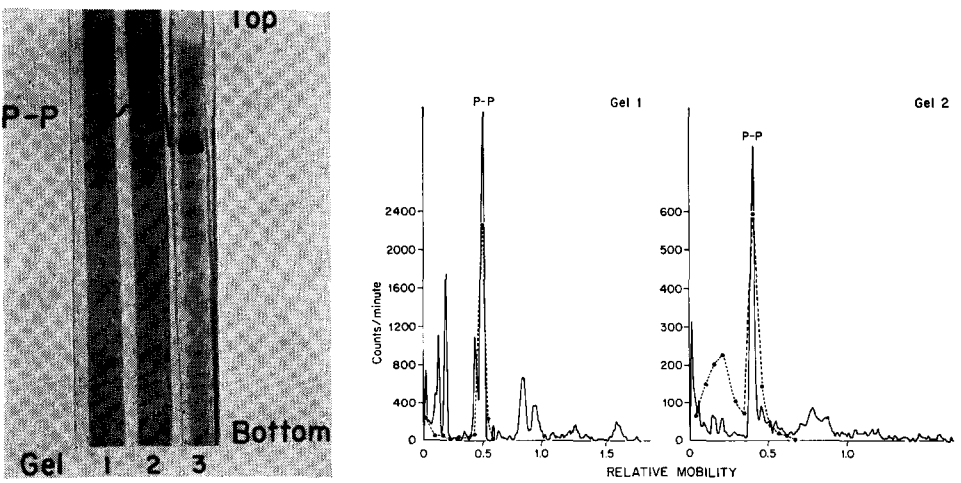

Fig. 9. Separation of sarcoplasmic reticulum proteins on soaked, acid gels containing 7% polyacrylamide. Gels were stained with Amido Schwartz. The densitometry tracings (———) and ^{32}P distribution (· · · · · ·) of ^{32}P charged membranes are also shown for Gel 1 (untreated sample) and Gel 2 (sample oxidized with performic acid). Sample of Gel 3 was reduced with 2-mercaptoethanol. The densitometry tracing of Gel 3 is not shown. Gels contained 16 μg sarcoplasmic reticulum protein. The relative mobilities of the phosphoprotein bands in Gels 1–3 were 0.48, 0.40 and 0.45 and were obtained using similar gels to which proteins of known molecular weight were added. ^{32}P distribution was obtained by slicing gels into about 2-mm segments immediately after the electrophoresis run and counting the radioactivity of the slices. Each point represents one 2-mm slice so that the overlap with the densitometry tracing is within experimental error.

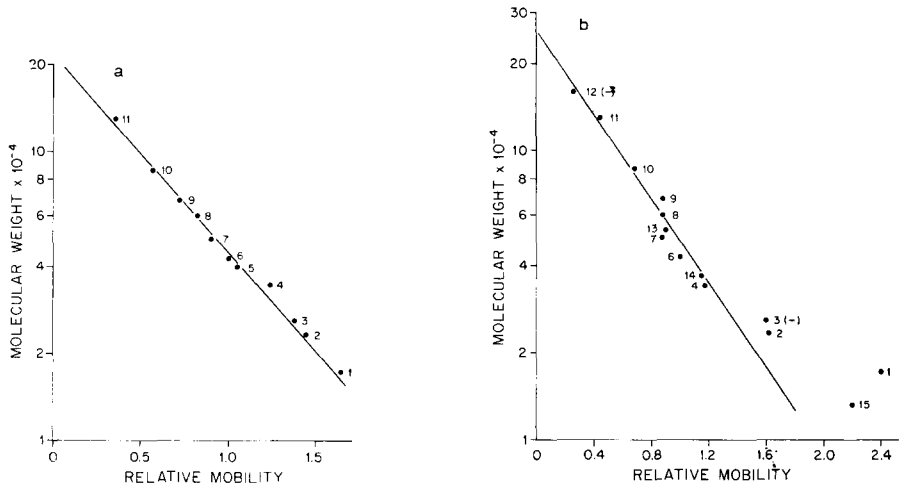

Fig. 10. Semilog plot of molecular weight against the distance relative to ovalbumin. a. 6% polyacrylamide gels containing 1% sodium dodecyl sulfate. b. 7% polyacrylamide gels, soaked in a mixture containing phenol. Proteins were reduced with 2-mercaptoethanol except those where the symbol (—) is added to the number. The following proteins were used (molecular weights are given in parentheses): 1, myoglobin (17200); 2, γ-globulin, light chain (23500); 3, chymotrypsinogen A (25700); 4, carboxypeptidase A (34600); 5, rabbit muscle aldolase (40000); 6, ovalbumin (43000); 7, γ-globulin, heavy chain (50000); 8, catalase (60000); 9, bovine serum albumin (68000); 10, conalbumin (86000); 11, β-galactosidase (130000); 12, γ-globulin (160000); 13, glutamate dehydrogenase (53000); 14, yeast alcohol dehydrogenase (37000); 15, ribonuclease (13700).

TABLE VI

ESTIMATION OF THE MOLECULAR WEIGHT OF THE MAJOR PROTEINS FROM SARCOPLASMIC RETICULUM USING GEL ELECTROPHORESIS

Mobilities relative to ovalbumin (R_m) are shown in the first column for soaked, acid gels and in the fourth column for sodium dodecyl sulfate gels. They were obtained from densitometry tracings of unreduced samples (Fig. 2). Reduced samples were run as well. Molecular weights were estimated using the plots in Figs. 10a and 10b. The major bands near the top of the gel ($R_m < 0.40$) showed inhomogenities indicating that each of them was composed of several proteins. The molecular weight corresponding to the peak for each of these bands is given. Values are averages of at least three determinations, S.D. was ± 10% or less.

Soaked, acid gels			*Sodium dodecyl sulfate gels*		
R_m	*Unreduced Mol. wt.*	*Reduced Mol. wt.*	R_m	*Unreduced Mol. wt.*	*Reduced Mol. wt.*
0.08	>200 000	**	0.05	Approx. 200 000	***
0.13	Approx. 200 000	**	0.09	Approx. 185 000	***
0.37	Approx. 140 000	**			
0.48	115 000	125 000	0.41	110 000	115 000
0.60*	96 000	96 000	0.475*	100 000	100 000
0.86	62 000	63 000	0.83	58 000	60 000
0.96	52 000	52 000	0.93	50 000	50 000

* This band is missing in preparations washed with 0.5 M LiBr.

** Appreciably decreased or removed when samples were treated with 2-mercaptoethanol or performic acid.

*** Appreciably decreased when samples were treated with 2-mercaptoethanol.

with a molecular weight of less than 75000 and were retained after reduction with 2-mercaptoethanol.

Coomassie Brilliant Blue was especially useful for detecting minor bands. However, this dye was not taken up in proportion to the amount of protein. Staining with Amido Schwartz provided better quantitation of protein bands in sodium dodecyl sulfate gels[42]. Preliminary studies showed that the same was true for soaked, acid gels. Within a given run, when Amido Schwartz was used as a dye, the area of any individual band was proportional to the amount of sarcoplasmic reticulum protein (10–30 μg) applied to the gel. We found in densitometry tracings of soaked, acid gels stained with Amido Schwartz, that 35–50 % of the total area under the curve was referable to the ^{32}P-labelled phosphoprotein band (R_m = 0.48, Gel 1 of Fig. 9).

When sarcoplasmic reticulum vesicles were reduced with 2-mercaptoethanol or oxidized with performic acid, the relative mobility of the most prominant band was shifted from 0.48 to 0.45 or 0.40, respectively (Gels 1–3 of Fig. 9). In both cases the pattern was simplified and several bands in the top of the gel disappeared. The question arises whether the protein of the ^{32}P-labelled phosphoprotein band was still present. The decrease in R_m after performic acid treatment might be due to an increase in the negative charge on the protein. To resolve this point ^{32}P-labelled sarcoplasmic reticulum vesicles were treated with performic acid overnight and then run on acid, soaked gels. About 50 % of the radioactivity of the ^{32}P-labelled phosphoprotein was stable to the performic acid treatment and was found in the gel. The band with R_m = 0.40 contained about 50 % of the recovered radioactivity in gels which were sliced immediately as well as in those which were stained and destained in 7 % acetic acid. The remainder was spread between the origin and the band with R_m = 0.40 (Gel 2, Fig. 9). A comparison of the densitometry tracings in Fig. 9 showed that the areas of the major band under the curve (R_m = 0.48 in the original sample, and R_m = 0.40 in the sample treated with performic acid) were approximately equal. These studies show that the band with R_m = 0.40 contained the oxidized form of the ^{32}P-labelled phosphoprotein.

As shown above, several large molecular weight bands were removed or decreased in intensity by the treatment with 2-mercaptoethanol or performic acid without an obvious appearance of new bands in 7 % gels run under standard conditions. To detect possible small molecular weight components such as "miniproteins"[43,44], samples, before and after treatment with 2-mercaptoethanol or performic acid, were applied to soaked, acid gels containing 10 % polyacrylamide (Gels 1–6, Fig. 11). Three of these six gels (Gels 1,3 and 5) also contained insulin, added as a marker for small molecular weight substances. We did not observe any appreciable bands in the region where the insulin migrated. This was true whether Coomassie Blue or Amido Schwartz was used as dye. Electrophoresis with sarcoplasmic reticulum vesicles labelled with ^{32}P and treated with performic acid showed that no radioactivity migrated into the small molecular weight region. Sodium dodecyl sulfate gels were also used to detect small molecular weight components. As in the soaked acid system, we observed in sodium dodecyl sulfate gels a drastic decreased of the intensity of bands with $R_m < 0.35$ after reduction of sarcoplasmic reticulum vesicles with 2-mercaptoethanol. We looked for the appearance of small molecular weight components in samples, before and after treatment with 2-mercaptoethanol on sodium dodecyl sulfate gels containing 10 % polyacrylamide. Gels of both unreduced and reduced samples (Gels

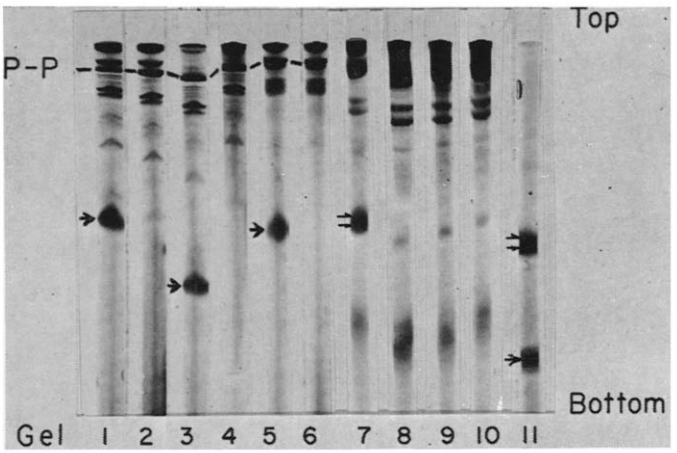

Fig. 11. Separation of sarcoplasmic reticulum proteins on soaked, acid and sodium dodecyl sulfate gels containing 10% polyacrylamide. Gels 1–6 in the soaked, acid system were prepared and run as described in MATERIALS AND METHODS with the exception that a 10% acrylamide solution was used instead of a 7% solution. Gels 1 and 2 contained untreated samples; Gels 3 and 4, samples treated with 2-mercaptoethanol; and Gels 5 and 6, samples treated with performic acid. Gels were stained with Coomassie Blue. All gels contained 20 μg sarcoplasmic reticulum protein. In addition 8 μg insulin was added to Gels 1, 3 and 5; the insulin band is indicated by an arrow. P-P refers to ^{32}P-labelled phosphoprotein. Sodium dodecyl sulfate gels (Gels 7–11) contained in addition to the polyacrylamide, 4 M urea, 0.1% sodium dodecyl sulfate and 0.1 M phosphate (pH 7.0). Gels were run for 30 min at 1 mA/tube and then for 7 h at 4 mA/tubes. They were fixed in 20% sulfosalicylic acid overnight, stained with 0.25% Coomassie Blue in 7% acetic acid for 4 h and then destained in 7% acetic acid. Samples were dissolved in a mixture containing 1% sodium dodecyl sulfate and 10 mM phosphate (pH 7.0) and either directly applied to the gels (Gel 7) or dialyzed for 24 h at 25° against a buffer containing 10 mM phosphate (pH 7.0) and 0.1% sodium dodecyl sulfate (Gel 8). In Gels 9 and 10, the sample was dissolved in a mixture containing 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol and 10 mM phosphate (pH 7.0) incubated for 3 h at 37° (Gel 9) or for 5 min at 100° (Gel 10) and then dialyzed for 24 h at 25° against a buffer containing 10 mM phosphate (pH 7.0), 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol[43]. Gels 7–10 contained 60 μg sarcoplasmic reticulum protein, Gels 7 and 11 contained 10 μg ribonuclease (unreduced), and Gel 11 contained 8 μg insulin (unreduced). Insulin is indicated by an arrow and ribonuclease is indicated by a double arrow.

7–11, Fig. 11), contained a band in the region where insulin migrates. Coomassie Blue or Amido Schwartz was used as dye in these experiments. However, the intensity of the fast moving band was not appreciably changed by the treatment with 2-mercaptoethanol and was less than that of the insulin band containing 8 μg protein. The gels contained 60 μg sarcoplasmic reticulum protein.

DISCUSSION

A highly purified preparation of vesicles which actively transport Ca^{2+} has been prepared from sarcoplasmic reticulum of skeletal muscle and characterized. The isolation procedure includes a sucrose step gradient and a salt wash. We estimate that more than 90% of our salt-washed preparation was derived from sarcoplasmic reticulum. This conclusion is based on estimation of enzymes marking contaminating cell fractions, cytochrome analysis, acrylamide-gel electrophoresis of membrane proteins, ^{32}P-labelled phosphoprotein formation and electron microscopy. In addition functional

vesicles were isolated by Ca^{2+} loading. The procedure was designed to isolate sarcoplasmic reticulum vesicles rather than specific components such as the Ca^{2+}-ATPase[31].

Characterization of the quality of a vesicle preparation is difficult. In principle, Ca^{2+} uptake should be the best test because in living muscle Ca^{2+} uptake from the cytoplasm is practically complete[45]. But our preparations and probably those of others are heterogeneous in this respect. We attribute the heterogeneity to differential leakiness of the vesicles. The heterogeneity of function was not reflected in any heterogeneity of composition or structure as far as we could estimate. In principle, we could have used the sucrose gradient to isolate for study only vesicles with the highest uptake, but a preliminary experiment gave such a low yield that characterization in other respects would have been impractical. Furthermore, we were afraid that accumulation of calcium oxalate might itself be injurious. For this reason it was necessary to include leaky vesicles in the preparation. Ca^{2+} uptake and Ca^{2+}-ATPase activities have been widely used to estimate the purity of vesicles. These activities reflect in part the leakiness of the vesicles. Supplementation of the ATPase assay with detergent requires exact conditions to ensure complete leakiness without inhibition of activity. In our experience the best test was the formation of the ^{32}P-labelled phosphoprotein of the Ca^{2+} pump in conjunction with electrophoresis of the proteins in a soaked, acid gel. The ratio of total phosphorus to protein was also useful as a preliminary test for changes in composition. The highest ratio in active preparations was 26 μg P per mg protein.

The main contaminants in our preparation were from mitochondria and were probably mitochondrial vesicles. The succinate–cytochrome *c* reductase activity and analysis of cytochrome ($a + a_3$) gave about 2–3 % contamination. Failure to detect 5′-nucleotidase activity[35] showed absence of sarcolemma and insignificant acid phosphatase activity showed negligible contamination with lysosomes.

Treatment with 0.5 M LiBr increased the phosphorus to protein ratio by about 20 % (to 23–25 μg P per mg protein) almost completely removing a protein with a molecular weight of about 100 000. This mild salt treatment did not appear to remove parts of the membrane essential for accumulation since removal of the protein did not lower the Ca^{2+} uptake capacity. However, the ATPase activity was frequently increased (*cf.* Table I) which indicates a slight impairment of membrane tightness. A clearly deleterious effect was observed at higher LiBr concentrations, with considerable loss of Ca^{2+} uptake capacity and ^{32}P-labelled phosphoprotein concentration.

Other investigators have used a sucrose gradient for purification of sarcoplasmic reticulum vesicles[5,10,46]. A direct comparison between their preparations and ours is difficult, since the two most reliable criteria for determining contamination by extraneous proteins, ^{32}P-labelled phosphoprotein formation and gel electrophoresis, have not been used consistently. MAKINOSE[8] reported a steady-state concentration of 2–3 nmoles ^{32}P per mg protein for sarcoplasmic reticulum vesicles purified on a sucrose gradient. We find 3–4 nmoles ^{32}P per mg protein (*cf.* Table I) for our preparations before the salt wash.

Our salt washed preparations have a lipid phosphorus to protein ratio of approx. 24 μg P per mg protein. This is the highest ratio reported and may be compared with those of SERAYDARIAN AND MOMMAERTS[10], FIEHN AND HASSELBACH[47] and YU *et al.*[48] who report ratios of 11, 18 and 19, respectively. No effort was made by these authors to free the vesicles from extraneous proteins which may have been present. Simple

differential centrifugation with subsequent KCl washes yields low lipid phosphorus to protein ratios (11 μg P per mg protein)[13] with a rather low steady-state concentration of ^{32}P-labelled phosphoprotein of 1.3–3.0 nmoles ^{32}P per mg protein[49] compared with 3.5–4.5 nmoles ^{32}P per mg protein in our preparation.

The lipid content of the salt extracted sarcoplasmic reticulum vesicles is 39 % by wt. Phospholipids alone account for 37 %. This is to be compared with 27 and 29 % reported by CARVALHO AND LEO[50] and FIEHN AND HASSELBACH[47], respectively. Sarcoplasmic reticulum has a unique and relatively simple lipid composition. It has already been reported that phosphatidylcholine is the major phospholipid accounting for 2/3 or more of the phospholipid[10,13,48,51]. Phosphatidylethanolamine and phosphatidylinositol are the other major phospholipids which together with phosphatidylcholine account for 95 % of the phospholipid.

Neutral lipids account for but 7 % of the lipid. This divided between fatty acid (1 %), cholesterol (2 %), and triglyceride (4 %). It may well be that the triglyceride is only a contaminant. Our neutral lipid content is considerably lower than the 20 and 38 % reported by FIEHN AND HASSELBACH[47] and CARVALHO AND LEO[50], respectively.

In agreement with INESI *et al.*[49] we find that maximal levels of phosphoprotein are formed within a few seconds at 0° and low ATP concentrations. As previously shown, this formation is strictly dependent on Ca^{2+}. The ^{32}P-labelled phosphoprotein intermediate was isolated at acid pH. At low pH it is rather stable[7–9], and it is therefore possible to characterize the phosphoprotein using gel electrophoresis. These experiments have been previously carried out by MARTONOSI[36] using the acid gel system of TAKAYAMA *et al.*[30] and sodium dodecyl sulfate gels. We repeated these studies. However, both gel systems have serious disadvantages. The acid system of TAKAYAMA *et al.* does not allow the ^{32}P-labelled protein to enter the gel and sodium dodecyl sulfate gels do not clearly resolve sarcoplasmic reticulum proteins. The system also produces appreciable hydrolysis of the ^{32}P-labelled phosphoprotein during electrophoresis. Recently MACLENNAN[31] reported that the acid system can be significantly improved by soaking the gels in a solution containing phenol prior to electrophoresis. The soaking step allowed the ATPase enzyme of sarcoplasmic reticulum to migrate into the gel. We modified MACLENNAN'S conditions to obtain a high resolution of sarcoplasmic reticulum proteins. Analysis of these soaked, acid gels showed that more than 90 % of the ^{32}P that entered the gel was recovered in a major protein band having a molecular weight of approx. 115000. The molecular weight of the Ca^{2+}-stimulated ATPase has been previously estimated to be 100000 with respect to protein based on the number of sulfhydryl-groups essential for Ca^{2+} transport[52] and to be 190000 overall on the basis of radiation inactivation studies[53]. MCFARLAND AND INESI[54] have characterized an enriched Ca^{2+}-stimulated ATPase preparation by sedimentation and electrophoresis in Triton X-100. The presence of proteins with a molecular weight of 80000 and multiples of this value were found[54]. However, the significance of these findings is uncertain, since the ATPase may not have been the only component present.

Gel electrophoresis showed that sarcoplasmic reticulum vesicles contained proteins in addition to the ATPase. In this regard, our studies agree with those of others[31,36,55]. In contrast YU AND MASORO[43] report one major protein band in sarcoplasmic reticulum membranes accounting for about 90 % of protein. YU AND MASORO[43] dissociated this protein into 6500 molecular weight units by reduction with β-mer-

captoethanol in the presence of sodium dodecyl sulfate. LAICO *et al.*[44] claim that a miniprotein is a major component of most membranes. In our hands performic acid oxidation or reduction with 2-mercaptoethanol of sarcoplasmic reticulum protein removed several of the large molecular weight proteins but it is not clear to what molecular weight these proteins were converted. A low molecular weight component was not observed in soaked, acid gels but was detectable as a minor component in sodium dodecyl sulfate gels whether or not sarcoplasmic reticulum vesicles were reduced with mercaptoethanol. It is significant that the major band representing the ATPase was not dissociated by this treatment.

Knowledge of the molecular weight of the ^{32}P-labelled phosphoprotein allows us to estimate the lower limit of the fraction of protein in the vesicles referable to the Ca^{2+} pump. Approx. 4.5 nmoles phosphorus were bound per mg sarcoplasmic reticulum protein (Table II). Using a molecular weight of 115000 (Table VI) for this component and assuming one phosphorylation site per enzyme molecule, we find that at least 50 % of the protein of sarcoplasmic reticulum is made up of the Ca^{2+} pump. By staining the soaked, acid gels with 1 % Amido Schwartz the area referable to ^{32}P-labelled phosphoprotein was 35–50 % of the total which is in fair agreement with the first estimate of 50 %. These two independent estimates indicate that the assumption of one phosphate binding site per molecule is valid.

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REFERENCES

1 W. HASSELBACH, *Prog. Biophys. Mol. Biol.*, 14 (1964) 167.
2 A. WEBER, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. 1, Academic Press, New York, 1966, p. 203.
3 S. EBASHI, M. ENDO AND I. OHTSUKI, *Q. Rev. Biophys.*, 2 (1969) 351.
4 W. HASSELBACH AND M. MAKINOSE, *Biochem. Z.*, 333 (1961) 518.
5 W. HASSELBACH AND M. MAKINOSE, *Biochem. Z.*, 339 (1963) 94.
6 S. EBASHI AND F. LIPMANN, *J. Cell Biol.*, 14 (1962) 389.
7 T. YAMAMOTO AND Y. TONOMURA, *J. Biochem. Tokyo*, 64 (1968) 137.
8 M. MAKINOSE, *Eur. J. Biochem.*, 10 (1969) 74.
9 A. MARTONOSI, *J. Biol. Chem.*, 244 (1969) 613.
10 K. SERAYDARIAN AND W. F. H. M. MOMMAERTS, *J. Cell Biol.*, 26 (1965) 641.
11 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 A. MARTONOSI, J. DONLEY AND R. A. HALPIN, *J. Biol. Chem.*, 243 (1968) 61.
14 S. FLEISCHER AND B. FLEISCHER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 406.

15 C. SCHNAITMAN, V. G. ERWIN AND J. W. GREENAWALT, *J. Cell Biol.*, 32 (1967) 719.
16 R. H. MICHELL AND J. N. HAWTHORNE, *Biochem. Biophys. Res. Commun.*, 21 (1965) 333.
17 R. WATTIAUX AND C. DEDUVE, *Biochem. J.*, 63 (1956) 606.
18 S. FLEISCHER, B. FLEISCHER AND W. STOECKENIUS, *J. Cell Biol.*, 32 (1967) 193.
19 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
20 G. ROUSER AND S. FLEISCHER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 385.
21 P. S. CHEN, T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
22 A. N. SIAKOTOS AND G. ROUSER, *J. Am. Oil Chemists' Soc.*, 42 (1965) 913.
23 G. ROUSER, G. KRITCHEVSKY AND A. YAMAMOTO, in G. V. MARINETTI, *Lipid Chromatographic Analysis*, Vol. 1, Marcel Dekker, New York, 1967, p. 99.
24 G. ROUSER, A. N. SIAKOTOS AND S. FLEISCHER, *Lipids*, 1 (1966) 85.
25 R. L. SEARCY, L. M. BERGQUIST AND R. C. JUNG, *J. Lipid Res.*, 1 (1960) 349.
26 R. T. HO, *Anal. Biochem.*, 36 (1970) 105.
27 S. FLEISCHER, B. FLEISCHER, A. AZZI AND B. CHANCE, *Biochim. Biophys. Acta*, 225 (1971) 194.
28 J. LENARD, *Biochemistry*, 9 (1970) 1129.
29 W. L. ZAHLER, B. FLEISCHER AND S. FLEISCHER, *Biochim. Biophys. Acta*, 203 (1970) 283.
30 K. TAKAYAMA, D. H. MACLENNAN, A. TZAGOLOFF AND C. D. STONER, *Arch. Biochem. Biophys.*, 114 (1966) 223.
31 D. H. MACLENNAN, *J. Biol. Chem.*, 245 (1970) 4508.
32 C. H. W. HIRS, in C. H. W. HIRS, *Methods in Enzymology*, Vol. 11, Academic Press, New York, 1967, p. 197.
33 O. S. PRIVETT, M. L. BLANK, D. W. CODDING AND E. C. NICKELL, *J. Am. Oil Chemists' Soc.*, 42 (1965) 381.
34 R. L. POST AND A. K. SEN, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 773.
35 D. L. FERDMAN, N. G. HIMMELREICH AND G. P. DYADYUSHA, *Biochim. Biophys. Acta*, 219 (1970) 372.
36 A. MARTONOSI, *Biochem. Biophys. Res. Commun.*, 36 (1969) 1039.
37 A. MARTONOSI, *J. Biol. Chem.*, 243 (1968) 71.
38 R. J. PODOLSKY, T. HALL AND S. L. HATCHETT, *J. Cell Biol.*, 44 (1970) 699.
39 A. L. SHAPIRO, E. VINUELA AND J. V. MAIZEL, JR., *Biochem. Biophys. Res. Commun.*, 28, (1967) 815.
40 A. K. DUNKER AND R. R. RUECKERT, *J. Biol. Chem.*, 244 (1969) 5074.
41 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.
42 C. A. SCHNAITMAN, *Proc. Natl. Acad. Sci. U.S.*, 63 (1969) 412.
43 B. P. YU AND E. J. MASORO, *Biochemistry*, 9 (1970) 2909.
44 M. T. LAICO, E. I. RUOSLAHTI, D. S. PAPERMASTER AND W. J. DREYER, *Proc. Natl. Acad. Sci. U.S.*, 67 (1970) 120.
45 R. J. PODOLSKY AND L. L. CONSTANTIN, *Fed. Proc.*, 23 (1964) 933.
46 B. P. YU, F. D. DEMARTINIS AND E. J. MASORO, *Anal. Biochem.*, 24 (1968) 523.
47 W. FIEHN AND W. HASSELBACH, *Eur. J. Biochem.*, 13 (1970) 510.
48 B. P. YU, F. D. DEMARTINIS AND E. J. MASORO, *J. Lipid Res.*, 9 (1968) 492.
49 G. INESI, E. MARING, A. J. MURPHY AND B. H. MCFARLAND, *Arch. Biochem. Biophys.*, 138 (1970) 285.
50 A. P. CARVALHO AND B. LEO, *J. Gen. Physiol.*, 50 (1967) 1327.
51 W. W. KIELLEY AND O. MEYERHOF, *J. Biol. Chem.*, 183 (1950) 391.
52 W. HASSELBACH AND K. SERAYDARIAN, *Biochem. Z.*, 345 (1966) 159.
53 K. VEGH, P. SPIEGLER, C. CHAMBERLAIN AND W. F. H. M. MOMMAERTS, *Biochim. Biophys. Acta*, 163 (1968) 266.
54 B. H. MCFARLAND AND G. INESI, *Biochem. Biophys. Res. Commun.*, 41 (1970) 239.
55 R. PANET AND Z. SELINGER, *Eur. J. Biochem.*, 14 (1970) 440.