ASSEMBLY OF ATPASE PROTEIN IN SARCOPLASMIC RETICULUM MEMBRANES

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ABSTRACT Three specimen preparation techniques for electron microscopy were used to investigate the incorporation of the ATPase polypeptide chains in the membranes of fragmented sarcoplasmic reticulum (SR) obtained from rabbit skeletal muscle. Observations were made of both normal vesicles and vesicles exposed to trypsin, which is known to cleave the ATPase protein and to alter the ultrastructure of the vesicles in predictable ways. Freeze-fracture replicas reveal the typical 90-Å particles on the concave (PF) faces with a density of 5,730 ± 520/μm^2. On the other hand both negatively stained and deeply etched preparations display outer projections, which are absent on trypsin-incubated vesicles. The etched specimens afford for the first time top views of the vesicles in the absence of any stain. These views reveal outer projections on the PS surface with a density of 21,000 ± 3,900/μm^2, a value nearly approximating the density of the ATPase polypeptide chains (106,000 mol wt) calculated on the basis of protein and membrane area determinations. On the other hand, this value is three to four times higher than that found for the density of the 90-Å particles on the concave fracture faces. Since both outer projections and 90-Å particles are identified with the ATPase protein, it is suggested that the ATPase polypeptide chains are amphiphilic molecules, with polar ends protruding individually as outer projections on the surface of the vesicles, and hydrophobic ends appearing as 90-Å particles on the concave fracture faces. The discrepancy between the densities of the outer projections and the 90-Å particles may be attributed either to variable penetration of the polypeptide chains into the membrane bilayer, or to formation of oligomers containing three or four hydrophobic ends and appearing as single 90-Å particles. Each ATPase chain forms a complex with 20–30 phospholipid molecules. The remaining phospholipids (~70% of the total SR phospholipids) account for less than half the membrane volume. It is proposed that the outer leaflet of the SR membrane is prevalently composed of the ATPase lipoprotein complex, and the inner leaflet is mostly a phospholipid monolayer.

INTRODUCTION

Fragments of sarcoplasmic reticulum (SR) membranes are particularly suitable for studying structure-function relationships since they display only one major activity: Ca^{2+} uptake coupled to ATP hydrolysis (Hasselbach and Makinose, 1961, 1963; Ebashi and Lipman, 1962; Weber et al., 1966). Previous studies have shown that the major (106,000 mol wt) protein component of SR is a Ca^{2+}-dependent ATPase (MacLennan, 1970; Meissner and Fleischer, 1971; Martonosi and Holpin, 1971; Inesi, 1972).

Furthermore, attempts have been made to identify the ATPase protein with ultrastructural details observed by electron microscopy on the SR vesicles (Deamer and Baskin, 1969; Hasselbach and Elfvin, 1967; Ikemoto et al., 1968; Inesi and Asai, 1968; Tillack et al., 1974; Inesi and Scales, 1974; Stewart and MacLennan, 1974; Sarzala et al., 1975; Thorley-Lawson and Green, 1973).

In this regard we have used negative stain, freeze-fracture and deep etch electron microscopy to investigate the assembly of ATPase in the membrane. Replicas of quickly frozen and deeply etched SR vesicles have permitted for the first time observation of the outer membrane surface in the absence of negative stains, which collapse the vesicles and in some cases produce denaturing effects (Deamer and Baskin, 1969). We were then able to evaluate quantitatively the ultrastructural elements observed in freeze-fractured and in deeply etched preparations. Furthermore, we attempted to interpret these findings in terms of molecular stoichiometry, considering estimates on the density of ATPase molecules per membrane unit area, obtained through chemical measurements on the same SR preparations.

METHODS

SR Preparations

SR vesicles were prepared by homogenization and differential centrifugation of rabbit skeletal muscle (Eletr and Inesi, 1972). Protein concentrations were measured with the biuret or Folin reagents. Protein solubilization in dodecylsulfate and gel electrophoresis were carried out as previously described (Inesi and Scales, 1974). ATPase activity was determined by measuring the release of inorganic phosphate according to Fiske and Subbarow (1925).

Trypsin Digestion of SR Vesicles

Trypsin digestion of SR vesicles was carried out at 25°C in the following reaction mixture: 1 mg of SR protein/ml, 10 mM MOPS, 100 mM NaCl, and 0.05 mg trypsin/ml. The incubation was interrupted after 3 h with soybeam trypsin inhibitor at a concentration twice as high as that of trypsin.

Negative Staining

Droplets of SR suspension (approximately 0.2 mg protein/ml) were placed on carbon-coated Formvar grids and stained with 0.5% uranyl acetate.

Freeze-Fracturing

Samples for freeze-fracturing were prepared by washing 8–10 mg SR protein in 80 mM KCl and 10 mM MOPS (pH 6.8) and resuspending in 0.2 ml of 60 mM KCl, 10 mM MOPS, and 20% glycerol. After 2 h in the glycerol solution, drops of the sample were frozen in liquid Freon 22, fractured at −100°C, and replicated with platinum-carbon. The replicas were cleaned for 1 h in bleach, washed twice, and mounted on uncoated copper grids for viewing in the electron microscope.

Freeze-Etching

Samples for freeze-etching were prepared as described for freeze-fracturing except that the washed sediments were not resuspended in glycerol. The samples were frozen in liquid Freon 22

and transferred to a Balzers freeze-fracture device (Balzers High Vacuum Corp., Santa Ana, Calif.). They were fractured and etched for 1 min at −100°C in a vacuum of 1×10^{-6} torr, with the liquid-nitrogen cooled knife directly above the fractured samples. Two samples of normal vesicles and two samples of the trypsin-incubated vesicles were etched and then replicated at the same time after removing the knife and shadowing immediately with platinum-carbon.

Electron Microscopy

All samples were examined in a Philips EM200 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, N.Y.) at 80 kV with a cold contamination device surrounding the specimen.

Optical Diffraction

The optical diffractometer used in these studies consists simply of a He-Ne laser (Spectra-Physics Inc., Mountain View, Calif.), a 135 mm converging lens, and a photomicroscope. Much more elaborate equipment has been described previously (Markham, 1968; Taylor and Lipson, 1964). Transparencies were placed 4 m in front of the lens and illuminated with the zero-order Airy disc arising from a 200 μm circular aperture at the laser. For all practical purposes this constitutes parallel illumination and eliminates the need for the condenser lenses described in the references.

Micrographs of freeze-fractured SR vesicles were enlarged to a final magnification of ×286,000. A sheet of black paper was placed behind the micrograph and holes were punched at the locations on the micrograph where there seemed to be a particle. The black mask, then, represents the arrangement of particles on the concave fracture face of the micrograph. The mask was removed and placed over a sheet of white paper to make the holes appear white. This was then photographed on a copy table with a positive film, Kodak High Speed Duplicating Film 2575, and developed to enhance the contrast in undiluted Dektol (Eastman Kodak) for 2 min. The resulting transparency reduced the size of the mask 11.7 times and was used as the object in the diffractometer. The transparency is black everywhere except at the particle locations where the emulsion is clear (see Fig. 4).

The diffraction patterns were calibrated with 200-mesh screen which gives a two-dimensional diffraction pattern whose first-order spatial frequencies correspond to the 125 μm separation on the object screen.

RESULTS

Electron microscopic observations of both normal SR vesicles and SR vesicles exposed to trypsin were made using three specimen preparation techniques.

Negatively Stained SR

Surface projections of negatively stained SR vesicles have been described previously (Ikemoto et al., 1968; Inesi and Asai, 1968). The projections are 35 Å in diameter, but they only appear on the edges of the vesicles, making it difficult to measure their separation. Fig. 1A shows a group of SR vesicles negatively stained with uranyl acetate.

Exposure to trypsin removes the outer projections and leaves vesicles with smooth edges as shown in Fig. 1B. The disappearance of the surface detail occurs simultaneously with extensive cleavage of the ATPase protein as monitored by electrophoretic analysis (Stewart and MacLennan, 1974; Inesi and Scales, 1974).

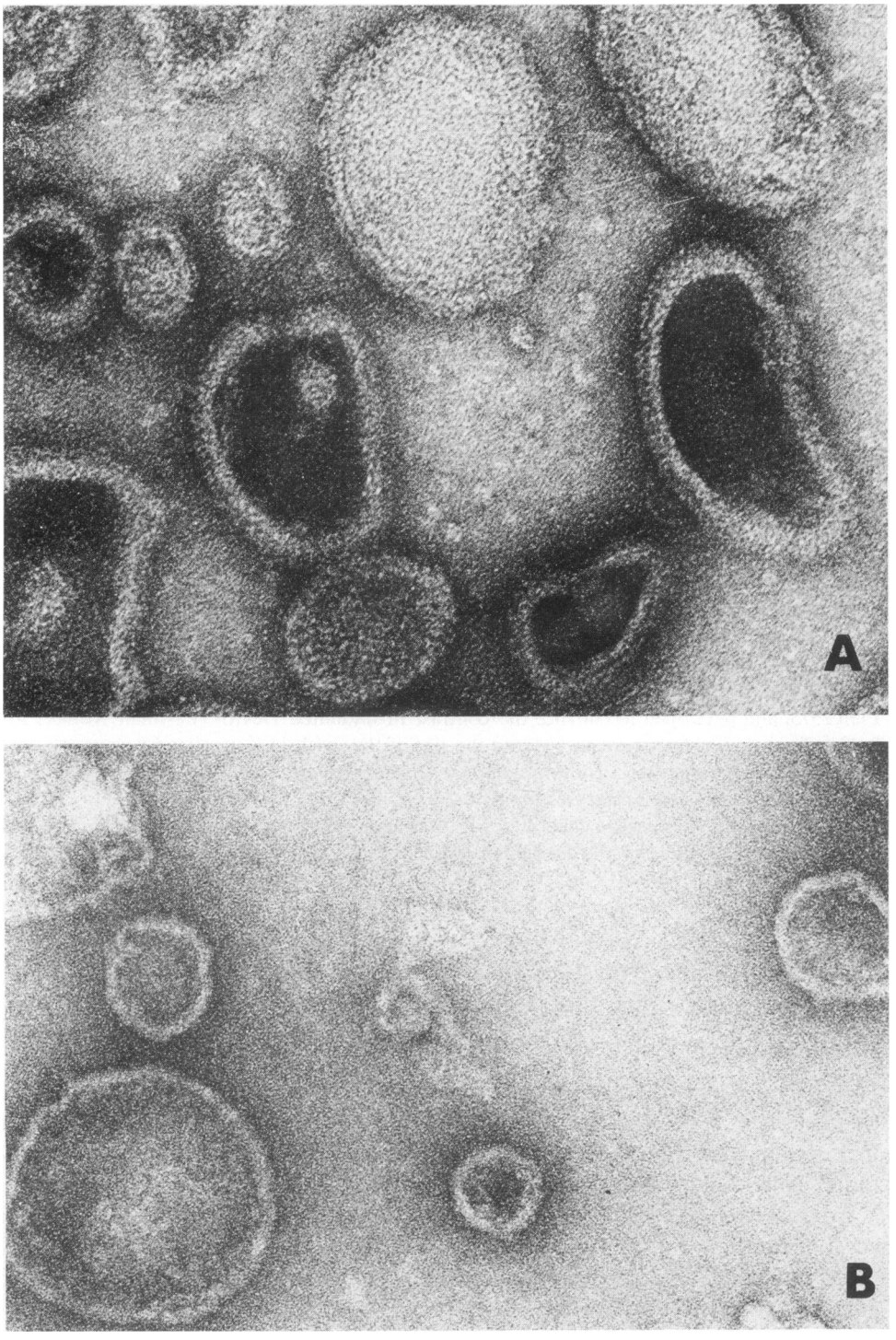

FIGURE 1 (*A*) SR vesicles negatively stained with 0.5% uranyl acetate. Outer projections are clearly seen extending from the edges of the vesicles. × 285.000. (*B*) After prolonged exposure to trypsin (SR protein/trypsin = 20 for 3 h), the outer projections are absent. × 285,000.

The surface projections seen around the perimeter of the native SR vesicles most likely originate from planes other than that of the surface, thereby becoming superimposed in the final two-dimensional micrograph. On the planar surface of the membrane the projections are less well outlined. Nevertheless, estimates on the density of the projections have been presented (Jilka et al., 1975) yielding interesting information. However, structural measurements from negatively stained images are not reliable by themselves since the vesicles are collapsed and severely distorted on the grid, yielding a complicated two-sided image as often found with other specimens (Klug and DeRosier, 1966). For this reason quantitation of surface area and surface detail apparent in the final two-dimensional electron image may be unrealistic.

It should be stressed that all high magnification electron microscope images of these specimens are phase images and have a characteristic granular appearance. This so-called phase grain is caused by coherent scattering from the stain, specimen and carbon support film, limiting the resolution of negatively stained specimens to 20 Å (Haydon, 1968).

The grain size depends on the plane of focus, being smallest near focus and increasing on either side of true focus (Thon, 1966). The granular appearance on the surface of negatively stained SR vesicles can, therefore, be enhanced by defocusing, but this granularity does not necessarily represent a legitimate ultrastructural detail as is easily demonstrated by comparing the structure in question with the neighboring granularity of the support film. Therefore, observations made on negatively stained preparations require verification by an independent technique.

Freeze-Etched SR

Vesicles from the same SR preparations used for negative-staining were washed, resuspended and frozen in the absence of glycerol as described in Methods for freeze-etching. After fracturing the samples were deeply etched for 60 s at −100°C. This removes a layer of ice and reveals the interface between the unfractured SR vesicles and the aqueous medium. We label this surface the PS surface which represents the external surface of the vesicles. Fig. 3A shows vesicles uncovered by the extended etch. We notice that this surface is rough and granular, each "particle" being about 50 Å in diameter.

Sometimes a vesicle is fractured and then etched. In this case, the fracture reveals the EF face and the etch uncovers the neighboring PS surface. Fig. 2B shows such a vesicle. The central smooth region is the typical EF face seen in freeze-fracture replicas (see Fig. 3A). This region, which is thought to represent the inner lipid leaflet of the membrane, is clearly smooth compared with the surrounding granular PS surface.

The advantage of freeze-etching is that the sample undergoes minimal pretreatment, and in fact our samples were prepared without the use of either fixatives or antifreeze. The replicas expose the top surface of the vesicles, which allows us to estimate the density of outer projections. Caution must be exercised when depositing platinum for these replicas to avoid bridging the spaces between the projections. This invariably happens, however, to the side of the vesicles nearest the platinum source, and we only

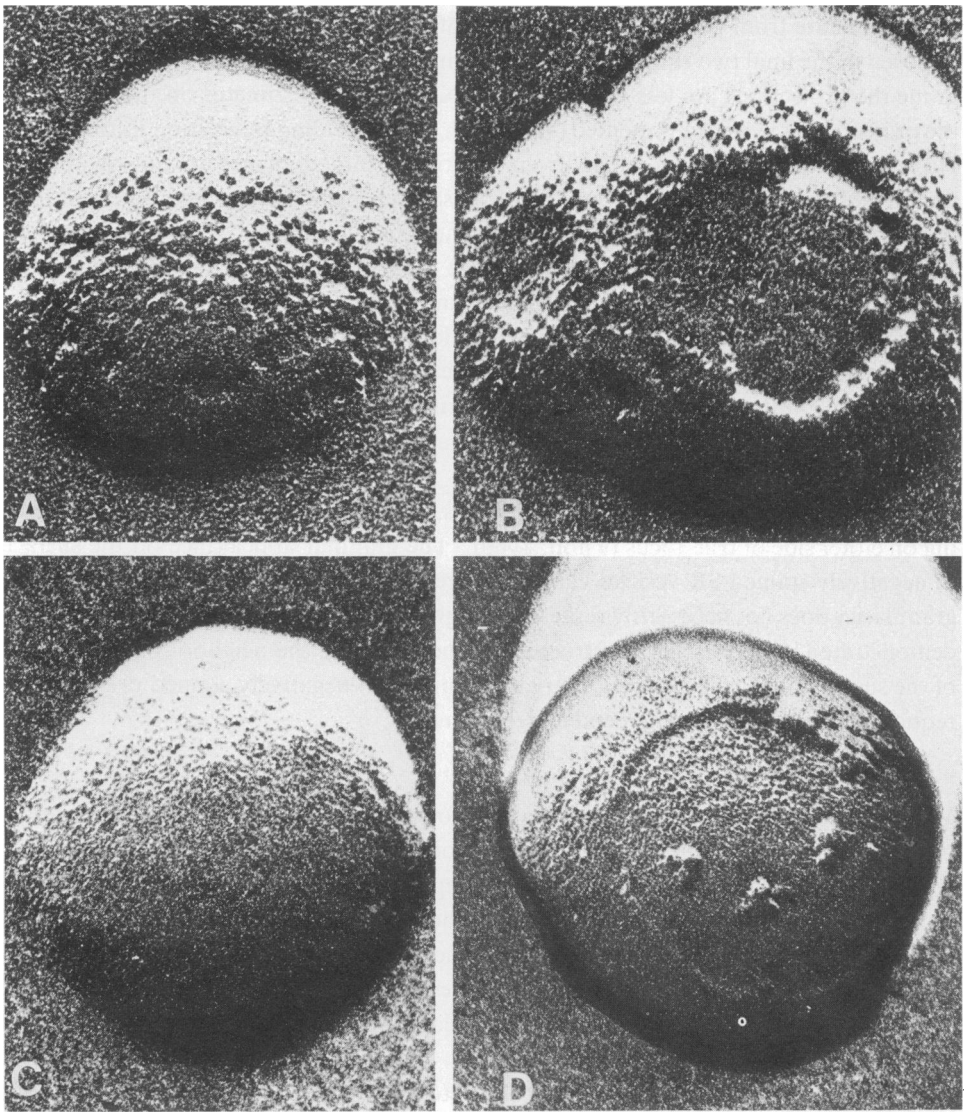

FIGURE 2 (*A*) A typical surface revealed after etching for 1 min at −100°C in the absence of glycerol. In this case an intact vesicle has been uncovered by the prolonged etch. × 285,000. (*B*) A vesicle that has been fractured, revealing the smooth EF fracture face, and then etched, showing the neighboring PS surface. × 285,000. The outer projections on this surface correspond to the projections seen on negatively stained specimens (Fig. 1A). Estimates of the density of outer projections from micrographs such as these give an average of 21,000 ± 3,900/μm^2. (*C*) A typical smooth surface revealed after etching of SR vesicles incubated in trypsin. This represents the freeze-etch view of the same preparation shown by negative staining in Fig. 1B. × 285,000. (*D*) A vesicle exposed to trypsin that has been fractured and etched, revealing a smooth external surface. × 285,000. Replicas of both trypsin-incubated and native SR were made simultaneously.

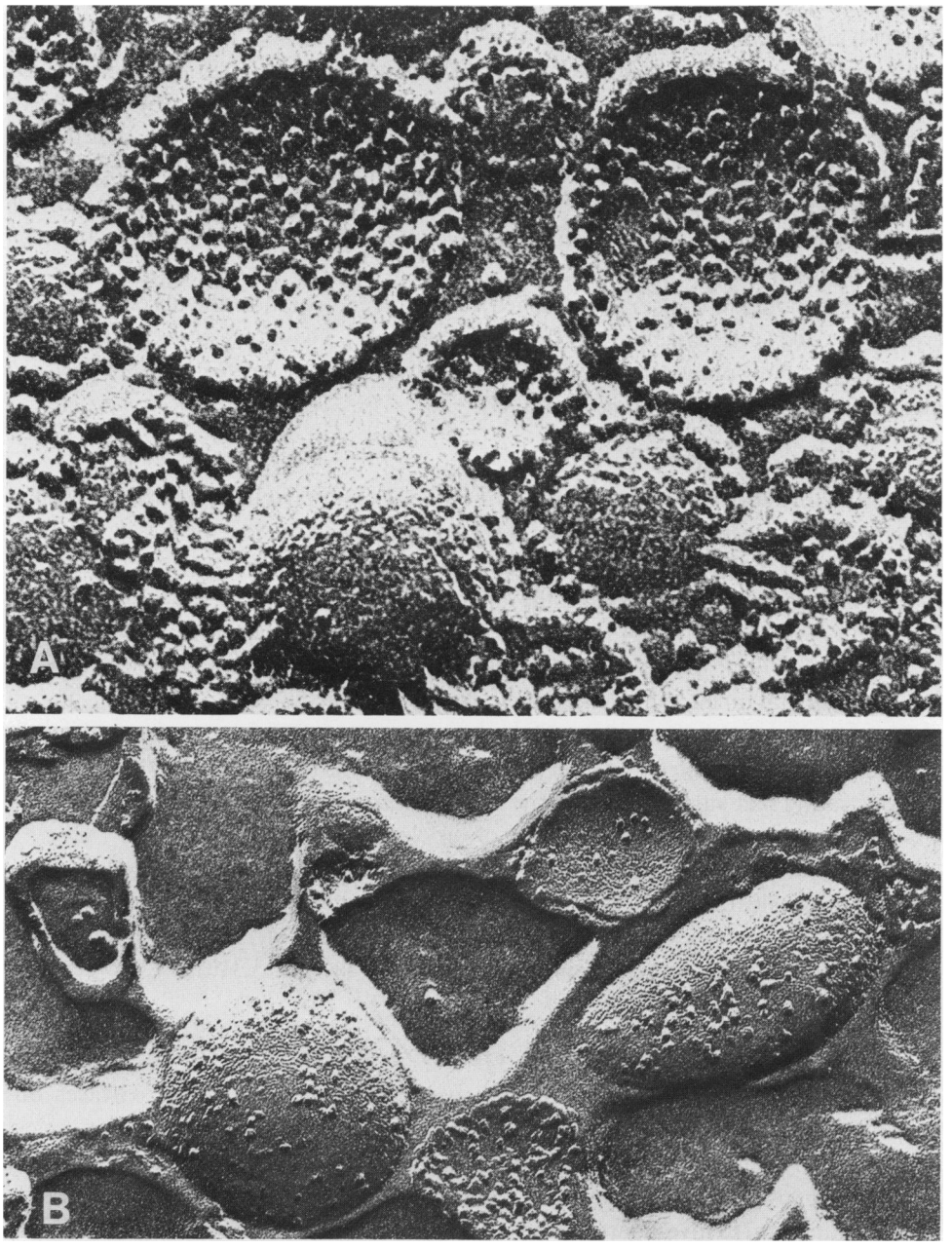

FIGURE 3 (*A*) Freeze-fractured SR membranes showing the association of 90-Å particles with the concave (PF) fracture faces. The average particle density is 5,730 ± 520/μm^2. Almost none of the convex faces (5%) have particles. × 285,000. (*B*) After trypsin digestion there is a dramatic alteration of the particle disposition. Now 80% of the convex faces have particles (1,380/μm^2) and the density on the concave faces decreases to 3,900/μm^2. × 122,000.

begin to see the projections on the top and far side of the vesicles. Although this makes a precise measurement of particle densities difficult, we were able to count particles on a number of favorable views obtained from six different preparations. The regions with best contrast and most distinct particles were used for counting. The irregularly shaped areas were measured by tracing them on paper and weighing the traced area with suitable calibration. The average density of outer projections obtained from 38 etched vesicles was measured in this way and found to be 21,000 ± 3,900/μm^2, a figure surprisingly close to that given by Jilka et al. (1975) for the outer projections of negatively stained SR.

Vesicles exposed to trypsin were washed, resuspended, frozen in the absence of glycerol and etched at the same time as the normal sample. These trypsin-treated vesicles, which do not show outer projections when negatively stained, present smooth surfaces also on freeze-etch replicas (Fig. 2C and D).

Freeze-Fractured SR

Freeze-fracture replicas were prepared from SR vesicles previously washed and resuspended in 20% glycerol. The samples were fractured and etched for 4 s at −100°C. Fig. 3A shows the typical fracture faces of SR: the concave cytoplasmic leaflet, PF face, containing 90-Å particles and the smooth convex inner face, EF face. In these preparations of rabbit SR, 93% of the PF faces contain 90-Å particles with an average density of 5,730/μm^2. 95% of the EF faces are smooth, and the remaining 5% have so few particles that no measurement of their density was attempted.

The prevalent association of the 90-Å particles with the PF fracture faces of longitudinal SR was found even in whole muscle preparations (Franzini-Armstrong, 1974), demonstrating that such an asymmetric distribution is a feature of the membrane *in situ,* before being fragmented in the form of vesicles.

After trypsin digestion the asymmetric disposition of particles is altered dramatically. The density of the PF faces decreases to 3,900 ± 880 per μm^2, while 80% of the EF faces display particles with a density of 1,380 ± 590 per μm^2. It should be noticed that the total particle density is conserved: PF + EF faces is 5,310 ± 1,000 per μm^2 (see Fig. 3B).

Optical Diffraction of Images of Freeze-Fracture SR

Information on the arrangement and the average distance among the particles observed on the concave (P) freeze-fracture faces was conveniently obtained by optical diffraction. This technique is most commonly used in electron microscopy to reveal order in negatively stained specimens. Electron micrographs were first analyzed by optical diffraction to investigate periodicities in the image of negatively stained tobacco mosaic virus (Finch et al., 1964) and to analyze the polyhead of T-4 bacteriophage (Klug and Berger, 1964).

Since diffraction patterns of freeze-fracture specimens are confused by both the platinum grain and the uneven appearance of the structures as produced by the platinum shadow, the particles' positions on micrographs (Fig. 4A) were mapped into holes

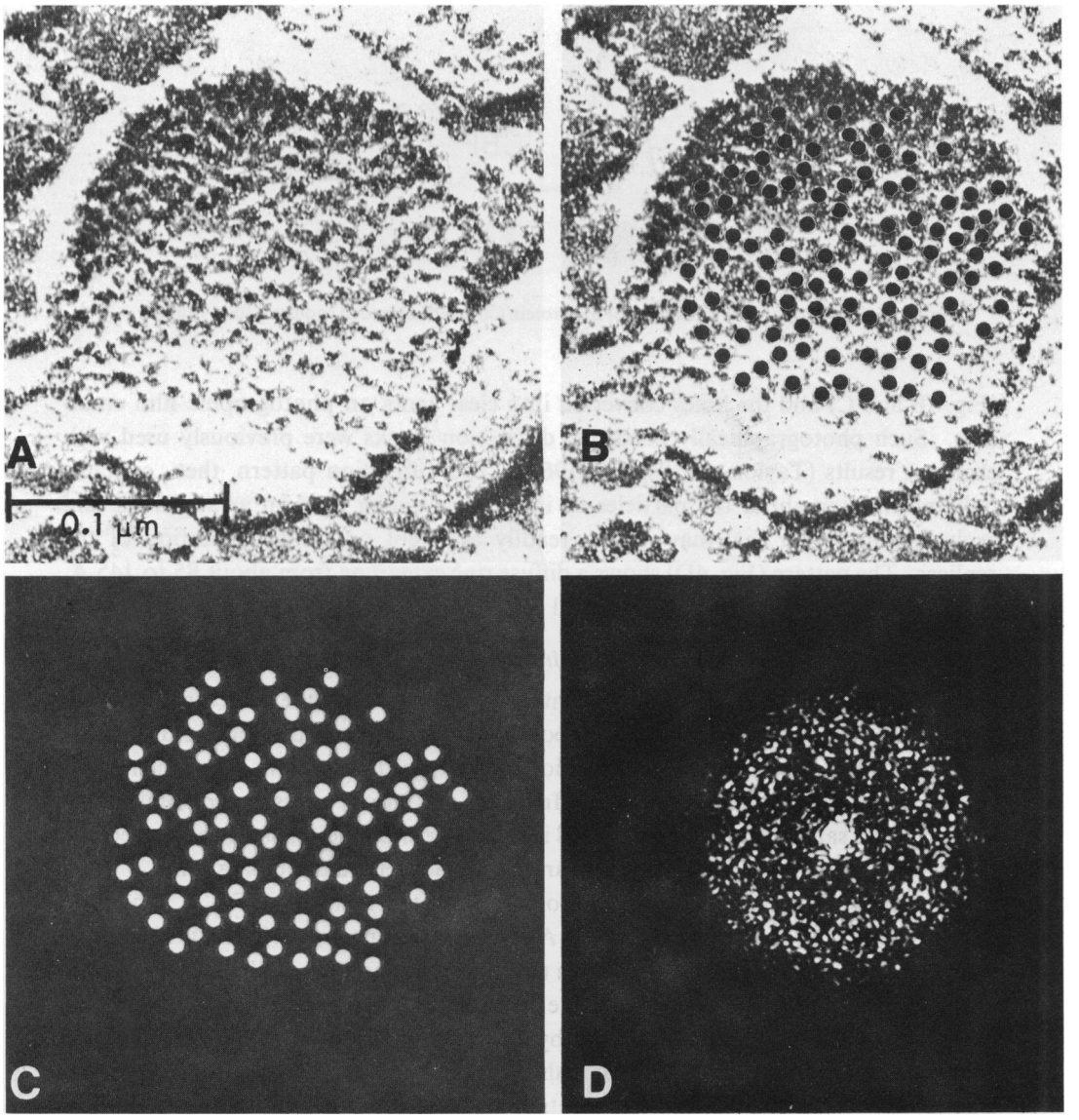

FIGURE 4 Optical diffraction of the particle distribution on a concave fracture face of a freeze-fractured SR vesicle. (*A*) Electron micrograph (× 286,000) of a concave fracture face from a replica similar to Fig. 3A. (*B*) Holes were punched at the particle positions in the micrograph. The micrograph was backed by an opaque screen so that holes were punched simultaneously into this screen. (*C*) When all of the particles had been punched out, the screen was separated from the micrograph and used to represent the particle distribution on the fracture face. This screen was then copied and made into a 35 mm slide and used as the object in an optical diffraction bench. (*D*) The optical diffraction pattern of *C*. The spatial frequencies fall predominantly within a ring whose average radius corresponds to a spacing of 110 Å. This also demonstrates a lack of order in the particle positions.

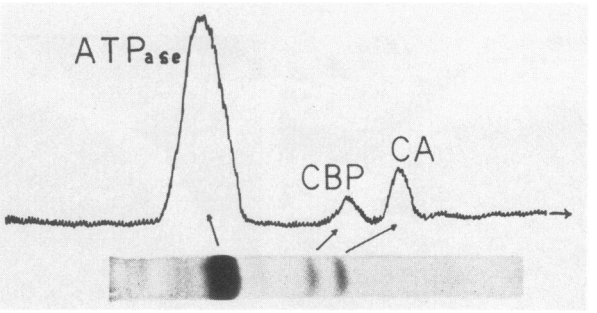

FIGURE 5 Electrophoretic separation of SR protein (30 μg) dissolved in sodium dodecylsulfate.

(Fig. 4B and C) and the holes converted into clear spots on photographic film emulsion. Such photographically produced diffraction masks were previously used with excellent results (Taylor and Lipson, 1964). The diffraction pattern, then, contains only information about the particles of interest, revealing random arrangement and excluding any order that may not be readily apparent on visual inspection of the replicas. The pattern (Fig. 4D) shows a diffuse ring extending from about 85 to 145 Å, with an average radius corresponding to 110 Å.

Membrane Composition of Protein and Phospholipid

One of the aims of our work was a quantitative evaluation of the structural observations in the light of the membrane composition of protein and phospholipid. Therefore, we characterized our SR preparations with regard to protein components and membrane area per protein unit weight. In fact, it was strictly necessary to obtain these values for the same SR preparations used in the structural studies, since the percentage of extrinsic protein associated with SR varies with the method of preparation.

An example of electrophoretic resolution of SR protein is shown in Fig. 5. The fractions appearing on the gel correspond to ATPase (MacLennan, 1970; Inesi, 1972), calcium-binding protein, and Calsequestrin (MacLennan and Wong, 1971; Ostwald and MacLennan, 1974). The fractional value of total SR protein corresponding to each electrophoretic fraction was determined by densitometry: 0.81 for the ATPase, 0.07 for calcium-binding protein, and 0.12 for Calsequestrin. The accuracy of the densitometric method was evaluated in experiments with standard protein mixtures of known composition, and the maximal deviation of the experimental from the expected values was found to be $\pm 10\%$.

Our SR preparations contained 0.53 μmol of lipid phosphate (phospholipid) per mg protein. It was previously reported that phospholipids are required for the functional integrity of the SR ATPase (Martonosi, 1964). In this regard we were able to determine the amount of phospholipid bound to the ATPase protein by dissolving the SR vesicles in the nonionic detergent Triton X-100 and separating on a sucrose gradient a peak containing ATPase activity, protein and bound phospholipid, from a peak containing the remaining phospholipid of the SR membrane (Fig. 6A). Electrophoretic

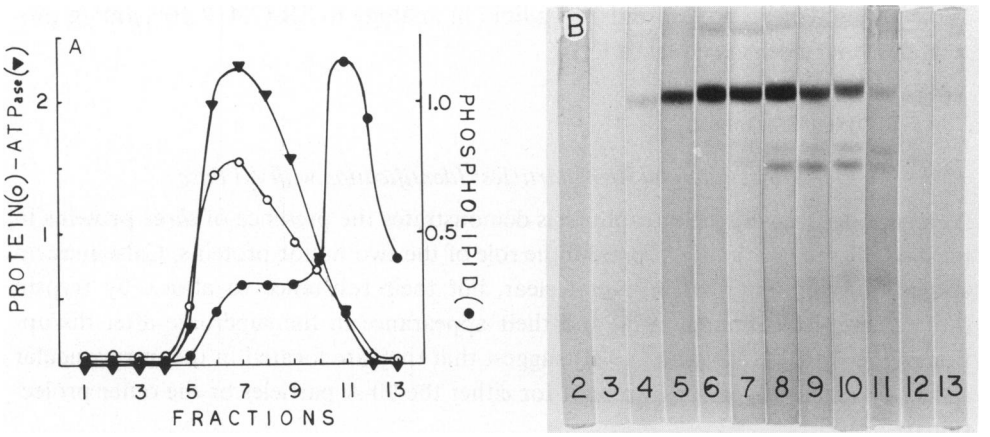

FIGURE 6 Separation of protein, phospholipids, and ATPase activity by sucrose gradient centrifugation of Triton-solubilized SR. For the solubilization 0.4 ml 10% Triton X-100 was added to a 2.0 ml sample containing 20 mg SR protein, 20 mM MOPS (pH 6.8), 5 mM $CaCl_2$, 10% glycerol, and 2.5 mM dithiothreitol. After incubation for 10 min in ice, the sample was centrifuged at 20,000 *g* for 1 h. 2 ml of the supernate were then placed on top of a 10–25% continuous sucrose gradient containing 20 mM MOPS pH 6.8, 5 mM $CaCl_2$, 2.5 mM dithiothreitol, 1 M KCl, and 0.5% Triton X-100. Centrifugation was carried out at 40,000 *g* for 24 h. Fractional samples were then taken from the bottom. (*A*) Protein (mg/ml), lipid phosphate (μmol/ml), and ATPase activity (μmol ATP/mg protein per min). (*B*) Electrophoretic analysis of the protein contained in the fractional samples of the sucrose gradient. The amount of protein on the gels is not proportional to the protein concentration on the fractional samples.

analysis (Fig. 6B) reveals that in some of the gradient fractions ATPase is satisfactorily purified. In these fractions the phosphate is 0.15 μmol/mg protein, corresponding to a phospholipid:protein molar ratio of approximately 20, based on a 106,000 mol wt for the ATPase protein. It should be pointed out that if the SR solubilization is carried out at low ionic strength and low calcium concentration, the protein loses both enzyme activity and bound phospholipid.

The volume displaced by the number of SR vesicles corresponding to the protein weight unit was estimated by determining the space excluded to [^{14}C]dextran (Duggan and Martonosi, 1970) and found to be 7.4 ± 0.2 ml/g protein. This value is slightly higher than that found by Duggan and Martonosi (1970), most likely due to a somewhat greater content in proteins other than ATPase in their SR preparation. From our value of 7.4 ml/g protein, the membrane area corresponding to 1 g SR protein (A_{tot}) can be calculated according to:

$$A_{tot} = (4\pi r^2) \cdot [V_{tot}/(4\pi/3)r^3],$$

where r is the average radius of one vesicle (750 Å) and V_{tot} is the space excluded to dextran. The thickness of the membrane is, here, considered to be negligible with respect to the radius of the vesicles.

In the SR preparations used in these studies, our estimate for A_{tot} is 2.9 × 10^{14} μm^2/g protein. Such a figure is very close to that calculated for a model membrane 62 Å thick

and composed of 1 g protein and 0.65 g lipid in analogy to SR (2.4×10^{14} μm^2/g protein, based on a density equal to 1.1).

DISCUSSION

Outer Projections and 90-Å Particles: Identification with ATPase

As mentioned above gel electrophoresis demonstrates the presence of *three* proteins in purified SR preparations. At present the role of the two minor proteins, Calsequestrin and calcium-binding protein, is not clear, but their resistance to attack by trypsin (Stewart and MacLennan, 1974) and their appearance in the supernate after disruption of the vesicles (Meissner, 1975) suggest that they are located in the intravesicular space and cannot, therefore, account for either the 90-Å particles or the outer projections.

Studies of the development of ATPase in embryonic SR have shown that there is a concurrent appearance of this enzyme activity with both 90-Å particles (Tillack et al., 1974; Baskin, 1974) on freeze-fracture replicas and 35-Å outer projections (Sarzala et al., 1975; Martonosi, 1975) on negatively stained preparations. Furthermore these two structural elements exhibit parallel behavior on stepwise trypsin digestion (Stewart and MacLennan, 1974; Inesi and Scales, 1974).

Finally, when the ATPase is isolated from SR vesicles by detergents and then reassembled to form membrane vesicles, electron microscopy reveals both outer projections (Hardwicke and Green, 1974) on negatively stained preparations, and 90-Å particles on freeze-fracture faces (Packer et al., 1974). Therefore, both the 35-Å projections and the 90-Å particles may be safely identified with the ATPase protein.

One of the aims of our work was to compare quantitatively the density (number per unit area) of the outer projections observed on the surface of the vesicle, the density of the 90-Å particles appearing on the PF freeze-fracture faces, and the calculated density of the ATPase polypeptide chains on the plane of the membrane.

Freeze-Etched SR: Density of Projections on the PS Surface

The density of the outer projections on the surface of the membrane can be best estimated in freeze-etched preparations. Until now, surface projections have been seen only on the edges of negatively stained vesicles where measurements of density cannot be obtained. Furthermore, thin sections of SR pellets do not show such surface detail (Baskin, 1971), raising the possibility of artifacts. The advantage of the freeze-etch technique is that the specimen undergoes minimal pretreatment, our samples being prepared without the use of either fixatives or glycerol. The fact that surface projections are shown on intact vesicles by both negative staining and freeze etching techniques, while they are not shown by either technique on vesicles treated with trypsin, indicates that these projections are native elements of the SR structure.

The advantage offered by the freeze-etch technique is exposure of the top surface of the vesicles, on which the density of the outer projections within the plane of the mem-

brane can be estimated. We find for this parameter a value of 21,000 ± 3,900/μm^2, which is greater than the density of the 90-Å particles observed on the concave freeze-fracture faces.

Freeze-Fractured SR: Density of Particles on the PF Face

The 90-Å particles, first observed in SR by Deamer and Baskin (1969), are almost exclusively associated with the concave fracture faces. Their density within the plane of the membrane is 5,730 ± 520/μm^2, a figure three to four times smaller than that found for the outer projections. Our optical diffraction analysis excludes any ordering of the particles, a finding consistent with high fluidity of the SR membrane (Davis and Inesi, 1971; McConnell et al., 1972). The average spacing of the adjacent particles is 110 Å.

Density of ATPase Polypeptide Chains: A Calculation

The density of ATPase polypeptide chains within the plane of the SR membrane may be calculated on the basis of protein and membrane area determinations. Several investigators have identified the SR ATPase with a 106,000 mol wt polypeptide chain (MacLennan, 1970; Meissner and Fleischer, 1971, 1973; Racker, 1972). The relative amount of the ATPase protein with respect to the total SR protein varies with the purity of the preparations and values ranging from 16% to 90% have been reported (MacLennan, 1970; McFarland and Inesi, 1971, 1970; Martonosi and Holpin, 1971; Meissner and Fleischer, 1971; MacLennan and Wong, 1971). In the SR vesicles used in our studies the 106,000 mol wt polypeptide chain identified with ATPase accounts for 81% of the total protein.

An average value for the membrane area (A_{ATPase}) occupied by one of the polypeptide chains identified with the ATPase is obtained by dividing the membrane area corresponding to the total protein weight (A_{tot}), by the number of polypeptide chains per total protein unit weight:

$$A_{ATPase} = A_{tot}/[F \cdot (N/MW)],$$

where F is the fraction of total SR protein accounted for by ATPase, N is the Avogadro's number, and MW is the molecular weight of the polypeptide chain (106,000). The value for A_{tot} was obtained as described in the Results section and is 2.9×10^{14} μm^2/g protein. The experimental value for F is 0.81; however we consider a possible error of 0.10 (F = 0.71–0.91) to account for inaccuracies of densitometry, evaluated with standard protein mixtures. Therefore our estimate for A_{ATPase} is $7.1–5.7 \times 10^{-5}$ μm^2.

Finally, the number of polypeptide chains per unit of SR membrane area (D) may be calculated according to:

$$D = 1/A_{ATPase},$$

and found to be $1.41–1.75 \times 10^4/\mu m^2$. This value represents the estimated density of the 106,000 mol wt polypeptide chains (ATPase) within the plane of the membrane.

This figure is quite close to that obtained for the density of the outer projections, while being three to four times greater than that obtained for the density of the 90-Å particles visible on freeze-fracture faces.

It is important to realize that the estimated value for the density of the ATPase polypeptide chains within the plane of the membrane is not significantly changed by sizable variations of the experimental parameters F and V_{tot}. In fact to obtain a density of polypeptide chains similar to that of the 90-Å particles (approximately 6,000/μm^2), F should be less than 30% (as opposed to our experimental value: 72–91%). Simple inspection of Fig. 5 shows that this cannot be the case.

On the other hand, if rather than our experimental value for V_{tot} (7 ml/g protein), a value closer (5 ml/g protein) to that obtained by Duggan and Martonosi (1970) is used, then the estimated density of the polypeptide chains is increased to approximately 22,000/μm^2. This value is almost identical to that found for the density of outer projections in the freeze-etch preparations. Such a relation of densities indicates that all the ATPase polypeptide chains appear individually as projections on the outer surface of the SR vesicles.

The lower density of the 90-Å particles on the freeze-fracture faces, with respect to the density of the outer projections may be due to variable penetration of the ATPase protein into the membrane bilayer, so that some of the ATPase molecules do not protrude from the concave fracture plane. On the other hand, it is possible that the hydrophobic ends of the ATPase chains join to form oligomers (three or four per 90-Å particle) within the interior of the membrane. This later hypothesis is consistent with molecular weight measurements of enzymatically active, detergent-solubilized ATPase, showing that the smallest particles retaining full activity contain three to four polypeptide chains (Le Maire and Tanford, 1976; Murphy, 1976).

It should be pointed out that the reported findings may be a feature of the native SR structure, as well as a result of the quick freezing procedure. Presently, we are unable to distinguish between these two possibilities. We have obtained identical findings with vesicles fixed in 1% glutaraldehyde before freeze-fracturing.

The Lipids of SR: Functional and Structural Roles

Finally, we wish to consider the relationship of ATPase protein with the SR lipids. It is well known that treatment of SR vesicles with phospholipase leads to enzyme inactivation (Martonosi, 1964). Fig. 6A shows that solubilized SR ATPase retains enzymatic activity only if associated with part of the membrane phospholipid (see also Warren et al., 1974, and Hardwicke and Green, 1974). It is estimated that each ATPase molecule must be associated with 20–30 phospholipid molecules to form an enzymatically active lipoprotein.

The fraction of SR phospholipids involved in the formation of this lipoprotein is approximately 0.28. It is reasonable to assume that the remaining phospholipids are structural components of the membrane bilayer. In fact, measurements of X-ray diffraction (Dupont et al., 1973; Liu and Worthington, 1974), and paramagnetic spectroscopy (Eletr and Inesi, 1972) observations are consistent with this assumption.

The volume (V_{memb}) of SR membrane corresponding to 1 g of protein can be estimated by multiplying the pertinent area (see definition of A_{tot} in results section) by the thickness of the membrane, which is 62 Å as determined by low angle X-ray diffraction (Liu and Worthington, 1974). The resulting value is 1.8×10^{12} μm^3/g protein. Since the SR membrane contains only 0.45 g of phospholipid/g protein, the contribution of the phospholipid to V_{tot} is 0.432×10^{12} μm^3, compared with 0.62×10^{12} μm^3 which is the ATPase protein contribution. The remaining volume may be accounted for by sterols, neutral lipids, proteins other than ATPase and water channels.

It is apparent from these estimates that a phospholipid bilayer can only make up less than half of the SR membrane structure. This can also be appreciated considering the area occupied by a phospholipid molecule (50 $Å^2$, based on a 4.5 Å band obtained by high angle X-ray diffraction) and the number of phospholipid molecules present in V_{tot}. This value is 1.78×10^{14} $Å^2$, which is less than necessary to cover with a *monolayer* the corresponding membrane area ($A_{tot} = 2.9 \times 10^{14}$ $Å^2$). This is in agreement with the estimate of Martonosi (1964).

In this regard, it is important to consider that low angle X-ray diffraction studies (Dupont et al., 1973; Liu and Worthington, 1974) have yielded asymmetric profiles for electron distribution along an axis perpendicular to the membrane plane, although these two studies disagree in the assignment of phases. This indicates that the SR protein is located prevalently on one side of the membrane. The electron microscopic evidence (cf. also Hasselbach and Elfvin, 1967) is consistent with an amphiphilic character of the ATPase protein and its prevalent association with the outer leaflet of the SR membrane.

Conceding the approximation of our figures, we propose that the outer membrane leaflet of the SR membrane is composed mostly of ATPase protein, while the inner leaflet is prevalently a lipid monolayer.

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REFERENCES

BASKIN, R. J. 1971. Ultrastructure and calcium transport in crustacean muscle microsomes. *J. Cell Biol.* **48**:49.

BASKIN, R. J. 1974. Ultrastructure and calcium transport in microsomes from developing muscle. *J. Ultrastruct. Res.* **49**:348.

DAVIS, D., and G. INESI. 1971. Proton nuclear magnetic resonance studies of sarcoplasmic reticulum membranes. Freeze-fracture electron microscopy studies. *Biochim. Biophys. Acta.* **241**:1.

DEAMER, D. W., and R. J. BASKIN. 1969. Ultrastructure of sarcoplasmic reticulum preparations. *J. Cell Biol.* **42**:296.

DUGGAN, P. F., and A. MARTONOSI. 1970. The permeability of sarcoplasmic reticulum membrane. *J. Gen. Physiol.* **56**:147.

DUPONT, Y., S. C. HARRISON, and W. HASSELBACH. 1973. Molecular organization in the sarcoplasmic reticulum membrane studied by x-ray diffraction. *Nature (Lond.).* **244**:555.

EBASHI, S., and F. LIPMAN. 1962. Adenosine triphosphate linked concentration of calcium ions on a particulate fraction of rabbit muscle. *J. Cell Biol.* **14**:389.

ELETR, S., and G. INESI. 1972. Phospholipid orientation in sarcoplasmic membranes: spin-label ESR and proton NMR studies. *Biochim. Biophys. Acta.* **282**:174.

FINCH, J. T., A. KLUG, and A. O. W. STRETTON. 1964. The structure of the "polyheads" of T4 bacteriophage. *J. Mol. Biol.* **10**:570.

FISKE, H., and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**:375.

FRANZINI-ARMSTRONG, C. 1974. Freeze fracture of skeletal muscle from the tarantula spider. *J. Cell Biol.* **61**:501.

HARDWICKE, P. M. D., and N. M. GREEN. 1974. The effect of delipidation on the adenosine triphosphatase of sarcoplasmic reticulum. *Eur. J. Biochem.* **42**:183.

HASSELBACH, W., and L. G. ELFVIN. 1967. Structural and chemical asymmetry of the calcium-transporting membranes of the sarcotubular system as revealed by electron microscopy. *J. Ultrastruct. Res.* **17**:598.

HASSELBACH, W., and M. MAKINOSE. 1961. Die Calciumpumpe der Erschlaffumsgrana des Muskets und ihre Abhängigkeit von der ATP-spalting. *Biochem. Z.* **33**:518.

HASSELBACH, W., and M. MAKINOSE. 1963. Über den Mechanismus des Calciumtransportes durch die Membranen des sarkoplasmatischen Reticulums. *Biochem. Z.* **339**:94.

HAYDON, G. B. 1968. On the interpretation of high resolution electron micrographs of marcomolecules. *J. Ultrastruct. Res.* **25**:359.

IKEMOTO, N., F. A. SRETER, A. NAKAMURA, and J. GERGELY. 1968. Tryptic digestion and localization of calcium uptake and ATPase activity in fragments of sarcoplasmic reticulum. *J. Ultrastruct. Res.* **23**:216.

INESI, G. 1972. Active transport of calcium ion in sarcoplasmic membranes. *Ann. Rev. Biophys. Bioeng.* **1**: 191.

INESI, G., and I. ASAI. 1968. Trypsin digestion of fragmented sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **126**:469.

INESI, G., and D. SCALES. 1974. Tryptic cleavage of sarcoplasmic reticulum protein. *Biochemistry.* **13**: 3298.

JILKA, R. L., A. MARTONOSI, and T. W. TILLACK. 1975. Effect of purified Mg^{2+} + Ca^{2+}-activated ATPase of sarcoplasmic reticulum upon the passive Ca^{2+} permeability and ultrastructure of phospholipid vesicles. *J. Biol. Chem.* **250**:7511.

KLUG, A., and J. E. BERGER. 1964. An optical method for the analysis of periodicities in electron micrographs and some observations on the mechanism of negative staining. *J. Mol. Biol.* **10**:565.

KLUG, A., and D. J. DEROSIER. 1966. Optical filtering of electron micrographs: reconstruction of one-sided images. *Nature (Lond.).* **212**:29.

LE MAIRE, M., and C. TANFORD. 1976. Molecular characterization of enzymatically active, detergent solubilized Ca^{++} ATPase from sarcoplasmic reticulum. *Biophys. J.* **16**:(2, pt. 2):82*a* (Abstr.); and *Biochemistry.* In press.

LIU, S. C., and C. R. WORTHINGTON. 1974. Electron density levels of sarcoplasmic reticulum membranes. *Arch. Biochem. Biophys.* **163**:332.

MCFARLAND, B. H., and G. INESI. 1970. Studies of solubilized sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **41**:239.

MCFARLAND, B. H., and G. INESI. 1971. Solubilization of sarcoplasmic reticulum with Triton X-100. *Arch. Biochem. Biophys.* **145**:456.

MACLENNAN, D. H. 1970. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* **245**:4508.

MACLENNAN, D. H., and P. T. S. WONG. 1971. Isolation of a calcium sequestering protein from sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1231.

MARKHAM, R. 1968. The optical diffractometer. Methods of Virology IV. Maramorosch and Koprowski, editors. Academic Press, Inc., New York. 503.

MARTONOSI, A. 1964. Role of phospholipids in ATPase activity and Ca^{2+} transport of fragmented sarcoplasmic reticulum. *Proc. Fed. Am. Soc. Exp. Biol.* **23**:913.

MARTONOSI, A. 1975. Membrane transport during development in animals. *Biochim. Biophys. Acta.* **415**: 311.

MARTONOSI, A., and R. A. HOLPIN. 1971. The protein composition of sarcoplasmic reticulum membrane. *Arch. Biochem. Biophys.* **144**:66.

MCCONNELL, H. M., K. L. WRIGHT, and B. G. MCFARLAND. 1972. The fraction of the lipid in a biological membrane that is in a fluid state: a spin label assay. *Biochem. Biophys. Res. Commun.* **47**:273.

MEISSNER, G. 1975. Isolation and Characterization of two types of sarcoplasmic reticulum vesicles. *Biochim. Biophys. Acta.* **389**:51.

MEISSNER, G., and S. FLEISCHER. 1971. Characterization of sarcoplasmic reticulum from skeletal muscle. *Biochim. Biophys. Acta.* **241**:356.

MEISSNER, G., and S. FLEISCHER. 1973. Ca^{2+} uptake in reconstituted sarcoplasmic reticulum vesicles. *Biochem. Biophys. Res. Commun.* **52**:913.

MURPHY, A. J. 1976. Cross-linking of the sarcoplasmic reticulum ATPase protein. *Biochem. Biophys. Res. Commun.* **70**. In press.

OSTWALD, T. J., and D. H. MACLENNAN. 1974. Isolation of a high affinity calcium-binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* **249**:974.

PACKER, L., C. W. MELHERD, G. MEISSNER, W. L. ZAHLER and S. FLEISCHER. 1974. The structural role of lipids in mitochondrial and sarcoplasmic reticulum membranes. Freeze-fracture electron microscopy studies. *Biochim. Biophys. Acta.* **363**:159.

RACKER, E. 1972. Reconstitution of calcium pump with phospholipids and a purified Ca^{2+}-adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* **247**:8198.

SARZALA, M. G., M. PILARSKA, E. ZUBRZYCKA, and M. MICHALAK. 1975. Changes in the structure, composition and function of sarcoplasmic reticulum membrane during development. *Eur. J. Biochem.* **57**:25.

STEWART, P. S., and D. H. MACLENNAN. 1974. Surface particles of sarcoplasmic reticulum membranes. *J. Biol. Chem.* **249**:985.

TAYLOR, C. A., and H. LIPSON. 1964. Optical transforms. Cornell University Press, Ithaca, New York.

THON, F. 1966. Zur Defokussierungsabhangigkeit des Phasenkontrastes bei der elektronmikroskopischem Abbilding. *Z. Naturforschung.* **21a**:476.

THORLEY-LAWSON, D. A., and N. M. GREEN. 1973. Studies on the location and orientation of proteins in the sarcoplasmic reticulum. *Eur. J. Biochem.* **40**:403.

TILLACK, T. W., R. BOLAND, and A. MARTONOSI. 1974. The ultrastructure of developing sarcoplasmic reticulum. *J. Biol. Chem.* **249**:624.

WARREN, G. B., P. A. TOON, N. J. M. BIRDSALL, A. G. LEE, and J. C. METCALFE. 1974. Complete control of the lipid environment of membrane-bound proteins: application to a calcium transport system. *FEBS Lett.* **41**:122.

WEBER, A., R. HERZ, and I. REISS. 1966. Study of the kinetics of calcium transport by isolated sarcoplasmic reticulum. *Biochem. Z.* **345**:329.