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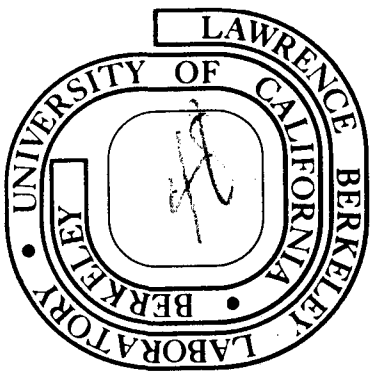

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THE STRUCTURAL ROLE OF LIPIDS IN MITOCHONDRIAL AND SARCOPLASMIC RETICULUM MEMBRANES

FREEZE-FRACTURE ELECTRON MICROSCOPY STUDIES

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SUMMARY

The role of phospholipid in the structure of the membranes of beef heart mitochondria and of the sarcoplasmic reticulum membranes from rabbit skeletal muscle has been investigated by freeze-fracture electron microscopy.

Progressive removal of membrane phospholipids, by phospholipase A treatment or detergent treatment, or by organic solvent extraction, results in loss of the smooth background seen in membrane fracture faces and decreased ability of membrane to undergo freeze fracture to yield fracture faces. Instead cross-sections of vesicles or particle clusters are observed.

Sarcoplasmic reticulum vesicles have a 9 to 1 asymmetry in the distribution of particles between the convex and concave fracture faces. There is also a wide range of particle size distribution in both of these fracture faces with 85-Å particles in greatest number. The removal of membrane associated proteins by detergent extraction does not appreciably change the distribution in particle size. Sarcoplasmic reticulum vesicles were dissolved with detergent and reassembled to form membrane vesicles containing mainly one protein (approx. 90 %),i.e. the Ca^{2+} pump protein, and with a ratio of lipid to protein similar to the original membrane. The reconstituted vesicles readily underwent freeze fracture but the asymmetric particle distribution between the fracture faces was no longer observed. The size distribution of particles in the reconstituted membrane, consisting mainly of Ca^{2+} pump protein, and phospholipid. was similar in heterogeneity to the original sarcoplasmic reticulum membrane. Thus the heterogeneity in particle size could reflect variation in the orientation of the Ca^{2+} pump protein within the membrane.

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanolsulfonic acid.

INTRODUCTION

A major function of biological membranes is the partitioning of cellular components, such that they provide vectorial directionality to metabolic processes. This implies an asymmetric organization of protein and/or of the lipid components in the membrane. Examples are the inwardly directed ATP-dependent Ca^{2+} transport systems of the inner mitochondrial and sarcoplasmic reticulum membranes.

The lipids which are present in the membranes can enter into both lipid-lipid and lipid–protein interactions and can influence protein–protein interactions. Biochemical approaches for the removal of lipids from membranes and their subsequent reinsertion have been successful in demonstrating the importance of lipids in enzyme and membrane functions [1–3].

One approach to studying membrane structure and formation is to perturb the membrane by selectively removing specific components. In the case of the mitochondrial inner membrane it is possible to deplete the membrane of practically all the lipid of the membrane ($> 95\,\%$), yet the trilaminar membrane structure remains, as viewed by conventional electron microscopy [4]. This observation seriously questioned a lipid bilayer as the supporting structure of all membranes as postulated by Danielli and Davson [5]. In order to explain the remarkable stability of this membrane, protein-cross bridges which buttress the membrane structure were postulated [4]. Indeed there is now good indication that a protein can span across the width of the membrane, and few particles observable in the fracture faces of membranes could be such protein [6, 7].

Protein in the membrane appears to be arranged in at least two levels of organization. The intrinsic membrane proteins are intimately associated with lipid and protein. The trilaminar structure of the membrane is disrupted when these proteins are extracted with the use of detergents. Some of the membrane proteins are more readily extractable and have been called "membrane associated proteins" [8–10] or peripheral proteins [11]. The extraction of these proteins results in loss of "surface fuzz" but not of the trilaminar structure of the membrane as seen by conventional electron microscopy [10]. The use of freeze-fracture electron microscopy, does not involve chemical fixation and provides another means of observing ultrastructural details of membranes in which the lipid and protein composition has been altered [12–15].

This investigation reports on the use of freeze-fracture electron microscopy to study normal and perturbed membrane vesicles derived from membranes of beef heart mitochondria and of rabbit skeletal muscle sarcoplasmic reticulum.

MATERIALS AND METHODS

Reagents

Sucrose (special enzyme grade) and ultrapure urea were purchased from Mann Research Lab. (New York), bovine serum albumin from Armour Pharmaceutical Co. (Chicago), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and dithiothreitol from Calbiochem (Los Angeles), and Tris from Sigma (St. Louis,). Phospholipase A (*Naja naja*) was obtained from the Miami Serpentarium Lab. (Miami) and was further purified as previously described [2]. Glutaraldehyde

(vacuum distilled in glass at low temperature and the distillate treated with a special grade of high surface active carbon and antacid to remove traces of glutaric acid) was obtained from Polysciences, Inc., (Paul Valley Industrial Park, Warrington, Penn.).

Preparation and perturbation of submitochondrial vesicles from beef heart mitochondria

Beef heart mitochondria were prepared [16] and then disrupted using N_2 decompression and shear in a Parr Bomb [17, 9]. Vesicles prepared by this method retain optimal respiratory activity from substrate to O_2.

Controlled removal of phospholipid from mitochondria or submitochondrial vesicles was achieved using a purified preparation of phospholipase A from snake venom as previously described [2]. The amount of phospholipase A and the time of incubation were varied to give different degrees of phospholipid degradation. All samples were washed 4 times with 1 % bovine serum albumin in 0.25 M sucrose to remove the lysophosphatides and fatty acid by-products and once with 0.25 M sucrose to remove bovine serum albumin. A control sample of mitochondrial vesicles was carried through the same procedure by substituting bovine serum albumin for phospholipase A in the incubation mixture.

The absorption of bovine serum albumin to the washed vesicles was checked using polyacrylamide-gel electrophoresis. Densitometry tracings of the gels showed no significant amount of bovine serum albumin (< 3 % of the protein) [9].

Mitochondrial vesicles were also depleted of lipid using extraction with aqueous acetone (10 % water in acetone) [2].

Preparation and perturbation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle using sucrose gradient centrifugation in zonal rotors [18, 19]. Following the second zonal centrifugation, the preparation was treated on ice overnight in the presence of 15 % sucrose with 0.6 M KCl to remove small amounts of extraneous muscle proteins. The purified sarcoplasmic reticulum vesicles have been characterized in detail and found to have a relatively simple lipid and protein composition [18, 20].

Sarcoplasmic reticulum vesicles were perturbed in three different ways: (1) Phospholipase A digestion or bile acids at relatively low concentrations were used to remove membrane associated protein and part of the phospholipid [18, 21]. The remaining membranous fraction was enriched with respect to the Ca^{2+}-pump protein (referred to also as Ca^{2+}-stimulated ATPase protein). Sarcoplasmic reticulum vesicles were treated with phospholipase A for various times to degrade different amounts of phospholipid [21]. All samples were then washed 4 times with 1 % bovine serum albumin to remove the degradation products (lysophosphatides and fatty acid) of the phospholipase A treatment. The membrane preparations were finally washed with 0.3 M sucrose –1 mM HEPES (pH. 7.4) to remove bovine serum albumin. Bile acids in the presence of salt were used to prepare partially purified Ca^{2+}-pump protein [18]. At detergent concentrations of approx. 2–4 mg/ml cholate or 0.5 mg/ml deoxycholate sarcoplasmic reticulum vesicles (2.5 mg protein/ml) are partially disaggregated. Membrane associated protein and part of the phospholipid are solubilized, while the particulate fraction contains the Ca^{2+}-pump protein with a purity of about 85 %. The amount of phospholipid associated with the Ca^{2+}-pump protein depends on the exact detergent concentrations used.

(2) Sarcoplasmic reticulum vesicles (3 mg/ml) were solubilized with deoxycholate (2 mg/ml) so that they were no longer membranous. They were then reassembled into membranous vesicles by removing the detergent using dialysis for 24 h [18].

(3) Sarcoplasmic reticulum vesicles were fixed for 2 h at 0 °C with 0.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). The fixed vesicles were washed in 0.25 M sucrose, 0.1 M cacodylate buffer (pH 7.4), frozen in liquid N_2 and kept at −70 °C until freeze fractured.

Freeze-fracture electron microscopy

Samples were thawed, supplemented with glycerol to a final concentration of 10 % and then processed for freeze fracture as previously described [22]. In brief, the biological specimen is placed on a paper disc, frozen first in liquid freon, then in liquid N_2 and fractured under high vacuum in a Balzer's freeze-fracture apparatus. The fractured surface is replicated with a thin layer of platinum which is supported by a thicker layer of carbon. The replica is freed from the biological specimen by dissolving away the organic matter in chlorox and is then washed in water. The samples were examined in a Siemen's 1A electron microscope.

The direction of platinum shadowing is indicated by a bar located in the lower left-hand corner of each electron micrograph. The length of the bar also is a calibration for 0.2 μm. In cases where the specimen was treated with phospholipase A, detergents or organic solvent, the amount (in μg) of phospholipid phosphorus/mg of membrane protein is given to indicate the extent of lipid depletion.

Our replicas were prepared in the presence of 10 % glycerol to prevent distortion due to ice crystal formation. During the cooling process, a eutectic forms. Under these conditions the non-membranous protein material becomes squeezed in between these eutectics which probably has the effect of augmenting the aggregation or clustering of particles observed in many of our replicas of lipid-depleted membranes.

Analyses

Protein was estimated by the method of Lowry et al. [23] using crystalline bovine serum albumin as standard. Total phosphorus was measured using a modification [24] of the method of Chen et al .[25] as an estimate of lipid phosphorus.

RESULTS

Mitochondrial membrane-lipid depletion studies

The appearance of beef heart mitochondria in freeze-fracture replicas is shown in Fig. 1A. About 50 % of removal of membrane phospholipids by the phospholipase A procedure results in significant difference in appearance (Fig. 1B). There is a decreased tendency of the membrane to fracture and fracture faces of smaller vesicles are also observed. Many regions consisting of amorphous or aggregated particles were seen along with curved or circular trails representing crossmembrane fractures. More extensive removal of lipid (not shown) results in the complete absence of observable fracture faces.

In beef heart mitochondria most of the membrane (approx 95 %) is referable to inner membrane. Submitochondrial vesicles are simple structures consisting only of

Fig. 1A. For legend see p. 164.

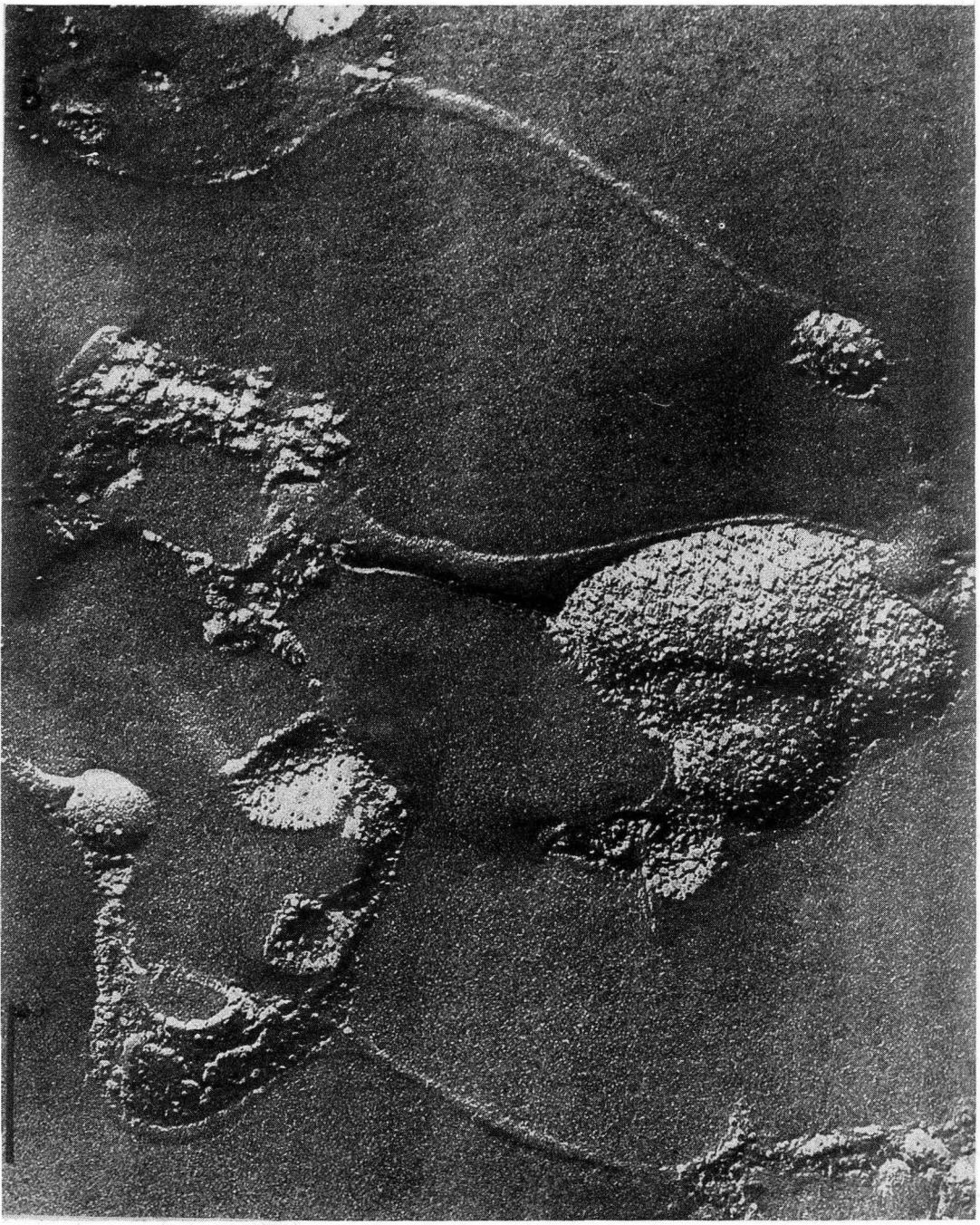

Fig. 1. Freeze-fracture electron micrographs of normal (18.6 μg P/mg protein) (A) and phospholipase A-treated (7.48 μg P/mg protein) (B) beef heart mitochondria (108 000 ·). The phospholipase A treated mitochondria retained only 45 % of their original phospholipid. This value is calculated using 13.7 μg P/mg protein as 100 % phospholipid since 3.5 μg P/mg protein of mitochondria is acid soluble and 1.4 μg P/mg protein is not lipid extractable [1].

single membrane and the compartment it encloses. The soluble matrix proteins of the mitochondria are largely removed during the preparation.

Examination of such mitochondrial vesicles after fracturing shows the presence of vesicles both in fracture faces and in cross-sections. Analysis of the frequency of occurrence of membrane faces shows slightly more convex than concave fracture faces. We find an asymmetry in the number of particles distributed between the A face (side facing matrix) and the B face (side facing the intermembrane space) of 3125 and 1400 particles/μm^2, respectively.

Treatment of vesicles under the conditions used in the phospholipase A procedure, but without adding phospholipase A, resulted in no detectable change in the structure of the membranes as judged by the distribution of membrane protein particles (Fig. 2A). However, removal of approx. 20 % of the phospholipid by the

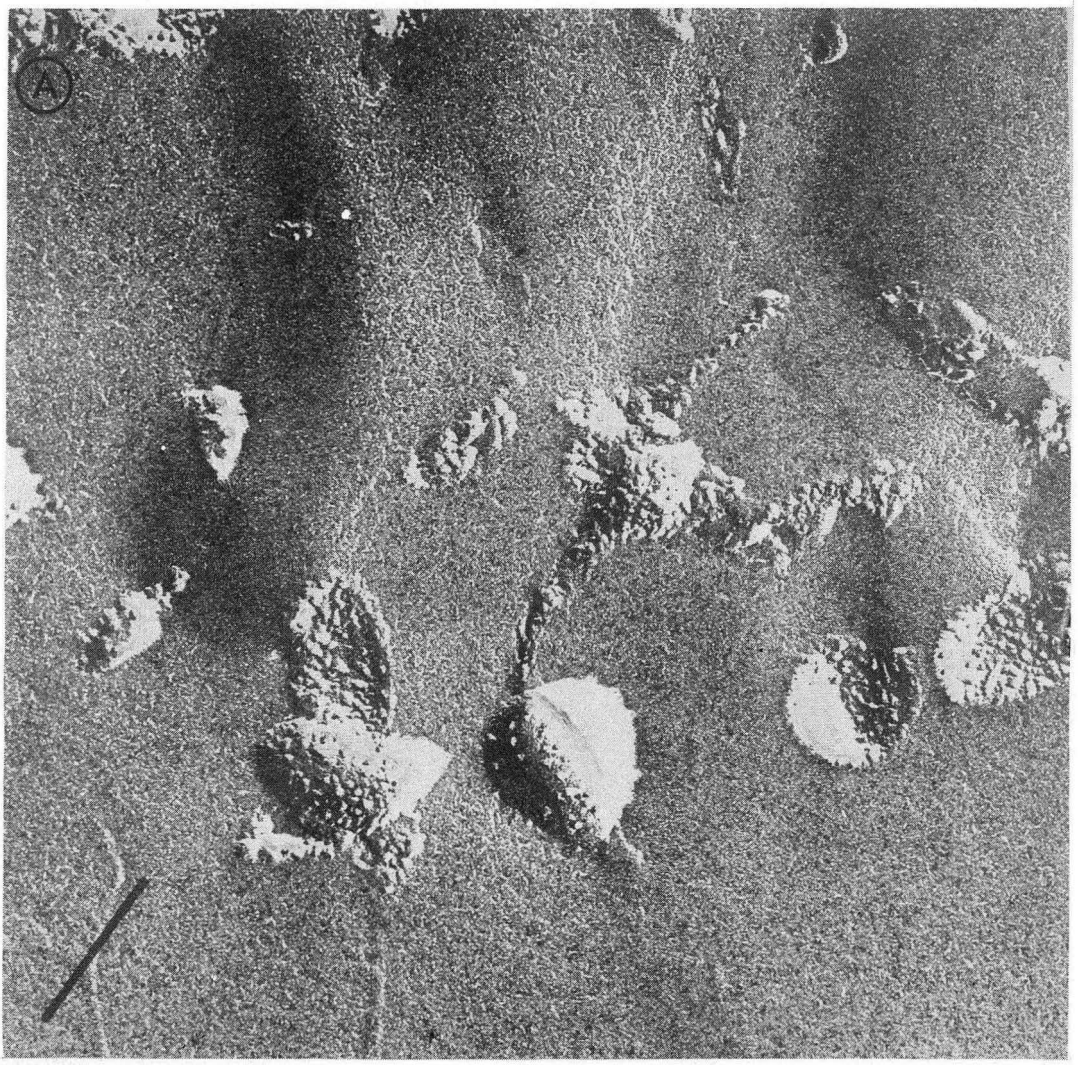

Fig. 2A. For legend see p. 167.

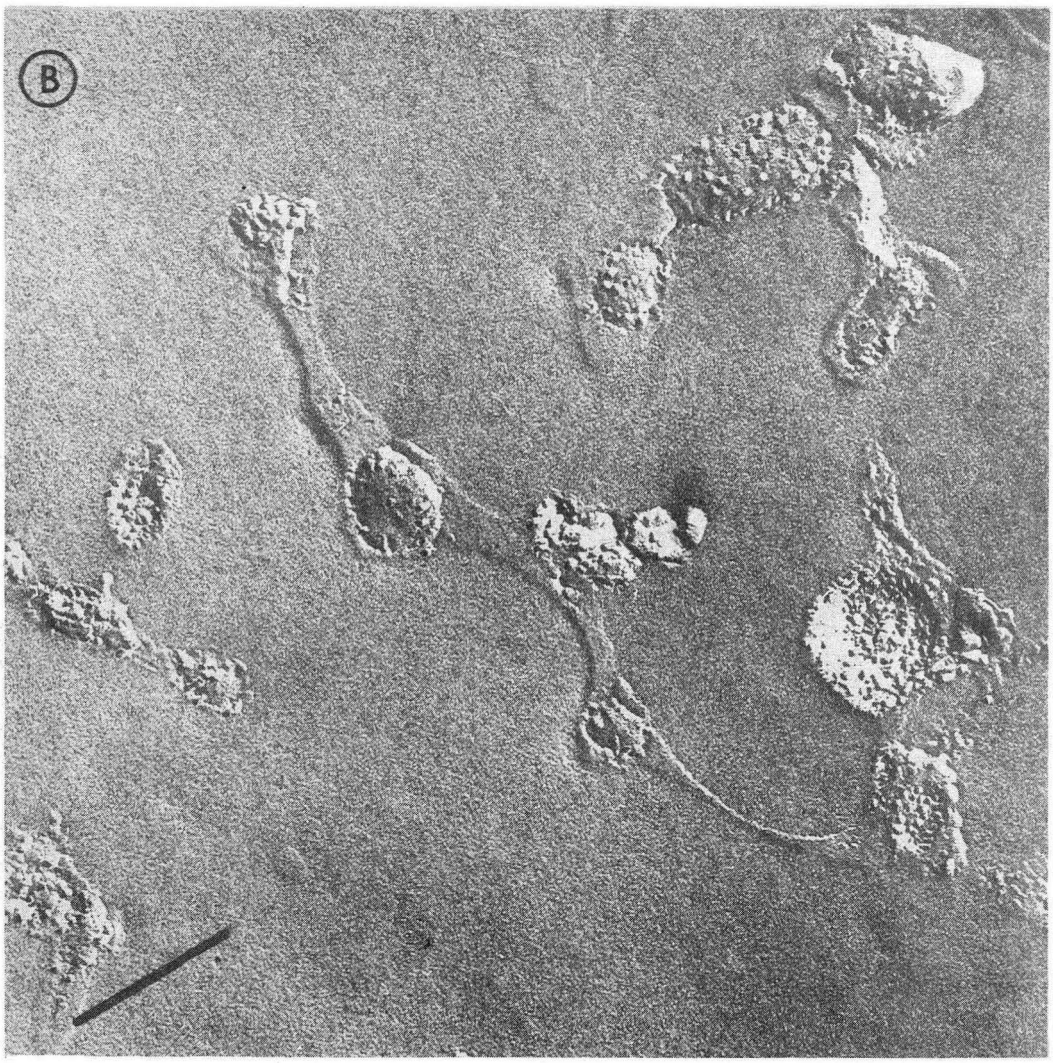

Fig. 2. Normal and lipid-depleted beef heart mitochondrial vesicles. The lipid was extracted using the phospholipase A procedure. (A) Control vesicles (phospholipase A omitted, four bovine serum albumin washes) (phosphorus to protein ratio of 19.4 μg P/mg protein). (B,C) Vesicles treated with phospholipase A: B, 12.9 μg P/mg protein (64 % phospholipid remaining) C, and 8.8 μg P/mg protein (41 % phospholipid remaining). The amount of phospholipase A used and the time of incubation were varied to give the controlled phospholipid depletion as indicated by the bound phosphorus and percentage phospholipid remaining, (108 000 ×) [2]. The vesicles contain about 1.5 μg P/mg protein which is not lipid extractable. The acid-extractable phosphorus is largely lost during the preparation of the submitochondrial vesicles [1, 17].

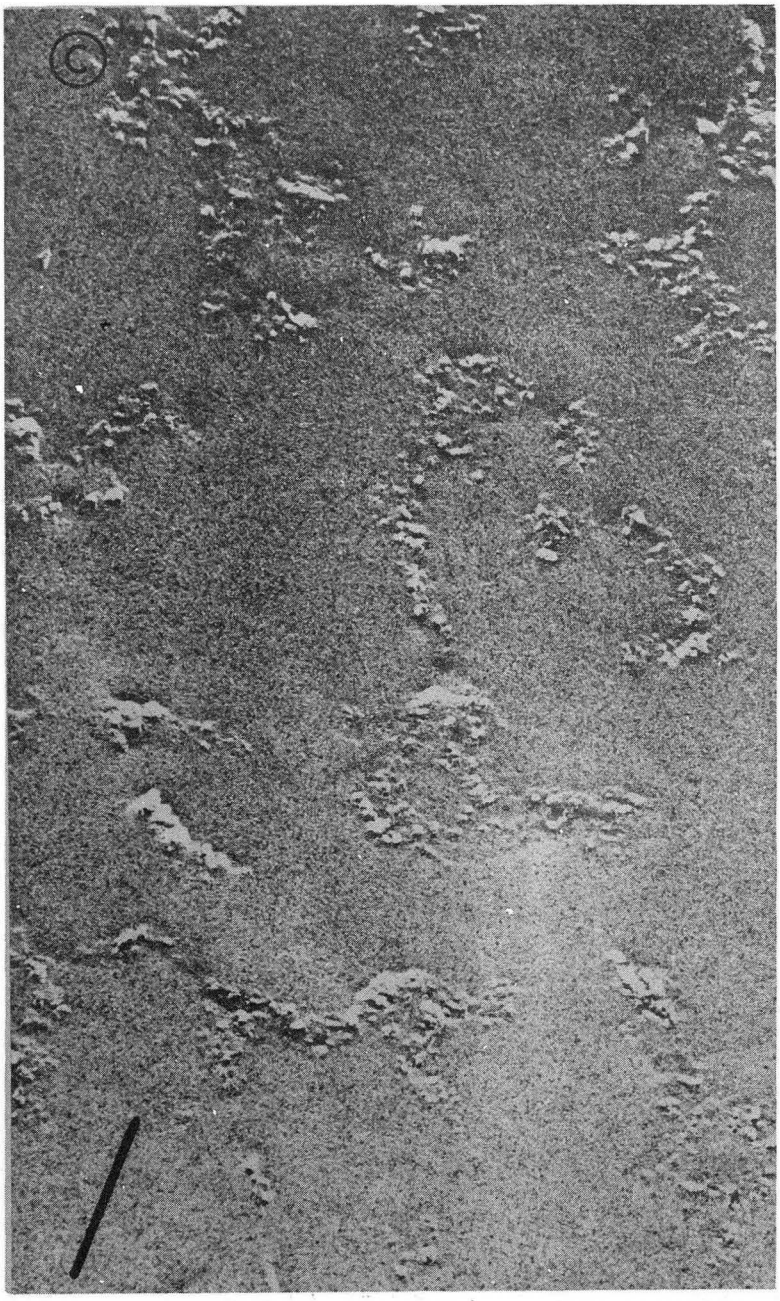

Fig. 2C. For legend see p. 166.

TABLE I

OCCURRENCE OF FRACTURE FACES IN NORMAL AND LIPID-EXTRACTED BEEF HEART SUBMITOCHONDRIAL PREPARATIONS

Freeze-fracture electron micrographs of a series of beef heart submitochondrial vesicles which were progressively depleted of lipid and characterized with respect to freeze-fracture capability. Preparations and treatments are given in Materials and Methods.

Extraction treatment	Bound phosphorus (μg P/mg protein)	Lipid remaining (%)	Profiles counted	Percent of total profiles			
				Fracture face		Cross sections*	Particle clusters**
				Concave	Convex		
Phospholipase A (control)***	19.4	100	574	35.0	48.4	9.7	7.0
Phospholipase A	18.2	93	691	20.6	21.4	13.5	44.5
Phospholipase A	12.2	60	128	5.7	14.9	14.5	65.0
Phospholipase A	6.6	28		0	0	0	100.0
Acetone (10 % water)		15		0	0	0	100.0

* Cross fracture appears as a circle of particles in the plane of the fractured surface.

** Particle clusters appear as groups of particles with no apparent geometrical shape.

*** Phospholipase A omitted, four bovine serum albumin washes (cf. Materials and Methods).

phospholipase A procedure caused an increase in the relative occurrence of cross-section as compared to fracture faces (not shown).

Fig. 2C shows that after removal of progressively more phospholipid (36 % removed) the smooth areas between membrane particles frequently become rough, suggesting loss of lipid. After removal of 60 % of the phospholipid, fracture faces are no longer seen, and the protein components appear as clusters of particles (Fig. 2C).

The occurrence of freeze-fracture profiles of normal and lipid-depleted vesicles were compared in a separate study (Table I). The number of fracture faces compared with cross sections and particle clusters decreases strongly with increased removal of lipid. Removal of greater than 85 % of the phospholipid with aqueous acetone represents an extreme case where only particle clusters are observable. The trilaminar appearance of the membrane remains after the removal of practically all of the lipid from mitochondrial vesicles [4]. Thus, the ability of the membrane to be fractured to yield fracture faces is decreased and eliminated as the lipid is removed.

Perturbation studies of sarcoplasmic reticulum vesicles

Freeze-fracture studies of original sarcoplasmic reticulum vesicles show a clearly asymmetric organization of particles in the membrane. Concave half-membrane fracture faces contain approx. 9 times more particles than the convex faces (Fig. 3. Table II).

The role of phospholipid in membrane structure has been investigated by removing increasing amounts of phospholipid. Two methods were used to partially deplete sarcoplasmic reticulum membranes of phospholipid, i.e. sarcoplasmic reticulum vesicles were treated with low amounts of bile acids or with phospholipase A followed by washing with bovine serum albumin. In both methods, two major protein components of sarcoplasmic reticulum, the Ca^{2+}-binding protein and M_{55} protein [18, 21], are released resulting in membrane preparations which are mainly composed of the Ca^{2+}-pump protein (about 85 % pure) and various amounts of phospholipid. The Ca^{2+}-pump protein preparations are enzymatically active in that they are capable of forming a phosphoenzyme intermediate. Their Ca^{2+}-stimulated ATPase activities decrease with increasing amounts of phospholipid removed during the detergent or phospholipase A treatment. The preparations are not capable of accumulating appreciable amounts of Ca^{2+}. Sarcoplasmic reticulum vesicles treated with phospholipase A or partially disaggregated with bile acid remain membraneous and have the typical trilaminar appearance seen in conventional electron micrographs [18, 21]. Some of the membranes, however, no longer form closed vesicles. In contrast the freeze-fracture studies show that the arrangement of the membrane components undergoes drastic changes when treated with bile acids or phospholipase A. As in the case of the mitochondrial vesicles, there appears to occur a loss of the ability of the vesicles to fracture along the plane of the membrane (Figs 4 and 5). These changes become more pronounced when increasing amounts of phospholipid are removed. Extensive removal of phospholipids leads to clusters of particles and complete loss of concave and convex fracture faces. (Tables II and III).

To study the possible importance of protein–protein interactions in the distribution of membrane particles seen in fracture faces, cross-linking studies were undertaken with the bifunctional reagent glutaraldehyde. Cross-linking with glut-

TABLE II

OCCURRENCE OF FRACTURE FACES IN RABBIT SKELETAL MUSCLE SARCOPLASMIC RETICULUM VESICLES

Freeze-fracture electron-micrographs of sarcoplasmic reticulum vesicle preparations which were progressively depleted of lipid and analyzed for modification of the membrane to undergo freeze fracture. Preparations and treatment are given in Materials and Methods.

Treatment	Bound phosphorus (μg P/mg protein)	Lipid remaining (%)	Profiles counted	Percent of total profiles			
				Fracture face		Cross sections*	Particle clusters**
				Concave	Convex		
None	23		65	33	32	5	30
Phospholipase A-control***	26	100	259	38	29	2	31
Phospholipase A†	11.3	43	201	7	15	0	78
Phospholipase A†	6.2	24		0	0	0	100
Phospholipase A†	1.7	7		0	0	0	100

* Cross fracture appears as circle of particles in the plane of the fractured surface.

** Particle clusters appear as groups of particles with no apparent geometrical shape.

*** Phospholipase A omitted, four bovine serum albumin washes.

† Vesicles exposed to phospholipase A for 0.5, 15, and 60 min, consecutively.

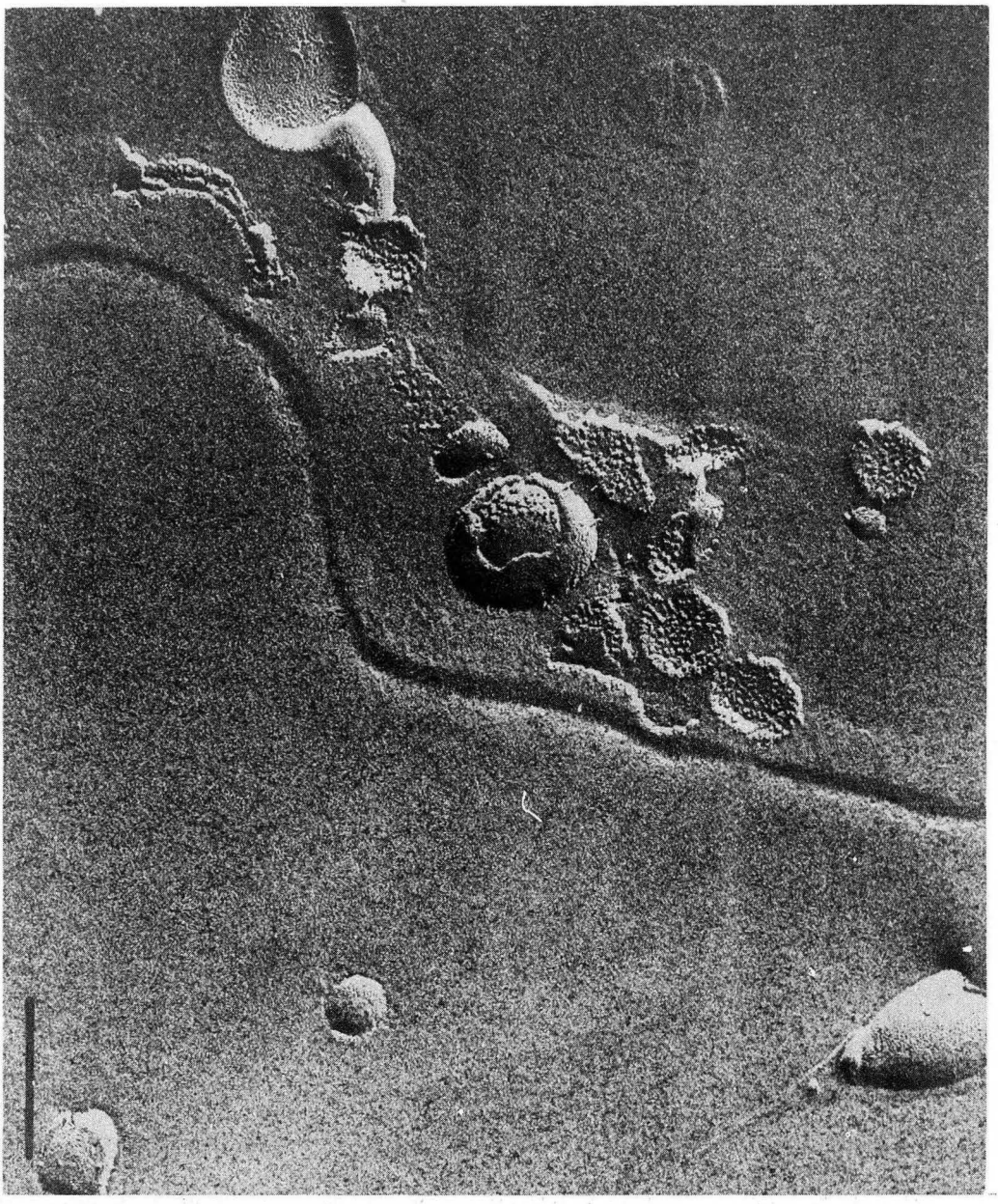

Fig. 3. Normal sarcoplasmic reticulum vesicles showing particle-covered concave and convex fracture faces (22.4 μg P/mg protein). (108 000 $\times$).

araldehyde would be expected to lead to the formation of polymers by stabilizing protein–protein and some lipid, mainly phosphatidylethanolamine–protein, interactions. It is therefore not surprising that changes in the characteristic pattern of fracturing are observed (Fig. 6). Occasionally, concave and convex fracture faces or

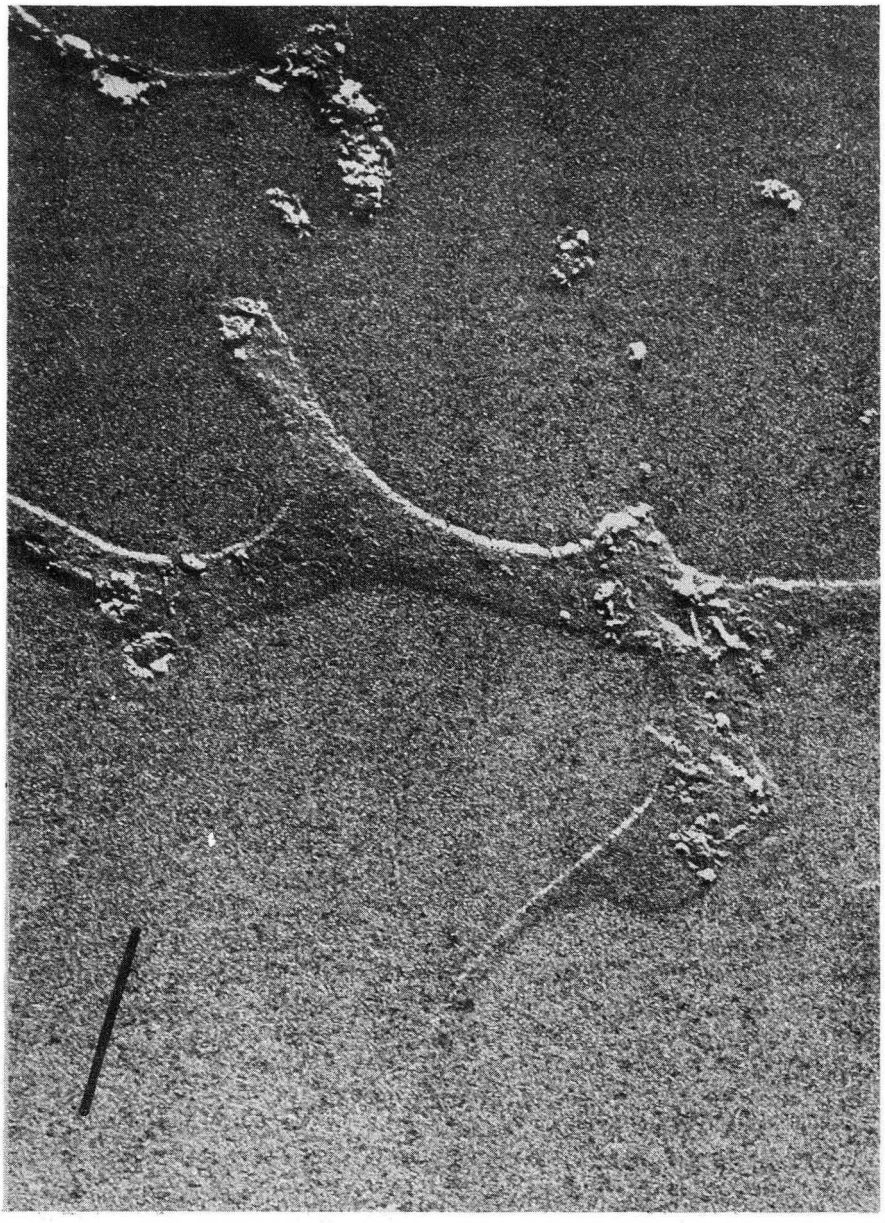

Fig. 4. Lipid-depleted sarcoplasmic reticulum vesicles prepared by treatment with phospholipase A. 10.9 μg bound P/mg protein remained with the membranous fraction after phospholipase A treatment and four bovine serum albumin washes (120 000 ×).

cross-sections of vesicles are seen, but there is a distortion in the manner in which some of the material fractures after cross-linking.

In a third type of perturbation experiment sarcoplasmic reticulum vesicles were first solubilized with deoxycholate so that they were no longer membranous. Subse-

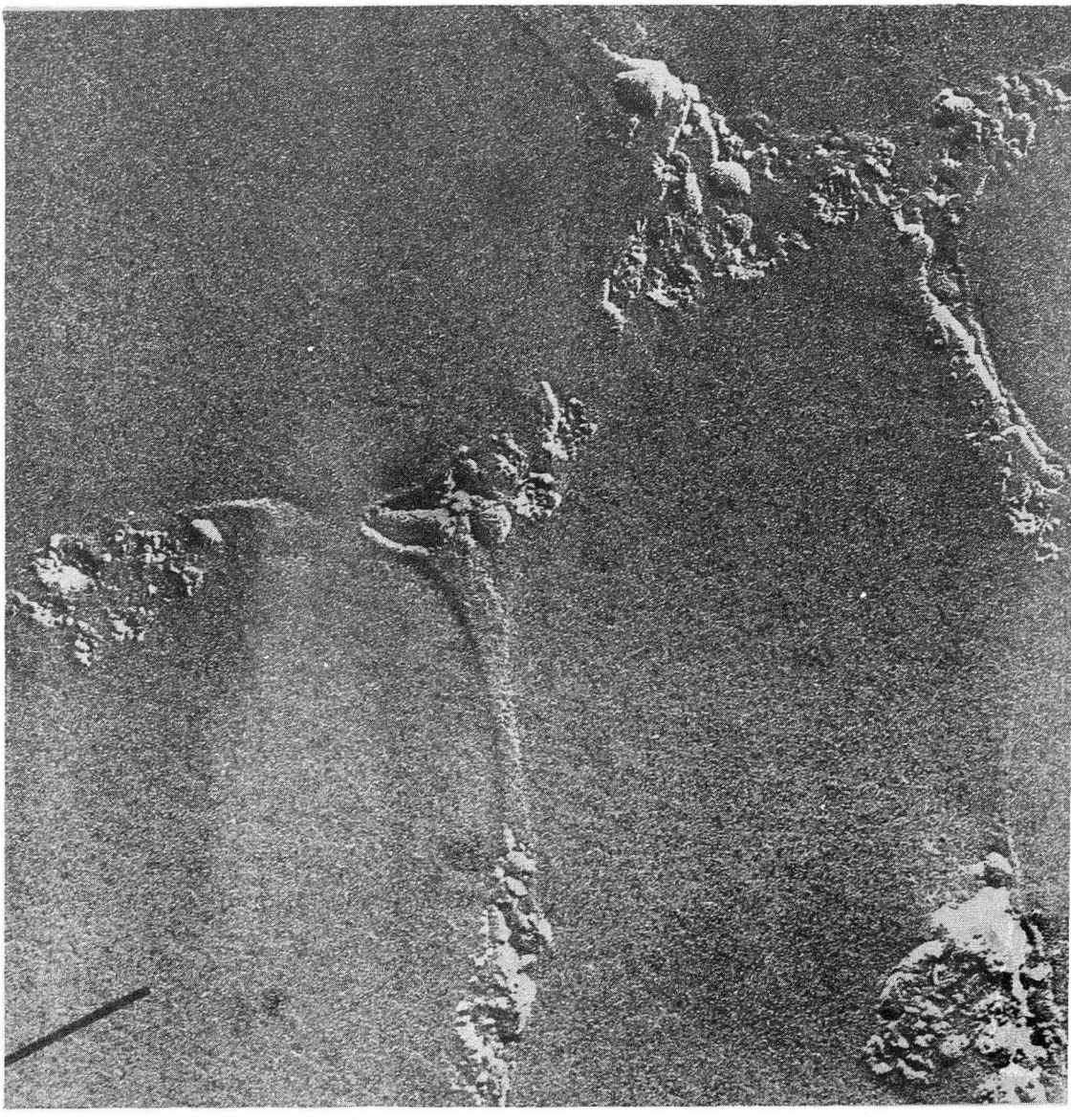

Fig. 5. Freeze-fracture electron micrographs of sarcoplasmic reticulum vesicles treated with detergent. 15.9 μg P/mg protein were bound to the membranous fraction after treatment of the vesicles with bile acid. (120 000 ×).

quent removal of the detergent by dialysis resulted in reconstitution of membrane vesicles as judged by conventional electron microscopy [18]. The reformed vesicles are composed mainly of the Ca^{2+}-pump protein (about 90 % pure) and amounts of phospholipid normally present in sarcoplasmic reticulum vesicles. Such reconstituted membranes readily undergo freeze fracture and electron micrographs of the fracture faces show membrane particles (Fig. 7). The asymmetric distribution of membrane

TABLE III

MEMBRANE PARTICLES IN FREEZE-FRACTURE FACES OF SARCOPLASMIC RETICULUM VESICLES

Preparation	Bound phosphorus (μg P/mg protein)	Fracture face	Area (μm^2)	Particle No.	Density (particles/μm^2)	Concave/convex ratio*
Control vesicles	22.4	Concave (A)	0.08	450	5625	8.65
		Convex (B)	0.005	36	650	
Vesicles extracted with detergent	21.2	Modified or absent	0.05	158	3285	
Vesicles solubilized with detergent and reassembled by dialysis**	27	Concave	0.20	936	4680	1.05
		Convex	0.16	711	4471	

* Ratio of concave to convex particle density.

** Data from two different preparations.

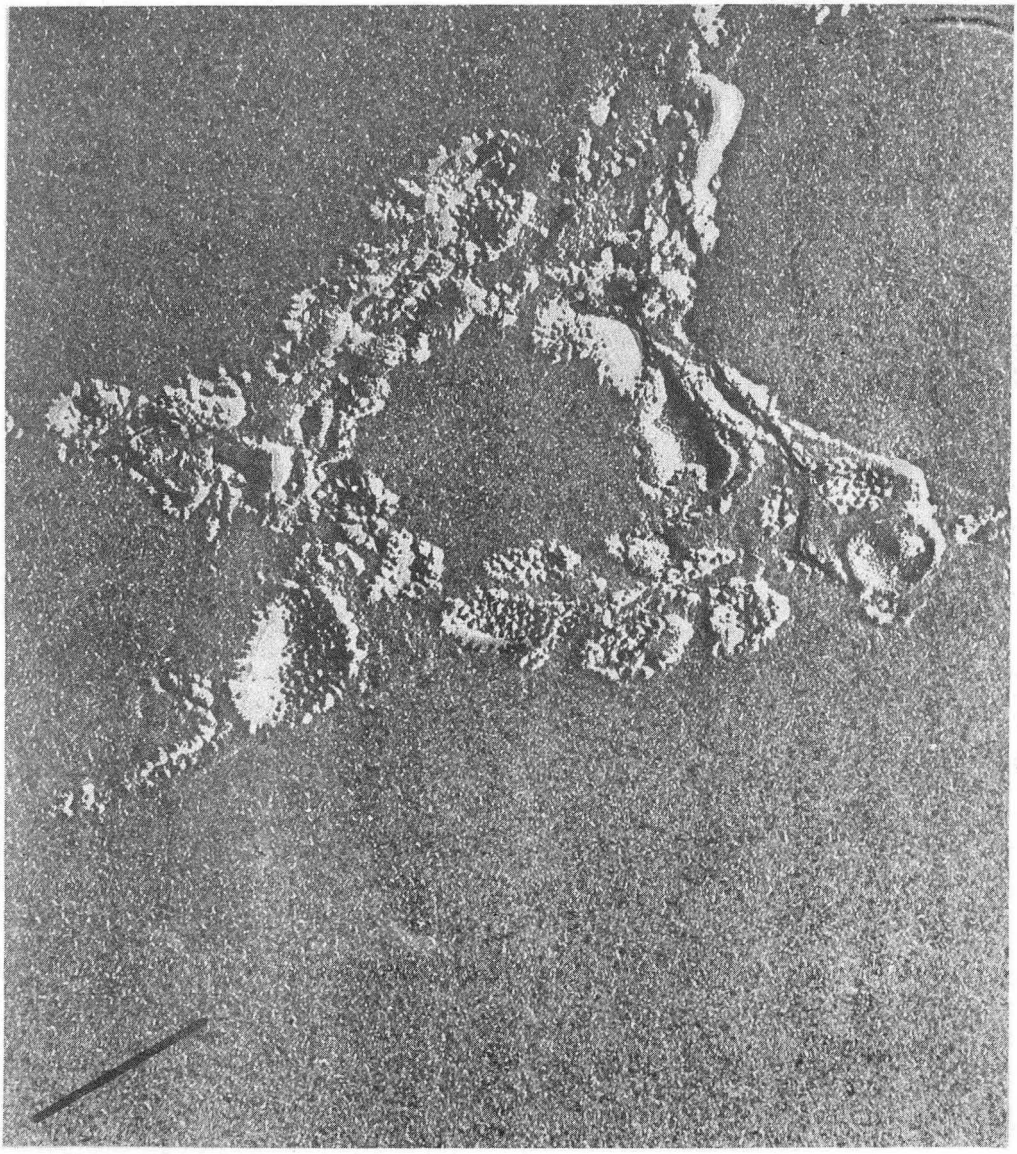

Fig. 6. Sarcoplasmic reticulum vesicles treated with glutaraldehyde. Sarcoplasmic reticulum vesicles were fixed with 0.5 % glutaraldehyde as described in Materials and Methods. (120 000 ×).

particles between the two fracture faces characteristic of control vesicles is completely lost in the reconstituted sarcoplasmic reticulum vesicles, i.e. the distribution of membrane particles seen in the half-membranes as indicated from examination of concave and convex fracture faces is approx, 1:1 (Table III).

There is a broad distribution of particle sizes in the fracture faces of normal sarcoplasmic reticulum membranes varying from 20 to 120 Å with particles of 85-Å

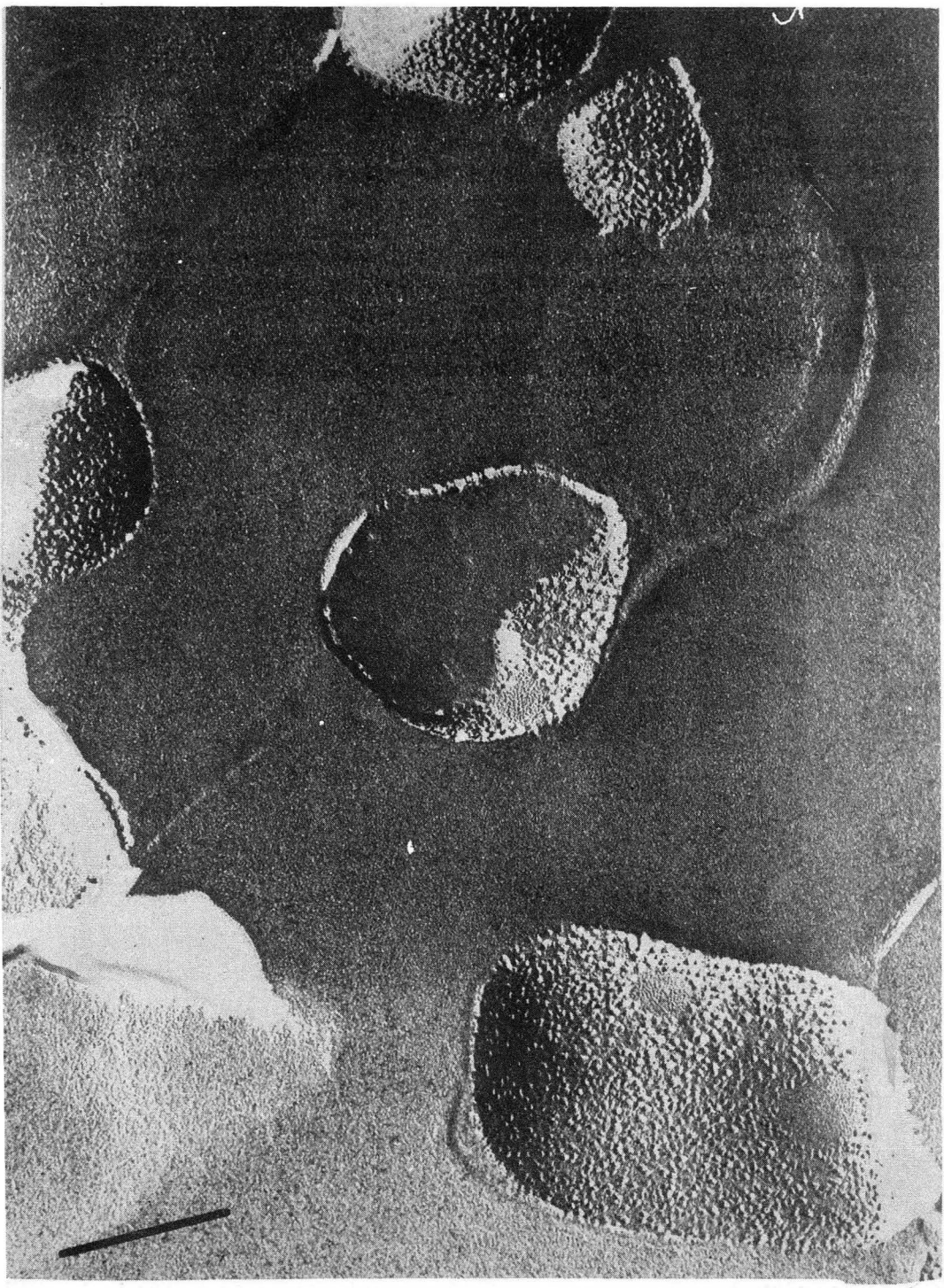

Fig. 7. Sarcoplasmic reticulum vesicles solubilized with deoxycholate and reassembled by dialysis. The reassembled vesicles contained 27 μg bound P/mg protein. (120 000 ·).

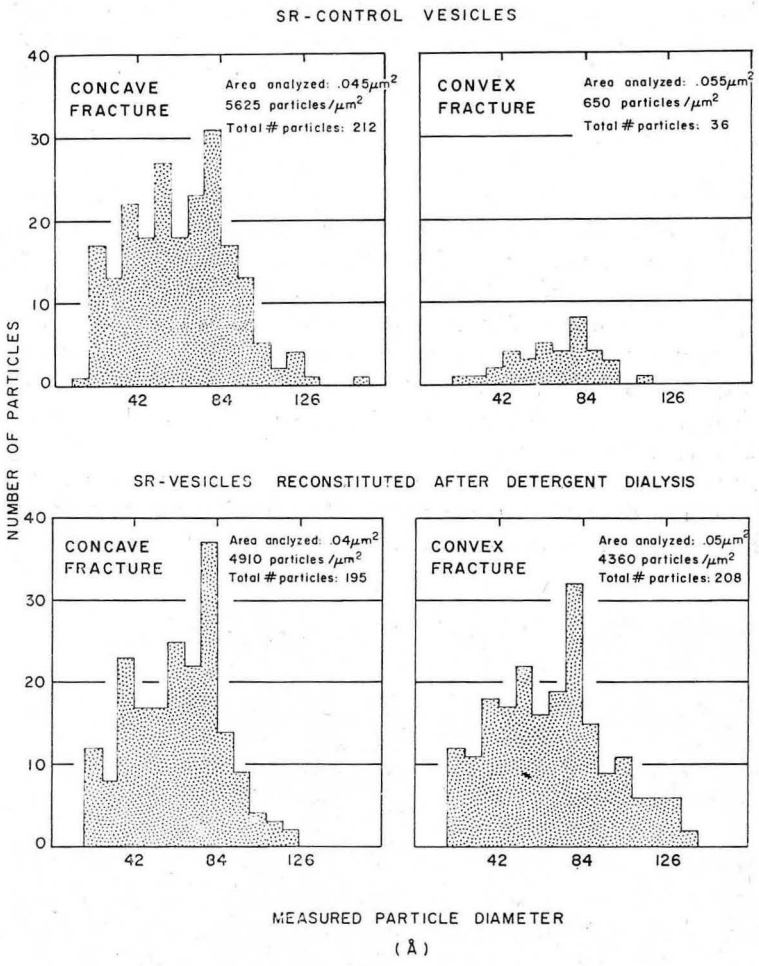

Fig. 8. Analysis of the size and distribution of the membrane particles seen in fracture faces of normal sarcoplasmic reticulum (SR) vesicles and vesicles which are solubilized with deoxycholate and then reassembled by dialysis to remove the detergent.

size present in greatest number (Fig. 8). The size distribution of particles in the reconstituted membranes, which are composed mainly of the Ca^{2+}-pump protein, also shows a size distribution typical of the original sarcoplasmic reticulum membrane.

DISCUSSION

Lipid depletion studies

This study has demonstrated that two biomembranes, with different function, complexity, and composition are similarly affected by the removal of phospholipid. Beef heart inner mitochondrial membrane vesicles and rabbit skeletal muscle sarcoplasmic reticulum vesicle membranes are particularly useful for such studies for several reasons. First, criteria for their purity, chemical composition, and appearance

by conventional electron microscopy have been established [1, 2, 4, 8, 9, 18, 21]. In addition, inner mitochondrial membranes provide an example of an extremely complex functional membrane where structure would be expected to have a commensurate complexity [26]. Sarcoplasmic reticulum is an example of a simple membrane with only three major protein components, two of which are membrane-associated proteins [18].

The lipid-depletion studies provide convincing proof that the particles seen in the fracture faces of the mitochondrial and sarcoplasmic reticulum membranes are indeed protein or lipoprotien components. Three different modes of lipid depletion were studied: phospholipase A treatment, detergent treatment, and extraction with acetone. Although these three means of lipid removal differ in the way in which they alter membrane structure, extensive lipid depletion by all three methods, as depicted in Fig. 9, resulted in the formation of particle clusters composed mostly of protein with loss of the smooth interparticle region of the fracture faces presumed to be the ordered lipid domains (possibly bilayers).

Since the structure of the membrane as observed by conventional electron microscopy is preserved in the main after removal of lipid [4, 21], it would appear that the primary effect of lipid removal is to decrease the ability of the membrane to

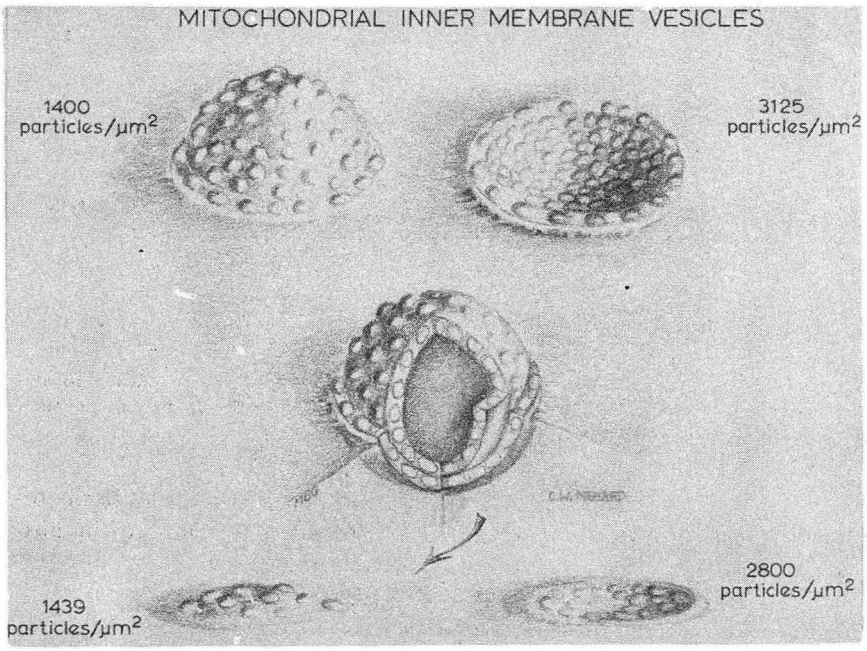

Fig. 9. Diagrammatic representation of the effect of increased removal of phospholipid from mitochondrial inner membrane vesicles on structure seen in freeze-fracture replicas. Original vesicles 19.4 μg P/mg. The partial depletion of lipid protein to 12.4 μg P/mg protein results in shallow convex and concave fracture faces. As lipid is further removed from the membrane there is a loss of smooth interparticle areas, and the fracture face disappears resulting in cross-fractures exposing a circular double row of particles indicating particles present in both half-membranes. With continued lipid depletion a single row of particles is observed in the preparations and eventually only particles are seen. In this sketch we do not mean to imply absence of protein–protein association.

be fractured. The reason for this may be that phospholipid contributes to the plasticity of the membrane and at least in part to the hydrophobic character of the membrane, which is believed to be required for freeze fracture. Speth et al. [27] have also found that treatment of erythrocyte membranes with phospholipase and detergents changes the normal fracture pattern. A further study of depletion and rebinding of various lipids may help to elucidate the means by which lipids are involved in the organization of membrane proteins. Indeed, earlier studies by Packer and Williams (cf. [28]) and by James and Branton [12] and James et al. [13] have shown that modifications of the unsaturated fatty acid composition of membranes change the organization of membrane particles and that such changes are associated with changes in functional properties such as membrane permeability.

Membrane asymmetry and particle arrangement

The present studies suggest that under the conditions of freeze fracture one has to distinguish between two levels of constraint within a membrane. The first is with respect to the asymmetric distribution of the particles between the two fracture faces. The second one concerns the particle size distribution within a fracture face. The arrangement of the particles within a membrane is not random in that an unequal number of particles in the convex and concave fracture faces is observed, as shown diagrammatically in Figs 9 and 10 for the mitochondrial inner and sarcoplasmic reticulum membranes, resp. In previous studies an asymmetric arrangement of particles has been also observed in these and other membranes [30, 31]. Using intact mito-

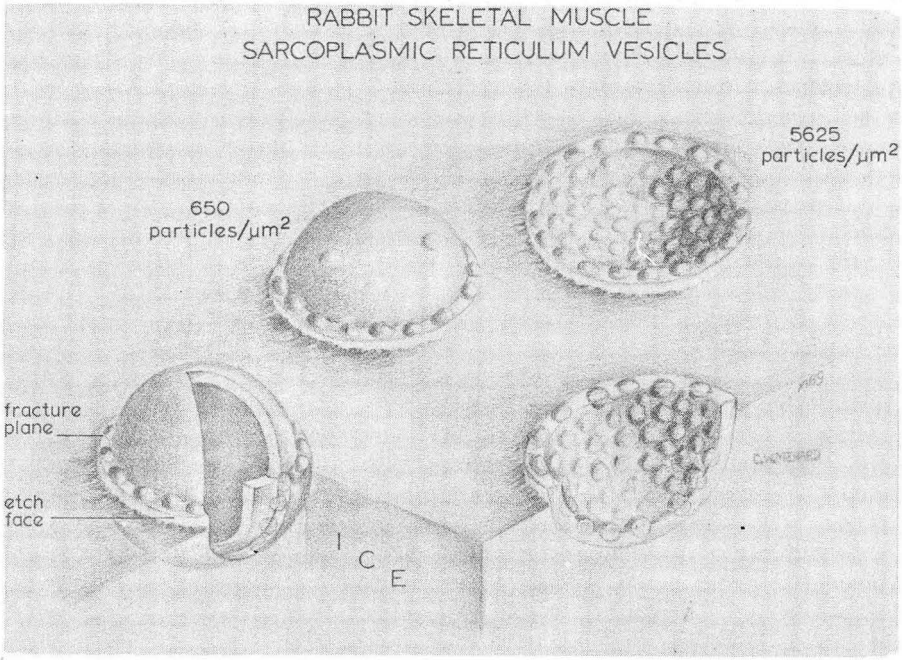

Fig. 10. Diagram of rabbit skeletal muscle sarcoplasmic reticulum vesicles showing the asymmetric arrangement of the Ca^{2+} pump protein in the two half-membranes. The concave (A Face) fracture face is the half-membrane with the greater density of particles; the convex (B Face) has fewer particles.

chondria [29] we have previously shown that particles of inner membranes have a 2:1 asymmetry, with approximately 4000 particles/μm^2 on the side facing the matrix (A Face) and 2000 particles/μm^2 on the side facing the intermembrane space (B Face). Heart submitochondrial vesicles examined here also show a 2:1 asymmetry in the A and B faces, respectively. Sarcoplasmic reticulum membranes have a very asymmetric distribution of particles between the two fracture faces. In the present study a 9:1 asymmetry of particles is observed in the A/B faces. We find a complete loss of particle asymmetry has taken place in reassembled sarcoplasmic reticulum vesicles similar to the studies of MacLennan et al. [30]. Several reasons may exist for this. For instance, the absence of membrane-associated protein might have led to a loss of constraint of particle orientation with respect to the fracture face, or the membrane particles might have arranged in random fashion during the actual process of membrane reassembly. Thus, the freeze-fracture technique is a very sensitive tool which reveals loss of particle asymmetry in reconstituted membranes which is not readily seen by conventional electron microscopy.

Analysis of the size distribution of membrane particles in the inner mitochondrial and sarcoplasmic reticulum membranes shows a spectrum of particle sizes, the predominant size in both membranes is about 85 Å, similar to the one observed by others [29–33]. In complex membranes, such as the inner mitochondrial membrane where many intrinsic proteins are present, the identity of these particles is unknown. The presence of particles in freeze-fracture faces of sarcoplasmic reticulum membranes and their possible relationship to the Ca^{2+}-pump protein was first pointed out by Deamer and Baskin [32]. The particles observed in freeze fracture seem to be referable to the Ca^{2+}-pump protein or a complex of the Ca^{2+}-pump protein with phospholipid. Our studies further show that there is a wide distribution in the size of these particles even though there is essentially only one intrinsic membrane protein present. This clearly means that there is no direct correlation between molecular weight and particle size as seen in the freeze-fracture faces. Heterogeneity in particle size might result from the shadowing technique [34] or may reflect orientation of the Ca^{2+}-pump protein within the fracture face. This distribution of sizes is similar for both the original and reassembled sarcoplasmic reticulum membranes (Fig. 8) with regard to the Ca^{2+}-pump protein.

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