Correlation of Ultrastructure of Reconstituted Sarcoplasmic Reticulum Membrane Vesicles with Variation in Phospholipid to Protein Ratio*

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We have previously described the reconstitution of functional membrane vesicles with lipid content similar to that of the normal sarcoplasmic reticulum membrane (~1.0 µmol of phospholipid/mg of protein). The present study describes methodology to prepare reconstituted membrane vesicles with defined phospholipid to protein ratio, both lower and higher than that of the original membrane. The Ca²⁺ loading rate and efficiency are greatest in the membranes of highest protein content (0.38 µmol of phospholipid/mg of protein), decline slowly as the lipid content is quadrupled, and decrease markedly as the lipid content is quadrupled again. Such membranes with defined composition can be used to study lipid-protein interaction and to correlate membrane structure with composition.

The number of particles observed by freeze-fracture electron microscopy can be correlated with protein content, whereas the percentage of smooth domain is proportional to the lipid content of the reconstituted membrane. Since 90% or more of the protein of the reconstituted membrane is the calcium pump protein, the number of particles observed by freeze-fracture is directly proportional to the amount of calcium pump protein in the membrane. The number of pump molecules calculated to be in the membrane is greater by a factor of two than the number of particles which we observed. This multiplicity ratio could be greater depending upon the assumptions made regarding the width of the membrane (see "Appendix"). Thus, it would appear that the particles consist of two or more molecules of pump protein. The change in protein concentration of the membrane is reflected also in thin sections and by negative staining. In thin sections, the broad inner and outer 70 Å bands become discontinuous and patchy and, in the limit, approach a symmetrical 20,20,20 Å trilayer as the protein content of the membrane becomes small. In an analogous fashion, the concentration of particles at the surface of the membrane, observed by negative staining, decreases with increasing lipid concentration in the membrane. Thus, the correlation of composition with structure can be observed by each of the three methods of sample preparation for electron microscopic analysis.

Sarcoplasmic reticulum is a highly specialized membrane system which regulates calcium ion fluxes during muscle contraction and relaxation. It is one of the most intensively studied membrane systems (1–4). The membrane consists of 60% protein and 40% phospholipid (5) of which the calcium pump, of Mₙ = 119,000 (6), comprises about 90% of the protein of the membrane (7). Membrane reconstitution provides a powerful approach to the study of membrane structure and function. We describe a procedure to reconstitute functional sarcoplasmic reticulum vesicles with different phospholipid to protein ratios, both lower and higher than that of the original SR membrane. Such reconstituted membranes are suitable for the study of the nature of lipid-protein interaction in the membrane. In this study, we correlate protein and phospholipid composition with membrane structure as observed by electron microscopy. A preliminary report has appeared (8).

EXPERIMENTAL PROCEDURES

Materials—[γ-³²P]ATP was prepared according to Post and Sen (9) and was a gift from Dr. Robert Post (Department of Physiology, Vanderbilt University). Nonradioactive ATP was obtained from P-L Biochemicals (Milwaukee, Wis.). Deoxycholate (Matheson, Coleman and Bell, Norwood, Ohio) was recrystallized before use (10). Dialysis was carried out with the use of standard cellulose nitrate tubing (Arthur H. Thomas Co., Philadelphia, Pa.) with a flattened width of 2.5 cm and an average pore diameter of 4.8 nm. The tubing was soaked in 10 mM Tris/acetate, 1 mM EDTA, pH 8.0, at 4°C for at least 24 h before use.

Assays—Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard. Total phosphorus was determined and used as a measure of lipid phosphorus (5, 12).

Energized calcium accumulation, i.e. Ca²⁺ loading and Ca²⁺ uptake was measured at 23°C in the presence and absence of calcium oxalate, respectively, as previously defined and described (13). Ca²⁺ uptake capacity was determined in a medium containing 200 µg of sarcoplasmic reticulum protein/ml of 0.1 M KCl, 5 mM MgCl₂, 5 mM ATP, 100 µM ⁵¹Ca²⁺, and 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.0. The reaction was started by addition of ATP and was terminated after 1 min by filtration using a type GS 0.2 µm Millipore filter. Ca²⁺ loading was measured in the presence of oxalate at 23°C in a medium containing 5 to 20 µg of sarcoplasmic reticulum protein/ml of 0.1 M KCl, 5 mM MgCl₂, 5 mM ATP, 100 µM ⁵¹Ca²⁺ (5 × 10⁵ cpm/µmol), 5 mM potassium oxalate, and 10 mM Hepes, pH 7.0. The reaction was initiated by addition of ATP and was terminated after 1- and 16-min periods by filtration using a type GS 0.22 µm Millipore filter. The amount of ⁵¹Ca²⁺ accumulated at 1 min gives the Ca²⁺ loading rate while the value at 16 min is taken as the Ca²⁺ loading capacity (13).

Ca²⁺-stimulated and Ca²⁺-independent (basic) ATPase activities were measured for 1 min at 23°C in a medium identical with the Ca²⁺ loading reaction mixture except that the latter assay was in the presence of 1 mM ethylene glycol bisth(β-aminoethyl ether)N,N,N',N''-tetraacetic acid with no added Ca²⁺. Inorganic phosphate was deter-

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† The abbreviations used are: SR, sarcoplasmic reticulum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mₙ, poly peptide of estimated molecular weight of 30,000 (5, 10).
mained by the method of Fiske and Subbarow (14) with Elson as a reducing agent. Phosphoenzyme formation was carried out at 0°C using conditions previously described (5).

Polyacrylamide gel electrophoresis was carried out in 0.1% sodium dodecyl sulfate on 11 and 5% polyacrylamide in the separating and stacking gel, respectively, according to Laemmli (15). Samples (approximately 40 μg of protein/gel) were reduced with 2.5% 2-mercaptoethanol by heating for 4 min at 100°C prior to electrophoresis. After electrophoresis, gels were stained with 1% amido black (10). Analysis of phospholipid classes of the lipid extracts of the normal and reconstituted SR vesicles was determined by thin layer chromatography (12, 16) using chloroform/methanol/H2O (65:25:4, v/v) as the solvent system. The bands were visualized by charring at 180°C for 30 min after spraying with concentrated H2SO4/formaldehyde (97.3, v/v). The results are expressed as percentage of total lipid phosphorus obtained by measuring the phosphorus of the separated phospholipids (5, 12).

**Electron Microscopy**

Samples for freeze-fracture were prepared by suspending 0.5 to 1 mg of protein of the pelleted vesicles in 25% (w/w) glycerol solution containing 0.3 M sucrose, 100 mM KCl, 1 mM Hepes, pH 7.1, at 0°C overnight. The pellets were then quick frozen from room temperature or from 0°C by immersing them into liquid freon at approximately -140°C. After freeze-fracturing, the specimens were floated onto 5% Clorox and cleaned with distilled water (17). The intramembranous particles on both fracture faces were counted and the particle density expressed as the number of particles/μ2. The interparticle distance and the area of smooth domains were measured using an electronic graphics calculator from Numonics Corp., Landsdale, Pa.

Thin sections of the membranes were prepared as described previously (17). All samples were prefixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 1% tannic acid (Mal- linckrodt analytical reagent) for 2 h in the cold (-4°C), washed, and postfixed for 2 h at 4°C in 1% osmium tetroxide in 0.1 M Veronal acetate buffer (pH 7.2) containing 2.4 mM CaCl2 and 0.06 M NaCl. The samples were block-stained with 0.5% uranyl acetate in Veronal acetate buffer (pH 6.0) for 2 h at room temperature. After dehydration and embedding in Epon-Araldite mixture, the thin sections were washed, diluted with an approximately equal volume of dialysis buffer and centrifuged. The pellet was washed twice using 10 ml of dialysis buffer/1 mg of protein prior to sucrose gradient centrifugation.

**RESULTS**

We have developed a procedure to vary the lipid content of reconstituted sarcoplasmic reticulum membrane vesicles (Fig. 1, Table I). The reconstituted vesicles, prepared by dialysis of the detergent-solubilized sample for 1 and 4 h at room temperature, consist of one-half and two-thirds of the lipid content, respectively, of the original sarcoplasmic reticulum vesicles. Membranes of higher lipid to protein ratios were obtained by supplementing the detergent-solubilized sample with added phospholipid prior to dialyzing for 4 h. The samples were recovered by sedimentation, purified on a sucrose gradient, and then washed two times with dialysis buffer. The reconstituted membrane vesicles of each lipid to protein ratio formed a narrow band at their buoyant density after sucrose centrifugation.
Correlation of Structure with Lipid Content of R-SR Membranes

Preparation of reconstituted sarcoplasmic reticulum vesicles of defined lipid to protein ratio

The reconstituted sarcoplasmic reticulum vesicles with varying lipid content were prepared and purified as described under "Experimental Procedures." The band containing the reconstituted preparations were less than 2 mm wide. The linear gradient was 15 to 40% sucrose as described under "Experimental Procedures" except for Samples F and G where a linear gradient of 5 to 20% sucrose was used. Data for A to E was averaged from 15 experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original SR</th>
<th>Reconstituted SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR lipid added †</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dialysis time (h)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Isopycnic density % Sucrose</td>
<td>29-32</td>
<td>38.0</td>
</tr>
<tr>
<td>g/ml at 17°C</td>
<td>1.129</td>
<td>1.167</td>
</tr>
<tr>
<td>Recovery of protein (%)</td>
<td>40</td>
<td>47</td>
</tr>
<tr>
<td>Bound phosphorus, μmol Pi/mg protein</td>
<td>0.78 ± 0.04</td>
<td>0.38 ± 0.04</td>
</tr>
</tbody>
</table>

† Added SR phospholipid is expressed in equivalents (designated X) where one equivalent is taken as 24 μg of phosphorus/mg of protein, i.e. the amount in normal SR. The phospholipid content of the SR membrane is somewhat higher, approximately 31 μg of phosphorus/mg of protein (value for light sarcoplasmic reticulum (7)).

Fig. 2. Polyacrylamide gel electrophoresis of original and reconstituted sarcoplasmic reticulum vesicles. Sodium dodecyl sulfate gels, containing 11 and 5% acrylamide in the separating and stacking gel, respectively, were prepared as described by Laemmli (15). The samples were reduced with 2.5% 2-mercaptoethanol by heating for 4 min at 100°C prior to gel electrophoresis. After electrophoresis, gels were stained with 1% amido black. NSR, original sarcoplasmic reticulum membranes; A to E, reconstituted sarcoplasmic reticulum membranes with phospholipid to protein ratio of 0.38, 0.57, 0.78, 1.03, and 1.29 μmol/mg of protein, respectively. CPP, calcium pump protein; CBP, calcium binding protein.

Gradient centrifugation. The isopycnic density of the reconstituted vesicles decreased with increasing lipid content in a coherent fashion. The protein recovery is increased with higher phospholipid content. The amount of lipid phosphorus in the reconstituted sarcoplasmic reticulum vesicles is directly proportional to the amount of phospholipid present prior to the 4-h dialysis (Fig. 1).

Characterization of Reconstituted Sarcoplasmic Reticulum Vesicles of Varying Lipid Content—The protein profile of the reconstituted sarcoplasmic reticulum vesicles was obtained by polyacrylamide gel electrophoresis using sodium dodecyl sulfate. The reconstituted vesicles with different lipid content are composed mainly of Ca2+ pump protein (≥90%) (Fig. 2), i.e. most of the Ca2+ binding and M55 proteins (10) are no longer present in the reconstituted vesicles. The decreased amount of these proteins, especially M55, as compared with our previous reconstituted preparation (13), is due to the shorter period of dialysis. The phospholipid composition of the reconstituted vesicles of varying lipid to protein ratio is essentially the same and is not different significantly from the original SR (Table II).

The functional characteristics of the reconstituted sarcoplasmic reticulum vesicles of different lipid to protein ratio are compared in Table III. The Ca2+ loading rate is highest in the reconstituted sample with lowest lipid content, the rate being approximately 30% that of normal sarcoplasmic reticulum vesicles. The Ca2+ loading rate decreases with increased lipid content of the membrane. The Ca2+ loading capacity is optimal in reconstituted membrane vesicles of lipid content similar to normal sarcoplasmic reticulum and decreases sharply when the lipid content becomes much larger. The Ca2+-stimulated ATPase activity increases with increased lipid content. Accordingly, the Ca2+ loading efficiency of these vesicles decreases with increasing lipid content. Nonenergized binding of calcium ions (in the absence of ATP) is the same for normal and reconstituted SR vesicles, but the energized Ca2+ uptake capacity is only about one-fourth that of the original sarcoplasmic reticulum vesicles. Phosphoenzyme formation of reconstituted sarcoplasmic reticulum vesicles is somewhat higher in reconstituted than the original SR, since the former are enriched in Ca2+ pump protein due to loss of M55 and Ca2+ binding protein.

Correlation of Structure with Lipid Content—Normal sarcoplasmic reticulum is a highly asymmetric membrane as revealed by electron microscopy. Freeze-fracture electron mi-

TABLE I

Phospholipid composition of normal and reconstituted sarcoplasmic reticulum vesicles

The data are averages of two different preparations (4 thin layer chromatography plates each).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Original SR</th>
<th>Reconstituted SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bound phosphorus μmol Pi/mg protein</td>
<td>0.78</td>
<td>0.38</td>
</tr>
<tr>
<td>% of total lipid phosphorus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>75</td>
<td>73</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Other †</td>
<td>10</td>
<td>11</td>
</tr>
</tbody>
</table>

† Contains mostly phosphatidylinositol, but also some phosphatidylserine + sphingomyelin (5).
TABLE III

Functional characteristics of reconstituted sarcoplasmic reticulum vesicles of varying lipid to protein ratio

The data for Samples A to E are the average of at least six experiments. Ca" uptake and loading were measured in the absence and presence of oxalate, respectively (13). The Ca" loading efficiency was calculated by dividing the Ca" loading rate by the Ca"-stimulated ATPase rate, assayed in the presence of oxalate (see "Experimental Procedures"). The Ca"-stimulated ATPase was obtained by subtracting "basic" ATPase from the total Ca"-stimulated ATPase. Basic ATPase was approximately 0.05 for normal sarcoplasmic reticulum and 0.02 or lower in the reconstituted preparations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original SR</th>
<th>Reconstituted SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Lipid content</td>
<td>µmol lipid phosphorus/mg protein</td>
<td>0.78</td>
</tr>
<tr>
<td>Ca&quot; uptake capacity</td>
<td>nmol Ca&quot;/mg protein</td>
<td>119</td>
</tr>
<tr>
<td>Ca&quot; binding + Ca uptake (+ATP)</td>
<td></td>
<td>23</td>
</tr>
<tr>
<td>Ca&quot; binding (−ATP)</td>
<td></td>
<td>96</td>
</tr>
<tr>
<td>Difference</td>
<td></td>
<td>5.36</td>
</tr>
<tr>
<td>Ca&quot; loading capacity</td>
<td>µmol Ca&quot;/mg protein/16 min</td>
<td>2.10</td>
</tr>
<tr>
<td>Ca&quot; loading rate</td>
<td>µmol Ca&quot;/mg/min</td>
<td>1.06</td>
</tr>
<tr>
<td>Ca&quot;-stimulated ATPase rate</td>
<td>µmol P_i/mg/min</td>
<td>2.0</td>
</tr>
<tr>
<td>Ca&quot;/ATP</td>
<td></td>
<td>5.8</td>
</tr>
<tr>
<td>Phosphoenzyme formation</td>
<td>nmol phosphoenzyme/mg protein</td>
<td>0.41</td>
</tr>
</tbody>
</table>

The asymmetry of the original sarcoplasmic reticulum membrane can also be visualized in thin sections with the use of tannic acid to enhance contrast (Fig. 4A). The width of the bands of the trilaminar membrane is 70 Å, and both inner and outer membranes are 20 Å for outer, mid, and inner layers, respectively (17). However, the trilayer has a symmetric appearance in the reconstituted membranes. Both inner and outer bands of the trilaminar membrane form a broad, 70 Å wide continuum, in the reconstituted membrane. The lipid vesicle structure appears to be modified by the presence of one type of protein, the density of intramembranous particles is directly proportional to the concentration of calcium pump protein of the membrane.

It is significant that the percentage of smooth area for each of the preparations is greater in the inner fracture face while the density of particles is numerically greater in the outer face. The greater number of particles in the outer versus inner fracture face is small, but undoubtedly significant, since it is consistent for each membrane of different lipid to protein ratio and there is the reciprocally greater extent of smooth surface in the inner fracture face containing the lower density of particles.

1.29 µmol of phosphorus/mg of protein. The outer and inner layers have smaller patches (cf. arrows) with much of the surface without patches (cf. triangle).

Fig. 5. Negative staining electron microscopy of normal and reconstituted sarcoplasmic reticulum vesicles (× 278,000). A, normal SR; B and C, reconstituted SR with phospholipid to protein ratio of 0.38 and 1.29 µg of phosphorus/mg of protein, respectively; D, lipid vesicles prepared from sarcoplasmic reticulum lipid. Uranyl acetate was used for negative staining. The 40 Å particles (arrows) are observed only in the outer surface of normal SR and on both inner and outer surfaces of reconstituted SR. The lack of particles (triangle) can be due to imperfect staining (A) or to decreased protein in the membrane. The lipid vesicle structure appears to be modified by the negative staining procedure (compare with Fig. 6D).

Fig. 6. Electron microscopy of reconstituted SR and lipid vesicles. A, (× 231,000) and B, (× 278,000) reconstituted membranes with phospholipid content of 2.5 µmol of phosphorus/mg of protein. C, (× 231,000) and D, (× 110,000) vesicles prepared from sarcoplasmic reticulum lipids. A and C, thin sections using tannic acid enhancement; B and D, samples were observed after negative staining and freeze-fracture electron microscopy, respectively. In B, the arrow points to surface particles, the triangles to areas devoid of particles; in D, the arrow indicates the direction of shadowing.
Correlation of Structure with Lipid Content of R-SR Membranes

Fig. 3
FIG. 4

Correlation of Structure with Lipid Content of R-SR Membranes
Correlation of Structure with Lipid Content of R-SR Membranes

FIG. 5
Correlation of Structure with Lipid Content of R-SR Membranes

FIG. 6
Correlation of Structure with Lipid Content of R-SR Membranes

TABLE IV

Freeze-fracture electron microscopy studies correlating particle density, interparticle distance, and the percentage of smooth area at the hydrophobic face of normal and reconstituted sarcoplasmic reticulum membranes of varying lipid content

Each value represents data obtained by counting at least 60 fracture faces. The number of pump molecules/p was calculated from the equation derived in the appendix. This is a minimum value which could be greater (see “Appendix”).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original SR</th>
<th>Reconstituted SR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Bound phosphorus μmol P/mg protein</td>
<td>0.78</td>
<td>0.38</td>
</tr>
<tr>
<td>Particles/p Convex face</td>
<td>5675 ± 655</td>
<td>6187 ± 1346</td>
</tr>
<tr>
<td>Convex face</td>
<td>723 ± 107</td>
<td>22,100</td>
</tr>
<tr>
<td>Pump molecules/p</td>
<td>12,200</td>
<td></td>
</tr>
<tr>
<td>Number of molecules calculated</td>
<td>1.91</td>
<td>1.91</td>
</tr>
<tr>
<td>Interparticle distance (Å)</td>
<td>90-116</td>
<td>90-116</td>
</tr>
<tr>
<td>Percentage of smooth area per fractured face area</td>
<td>6.2 ± 0.9</td>
<td>5.8 ± 0.8</td>
</tr>
<tr>
<td>Convex face</td>
<td>9.0 ± 1.2</td>
<td>14.8 ± 1.8</td>
</tr>
</tbody>
</table>

*The interparticle distance represents the range of center-to-center distances of all the neighboring particles surrounding any one particle but not across smooth areas. Only the concave fracture face of the original SR membrane was measured for the interparticle distance; both faces were measured in the reconstituted preparations.

*The percentage of smooth area per total fracture face area was obtained by measuring at least 10 fracture faces. Only the concave face was measured in the normal sarcoplasmic reticulum.

vesicles with approximately half the lipid content of the original sarcoplasmic reticulum (0.38 μmol of phospholipid/mg of protein) (Fig. 4D). When the lipid content of the reconstituted membranes is increased to equal and greater than that of the original membrane, the broad outer and inner layers become discontinuous patches, then smaller patches are observed and, in the limit, approach the appearance of a single layer which is carried out at room temperature. The procedure used is similar to that described by Meissner and Fleischer (13) except for dialysis times of 18 h instead of 4 h, which is more effective in solubilizing the sarcoplasmic reticulum membrane, i.e. 60% protein and 40% lipid (Fig. 4C and D). For phospholipid vesicles with lower lipid content to protein ratios in the range of normal sarcoplasmic reticulum, i.e. both lower and higher lipid content within a factor of two. The procedure used is similar to that described previously by us (13), with some modifications. The time of dialysis has been decreased. A shortened time of dialysis, 1 to 4 h instead of 18 h, gives a lower lipid to protein ratio for the reconstituted membrane vesicles. We have defined a procedure to prepare membranes with lipid to protein ratios containing 0.38 μmol of phospholipid/mg of protein or greater. The higher lipid content is achieved by supplementation with added SR phospholipid as described. A lower lipid content is achieved by shortening the time of dialysis. As before, dialysis is carried out at room temperature.

Membranes prepared using 4 h or less time for dialysis are practically devoid of M₉ and Ca²⁺ binding protein. Yet, such reconstituted membranes have similar functional characteristics as those prepared by dialysis for the longer time (18 h). It would appear, therefore, that the Ca²⁺ binding protein and M₉ do not affect the Ca²⁺ pumping rate or efficiency.

Another modification is that we now use a lower protein concentration of SR protein (5.6 instead of 7.0 mg/ml) for solubilization with dissociation buffer. These conditions are more effective in solubilizing the sarcoplasmic reticulum membrane, i.e. close to 100% of the membrane lipid and 95% of the

DISCUSSION

Biological membranes, such as sarcoplasmic reticulum, consist mainly of protein and phospholipid. For most membranes, the protein content is equal to or greater than that of the lipid. Yet, most reconstitution studies described to date have used a large excess of phospholipid in order to prepare membranes with function (25). The procedure described by Meissner and Fleischer (13) was the first in which functional membrane vesicles were reconstituted with lipid content similar to that of the original membrane, i.e. 60% protein and 40% lipid or 1 μmol of phospholipid/mg of protein. The procedure has recently been confirmed by Repke et al. (26). We now describe methodology to prepare functional membrane vesicles of varying lipid to protein ratios in the range of normal sarcoplasmic reticulum, i.e. both lower and higher lipid content within a factor of two. The procedure used is similar to that described previously by us (13), with some modifications. The time of dialysis has been decreased. A shortened time of dialysis, 1 to 4 h instead of 18 h, gives a lower lipid to protein ratio for the reconstituted membrane vesicles. We have defined a procedure to prepare membranes with lipid to protein ratios containing 0.38 μmol of phospholipid/mg of protein or greater. The higher lipid content is achieved by supplementation with added SR phospholipid as described. A lower lipid content is achieved by shortening the time of dialysis. As before, dialysis is carried out at room temperature.

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For phospholipid vesicles, both negative staining (Fig. 5D) and glutaraldehyde-tannic acid (24) fixation for preparation of thin sections (Fig. 6C) seem to induce formation of multilayers. Freeze-fracture electron microscopy gives the most reliable image of the phospholipid vesicles (Fig. 6D).
protein are solubilized. We have also expanded the washing procedure to insure membranes of uniform function and quality.

The reconstituted sarcoplasmic reticulum vesicles with different phospholipid content are homogeneous preparations consisting mainly of the calcium pump protein (>95%) and phospholipids. The preparations form a narrow band on a sucrose gradient by isopycnic centrifugation. The isopycnic density of the membrane vesicles is decreased with increased lipid content in the expected manner (Table I).

Normal SR is a highly asymmetric membrane as viewed by electron microscopy using either freeze-fracture, negative staining, or thin sections. The asymmetry of the normal membrane has been lost, at least in part, in the reconstitution procedure. This asymmetry is referable to the anisotropic orientation of the calcium pump protein (17).

The lipid to protein ratio of the reconstituted membranes can be varied over a wide range. However, it is in the range of lipid content of the original membrane, both lower and higher lipid content within a factor of two, that is most useful for correlating composition with structure. In this study, we could demonstrate a direct correlation of the number of particles at the hydrophobic face, observed by freeze-fracture electron microscopy, with the protein content of the membrane and the extent of smooth area into which the particles are inserted with the lipid content of the membrane.

We estimated the number of calcium pump protein molecules/μ² in normal sarcoplasmic reticulum and in the reconstituted membrane vesicles of different lipid to protein ratio using Equation 6 (see "Appendix" for derivation). The number of calcium pump protein molecules in the membrane for each of the preparations was calculated to be a factor of two greater than the sum of particles at both fracture faces, observed by freeze-fracture electron microscopy (Table IV). The value calculated for the number of calcium pump protein molecules in the membrane is a minimal value since the derived equation is based on the simplifying model that all of the protein in the membrane resides within the width of the bilayer. However, at least a portion of the calcium pump protein extends out from the surface of the membrane (17, 27), i.e., the width of the membrane, d, in Equation 6, is greater than the width of the bilayer. Therefore, Pₚ, the calculated number of pump molecules/μ² has been underestimated. We conclude that, on the average, several pump protein molecules, at least two and more likely three, are associated per particle which is observed. Further, if some of the particles on the inner and outer fracture faces derive from the same transmembrane pumping structure, then the average number of pump molecules per transmembrane structure would be even greater. A similar conclusion, that there are several pump molecules per structural unit, has been reached by others based on separate considerations (28, 29).

In thin sections, the outer and inner bands of the reconstituted membrane can also be observed to change as the lipid content of the membrane increases (Fig. 4). The reconstituted membrane of high protein content has a continuous broad outer and inner layer instead of the asymmetric appearance of normal SR which has only a broad outer (70 Å) layer. As the protein content of the membrane is decreased, the continuity of the broad outer and inner bands is decreased and only small patches can be visualized on either inner or outer faces of the membrane. In an analogous fashion, the concentration of particles at the surface of the membrane, observed by negative staining, decreases with increasing lipid concentration in the membrane. Thus, the correlation of composition with structure can be observed by each of the three methods of sample preparation for electron microscopic analysis.

The method for preparation of reconstituted vesicles of varying lipid to protein ratios and its use in correlating composition with structure is an illustration of the value of the reconstitution approach. Such reconstituted preparations are currently being used to study the structure and motional characteristics of the protein and phospholipid components of the membrane (30-32).

**APPENDIX**

The number of pump molecules per surface area of the membrane (n/μ²) was calculated using Equation 6. The number of pump molecules/μ² of surface area (Pₚ) can be estimated from the composition of the membrane:

Volume of 1 cm² area of membrane

\[
V(\text{cm}^2) = 1 \text{ cm}^2 \cdot d (\text{Å}) \cdot 10^{-4} \text{ cm/Å}
\]

(1)

Mass of 1 cm² area = \(M(g) = V \cdot \rho\)

(2)

Mass of protein/μ² area = \(M_p(g) = V \cdot \rho \cdot P_p\)

(3)

Number of pump molecules/μ² area

\[
P_{cm} = \frac{M_p \cdot PE}{(\text{nmol/mg of } \text{g}) \cdot (1 \cdot 10^{-5} \cdot \text{mg} \cdot 1 \cdot 10^{-9} \cdot \text{mol/nmol})} \cdot 6.02 \times 10^{23} \text{ molecules/mol}
\]

(4)

\[P_{cm} = 1 \cdot d \cdot 10^{-4} \cdot P_p \cdot PE \cdot 10^{-5} \cdot 6.02 \times 10^{23}\]

(5)

To convert \(P_{cm}\) to \(P_p\), the number of pump molecules/μ², multiply by \(10^{-8} \text{ cm}^2/\mu^2\)

\[P_p = d \cdot P_p \cdot PE \cdot 10^{-9.2}\]

(6)

where \(P_p\) = concentration of pump molecules (n/μ²); \(d\) = thickness of membrane, taken as 65 Å; \(\rho\) = density of membrane (g/cm²) obtained from the isopycnic density (see Table I); \(F_p\) = fraction of mass which is protein (values from Table I and assuming 1.29 μmol of phospholipid = 1 mg of lipid); \(PE\) = phosphenzyme formation (nmol/mg of protein). One pump molecule is assumed to form 1 eq of phosphoenzyme (see Table III).

This derivation is based on the simplifying model that all of the protein resides within the width of the bilayer. Approximately half of the calcium pump protein seems to extend out from the bilayer (17, 27). Thus, \(P_p\) in Table IV is a minimum value since the effective width of the membrane, \(d\), is greater (see Equation 6) than the 65 Å which we have used in our calculations.

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