Lipid Analysis and Freeze-Fracture Studies on Isolated Transverse Tubules and Sarcoplasmic Reticulum Subfractions of Skeletal Muscle*

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Comparative studies on the properties of isolated vesicles from the transverse tubules, longitudinal sarcoplasmic reticulum, and the light and heavy bands of the terminal cisternae are presented in this report. Lipid analysis shows that the longitudinal sarcoplasmic reticulum, and the light and heavy bands of terminal cisternae have a phospholipid content of 0.87, 0.64, and 0.53 μmol/mg of protein, whereas that of the transverse tubule is 1.57. The sarcoplasmic reticulum subfractions have an identical phospholipid composition. The percentage ratio of phosphatidylcholine/phosphatidyl-ethanolamine/phosphatidylinositol/phosphatidylserine/sphingomyelin is 68/17/8/2/4. The phospholipid composition in the transverse tubule is different, having values of 45/26/4/8/16 for the percentage ratio of the above phospholipids. The molar ratios of cholesterol to phospholipid in the various sarcoplasmic reticulum subfractions are identical and have a value of 0.1, whereas the molar ratio for the transverse tubules is 0.4.

Freeze-fracture studies reveal that the isolated transverse tubular vesicles have a disc shape. The concave fracture faces of these vesicles are smooth and have a low density of particles, whereas aggregation of particles forming ridges are often observed in their convex faces. This is in contrast to the sarcoplasmic reticulum which has concave faces with densely packed particles and smooth convex faces. Stereological analysis of freeze-fracture replicas reveals that the sarcoplasmic reticulum contamination in the transverse tubular fraction is approximately 12.5% on a milligram of protein basis.

This work represents a continuation of our studies on the biochemical properties and physiological functions of the microsomal fractions isolated from rabbit skeletal muscle. We have established a method for further fractionation of skeletal muscle microsomes into two fractions (1). One of these fractions contains essentially fragmented vesicles from the longitudinal SR. The other contains mainly vesicles derived from the TC and intact triad junctions. We have further separated the terminal cisternae; T tubule, transverse tubule.

Preparations of T-tubular Vesicles and Sarcoplasmic Reticulum Subfractions

The procedure for fractionation of rabbit sacrospinalis muscle microsomes into longitudinal SR, TC light band, TC heavy band, and T-tubular vesicles was as described previously (1, 2).

Phospholipid Analysis

Total lipid phosphorus in various microsomal fractions was determined by the method of Ames (3). For analysis of the phospholipid composition, the total lipid from the microsomal fraction was first extracted according to the procedure of Folch et al. (4) with some modification. Microsomes were extracted with chloroform/methanol (2:1 v/v) containing 10 μg of butyraldehyde hydrxytoluene/ml. The extracts were dried by a stream of dry nitrogen and redissolved in chloroform/methanol (2:1 v/v) at a concentration of 6 to 12 mg of lipid/ml.

Two-dimensional thin layer chromatography used to resolve the phospholipid composition in the extract was as described by Rouser et al. (5) except for the following modification. The two-solvent system consisted of (i) chloroform/methanol/28% aqueous ammonia (65/85/5, by volume) followed by (ii) chloroform/acetone/methanol/acetic acid/water (100/40/20/20%, by volume). Precoated silica gel chromatographic plates (20 x 20 cm) were purchased from EM Laboratories. Lipids on the plates were detected by iodine vapor. The phospholipid at each spot was extracted by the procedure of Skipski and Barclay (6) and the phosphorus content was determined according to the method of Ames (3). The individual phospholipid was identified by co-chromatography with standards obtained from Supelco, Inc. Furthermore, the presence of phosphatidylethanolamine and phosphatidylserine was confirmed by the ninhydrin-sensitive test (6). No distinction was made in these analyses between phosphatidyl lipids and plasmalogens.
The light and heavy TC fractions were separately pelleted by centrifugation at 77,000 × g for 25 min. The T-tubular vesicles were pelleted by centrifugation at 77,000 × g for 1 h. All pellets were fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.4, at 4°C for 6 h or more. The pellets were then immersed in 30% glycerol at 4°C for 18 h. Freeze-fracture was performed on a Balzers 360 M and replicas were examined in a Phillips 300 electron microscope.

Precaution was taken to ensure that the replica is representative of the whole population of the vesicles in the pellet. Before being frozen in Freon 12, the pellets of 0.6-mm thickness were cut perpendicular to their surface into blocks measuring 0.6 mm. The blocks were then placed into specimen holders with the vertical plane of the original pellets (0.6 × 1 × 1 mm²) facing upwards. Hence, the fracture plane was oriented to cut across vesicles from top to bottom of each pellet. The replica covering the entire fracture plane is representative of all vesicles in the pellet.

For determining the degree of purity of the T-tubular preparation, 15 electron micrographs were taken randomly throughout the surface area of the replica. The procedure for counting T-tubular and SR vesicles in the fraction and the stereological estimation of membrane area are described in detail under "Results." For intact muscle studies, rabbit back muscle was exposed and fixed tissue was sliced into small blocks and immersed in 30% glycerol. Subsequent freeze-fracture procedure was carried through as described above.

Thin Sectioning—The T-tubular pellet was fixed in 2.5% glutaraldehyde in 0.05 M sodium cacodylate, and postfixed with 1% osmium tetroxide in the same buffer. Subsequent procedures for electron microscopy were as described previously (1).

RESULTS

Phospholipid and Cholesterol Analysis—Table I shows the phospholipid and cholesterol contents of the various microsomal fractions. The longitudinal SR, TC light band, and TC heavy band have 0.87, 0.75, and 0.53 µmol of lipid phosphorus/mg of protein, respectively. The phospholipid content of the T-tubular vesicles is 1.57 µmol/mg; this value is much higher than that found in the SR subfractions. The cholesterol and cholesterol ester content in the SR subfractions ranges from 0.05 to 0.08 µmol/mg of protein. The cholesterol content of T tubules is 0.64 µmol/mg; again, this value is much higher than that of the SR subfractions. The molar ratio of cholesterol to phospholipid in the T-tubular fraction is about 4 times higher than that of the SR subfractions.

The composition of phospholipids extracted from the SR subfractions and T-tubular vesicles has been determined by two-dimensional thin layer chromatography. Table II shows that the phospholipid composition in the longitudinal SR and the two TC subfractions are almost identical. On the other hand, the membrane of T-tubular vesicles has a different phospholipid composition compared with that of the SR subfractions. While phosphatidylcholine remains the dominant phospholipid in the T tubule, it contributes only 45% as compared to about 68% of total phospholipid in SR. Phosphatidylethanolamine contributes about 25% of the total phospholipid in T tubules, which is about 1.5 times that found in the SR. Phosphatidylserine and sphingomyelin in the T-tubular fraction are 7.5 and 16.3% of the total phospholipid, respectively. These values are about 4 times higher than those found in the SR. Phosphatidylinositol in the T tubules is 4.4%, which is about one-half of the percentage found in SR. The other unidentified phospholipids in both the SR and T-tubular vesicles are 1.7 and 1.2% of the total phospholipids in each fraction, respectively.

Morphological Studies—Fig. 1 shows the freeze-fracture replica of the triad junctions of intact rabbit skeletal muscle. The TC are characterized by an extended membrane area

### Table I

<table>
<thead>
<tr>
<th></th>
<th>Longitudinal SR</th>
<th>TC and triads</th>
<th>T tubules</th>
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<tbody>
<tr>
<td></td>
<td>Light band</td>
<td>Heavy band</td>
<td>T tubules</td>
</tr>
<tr>
<td>Phosphorus/protein (µmol/mg)</td>
<td>0.87 ± 0.03</td>
<td>0.64 ± 0.01</td>
<td>0.75 ± 0.01</td>
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<tr>
<td>Cholesterol plus cholesterol esters/protein (µmol/mg)</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.01</td>
<td>0.06 ± 0.01</td>
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<td>Molar ratio of cholesterol and esters/phospholipid</td>
<td>0.09</td>
<td>0.10</td>
<td>0.08</td>
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</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th></th>
<th>Longitudinal SR</th>
<th>Longitudinal SR</th>
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<tbody>
<tr>
<td></td>
<td>Light band</td>
<td>Heavy band</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>68.2 ± 3.1</td>
<td>66.7 ± 0.3</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>16.8 ± 3.1</td>
<td>18.1 ± 0.5</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>7.6 ± 0.9</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>2.0 ± 1.0</td>
<td>1.7 ± 0.9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>3.7 ± 1.1</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>Unidentified phospholipids</td>
<td>1.7 ± 0.7</td>
<td>1.7 ± 0.5</td>
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The phospholipid composition of microsomal subfractions are shown in Table II. Values are the percentage of total lipid phosphorus, mean ± S.D., of three chromatographic plates from two microsomal preparations.
Fig. 1 (upper). Freeze-fracture replica of triad junctions in intact muscle. Panels a and b show that the convex fractured face of the T tubules has a high number of intercalated particles, whereas the concave fracture faces (Panels c and d) are relatively smooth. The branching into the longitudinal reticulum and lying on either side of the central narrow T tubule. The concave faces of the TC (Fig. 1, a, c, and d) show a high density of intercalated particles covering the whole surface. On the other hand, the convex faces of the TC (Fig. 1, a, b, and c) are smooth and display a very low density of particles. This distribution of particles on the SR fracture faces is characteristic for skeletal muscle (8, 9). The T tubules are narrow tubes of uniform width and are characterized by having a smooth concave face containing a low density of particles (Fig. 1, c and d) while the convex face displays a higher density of intercalated particles (Fig. 1, a and b). The high degree of curvature of the T tubule
Properties of Transverse Tubules and Sarcoplasmic Reticulum

**Fig. 4 (upper).** Survey field of isolated T-tubular fraction. The presence of a SR vesicle in this replica is indicated by the arrow.

**Fig. 5 (lower left).** Higher magnification freeze-fracture replica of some T-tubular vesicles. Aggregation of particles that resemble ridges are observed on convex faces (arrowheads). Also, indentations are present on the fractured faces of some vesicles, giving the appearance that the ridges have been removed during freeze-fracture (arrows). Ridge on the concave face (double-headed arrow) is less frequently observed.

**Fig. 6 (lower right).** Thin section micrograph of T-tubular vesicles. Cross-sectional view of many of the vesicles shows an elongate form with luminal electron dense matter confined to their endings.

makes it difficult to obtain accurate estimates of average particle densities since only small regions of the fractured surface are discernible.

Figs. 2 and 3 show the freeze-fracture replica of the TC light band and the TC heavy band. Apart from the size difference, the membrane morphology of these two populations of vesicles is similar. Densely packed particles are seen on virtually all concave faces. The convex faces are smooth and contain
very few particles. This asymmetric distribution of particles on the two leaflets of the fractured faces is characteristic of SR both in the intact muscle and after isolation and has been employed as a means of identifying the origin of SR vesicles in the microsomal fraction (9, 10).

The appearance of the isolated T-tubular vesicles revealed both by freeze-fracture (Fig. 4) and by thin sectioning (Fig. 6) is in striking contrast to that of the SR. In our previous paper (2), we concluded from thin section studies that the shape of the isolated T-tubular vesicles is either disc-shaped or banana-shaped. The replica of the T-tubular vesicles shows that when the fracture plane runs along the membrane surface of these vesicles, round-shaped convex and concave fracture faces are observed, whereas when the fracture plane cuts across the vesicles, the majority of the cross-section views of these vesicles have an elongate shape with a very high membrane curvature at the ends. These morphological features can be accounted for if the shape of the T-tubular vesicles is like a disc or a discus. Some of the vesicles with hemispherical convex and concave fracture faces or with round-shaped cross-sectional planes may be derived from swollen T-tubular vesicles. The presence of a small number of concave faces of SR in this fraction is clear such as that indicated by the arrow in Fig. 4.

The intercalated particle distribution of the convex and concave fractured faces of the isolated T-tubular vesicles closely resembles that of the T tubules in intact muscle and differs from that of the SR vesicles. Like those in the intact muscle, the concave faces of the T-tubular vesicles are smooth and have a low density of particles as compared to the densely packed particles on the concave faces of SR. Many of the convex faces of the T-tubular vesicles show the presence of particles, some of which form arrays that resemble ridges. This is in accordance with the presence of particles in the convex face of the 'T' tubule in the intact fiber. On the other hand, these convex faces are distinct from the smooth convex fractured faces of the SR vesicles. Fig. 5 shows the aggregation of particles in some isolated T-tubular vesicles at higher magnification. On the convex fracture face, the aggregation of particles appears to form a ridge that circumscribes the vesicles as a band (indicated by arrowheads). The ridges, though more frequently observed on the convex face, are also found on the concave faces (indicated by double-headed arrow). Sometimes, indentations are observed on the fractured face with the appearance that ridges have been stripped off during freeze-fracture (indicated by arrows).

The sharp membrane curvature of these disc-shaped vesicles is not favored on energetic grounds for a lipid bilayer. For this reason, we have sought morphological indications for the presence of constraining material which holds the shape of the vesicles in a disc-like conformation. The thin section of the T-tubular fraction (Fig. 6) shows electron denser matter confined within the endings of these vesicles. Such matter may be attached to the adjacent membrane and thus bridge between the two sides of the vesicle to constrain them in close apposition. A correspondence may exist between this electron dense matter and the ridges or particle aggregates observed in vesicles in the freeze-fracture replica.

**Purity of the Isolated T Tubules**—The degree of contamination of SR in the T-tubular fraction was determined as follows. Fifteen electron micrographs were taken randomly throughout the area of the replica. The types of vesicles seen on the micrographs were classified into the following categories: A, elongated vesicles; B, vesicles with few particles on concave faces; C, vesicles with densely packed particles on concave faces; D, vesicles showing convex faces; and E, vesicles of indeterminate nature (i.e. vesicles in which the fracture plane or the shadowing were unfavorable for identification). The number of vesicles in each category was counted independently by four investigators. In Table III, A, B, C, D, and E represent the average percentage ± S.D. of the various types of vesicles defined above in the 15 micrographs.

Further analysis was based on the following considerations. We conclude that A and B vesicles are of T-tubular origin; type C vesicles are from the SR. However, the origin of some of the type D vesicles, particularly those with low density of particles on their convex faces, cannot be determined. Hence, in calculating the number of SR vesicles showing convex faces, we assume that there is an equal ratio of SR to T-tubular vesicles in convex faces as in concave faces. Since the number of vesicles showing convex faces is equal to the number of vesicles showing concave faces (Table III), the total percentage of SR vesicles is 2C. Type E vesicles have been excluded from the calculation. Hence, SR contamination = (2C/(A + B + C + D)) × 100%. From data in Table III, we calculated that the contamination was 7.92%.

A more useful representation of SR contamination in the T-tubular fraction is an expression of the contamination on a protein to protein ratio. In order to obtain this value, we have converted the data to percentage of membrane volume occupied by the SR in the T-tubular fraction. Assuming that the membrane thickness of SR and T tubule are identical, the membrane volumes of the two types of vesicles are proportional to their membrane surface area. The ratio of membrane surface area per vesicle can be estimated by stereological techniques (11). A grid of lines was placed randomly on the top of electron micrographs of the T-tubular replica. The number of intersections of the lines of the grid with types A, B, and C vesicles were separately recorded. Then, the number of vesicles in each category was also counted. Great care was taken to count all vesicles of all sizes which could be identified in order to avoid bias in the sizing. The average number of intersections per vesicle in each category from six randomly chosen micrographs is shown in Table III. The average surface area ratio of SR vesicle to T-tubular vesicle was determined by the following equation:

$$c/((Aa + 2Bb)/(A + 2B))$$

where a, b, and c are the average intersections per vesicle in each category, respectively, and A and B are the number of vesicles in the categories as defined before. From the above equation, and the data of Table III, the average surface area ratio per vesicle of SR compared with T tubule is calculated as 1.02. Hence, the contamination of SR on a membrane volume basis is 8.08%.

The conversion from membrane volume contamination to milligrams of protein contamination is effected through the information of Table I. This permits a determination of the lipid to protein ratio of T tubules and the SR. The data of the TC light band is chosen for the calculation, since the contamination was 7.92%.

**TABLE III**

Vesicle counts and stereological analysis of the T-tubular fraction

<table>
<thead>
<tr>
<th>Type and percentage of vesicles in the population*</th>
<th>Number of line intersections per vesicle*</th>
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<tr>
<td></td>
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<tr>
<td>A</td>
<td>22.0 ± 2.5</td>
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<tr>
<td>B</td>
<td>27.4 ± 3.9</td>
</tr>
<tr>
<td>C</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>D</td>
<td>30.6 ± 7.4</td>
</tr>
<tr>
<td>E</td>
<td>16.7 ± 3.5</td>
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</table>

* The average percentage ± S.D. determined in 15 micrographs.

* Values ± S.D. determined from 6 micrographs.
subfractions, due to the proximity of the TC light band and T-tubular band in the sucrose gradient. We have not estimated the neutral lipids other than cholesterol and therefore have made the small correction necessary by reference to values in literature. Total neutral lipid in SR is taken as 23.5% (w/w) of the total lipid content; this is the average of the three values of 22, 28.5, and 20% reported by Fiehn et al. (12), Mahila and Zachar (13), and Fiehn and Hasselbach (14), respectively. While there are no reported data about the neutral lipid content in T tubules, we have assumed that its composition is similar to that found in skeletal muscle sarcolemma by others; the average value of the cholesterol content is about 56% that of the total neutral lipid (13, 14). Hence, we have calculated that the total neutral lipid per mg of protein in the TC light band and T-tubular band is 0.16 and 0.45 mg, respectively.

In converting the ratio of protein to lipid on a weight basis (w/w) to a volume basis (v/v), we again used the data in literature that the density of globular protein is 1.35 g/ml (15) and the density of phospholipid in a bilayer membrane is 1 g/ml (16). Furthermore, we assumed that the density of neutral lipids is the same as that of phospholipids. From these data, we have calculated that the SR contamination on a milligram of protein basis is 12.5%.

**DISCUSSION**

The phospholipid content varies from 0.57 to 0.87 μmol/mg of protein within the subfractions of the SR network. A similar finding has been reported by Meissner (17) for the light and heavy SR subfractions. The lower phospholipid to protein ratio observed in the TC vesicles may be due to the fact that calsequestrin and high affinity calcium-binding protein are confined to this region of the SR network (2, 17) and the remaining major protein, the Ca\(^{2+}\) pump protein, may therefore be uniformly distributed in the longitudinal SR and TC. The T-tubular vesicles have a phospholipid content of 1.57 μmol/mg of protein. This value is about 2- to 3-fold those found in the SR subfractions. Also, this value is about 3 to 4 times the phospholipid content reported in isolated sarcolemma from rat (12) and rabbit (18) skeletal muscle.

The phospholipid composition in these SR subfractions are in agreement with the determinations by Meissner and Fleischer (19) and Owens et al. (20). The phospholipid composition of the T tubules is distinct from that of the SR. Its relatively high content of sphingomyelin and phosphatidylserine is characteristic of the phospholipid composition of plasma membrane. Our values for the T-tubular phospholipid composition are similar to those of plasma membranes of rabbit skeletal muscle (13) and rat skeletal muscle (12). The molar ratio of cholesterol to phospholipid is nearly identical in the longitudinal SR, TC light band, and TC heavy band. The molar ratio of cholesterol to phospholipid in the T tubules is 0.4, about 4-fold the value found in SR. Coleman and Finean (21) reported that a high molar ratio of cholesterol to phospholipid is a property common to a variety of plasma membrane preparations and is high compared with the ratio found in other membrane systems of the same tissue. The molar ratio of cholesterol to phospholipid in sarcolemma of rabbit skeletal muscle has been estimated to be about 0.6 (18). Thus, the lipid composition of T tubules resembles that of plasma membrane in many respects. The phospholipid composition and cholesterol contents are characteristic of plasma membrane. On the other hand, the high phospholipid to protein ratio in the T tubules contrasts with that found in plasma membrane preparations.

The homogeneity of the lipid composition throughout the SR network fits well with the fact of membrane continuity between longitudinal SR and the TC and the concept of lipid diffusibility in a fluid membrane. On the other hand, though the T tubules are in close apposition with the TC and are physically connected with them through "feet-like" structures (22), the T-tubular membrane has a lipid composition that is distinct from that of the SR. The similarity of lipid composition of T tubules and plasma membrane is in accord with the continuity of these structures and lipid fluidity.

Freeze-fracture electron microscopy reveals that the surface structure of the T-tubular membrane can be distinguished from that of the SR. The isolated T-tubular vesicles have a disc or a discus shape; hence, the cross-section of these vesicles shows an elongate form. Comparison of the isolated T tubules with those in the intact muscle shows a considerable retention of structure by the former. The general pattern of interrelated particles is similar although there may be a greater degree of aggregation of particles in isolated T tubules. It is not immediately clear how the flattened tubular shape of intact T tubules is converted to a disc shape in the isolated preparation. The migration of lipid or protein appears to be necessary. It is possible that the same constraining structure which is responsible for retaining the tubular shape of the intact organelle is also responsible for preventing the formation of spherical vesicles in the isolated preparation. In this view, the isolated vesicles undergo lipid or protein migration but within the confines of a restraining structure such as a protein which may bridge between the two sides of the vesicle. Electron dense matter within the lumen of junctional T tubules of rabbit skeletal muscle has been recorded (23). We emphasize that by employing a mild protocol in the isolation of T tubules, we have been able to retain in good measure the structure exhibited by T tubules in the intact muscle.

Since the T tubules are isolated by fractionation of the triad junctions, the major source of contamination in the T-tubular fraction is SR vesicles. One of the complications in estimating the purity of the T-tubular preparation is the absence of a suitable enzyme or protein marker for the SR. However, since the surface membrane structure of the T tubules as revealed by freeze-fracture is distinct from that of the SR, we have employed their morphological difference as a means to determine the degree of contamination. We found that the SR contamination on a protein basis is 12.5%.

An estimation of the degree of purity of our T tubule preparation is likely to prove critical in many areas of biochemical study. This is particularly true in the area of Ca\(^{2+}\) permeability and active transport. One of the major physiological problems of muscle contraction concerns the role of calcium influx during excitation. The employment of Ca\(^{2+}\)ATPase as a marker for SR is clearly inappropriate since we do not know the morphological distribution of this enzyme. Other SR proteins such as calsequestrin or the M55 protein are not evenly distributed within the SR. The employment of freeze-fracture particle distribution, therefore, remains the currently practicable marker for SR contamination.

**Acknowledgments**—We wish to thank Dr. D. S. Smith for use of electron microscope facilities and for valuable help in interpretation of the micrographs. We thank the Papanicolaou Cancer Research Institute for use of their freeze-fracture facilities. We are grateful to Dr. H. L. Lo for discussion of the phospholipid analysis and to Dr. S. L. Haïa and Ms. S. Warren for discussion and generous supply of reagents for the cholesterol assay.

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