Isolation of Transverse Tubules by Fractionation of Triad Junctions of Skeletal Muscle*

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Using the fractionation procedure of rabbit skeletal muscle described previously (Caswell, A. H., Lau, Y. H., and Brunschwig, J.-P. (1976) Arch. Biochem. Biophys. 176, 417-430), a microsomal preparation can be further separated into two fractions in a sucrose density gradient. The "light" microsomal fraction contains mainly vesicles of longitudinal sarcoplasmic reticulum. The "heavy" microsomal fraction contains mainly terminal cisternae vesicles and intact triad junctions. We have employed [3H]ouabain directly injected into the muscle or intravenously injected into the muscle in order to mark and track transverse tubules during muscle fractionation. The transverse tubules can be detached from the terminal cisterna by a French press treatment. The detached T-tubular vesicles with the [3H]ouabain trapped within, migrate as a distinct band separate from the terminal cisternae vesicles on both a sucrose gradient and a sucrose gradient with 0.6 M KCl. Electron microscopy reveals that the isolated transverse tubules are mainly elongated vesicles with a length of 2000 Å and a width of 200 Å. Their morphology closely resembles that of transverse tubules from triad junctions. (NaK)-ATPase and CaATPase are demonstrated in these vesicles. Sodium dodecyl sulfate gel electrophoresis shows that the protein composition of the transverse tubules is distinct from that of the terminal cisternae. The terminal cisternae vesicles fractionate into two populations of vesicles after the French press treatment. Vesicles from the lighter region of the sucrose gradient appear empty in electron micrographs and the \( M_r = 55,000 \) protein is present primarily in this fraction. Terminal cisternae vesicles from the denser region of the gradient have extensive electron dense matter within and the \( M_r = 45,000 \) and 42,000 calcium-binding proteins are present primarily in this fraction.

This paper presents the second portion of our work on the study of organelles and membrane systems that are directly involved in excitation-contraction coupling of skeletal muscle. The least understood aspect in the contraction cycle is the mechanism that leads to the release of \( \text{Ca}^{2+} \) from the sarcoplasmic reticulum. There is much evidence that pinpoints the role of the transverse tubule in "triggering" \( \text{Ca}^{2+} \) release from the SR (1-3), but the details are largely unknown.

We have reported previously a procedure for obtaining an enriched preparation of intact triad junctions from rabbit skeletal muscle (4, 5). [3H]ouabain, used as an external membrane marker, proved to be a useful tracer of the fragmented T-tubules in the purification process. A brief summary of these findings is as follows. A microsomal preparation from skeletal muscle was separated into two fractions on a sucrose density gradient. The "light" vesicles with isopycnic point at 31% sucrose contain fragments of longitudinal reticulum. The "heavy" vesicles with isopycnic point at 40% sucrose are primarily of terminal cisternae (TC) origin. Similar findings have been reported by Meissner (6). Electron micrographs revealed that in our preparation the heavy vesicle fraction contained many intact triad junctions. When [3H]ouabain was either injected directly into the muscle or intravenously infused before the isolation procedure, only the heavy vesicle fraction was specifically labeled. Then through a series of experiments, we concluded that the [3H]ouabain-binding vesicles were mechanically attached to TC. We presented evidence that these [3H]ouabain-binding vesicles were T-tubules. The [3H]ouabain was trapped within the vesicular T-tubules of the triad junctions during homogenization of the muscle and could therefore be employed as a tracking technique for identifying T-tubules following muscle homogenization.

The purpose of this paper is: (a) to describe modifications of the isolation and fractionation procedures which give an enriched fraction of [3H]ouabain-binding vesicles; (b) to provide further supporting evidence that [3H]ouabain vesicles are T-tubules through morphological study of this fraction; (c) to present data concerning the enzymatic properties and the protein components of the isolated T-tubules and TC vesicles.

MATERIALS AND METHODS

Labeling and Fractionation of Muscle

[3H]Ouabain labeling of rabbit skeletal muscle and microsomal preparation were as described in the previous communication (6). For experiments of larger scale microsomal preparations, a Beckman zonal rotor (TI-14) was used. A 450-ml continuous gradient of 20 to 45% sucrose was formed by mixing 450 ml of 90% w/w sucrose with 0.01% NaNO₃ into a 500-ml mixing chamber which initially contained 20% w/w sucrose with 0.01% NaNO₃. In this case up to 500 mg of protein in 50 ml was loaded onto the gradient. This was centrifuged overnight at 30,000 \( \times g \) (average). Fractions containing 15 ml were drawn off the gradient by displacement with 65% sucrose.

* The abbreviations used are: SR, sarcoplasmic reticulum; TC, terminal cisternae; SDS, sodium dodecyl sulfate; T-tubule, transverse tubule.
The microsomes separated into two fractions in the sucrose gradient. The light microsomal fraction was located at 30 to 33% sucrose. The heavy microsomal fraction was located at 39 to 41% sucrose. This heavy microsomal fraction was taken from the gradient and diluted with 4 volumes of 0.5 M sucrose. This suspension was pelleted by centrifugation at 70,000 \( \times g \) for 1 h. The pellet was resuspended in 0.25 M sucrose and diluted to a protein concentration of 3 to 4 mg/ml. This homogenate was then passed through an Amicon French pressure cell. The proteins employed were isolated by sedimentation at 70,000 \( \times g \) for 30 min and resuspension in 1 mM Tris/Cl (pH 7.2). EGTA. The termination of the reaction and the determination of liberated phosphate were as described in the NaK-ATPase assay. The presence of 10e-4 M ouabain. The nonspecific ATPase is defined as the basal activity in the presence of 1 mM EGTA and 10e-4 M ouabain. The reaction was stopped by the addition of 0.3 ml of 2 N HCl and the assay for deaminated 3H-tyramine (New England Nuclear). An equal amount of boiled protein sample was similarly treated as a control. The reaction was diluted with cold distilled water to 0.18 M NaI concentration. The membranous fraction was washed two times by centrifugation at 70,000 \( \times g \) over night.

In experiments where KCi treatment was used, 0.6 M KCi was employed instead of the 0.25 M sucrose in the dilution and resuspension procedures described above. Also, the sucrose gradient contained 0.6 M KCi.

Electron Microscopy

Pooled samples from the density gradients were diluted with 4 volumes of 0.25 M sucrose. The heavy microsomal vesicles and the TC vesicles obtained after the French press treatment and sucrose gradient centrifugation were separately pelleted by centrifugation at 77,000 \( \times g \) for 20 min. The ouabain-binding vesicles were pelleted by centrifugation at 77,000 \( \times g \) for 1 h. All pellets were then processed for microscopy as described previously (5). Pellets for thin sectioning were always less than 0.5 mm in depth and were cut perpendicular to the surface of the pellet in blocks measuring about 0.5 \( \times \) 1 \( \times \) 1 mm\(^3\). Sections were cut from the block such that the sections encompassed the whole depth of the original pellet. Also sections were obtained from different areas of the pellet so as to give representative fields throughout each pellet. The fields of the various sections observed from each pellet and from the same band in different preparations showed a high degree of homogeneity.

Enzymatic Assays

ATPase - The basic medium for ATPase (Medium A) consisted of 100 mM NaCl, 10 mM Tris, 10 mM MgCl\(_2\), 1 mM ATP, and 10 mM histidine at pH 7.4 and 37\(^\circ\). Ten to fifty micrograms of protein were added to 1 ml of the medium and incubated for 10 to 15 min.

When assaying for (NaK) and nonspecific ATPase, Medium A was supplemented with 1 mM EGTA. The specific activity was determined from the difference of the total activities in the absence and presence of 10\(^{-4}\) M ouabain. The nonspecific ATPase is defined as the basal activity in the presence of 1 mM EGTA and 10\(^{-4}\) M ouabain. The reaction was stopped by the addition of 0.3 ml of H\(_2\)SO\(_4\) (6 N) with 20% HCl. The liberated inorganic phosphate was measured by the method of Eibl and Lands (8). In certain (NaK)-ATPase assays, the protein samples were subjected to treatment with 2 M NaI according to the procedure of Nakao et al. (9) and Hegyvary et al. (10), with slight modification, before assaying for (NaK)-ATPase. The NaI solution was added to 1 ml of the medium and incubated for 10 to 15 min. The NaI concentration was adjusted to 0.5 M by addition of 0.2 ml of 2 N HCl and the assay for deaminated 3H-tyramine (New England Nuclear). An equal amount of boiled protein sample was similarly treated as a control. The reaction was diluted with cold distilled water to 0.18 M NaI concentration. The membranous fraction was washed two times by centrifugation at 70,000 \( \times g \) for 30 min and resuspension in 1 mM Tris/Cl (pH 7.2).

The CaATPase activity was the difference of the activity in Medium A plus 0.5 mM CaCl\(_2\) compared with Medium A plus 1 mM Tris EGTA. The termination of the reaction and the determination of liberated phosphate were as described in the (NaK)-ATPase assay.

Monoamine Oxidase - Monoamine oxidase activity was determined according to the method of Wurtman and Axelrod (11) with some modifications. One hundred to two hundred micromolars of duplicate protein samples were each incubated at 37\(^\circ\) for 20 min in 0.25 ml of 0.5 M phosphate buffer, pH 7.4, in the presence of 2.5 \( \times \) 10\(^{-5}\) M [\(^{3}H\)]tyramine (New England Nuclear). An equal amount of boiled protein sample was similarly treated as a control. The reaction was stopped by addition of 0.2 ml of 2 N HCl and the assay for deaminated material was as described (12).

Succinate-tetrazolium Reductase - Succinate-tetrazolium reductase activity was determined on 50 to 100 \( \mu \)g of triplicate protein samples by the procedure of Pennington (12). An equal amount of boiled protein sample was identically treated to serve as control.

5'-Nucleotidase - A series of triplicate samples containing 100 to 300 \( \mu \)g of protein were assayed for 5'-nucleotidase by the method of Atronson and Toustek (13).

Inorganic phosphate released was estimated by the method of Lowry and Lopez (14). The preparation was used in all enzymatic assays such that the enzymatic activities were directly proportional to the protein concentration, with the exception of succinate reductase assay where no activity was detectable.

Gel Electrophoresis - The protein components and their molecular weights were evaluated by sodium dodecyl sulfate gel electrophoresis according to the method of Weber and Osborn (15), using mercaptoethanol as sulfur bridge reducing agent. The 10% acrylamide gel was used in this experiment. Protein (0.02 to 0.07 mg) was loaded on each gel. Molecular weight standards were run simultaneously with the samples. The standards were bovine serum albumin, egg subu- men, and \( \gamma \)-globulin, obtained from Sigma Co., and phosphorylase A obtained from Worthington Biochemical Corp. Molecular weights were estimated from a graph plotting mobilities against the loga- rithm of the molecular weight.

RESULTS

Isolation of Ouabain-binding Vesicles

We reported previously (5) that the microsomes from skeletal muscle could be separated into light and heavy microsomal fractions on a sucrose gradient. Experimental data suggested that ouabain-binding vesicles present in the heavy microsomal fraction were T-tubules attached to TC as intact triad junctions. These ouabain-binding vesicles could be separated from the bulk of TC vesicles by a French press with chamber pressure at 20,000 p.s.i. and subsequent sucrose density gradient centrifugation. However, we found that this procedure caused considerable fragmentation of the terminal cisterna and some of the fragments migrated to a similar region in the sucrose gradient as the ouabain-binding vesicles. We considered that a French press with less pressure might be effective in detaching the ouabain-binding vesicles from the TC but would avoid the breakup of the TC vesicles and consequent contamination of the ouabain-binding vesicles. No significant detachment of the \(^{3}H\)ouabain-binding activity from the triad vesicles were observed if the applied pressure in the French press was 1000 p.s.i. or lower. When the pressure was 2500 p.s.i. only about 50% of the \(^{3}H\)ouabain-binding vesicles were detached from the TC vesicles. Hence, the detachment of the \(^{3}H\)ouabain-binding activity is confined to a single sharp peak in the region of 22 to 26% sucrose.
the heavy microsomal vesicles after French press treatment at point of 40% sucrose (Band A2) contains vesicles of TC and distinctly separated from the other band of the gradient. The TC vesicles form a rather diffuse band at 30 to 40% sucrose of the gradient. The heavy microsomal fraction with isopycnic KC1 is present in the sucrose gradient and in the suspension that of Tube B is seen between the ouabain-binding vesicles throughout the purification of the ouabain-binding vesicles (Band B1) corresponds to the ouabain-binding vesicles. This band is intact triad junctions. Tube B shows the migration pattern of the vesicles and their degree of separation in the density gradient. This preparation can be separated into two bands of density gradient fractionation of a crude microsomal preparation. This preparation can be separated into two populations of vesicles in the gradient. The heavy microsomal fraction from the sucrose gradient was diluted with 0.6 M KC1 and pelleted by centrifugation. The pellet was resuspended in 0.6 M KC1, subjected to the French press and loaded back on a sucrose gradient that contained 0.6 M KC1. The protein profile and the [3H]ouabain-binding activity after such treatment are shown in Fig. 1B. There is substantial separation between the ouabain-binding vesicles and the bulk of the TC vesicles. The ouabain-binding vesicles now migrate to 16% sucrose, whereas the major protein peak of TC vesicles is at 29%. Since the specific gravity of 0.6 M KC1 is equivalent to about 6.5% sucrose, the isopycnic point of the ouabain-binding vesicles is therefore equivalent in density to 22.5% sucrose. Hence, the ouabain-binding vesicles have a slightly lower isopycnic point in a sucrose gradient which contains 0.6 M KC1 as compared with a pure sucrose gradient. The isopycnic point of the major TC vesicles is equivalent in density to 35.5% sucrose which is greater by 3.5% sucrose than the isopycnic point of these vesicles in a pure sucrose gradient. It appears from this figure that better separation of the ouabain-binding vesicles from the TC vesicles has been achieved by the inclusion of KC1 in the isolation medium and the density gradient.

Fig. 2 gives a visual indication of the migration patterns of the vesicles and their degree of separation in the density gradient in our isolation procedures. Tube A shows a sucrose density gradient fractionation of a crude microsomal preparation. This preparation can be separated into two bands of vesicles. The light microsomal fraction with isopycnic point of 32% sucrose (Band A1) contains mainly vesicles of longitudinal SR origin. The heavy microsomal fraction with isopycnic point of 40% sucrose (Band A2) contains vesicles of TC and intact triad junctions. Tube B shows the migration pattern of the heavy microsomal vesicles after French press treatment at a pressure of 5000 p.s.i. The upper band on the gradient (Band B1) corresponds to the ouabain-binding vesicles. This band is distinctly separated from the other band of the gradient. The TC vesicles form a rather diffuse band at 30 to 40% sucrose (Band B2). Tube C is a similar preparation except that 0.6 M KC1 is present in the sucrose gradient and in the suspension medium for French press treatment. A larger separation than that of Tube B is seen between the ouabain-binding vesicles (Band C1) and the TC vesicles (Band C2) in this preparation.

The protein yield and [3H]ouabain-binding activities throughout the purification of the ouabain-binding vesicles are shown in Table I. This shows that the [3H]ouabain activity of the heavy microsomal vesicles is approximately 2-fold higher than the crude microsomal preparation. A further 6-fold increase of the [3H]ouabain activity per mg of protein is found in the isolated ouabain-binding vesicles. If the fractionation procedure is carried out in high KC1 medium, the [3H]ouabain activity per mg of protein of these isolated ouabain-binding vesicles increased by about 7.4-fold over the heavy microsomal fraction. The [3H]ouabain activity of the isolated heavy microsomal vesicles remains fairly constant when subjected to washing and repelleting. This indicates that the ouabain activity does not deteriorate due to leakiness of the vesicles during isolation.

The final protein yield of the ouabain-binding vesicles is about 1% of the crude microsomal preparation. About 3 to 4 mg of protein is obtained in our preparation from 80 g of rabbit skeletal muscle. The yield of [3H]ouabain activity is approximately 13% of that of the crude microsomal preparation.
Morphological Studies of Bands from Gradients

Fig. 3 shows an electron micrograph of the heavy band obtained by fractionating microsomes in a sucrose density gradient. The band contains many vesicles with electron dense matter within which may be identified as TC vesicles. Some of these vesicles are attached as triads with elongated vesicles apposed on both sides by the TC vesicles (indicated by arrows). The elongated vesicles may be identified by reference to the morphology of intact muscle as being T-tubules. Some vesicles are also present which lack distinct properties which would permit identification. The T-tubular vesicles show some variety of morphology. Most are characterized by a long narrow shape. Some vesicles are more expanded, but exhibit pinched narrow ends. Visible in the field is also a T-tubule which has expanded to such an extent that it may only be identified as being T-tubular by the apposing terminal cisternae vesicles (indicated by double-headed arrows).

This dense band of heavy microsomes may be fractionated into three bands after French press homogenization. Their morphology is shown in Figs. 4 to 6. The distinct band of vesicles with isopicyn point between 22 and 25% sucrose which contains the ouabain-binding activity is shown in Fig. 4. A highly characteristic feature of this band is the presence of many elongated vesicles. These have a length of approximately 2000 to 2500 Å and a width of 180 to 200 Å. This pattern is distinct for the T-tubular vesicles observed in intact triad junctions in Fig. 3. The band also contains curved elongated vesicles which have the appearance of two concentric circles (indicated by long arrows) and many vesicles which are circular, but lack the definition of distinct membranes (indicated by short arrows). We have sought to analyze the appearance of these sections to determine the three-dimensional shape of the vesicles. Two alternative morphologies can explain the appearance of these sections. The vesicles may be mainly elongated sausage or banana-shaped vesicles or they may be flattened disc-shaped vesicles. In either case many sections will appear as elongated tubes. However, in the third plane of sectioning banana-shaped vesicles would appear as circles of diameter about 200 Å while disc-shaped vesicles would appear as circles of diameter about 2000 Å. The concentric circles can occur from sausage-shaped vesicles by bending of the vesicles until the ends make contact and fuse to form donut-shaped vesicles. Alternatively the concentric circles may arise from sections of thin discs, if the discs have become dimpled into a saucer shape. The indeterminate grey circles may be grazing sections of disc-shaped vesicles. The morphology of T-tubules in rabbit skeletal muscle is that of a flattened tube with width about three times the height. A flattened tubular morphology of the T-tubule is also reported in guinea pig skeletal muscle (16). Either flattened discs or banana-shaped vesicles may arise from T-tubules after homogenization if some migration of lipid in the bilayer has occurred. However, we conclude from this micrograph that a specific constraint is imposed within the membrane to prevent complete alteration of the morphology to a sphere.

Fig. 5 shows the fraction of vesicles taken between 30 to 33% sucrose. This fraction has low [13H]ouabain activity and can be seen to consist mainly of intact spherical vesicles. A few elongated vesicles are also present, but these represent a small minority in the total population.

Fig. 6 shows vesicles obtained between 39 to 41% sucrose. Electron dense material is localized within one side of the great majority of these vesicles. A comparison of their morphology with that of the heavy microsomal vesicles in Fig. 3 strongly suggests that they are derived from the portion of the TC membrane which is in apposition to the T-tubules. No elongated tubules are seen in this fraction.

We wish to emphasize that when the three bands of Figs. 4 to 6 are compared with their parent vesicles before French press treatment, all the morphologies present in Fig. 3 are found in Figs. 4 to 6.

The morphologies of the bands of vesicles derived from the microsomes after French press and centrifugation in a sucrose gradient that contains 0.6 M KCl is shown in the next three micrographs. The ouabain-binding vesicles taken from 15 to 17% sucrose with 0.6 M KCl are shown in Fig. 7. This fraction contains numerous small vesicles, only a few of which are elongated and resemble the T-tubules of the heavy microsomal fraction. It is possible that the high concentration of KCl in the sucrose gradient and in the medium during the French press is responsible for this structural alteration. This may cause a weakening of the membrane structure such that the elongated vesicles disintegrate in the French press and re-form into small spherical vesicles. The loss of the elongated configuration makes morphological identification difficult. However, these vesicles still maintain their [13H]ouabain content indicating that they are from the same source as the elongated [13H]ouabain-binding vesicles of the pure sucrose gradient.

Fig. 8 shows the vesicles from 28 to 30% sucrose with 0.6 M KCl. The morphology of these vesicles is similar to vesicles of the 31 to 33% band from the pure sucrose gradient. Occasionally a few elongated vesicles are seen.

The vesicles from 33 to 35% sucrose with 0.6 M KCl are shown in Fig. 9. These vesicles appear to be intact and their size is similar to that of the vesicles from 39 to 41% of the pure sucrose gradient. However, the quantity of electron dense matter within these vesicles appears to be less and more diffuse. Again, we consider that the 0.6 M KCl treatment is responsible for the loss of the localized electron dense matter that is characteristic of the TC vesicles.

SDS-Gel Electrophoresis

Fig. 10 shows two sets of gels. The first set (Gels A to C) are gels showing protein components of vesicles from the pure sucrose gradient. The second set (Gels D to F) are protein components of vesicles from the sucrose gradient with 0.6 M KCl. These fractions of vesicles taken for SDS-gel electrophoresis are equivalent to those taken for electron microscopic study shown in Figs. 4 to 9. Gel A shows the protein components of ouabain-binding vesicles while Gels B and C show the protein components of vesicles from 31 to 33% and 39 to 41% sucrose, respectively. MacLennan et al. (17, 18) have shown the existence of a Ca2+ pump protein at Mr = 102,000, a high affinity Ca2+-binding protein at Mr = 55,000 and two calsequestrin isozymes at Mr = 46,500 and 44,000. In our gels a dense protein band at Mr = 100,000 is seen in all three fractions from the gradient which corresponds to the calcium pump protein. Two closely spaced and heavily stained protein bands which correspond to calsequestrins are primarily seen in Gel C of fragmented TC vesicles from 39 to 41% sucrose. These two protein bands are either not distinct or absent from Gels A and B. A band equivalent to the high affinity Ca2+-binding protein (Mr = 55,000) is present mainly in Gel B. The ouabain-binding vesicles (Gel A) show clearly the presence of a protein band in similar position to the Ca2+ pump protein. However, many of the other protein bands do not correspond to proteins from the TC vesicles. For example, the three high molecular weight protein bands (i e Mr = 160,000, 140,000,
FIG. 3 (upper). Survey field of the "heavy" microsomal fraction (39 to 41% sucrose). Some triad junctions are indicated by arrows. Expanded T-tubule is indicated by double-head arrow. The bar line in this and subsequent electron micrographs represents 250 nm.

FIG. 4 (lower). Ouabain-binding vesicles (at 22 to 25% sucrose) isolated from the "heavy" microsomal fraction after French press treatment and sucrose gradient centrifugation. Long arrows indicate vesicles with the appearance of two concentric circles. Short arrows indicate vesicles that lack the definition of distinct membranes.
Fig. 5 (upper). Fragmented TC vesicles (at 31 to 33% sucrose) isolated from the "heavy" microsomal fraction after the French press treatment and sucrose gradient centrifugation.

Fig. 6 (lower). Fragmented TC vesicles (at 39 to 41% sucrose) isolated from the "heavy" microsomal fraction after the French press treatment and sucrose gradient centrifugation.
Isolation of Transverse Tubules

Fig. 7 (upper). Ouabain-binding vesicles (at 15 to 17% sucrose) isolated from the "heavy" microsomal fraction after KCl treatment, French press, and centrifugation in sucrose gradient with 0.6 M KCl.

Fig. 8 (lower left). Fragmented TC vesicles (at 28 to 30% sucrose) isolated from the "heavy" microsomal fraction after KCl treatment, French press, and centrifugation in sucrose gradient with 0.6 M KCl.

Fig. 9 (lower right). Fragmented TC vesicles (at 33 to 35% sucrose) isolated from the "heavy" microsomal fraction after KCl treatment, French press, and centrifugation in sucrose gradient with 0.6 M KCl.
isolation of transverse tubules

and 125,000) and a heavily stained band with apparent $M_r = 68,000$ of the ouabain-binding vesicles are clearly absent from the TC vesicles (Gels B and C). Bands of $M_r = 31,000$ and 24,000 proteins are present in both the ouabain-binding vesicles and TC vesicles. It is possible that these are junctional proteins between the TC vesicles and the T-tubule vesicles or they may be different proteins of similar molecular weights which are not adequately resolved by single dimensional electrophoresis.

Gels D, E, and F show the protein components from the ouabain-binding vesicles and the TC vesicles from 28 to 30% and 31 to 33% of the sucrose gradient in the presence of 0.6 M KCl. The major protein bands of the TC vesicles (Gels E and F) are similar to those described from the pure sucrose gradient (Gels B and C) except for the absence of the $M_r = 79,000$ protein. The ouabain-binding vesicles show some differences from those of the pure sucrose gradient. Apart from the absence of the $M_r = 79,000$ and 68,000 protein bands, the migration pattern of some of the minor bands are different. The loss of these protein components from the ouabain-binding vesicles and the TC vesicles may be due to the solubilization of these proteins by 0.6 M KCl treatment. However, the 100,000-dalton protein as well as the higher molecular weight protein components of these ouabain-binding vesicles are comparable to those from the pure sucrose gradient.

**Enzyme Activities of Fractions from Gradients**

Ouabain-sensitive (NaK)-ATPase and CaATPase from the various fractions of vesicles are presented in Table II. As indicated in the table, the vesicles from the pure sucrose gradient have been subjected to treatment with 2 M NaI in order to expose the latent (NaK)-ATPase. The NaI treatment was found to be unnecessary for detecting ouabain-sensitive (NaK)-ATPase in the preparation with 0.6 M KCl. Table II shows that the ouabain-binding vesicles have a (NaK)-ATPase of 0.07 to 0.1 $\mu$mol of P$_i$/mg/min, which is about 10-fold higher than that found in the TC vesicles. The (NaK)-ATPase from skeletal muscle sarcolemma has been reported in the range of 0.25 to 0.3 $\mu$mol of P$_i$/mg/min (19, 20); CaATPase is also observed in the ouabain-binding vesicles with an activity of about 1.0 $\mu$mol of P$_i$/mg/min. The TC vesicles in both fractions have a CaATPase of 2.0 to 2.4 $\mu$mol of P$_i$/mg/min, which is similar to the reported activity in SR (6, 21). The nonspecific ATPase of the ouabain-binding vesicles is about 4-fold higher than that of the TC vesicles.

Marker enzyme activities for other organelles in the isolated fraction of ouabain-binding vesicles have been determined. Monoamine oxidase as marker for outer membrane of mitochondria has an activity of 0.005 ± 0.001 (S.D.) nmol/min/mg in this fraction. This value is insignificant when compared with an activity of 35 nmol/mg/min in isolated mitochondria (22). Succinate dehydrogenase as marker for inner mitochondrial membrane is not detectable in this fraction. The activity

**Table II**

| Enzyme Activities of Ouabain-Binding Vesicles and Terminal Cisternae Vesicles ($\mu$mol P$_i$/mg protein/min, average values from 3 to 4 experiments ± S.D.) |
|------------------|-----------------|-----------------|
| **Preparation from Sucrose Gradient** | **(NaK)-ATPase** | **CaATPase** | **Nonspecific ATPase** |
| Ouabain-binding vesicles | 0.103 ± 0.013 | 0.922 ± 0.079 | 0.431 ± 0.009 |
| TC vesicles (light band) | 0.012 ± 0.004 | 2.05 ± 0.03 | 0.110 ± 0.006 |
| TC vesicles (dense band) | 0.008 ± 0.005 | 2.09 ± 0.00 | 0.046 ± 0.004 |
| **Preparation from Sucrose Gradient with 0.6 M KCl** | | | |
| Ouabain-binding vesicles | 0.072 ± 0.001 | 1.110 ± 0.053 | 0.454 ± 0.001 |
| TC vesicles (light band) | 0.004 ± 0.001 | 2.14 ± 0.054 | 0.196 ± 0.002 |
| TC vesicles (dense band) | 0.013 ± 0.001 | 2.40 ± 0.025 | 0.063 ± 0.002 |

The light band of TC vesicles is either from 31 to 33% region of the pure sucrose gradient or the 28 to 30% region of the sucrose gradient with 0.6 M KCl. The dense band of TC vesicles is either from 39 to 41% region of the pure sucrose gradient or 33 to 35% region of the sucrose gradient with 0.6 M KCl.
of 5'-nucleotidase is 0.009 ± 0.002 (S.D.) µmol/mg/min in the fraction of ouabain-binding vesicles. This value is 1 to 10% of the activity reported in sarcolemma preparations (19, 23). However, the significance of the values obtained by other authors depends on the purity of their preparations. It is not clear a priori whether 5'-nucleotidase is expected to be a T-tubular enzyme. If all the activity is a consequence of the presence of contaminating plasma membrane, then the degree of contamination is between 1 and 10%. However, we emphasize that this value represents the maximum extent of contamination and the true value may be considerably lower.

**DISCUSSION**

In our previous communication (5), we reported that using a relatively simple procedure, i.e. density gradient centrifugation and 3HJouabain as external membrane marker, we obtained a heavy microsomal fraction that contained 3HJouabain vesicles that were attached to TC vesicles. We concluded that when the T-tubules were fractionated the 3HJouabain was trapped inside and was used as tracer in the further purification of the T-tubules.

We now report that these ouabain-binding vesicles can be isolated from the TC vesicles through detachment by French press treatment and subsequent density gradient centrifugation. The characteristics of these ouabain-binding vesicles can be summarized as follows.

1. Ouabain-binding vesicles are of external membrane origin. This is based on their accessibility to bind 3HJouabain from extracellular fluid and the subsequent trapping of 3HJouabain as the organelles are homogenized and resealed to form vesicles. Also, (NaK)-ATPase activity is demonstrated in these vesicles. This is in accordance with their ouabain-binding property.

2. Ouabain-binding vesicles are originally attached to TC. We have demonstrated that the ouabain-binding vesicles always migrate together with the TC vesicles as a single band in the density gradient under various conditions (5). Electron microscopy provides morphological evidence that this microsomal fraction contains elongated vesicles (with similar size and shape to isolated ouabain-binding vesicles) and each is attached to two TC vesicles in the conformation of triad junctions.

3. Ouabain-binding vesicles migrate as a distinct band after detachment from the TC vesicles. The attachment of these ouabain-binding vesicles to the TC vesicles can be disrupted by French press treatment. After the disruption, the ouabain-binding vesicles migrate as a distinct band clearly separated from the TC vesicles in both the sucrose gradient and sucrose gradient with 0.6 M KCl.

4. Ouabain-binding vesicles are distinct from the TC vesicles in both their enzymatic activities and protein composition. The (NaK)-ATPase is primarily present in the ouabain-binding vesicles. The SDS-gel electrophoresis shows the presence of several proteins in the ouabain-binding vesicles which are absent from the TC vesicles.

5. Ouabain-binding vesicles have similar size and shape to T-tubules in intact triad junctions. Electron microscopy reveals that the isolated 3HJouabain binding fraction contains numerous elongated vesicles. Most of these elongated vesicles have a length of 1500 to 2500 Å and a width of 180 to 200 Å. Our micrographs of intact muscle, of isolated triad junctions and of vesicles after disruption in a French press show that we can track morphologically the vesicles of the triad junction throughout the whole procedure of isolation. Our ability to track not only the elongated vesicles but also the terminal cisternae and the intact triad junctions and to account for all the bands following French press treatment permits us to identify the ouabain-binding vesicles without ambiguity.

From the above criteria, we conclude that the isolated ouabain binding vesicles are T-tubular vesicles.

The calculations of the specific 3HJouabain-binding activity indicate that we have achieved about 13- to 19-fold purification of the T-tubular fraction from the crude microsomal preparation. Our marker enzyme assays indicate negligible mitochondrial contamination and a low or negligible contamination from plasma membrane.

The SDS-gel electrophoresis study shows the presence of a protein band of $M_r = 100,000$ that is similar to the CaATPase protein in the T-tubular fractions from both the pure sucrose gradient and the sucrose gradient with 0.6 M KCl. About 50% of the CaATPase activity found in the TC vesicles is demonstrated in the T-tubular fractions of both preparations. We believe that the CaATPase activity found in the isolated fraction is intrinsic to the T-tubules rather than originating from contaminating TC vesicles. The reasons are as follows:

(a) if the CaATPase activity in the T-tubular band originates solely from contaminating TC vesicles and since this activity is about 50% of that found in the TC fraction, we ought to be able to identify a large fraction of the spherical vesicles in the electron micrographs that correspond to the TC. However, there are only a few such circular vesicles in the T-tubular fraction and some of these vesicles may be of T-tubular origin.

(b) The separation on the gradient between the T-tubular fraction and the fraction of TC vesicles in the sucrose gradient which contains 0.6 M KCl is considerably greater than that observed in the pure sucrose gradient (Fig. 2). However, the CaATPase activity in the T tubular fraction from the sucrose gradient with 0.6 M KCl is again 50% that of the TC vesicles. We therefore consider that the CaATPase activity detected in the T-tubular fraction is intrinsic, but further experiments will be required to establish this unequivocally.

The use of 0.6 M KCl treatment and sucrose density gradient with 0.6 M KCl is probably not the method of choice for separating the T-tubules from the TC vesicles. While there is a greater separation between the vesicles in the sucrose gradient with 0.6 M KCl as compared to that in the pure sucrose gradient, our data show both morphological alteration and loss of proteins by the KCl treatment.

The SDS-gel electrophoresis study reveals that the protein composition of the lighter band of TC vesicles (31 to 33% sucrose region) is not the same as that of the TC vesicles from the heavier band of TC vesicles (39 to 41 sucrose region). The $M_r = 55,000$ protein is present mainly in the lighter band; whereas the $M_r = 45,000$ and 42,000 proteins are present mainly in the heavier band. Similar findings are also observed in the preparation from the sucrose gradient with 0.6 M KCl. An interpretation of these observations is that the French press causes further disruption of the TC vesicles such that each TC vesicle is broken into fragments which reseal to form separate vesicles. Thus the region of the TC which contains electron dense matter becomes the dense band in the gradient while the relatively empty region of the TC vesicles becomes the light band. It is possible then that $M_r = 45,000$ and 42,000 proteins have some association with the electron dense matter of the TC as described by Meissner (6).

Our aim in isolating T-tubules has been to assist us in understanding the mechanism of excitation-contraction coupling of muscle. This necessary preliminary work is therefore
the first stage of our investigations of the physiology, biochemistry, and anatomy of organelles of excitation-contraction coupling in muscle.

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