Identification and Extraction of Proteins That Compose the Triad Junction of Skeletal Muscle

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ABSTRACT Treatment of both transverse tubules and terminal cisternae with a combination of Triton X-100 and hypertonic K cacodylate causes dissolution of nonjunctional proteins and selective retention of membrane fragments which are capable of junction formation. Treatment of vesicles with Triton X-100 and either KCl or K gluconate causes complete dissolution of all components. Therefore K cacodylate exerts a specific preservative action on the junctional material. The membrane fragment from treatment of transverse tubules with Triton X-100 + cacodylate contains a protein of M_r = 80,000 in SDS gel electrophoresis as the predominant protein while lipid composition is enriched in cholesterol. The membrane fragment retains in electron microscopy the trilaminar appearance of the intact vesicles. Freeze fracture of transverse tubule fragments reveals a high density of low-profile, intercalated particles, which frequently form strings or occasional small arrays. The fragments from Triton X-100 plus cacodylate treatment of terminal cisternae include the protein of M_r = 80,000 as well as the spanning protein of the triad, calsequestrin, and some minor proteins. The fragments are almost devoid of lipid and display an amorphous morphology suggesting membrane disruption. The ability of the transverse tubular fragment, which contains predominantly the M_r = 80,000 protein, to form junctions with terminal cisternae fragments suggests that it plays a role in anchoring the membrane to the junctional processes of the triad.

The junctional proteins may be solubilized in a combination of nonionic detergent and hypertonic NaCl. Subsequent molecular sieve chromatography gives an enriched preparation of the spanning protein. This protein has subunits of M_r = 300,000, 270,000, and 140,000 and migrates in the gel as a protein of $M_r = 1.2 \times 10^6$ indicating a polymeric structure.

The triad junction or dyad junction of muscle is the only known intracellular membrane junction. The morphology of this junction has been extensively studied in intact muscle by electron microscopy and has been demonstrated to display a unique organization very different from such intercellular junctions as the gap junction (1). The triad junction almost certainly is also the site of message transmission in excitation–contraction coupling (2, 3). Our knowledge of the composition, molecular conformation, and dynamics of the junction is however extremely limited. For these reasons the resolution of the structure of the junction offers an intriguing challenge.

In an earlier article we presented evidence that a protein doublet observed in PAGE with molecular weights of ~325,000 and 300,000 was a constituent of the junction (4). This protein could be transferred in part from the transverse tubules to the terminal cisternae (TC)[1] by forming and then breaking the triad; hence we proposed that the protein spanned the gap between the two organelles. The fact that the junction could be disrupted without apparent damage to the associated organelles suggested that the spanning protein resided external to the membrane and was associated with it through a noncovalent interaction with a recognition site in the membrane. Hence it is likely that the junction is composed not only of a spanning protein, but also of an anchoring protein that is attached or embedded in the membrane and that recognizes and binds the spanning protein.

This article describes a further characterization and extrac-

[1] *Abbreviations used in this paper:* HTC, "heavy" terminal cisternae; LTC, "light" terminal cisternae; TC, terminal cisternae; T-tubules, transverse tubules.

tion of proteins that form the triad junction and suggests a molecular structure of the triad.

MATERIALS AND METHODS

The preparation of microsomes, TC/triads, "heavy" terminal cisternae (HTC), and transverse tubules (T-tubules) has been described before (5, 6). A brief description is as follows: TC/triads are prepared from a microsomal fraction from rabbit sacrospinalis muscle by centrifugation on a continuous sucrose-density gradient. The band with isopycnic point ~40% sucrose contains free TC and intact triad junctions consisting of two TC vesicles apposed on each side of a T-tubule vesicle. Broken triads are prepared by passing TC/triads through a French press at 6,000 psi. HTC and T-tubules are prepared by centrifuging the broken triads in a continous sucrose-density gradient on a Sorvall TV850 vertical rotor (DuPont Instruments, Sorvall Biomedical Div., Newtown, CT) for 1½ h at 130,000 *g*. Of the three bands formed the lightest is T-tubules (22–28% wt/wt sucrose), the intermediate band (30–35% sucrose) is light terminal cisternae (LTC), and the heaviest is HTC (38–42% sucrose). The organelles were concentrated by centrifugation and resuspended in a medium of 250 mM sucrose, 2 mM histidine, pH 7.0.

Protein was estimated by the Bradford assay (7) and, where stated, 2 mg of Triton X-100/mg of protein was added followed by the salt. The sample was layered on a linear continuous sucrose-density gradient between 12.5 and 60% sucrose in a Beckman SW 41 Ti rotor (Beckman Instruments, Inc., Fullerton, CA). It was centrifuged for 3 h at 200,000 *g*. Samples (1.3 ml) were withdrawn sequentially from the top of the gradient for subsequent analysis.

The samples from the gradient were assayed as follows: protein was assayed by the Folin method. Total lipid phosphorus was assayed by a modified Ames assay (8). Aliquots (50 μl) were diluted to 1 ml with water in a small test tube and lipid was extracted into the organic phase by twice partitioning with 1 ml of a 2:1 ratio of chloroform/methanol. The solvent was removed by passing a stream of air over the sample. H_2O_2 (30%, 100 μl) was added and allowed to incubate in an oven at 80°C overnight. To the dry sample was added 0.8 ml of 0.5 M HCl and the phosphorus was assayed subsequently by the method of Ames (8). Cholesterol and cholesterol ester were estimated from 100-μl samples using a SmithKline Instruments reagent cholesterol kit SV/28 (SmithKline Instruments, Inc., Sunnyvale, CA). This assay employs enzymic hydrolysis of cholesterol ester followed by cholesterol oxidase to produce H_2O_2 which was assayed colorimetrically at 500 nm in a 1-ml cell in a Zeiss spectrophotometer (Carl Zeiss, Inc., New York). All parts of the reaction are present in a single reagent sample. A cholesterol standard (0.052 μmol) was run simultaneously. PAGE slab gels were those of Laemmli (9). Most gels used 9% acrylamide, 0.24% *N,N'*-methylene-*bis*-acrylamide BIS separating phase. The analysis of the spanning protein employed 5% acrylamide, 0.133% *N,N*-methylene-*bis*-acrylamide. Samples were dissolved in an equal volume of sample buffer and incubated in a boiling water bath for 1 min, and between 100 and 250 μl was layered in the wells of the gel. Standards are the high-molecular-weight standards of Bio-Rad Laboratories (Richmond, CA) which contained myosin, β-galactosidase, phosphorylase *b*, BSA, and ovalbumin, whereas calsequestrin and the Ca pump protein served as internal standards. Standards for the spanning protein gel included cross-linked albumin (Sigma Chemical Co., St. Louis, Mo) and high-molecular-weight standards (Bio-Rad Laboratories). M_r calculations are based on a plot of log M_r against R_f.

The extraction of the spanning protein was carried out by first separating nonjunctional proteins by treating a suspension of 2 mg of protein/ml of TC/triads in 250 mM sucrose, 2 mM histidine, pH 7.0, 10 μM phenylmethylsulfonyl fluoride (PMSF) with 2 mg of Triton X-100/mg of protein, followed by centrifugation at 100,000 *g* for 1 h. The pellet was resuspended to 5 mg of protein/ml as assayed by the Bradford assay (7). The suspension medium contained 1 M NaCl, 125 mM sucrose, 1 mM histidine, pH 7.0, 100 μM phenylmethylsulfonyl fluoride, and 2 mg of Zwittergent 3–14 (Calbiochem-Behring Corp., San Diego, CA)/mg of protein. The samples was run on a Sephacryl S400 (Pharmacia Fine Chemicals, Piscataway, NJ) column 2.5-cm diam × 60 cm long. The column was pre-equilibrated and run with 1 M NaCl, 2.5 mM histidine, pH 7.0 at 4°C. The column eluant was monitored with a Pharmacia densitometer at 280 nm. The column was calibrated separately under identical conditions using a peristaltic pump to control elution rate. The proteins employed for the calibration were rabbit skeletal muscle phosphorylase kinase, phosphorylase *a* and phosphorylase *b*, which were gifts from Dr. Marukh Ganapathi, bovine thyroglobin (Sigma Chemical Co.), and horse spleen ferritin (Miles Laboratories Inc., Elkhart, IN).

Samples for thin sectioning and freeze fracture were suspended in 250 mM sucrose and 2 mM histidine, pH 7.0, and centrifuged in a Beckman airfuge (Beckman Instruments, Inc., Palo Alto, CA) at 12 psi for 5 min. For thin sectioning the pellet was incubated with 2.5% glutaraldehyde, 3% sucrose, 0.1 M Na cacodylate, 4% tannic acid, pH 7.2. Further processing was as described before (10). For freeze fracture the fixation of the tissue was with 2.5% glutaraldehyde, 3% sucrose, 50 mM Na cacodylate, pH 7.2. The subsequent preparation has been described previously (11). For negative staining, a drop of the preparation (~0.05 mg/ml) was placed on top of a 400-mesh parlodion (0.75% in amyl acetate)-coated grid and allowed to stand for 1 min. The drop was then blotted and replaced by a drop of 1% K phosphotungstate, pH 6.7, allowed to stand for 1 min, and then gently blotted until almost dry. Specimens were observed under a Philips EM-300 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) operating at 80 kV.

RESULTS

Distribution of Proteins between Subcellular Organelles

In previous work we have employed specific ligand or enzyme markers to delineate T-tubules and sarcoplasmic reticulum subfractions. Several proteins play a role in the triad junction which do not exhibit defined enzyme activities. Fig. 1 shows the gel electrophoretic pattern and the densitometric assay of some proteins, which are identified as bands on SDS PAGE. A preparation of TC/triads was passed through a French press to break the triad junction and then centrifuged to equilibrium on a sucrose-density gradient. Three distinct vesicle bands are discernible with isopycnic points at 26, 32, and 42% sucrose which we have previously identified as T-tubule, LTC, and HTC, respectively (6). The first three panels of densitometric analysis show the distribution of proteins that are representative markers for the three organelles. The 72,000 M_r protein is distributed specifically in the T-tubules in accord with our earlier conclusions that this is a T-tubule specific protein (6). The distribution of this protein matches perfectly to that of the [^{3}H]ouabain entrapment assay we have employed before (data not shown). The long tail of activity in the denser region of the gradient reflects, in part, the difficulties in assaying this protein at low concentration because it is not a major component of the triad and, in part, a possible low extent of T-tubules which were not broken from the triad by French press treatment. The 53,000 M_r protein that has been identified by Michalak et al. (12) as an intrinsic glycoprotein shows a preponderant distribution towards the LTC although some activity may also exist in the HTC. Little activity appears to be associated with T-tubules and the presence of this protein in the T-tubule region probably represents contamination. Calsequestrin with M_r = 62,000 is predominantly a marker for HTC. The Ca pump protein of sarcoplasmic reticulum (102,000 M_r) is not a good marker in gel electrophoresis in that Hidalgo et al. (13) have shown that a protein of similar M_r with predominant Mg ATPase activity is present in T-tubules.

The lower three panels show the distribution between organelles of three proteins that are subject to further analysis in this paper. The protein of M_r = 300,000 is part of a doublet that has been identified previously as the spanning protein of the triad (4). The organelle distribution shows that the protein is associated predominantly with the HTC with very little content in the T-tubules or LTC.

A protein of M_r = 80,000 has a wide distribution between organelles. This protein is a major constituent of T-tubules, but its distribution is more widespread. A second peak of content is associated with the LTC and some protein is also present in the HTC. Thus, either the protein is a constituent both of the surface membrane and of the internal organelles, or there are two different proteins of identical M_r. The protein is not an extrinsic protein because it is not released by KCl treatment. The distribution of a protein of M_r = 34,000 is

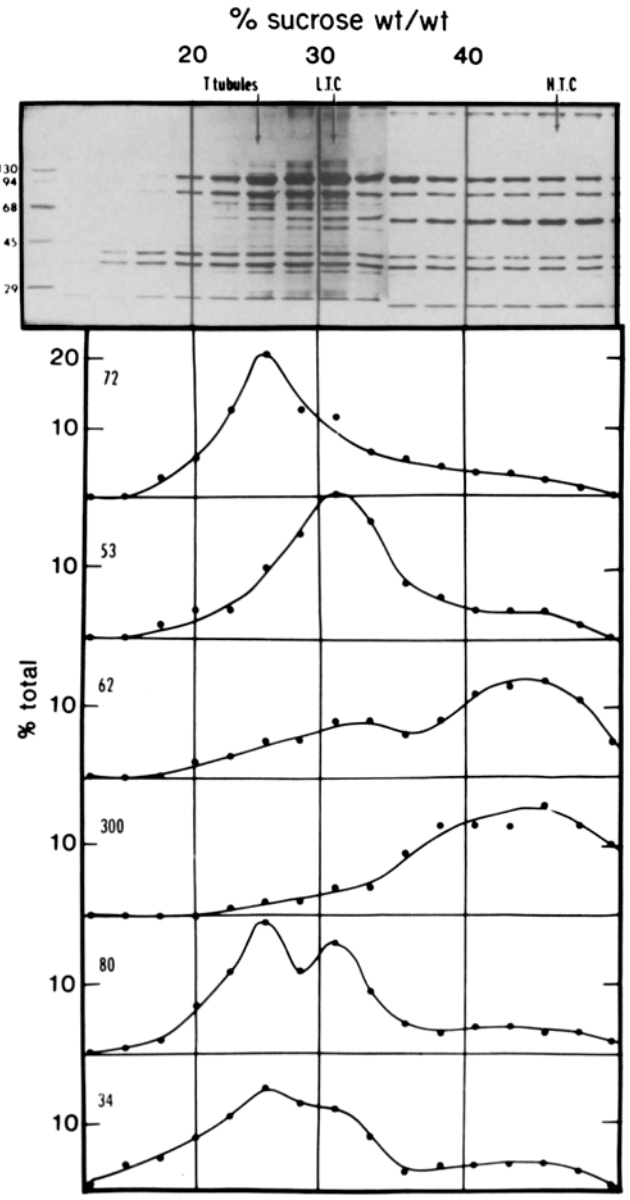

FIGURE 1 Densitometer distribution profile of SDS PAGE proteins from density gradient of broken triads. Broken triads were prepared as in Materials and Methods and centrifuged on a 12.5-50% linear sucrose gradient in a Sorvall TV 850 vertical rotor for 1.5 h at 150,000. Samples (1.8 ml) were withdrawn from the gradient and 10 μl were employed per lane on the Laemmli gel (9%). The gel was stained with Coomassie Brilliant Blue G250 (1% in 25% trichloracetic acid for 3 h) and destained in 5% methanol 7.5% vol/vol acetic acid. The lanes were scanned on a Zeineh soft laser densitometer (LKB Instruments, Inc., Rockville, MD). The intensity of the strongly stained bands was determined using the integrator of the densitometer (calsequestrin, 80,000 M_r protein) while that for the less intense bands was based on peak height. Values at upper left of graph are molecular weights ($\times 10^{-3}$).

also shown. This protein, which has been identified as an extrinsic constituent of the TC with a possible role in the triad junction (14), is distributed with a discrete peak associated with the T-tubules and a shoulder at the position of the LTC. The protein is also present in the HTC. Although not presented here, we have observed in a large number of SDS gel scans of microsome subfractions that the 80,000 and 34,000 M_r proteins are present in considerably lower concentrations in the longitudinal reticulum subfractionation and are essentially absent from some preparations.

Extraction of Membranes by Detergent and Salt

Free T-tubules and HTC were prepared by density gradient centrifugation of French press-treated TC/triads. This preparation yields pure HTC whereas T-tubules may contain up to 18% contamination by nonjunctional TC based upon freeze-fracture stereology (11) and Ca-stimulated hydrolysis of 3-O-methylfluorescein phosphate (15). The isolated organelles were separately treated with Triton X-100 and K gluconate or K cacodylate and centrifuged on a continuous sucrose-density gradient. When the individual organelles were treated with detergent plus K gluconate (0.5 M) complete dissolution of the organelles occurred. All of the protein detected either by direct assay of protein in the density gradient or by gel electrophoresis remained at the top of the sucrose gradient (data not shown). On the other hand when T-tubules and HTC, respectively, were treated with Triton X-100 plus K cacodylate, an opaque band was observed in the gradient and the gels show that some protein remained undissolved (18% of total protein for T-tubules and 30% for HTC, Fig. 2, *A* and *B*). The position of the bands is different for the two organelles. T-tubules give a band of isopycnic point 40% sucrose whereas the TC band is precipitated through 55% sucrose. In each case the isopycnic point is materially heavier than that of the native organelles (25% for T-tubules and 40% for heavy TC), suggesting considerable loss of lipid. SDS PAGE shows that the major protein preserved in the band by the cacodylate in both organelles is the 80,000 M_r protein identified by *3* in the figure. In the T tubules a small quantity of 102,000 M_r protein (*2*) is also preserved which may be the contaminating Ca pump from nonjunctional TC or Ca-Mg ATPase from T-tubules. The gel pattern in the region of the gradient containing undissolved material is virtually devoid of other proteins, and hence we may conclude that the 80,000 M_r protein is specifically preserved from dissolution by cacodylate. On the other hand, a number of other proteins from the T-tubules are fully dissolved by Triton X-100 and K cacodylate including the T-tubule-specific 72,000 M_r protein. A small portion of the 80,000 M_r protein is seen at the top of the gradient indicating that some of it has been dissolved by this protocol. Fig. 2*B* shows that in the undissolved TC fragments the 80,000 M_r protein is accompanied by the spanning protein doublet (*1*) and calsequestrin (*5*). Low amounts of some other proteins are also observed.

Fig. 3 shows the effect of salt and Triton X-100 treatment on TC/triads that have been passed through a French press to break the triad junction. The procotol is similar to Fig. 1 except that the gradient is steeper and therefore the organelle separation is less. In Fig. 3*A* this preparation has been treated with K gluconate alone and then centrifuged on a continuous density gradient. No protein is present at the top of the gradient indicating that K gluconate does not dissolve any of the proteins. However, it may have altered the disposition of calsequestrin (*5*) in the gradient because this protein is normally associated with the HTC at 40% sucrose. The marker for T-tubules is the 72,000 M_r protein (*4*) while that of HTC is the spanning protein doublet (*1*). The 80,000 M_r protein (*3*) can be seen to be distributed in both organelles as was demonstrated in Fig. 1. Fig. 3*B* shows the influence of Triton X-100 on the integrity and isopycnic density of the disrupted

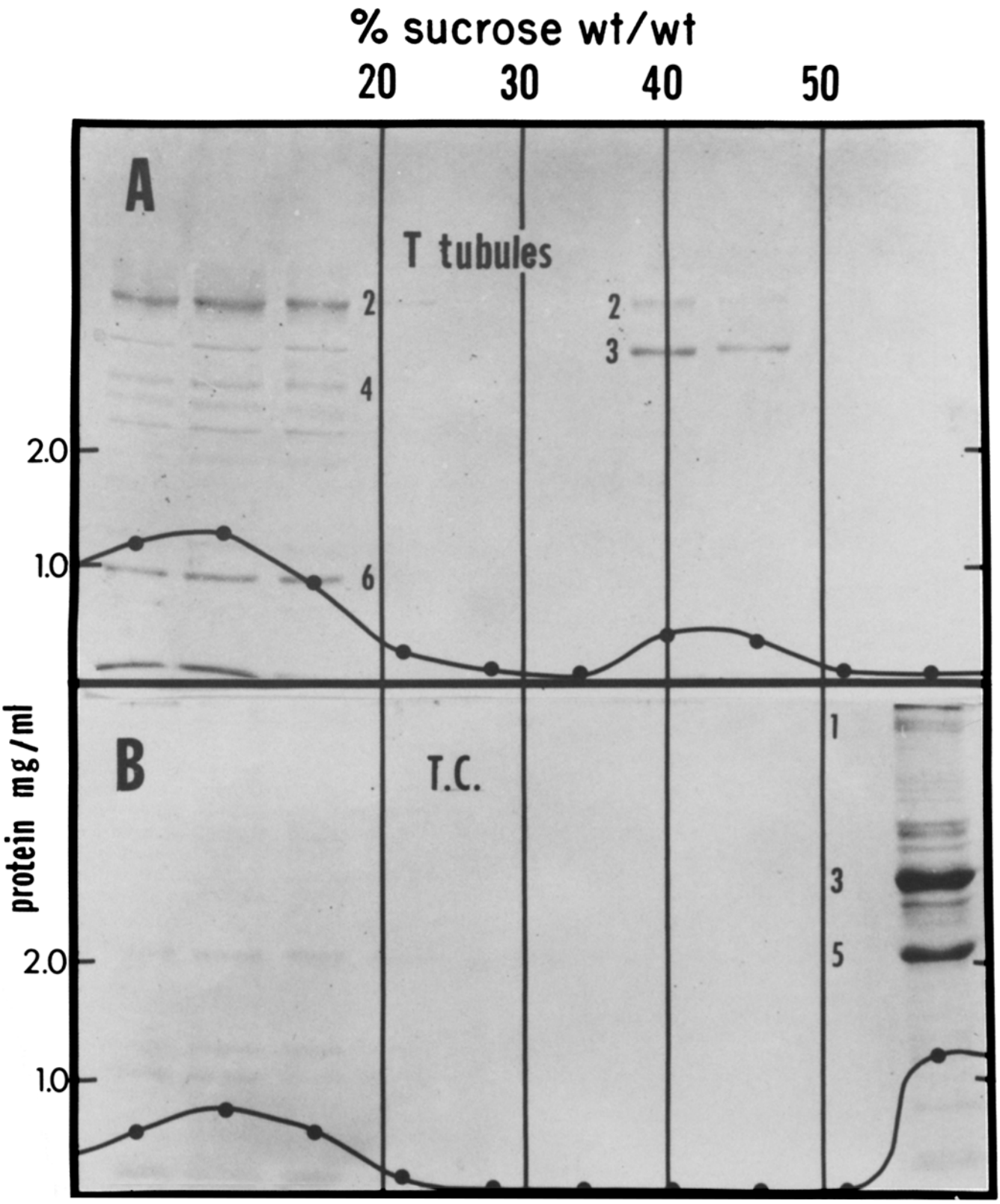

FIGURE 2 Gel electrophoretic patterns of density gradient profile of Triton plus cacodylate treated T-tubules (*A*) and HTC (*B*). The vesicles were prepared as described before (6) and resuspended to 2 mg protein/ml in 250 mM sucrose 2 mM histidine, pH 7.0. To this was added 2 mg/ml of Triton X-100 and 0.5 M K cacodylate, pH 7.0. The vesicles were overlayed on a 13 ml 12.5–65% sucrose density gradient in tubes of Beckman SW 41 Ti rotor (Beckman Instruments, Inc.). The suspension was centrifuged for 3 h at 210,000 *g*. Aliquots (1.2 ml) were withdrawn starting from the top of the gradient using a Buchler Autodensiflow probe. Sucrose density from the fractions was estimated using a Bausch and Lomb refractometer. 100 μl from each gradient tube was run on a 9% Laemmli SDS electrophoresis plate.

The numbers on the gels in this and subsequent figures refer to the positions of the following proteins: *1*, spanning protein doublet; *2*, 102,000 M_r protein; *3*, 80,000 M_r protein; *4*, 72,000 M_r potein; *5*, calsequestrin; *6*, 34,000 M_r protein.

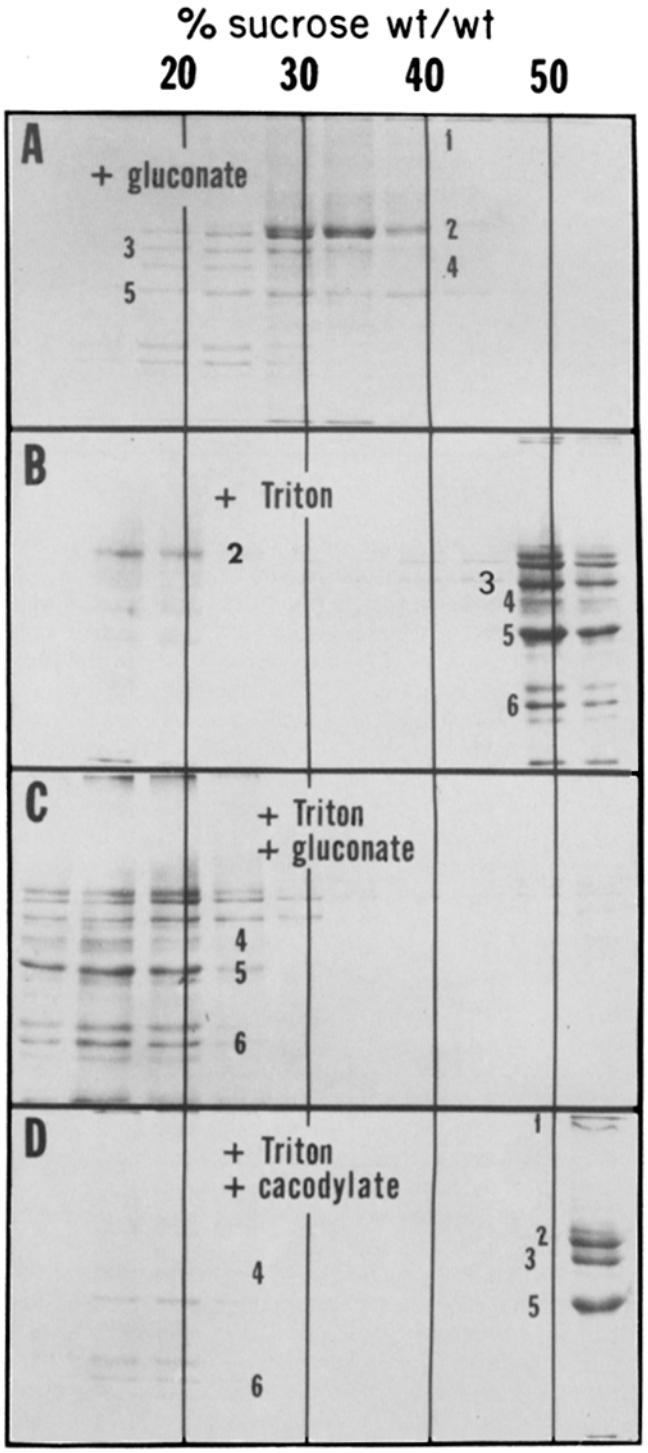

FIGURE 3 Gel electrophoretic patterns of density gradient profile of broken triads treated with salts and detergent. The broken triads (2 mg of protein/ml) were prepared by passing TC/triads through a French press at 6,000 psi. They were treated as indicated with 2 mg/mg of Triton X-100 and 0.5 M K gluconate or K cacodylate. The subsequent density gradient and assays were as described in Fig. 2.

vesicles from the triad junction. The Ca pump protein is the major protein seen at the top of the gradient indicating that it has dissolved in the Triton X-100. Nevertheless, a large proportion of protein has resisted dissolution in Triton X-100 and migrated to a dense region of the gradient. This includes TC proteins such as calsequestrin as well as T-tubule proteins such as the 72,000 M_r protein. In addition, the 80,000 M_r protein, which is present in both organelles, appears at a single isopycnic point in the gradient. Inasmuch as Fig. 2 shows that the Triton X-100-resistant fragments from T-tubules have a different isopycnic point than those from TC, the data of Fig. 3*B* suggest that treatment with Triton X-100 has promoted reformation of the triad junction. The reformation of the junction does not always occur in the presence of Triton X-100 because we have sometimes found the 72,000 and 80,000 M_r proteins to be distributed in a gradient with a different isopycnic point than calsequestrin. Fig. 3*C* shows the influence of K gluconate on the solubility of TC and T-tubule proteins in the presence of Triton X-100. The gradient profile indicates that almost full dissolution of all proteins has occurred. A similar observation has been made with a mixture of KCl and Triton except that KCl is even more potent. Fig. 3*D* is the same experiment as Fig. 3*C* except that the salt K cacodylate has been employed in place of K gluconate. This treatment causes considerably less dissolution of proteins than K gluconate. In particular, calsequestrin, the 80,000 M_r protein, a protein of $M_r \sim 100{,}000$, and the spanning doublet are found in the dense region towards the bottom of the gradient. Nevertheless, the dissolution is more complete than that with Triton X-100 alone because the 34,000 protein (*6*) and the T-tubular 72,000 protein (*4*) are dissolved. Thus, cacodylate preserves from dissolution a select range or proteins. The appearance of the resistant fraction as a single band in the gradient with an isopycnic point of ~52% sucrose contrasts with the two different isopycnic points of isolated T-tubules and TC similarly treated in Fig. 2 and gives evidence that the junction has been reformed. Therefore, each organelle contains in the Triton X-100 and cacodylate-resistant fraction the necessary constituents for recognition and binding of the two organelles. We have previously described a specific role of cacodylate in promoting the formation of the triad junction from isolated organelles (16). It has not proved feasible to isolate the Triton X-100-resistant fragments from T-tubules and TC and thus cause them to rejoin since the rejoining facility is labile with time.

Fig. 4 shows that when K gluconate is added to triads, which have been disrupted in a French press and treated with Triton X-100, very little protein is observed in the denser portion of the gradient indicating that Triton X-100 plus K gluconate have dissolved most of the protein. In contrast considerable retention of protein complex from dissolution is observed if the vesicles are treated with Triton X-100 plus K cacodylate. When both salts are present, the protein retention in the denser portion of the gradient is intermediate between that of each salt separately. Therefore cacodylate is actively preserving from dissolution some of the proteins that are normally dissolved by gluconate in the presence of Triton X-100 despite the high ionic strength of the medium.

Fig. 5 shows thin-section electron micrographs of the membrane fragments from isolated T-tubules, TC, and reformed triad junctions, respectively, which have resisted Triton X-100 plus cacodylate dissolution. Fig. 5*A* shows that fragments from T-tubules retain to a remarkable degree the membrane integrity of the intact vesicle although the vesicles are no longer closed but display broken ends. The membrane shows a well-developed trilaminar structure with a total thickness of ~100Å. There is no evidence of electron-dense matter broaching or crossing the translucent interior. In views in which the

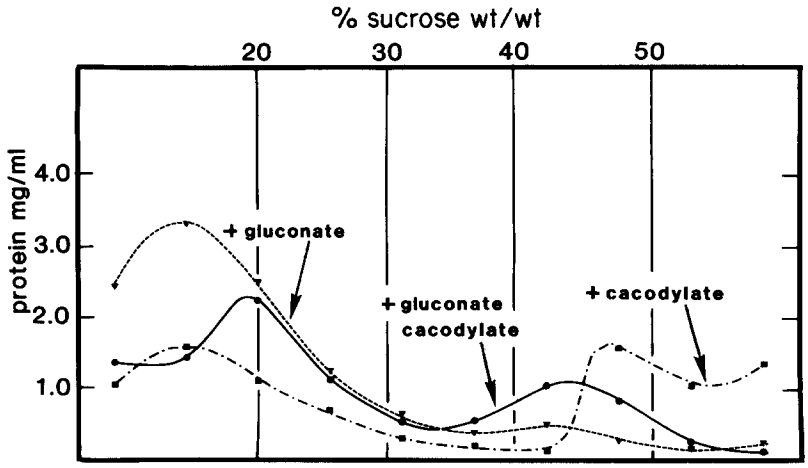

FIGURE 4 Density gradient profile of broken triads treated with salts and detergent. Conditions for this experiment are the same as those of Fig. 3.

trilaminar structure is not apparent, this may be associated with an oblique sectioning angle relative to the membrane surface, although we cannot preclude the possibility that some amorphous material is attached to the membrane. The membranes are frequently curved suggesting that they represent domains that have survived extraction from an intact vesicle and have retained in part the original vesicle shape. In sharp contrast, no discernible membrane organization is present in the Triton X-100 plus cacodylate-resistant fragment from HTC (Fig. 5*B*). The original HTC contained an electron-dense interior that was condensed and attached to the membrane but was otherwise apparently amorphous and consisted predominantly of calsequestrin. The amorphous material appears to have survived. However, because other proteins including the 80,000 M_r protein also survive Triton X-100 plus cacodylate extraction and are major components of the fragment, it is unlikely that the fragments observed in electron microscopy represent solely the interior of the HTC. The membrane organization of the junctional fragments has been destroyed by the treatment. Fig. 5*C* shows thin sections of fragments from broken triads that have been treated with Triton X-100 and cacodylate and subsequently centrifuged on a linear gradient to produce a single opaque band at 50% wt/wt sucrose. The well-defined trilaminar membranes are T-tubule fragments and the amorphous material is surviving TC fragments. Connection between the two types of fragment is seen, but individual feet cannot be discerned. This micrograph gives evidence of a reassociation of T-tubule and TC fragments induced by Triton X-100 plus cacodylate treatment confirming the evidence of Fig. 3*D* that the junction may be formed between the fragments.

The difference between the appearance of the Triton X-100 plus cacodylate-resistant T-tubule and TC fragments in the electron micrographs suggests a significant difference in biochemical composition although the major protein is the same in each case. Fig. 6 shows the protein, phospholipid, and cholesterol (plus esters) from density gradients of T-tubules and HTC treated with Triton X-100 plus cacodylate. The protein profile from T-tubules in Fig. 6*A* shows dissolved protein at the top of the gradient and a substantial protein band associated with fragments at 40% sucrose. Cholesterol is distributed similarly between the soluble fraction at the top of the gradient and the resistant fragments. Enrichment of cholesterol in the fragments is such that the cholesterol/protein ratio is 2.3 times higher than in the supernatant. On the other hand little of the phospholipid is retained in the resistant fragments. The phospholipid/protein ratio is 3.2 times higher in the supernatant than in the fragments suggesting a specific association of the 80,000 M_r protein with cholesterol. The molar cholesterol/protein ratio in the fragments is 1.6. The composition of the supernatant on a weight basis is 68% protein, 29% phospholipid, and 3% cholesterol. The fragments are composed 80% of protein, 11% phospholipid, and 9% cholesterol on a weight basis. The isopycnic density of this composition based on a protein density of 1.34, phospholipid density of 1.0, and cholesterol density of 1.07 is expected to be equivalent to 54% sucrose compared with the density of 40% sucrose obtained here. This implies the existence of other constituents in the fragments which could include neutral lipid and water.

The pattern of protein and lipid distribution of HTC treated with Triton X-100 and cacodylate is shown in Fig. 6*B*. The resistant fragments from HTC have an isopycnic point of 55% sucrose. The TC contain very little cholesterol (11). Here both cholesterol and phospholipid are fully dissolved by Triton X-100 plus cacodylate. Therefore, the resistant fragment is composed almost exclusively of protein which may account for the loss of discernible membrane organization.

Fig. 7*A* is a freeze-fracture replica of the resistant fragments derived from T-tubules that have been treated with Triton plus cacodylate. This figure shows the fracture face of the membrane and confirms the thin-sectioning observations that the membrane integrity is preserved after detergent plus cacodylate treatment. The broken ends of the fragments may sometimes be seen exposed on the fracture surface such that the membrane is divided into two layers with a discontinuity in the center (arrow). All membranes contain a large number of low-profile, intercalated particles. The particles therefore break evenly between the two faces. This appearance is different from that of intact T-tubules in which a small number of high profile particles are seen (3), although some intact T-tubules contain regions where low-profile particles are discernible (11). A characteristic feature of the particle organization shown here is the presence of linear strings and occasionally patches of arrays (arrowheads). These strings suggest at least partial organization of the protein structure of the fragment. The extent of organization is hard to determine inasmuch as the particles may have separated randomly between the two fracture faces. The linear separation of particles in the strings is approximately 90Å. The high density of the

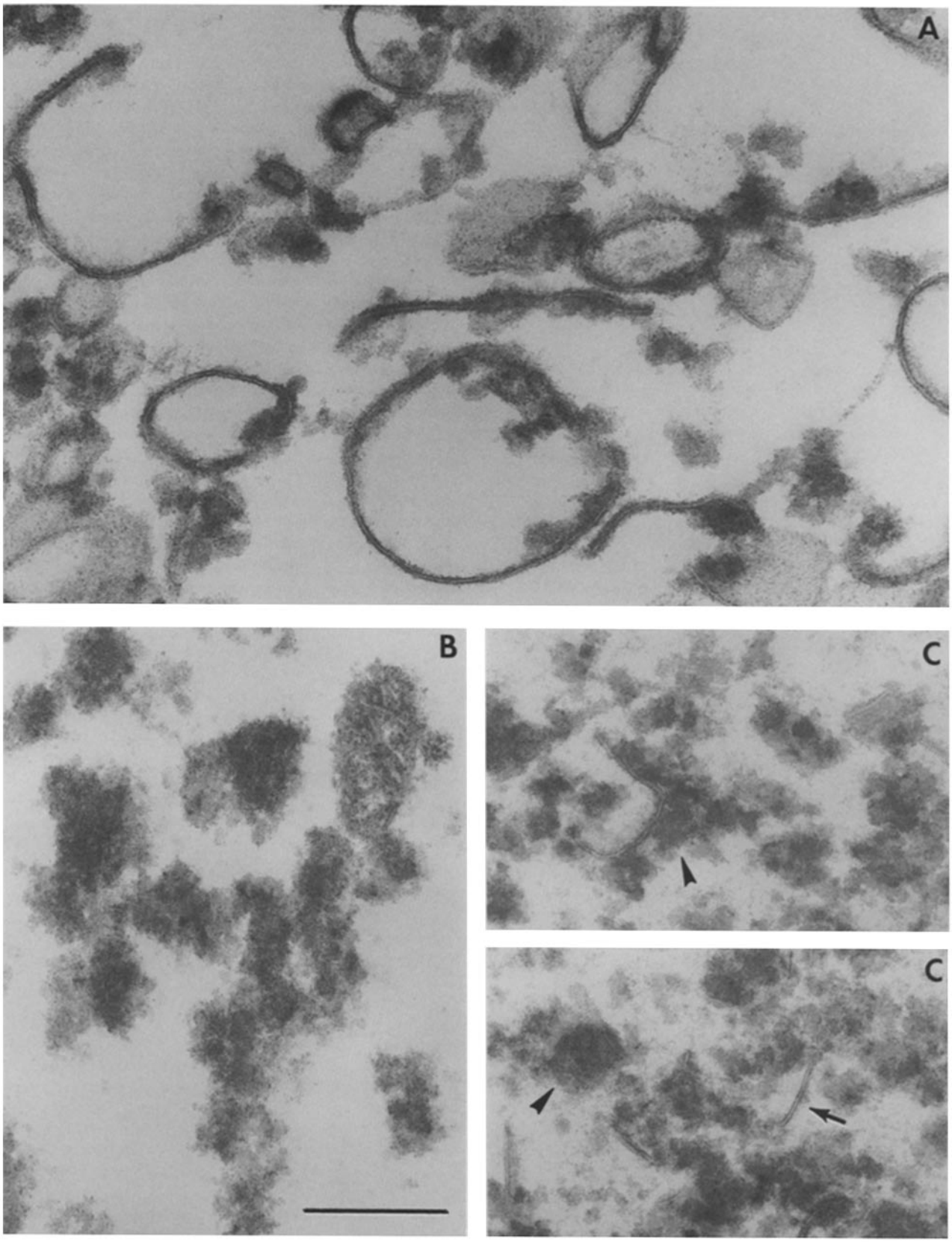

FIGURE 5 Thin section electron micrographs of Triton X-100 plus cacodylate-resistant fragments from T-tubules (*A*), heavy TC (*B*), and broken triads (*C*). The preparation of resistant fragments from T-tubules and heavy TC is the same as that of Fig. 2. The visible band from the gradient after Triton X-100 plus cacodylate treatment was removed and the vesicles pelleted by centrifugation at 120,000 *g* for 60 min. The samples were resuspended in 5 ml of 250 mM sucrose, 2 mM histidine, centrifuged at 77,000 *g* for 20 min, and the pellet was overlaid with 2.5% glutaraldehyde, 3% sucrose, 0.1 M Na cacodylate, and 4% tannic acid. Triton X-100 plus cacodylate-resistant fragments from broken triads were prepared as described in Fig. 3. The visible band was withdrawn, pelleted by centrifugation at 120,000 *g* for 60 min, and resuspended in a small volume of 250 mM sucrose, 2 mM histidine. The suspension was centrifuged in a Beckman airfuge for 5 min at 12 psi pressure. The pellet was treated as described above. In Fig. 5*C* the arrows indicate the well-preserved trilaminar appearance of T-tubule fragments whereas the arrowhead delineates the amorphous appearance of TC fragments. The bar line in this and subsequent micrographs represents 250 nm.

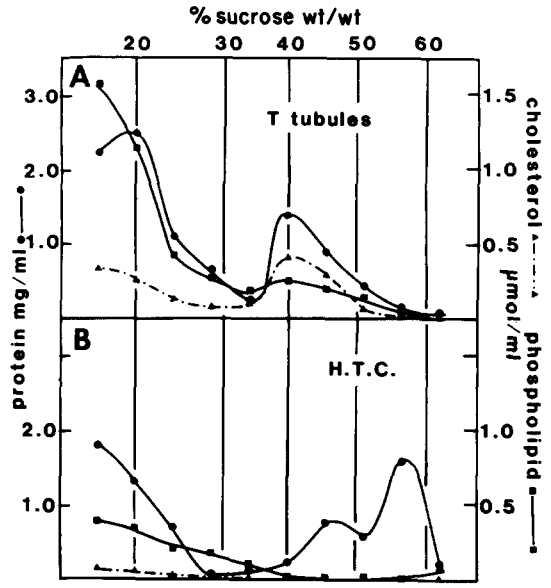

FIGURE 6 Protein, phospholipid, and cholesterol scan of density gradient from Triton plus cacodylate-treated vesicles. The preparation of the gradients for T-tubule and HTC fragments is that of Fig. 2. The protein was assayed by the Folin reagent; the total phosphorus and cholesterol (plus esters) were assayed as described in Materials and Methods.

intercalated particles is of the order to be expected of a membrane composed of 80% protein, and therefore the majority of the particles may be attributed to the major protein of the fragments, the 80,000 M_r protein. Fig. 7*B* shows negatively stained images of the Triton X-100 plus cacodylate-resistant T-tubule fragments. The membrane boundary is clearly defined. However, little or no discernible structure is observed either in the bulk membrane or at the edge. This is in accord with the freeze-fracture data implying that the low-profile particles are too short to penetrate beyond the membrane surface and that the 80,000 M_r protein is deeply immersed in the membrane structure. Alternatively, the 80,000 M_r protein may be considered to dominate the membrane structure with the interstices filled with cholesterol and phospholipid.

Extraction of Junctional Proteins

The proteins that constitute the junctional region of the triad may be readily dissolved from intact triadic vesicles by a combination of a nonionic detergent and high ionic strength. However, if the preparation has been previously enriched by treatment of vesicles with detergent plus K cacodylate, then the junctional material is frequently resistant to subsequent solubilization by detergent and high ionic strength. At present, the techniques available for further enrichment of the solubilized protein are limited by the need to maintain a high ionic strength. We have therefore not yet been successful in purifying the dissolved 80,000 M_r protein beyond that obtainable in the Triton X-100 plus cacodylate-resistant fraction from T-tubules.

A substantial enrichment of the spanning protein of the triad may, however, be obtained as is demonstrated in Fig. 8. TC/triads were treated with Triton X-100 to extract nonjunctional material and centrifuged, and the precipitate was extracted with Zwittergent 3–14 and 1 M NaCl. This sample was loaded on a Sephacryl S400 column and eluted with NaCl. The protein eluant profile indicated by the solid line trace has three peaks. The first peak represents insoluble and aggregated material running in the void volume. The second peak is of material of molecular weight 1.2×10^6 as estimated by calibration of the gel. This estimate is approximate and applies most accurately for a spherical protein lacking carbohydrate and detergent. The gel electrophoretic pattern from the gradient indicates that this peak is composed of three subunits of M_r = 300,000, 270,000, and 140,000 (indicated by *1*). These subunits clearly correlate with the position of the protein peak and their distribution corresponds with each other throughout the elution. They are present as a sharp homogenous peak. We have previously identified the 300,000 and 270,000 bands as the doublet of the spanning protein (4). (More complete analysis of molecular weights has caused us to give slightly reduced M_r from the previous paper.) We may therefore identify the peak from the column as the spanning protein. We have estimated previously from electron microscopy that the expected molecular weight of junctional feet would be 1.5×10^6 (10), which agrees well with our findings here. Attempts to deduce the stoichiometric ratio of these three bands were unsuccessful both in the enriched band from the gradient and in isolated triads because the ratio varied significantly from one preparation to another. We propose either that the subunits represent the expression and synthesis of different proteins of similar properties and are therefore equivalent to isozymes, or that a small degree of proteolysis has occurred immediately after muscle homogenization. The gel pattern indicates considerable enrichment in this protein and that the peak eluted from the column is in the range of 50% pure. This value varies from one experiment to another and we have occasionally obtained more pure samples.

DISCUSSION

Effects of Cacodylate

Our approach in this paper has been to elucidate further the composition of the triad junction by breaking the vesicles into the minimum components that still can form an intact junction. We have demonstrated previously that treatment of triads with Triton X-100 destroys nonjunctional membrane but does not solubilize the junction and leaves the membrane in the vicinity of the junction intact by electron microscopic criteria (10). In this paper we have dissected junctional from nonjunctional material still further by including K cacodylate with Triton X-100 and by separating the junctional organelles using a French press. We have shown before that cacodylate exerts a specialized role in promoting the formation of the triad junction from the isolated organelles (16). We report here that cacodylate specifically preserves certain proteins from the dissolution which is normally effected by the combination of Triton X-100 and high ionic strength. K cacodylate presumably exerts its primary action by preserving junctional protein from dissolution and secondarily promotes junction formation consequent upon its action in preserving junctional protein. A mechanism for this action might be through causing membrane protein of either the T-tubule or TC to form an organized paracrystalline array. This protein then associates with the spanning protein holding the subunits of the spanning protein in a configuration suitable for junction formation. Thus, for example, each subunit of the spanning protein may weakly associate with a protein in T-tubules. If

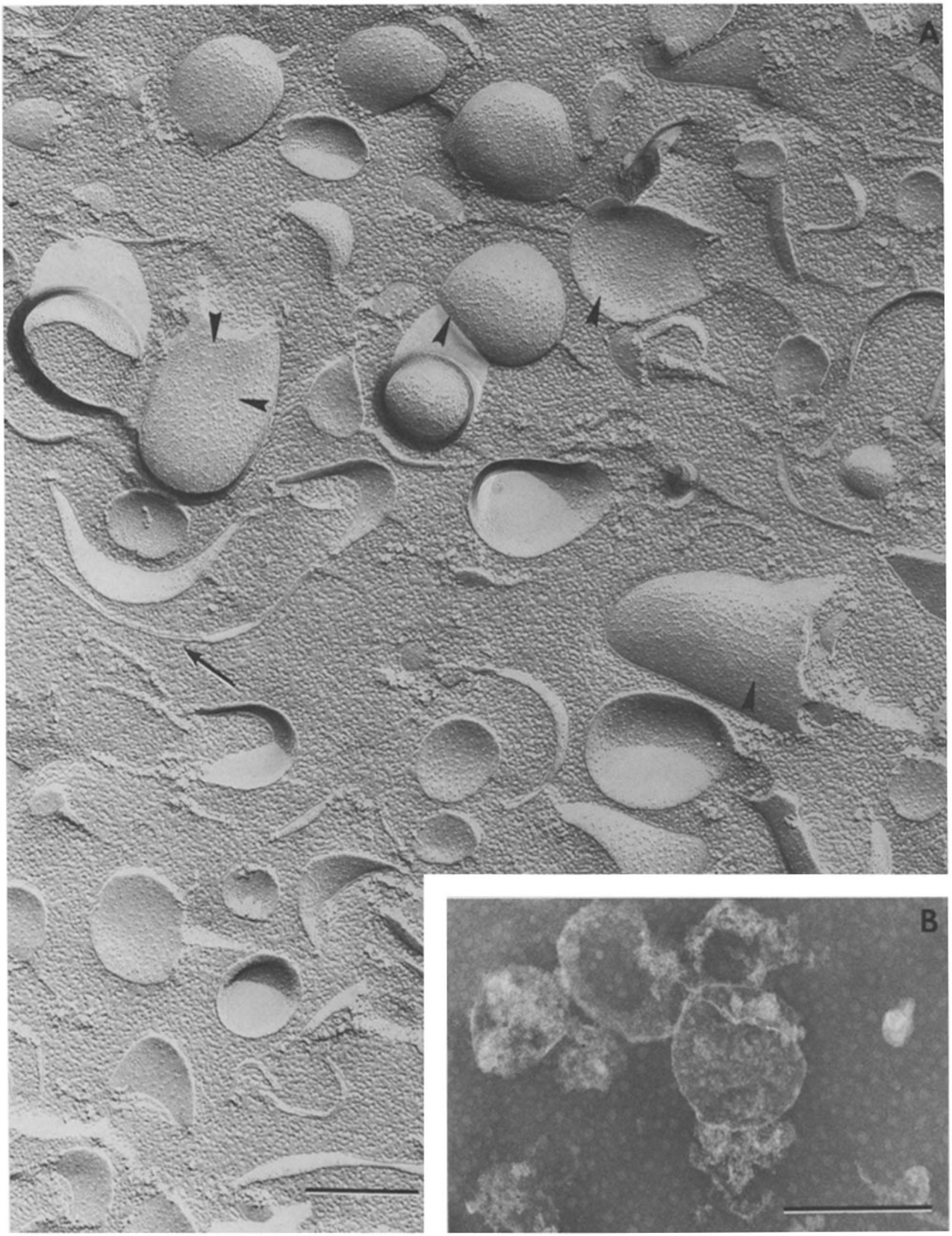

FIGURE 7 Freeze fracture (*A*) and negative stain (*B*) electron micrographs of Triton X-100 plus cacodylate treated T-tubules. T-tubule fragments were prepared as described in Fig. 2 and pelletted for freeze-fracture by centrifugation in an airfuge for 5 min at 15 psi pressure. The pellet was overlayed with fixative as described in Materials and Methods. The arrow represents broken ends of membrane fragment showing split membrane. Arrowheads delineate regions of freeze-fracture particle strings or small arrays.

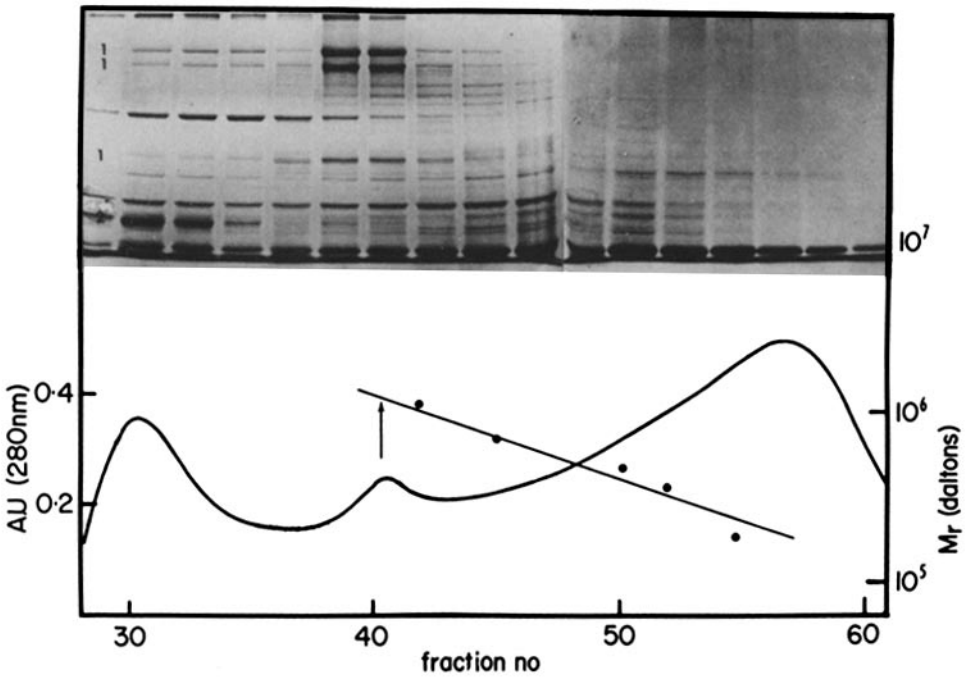

FIGURE 8 Molecular sieve column chromatography to enrich extracted spanning protein of triad. The conditions of the experiment have been described in Materials and Methods. The arrow shows the position of the peak of the protein band on the molecular weight calibration curve.

both the subunits of the spanning protein and the integral T-tubule junctional protein are held in an organized association, then the strength or rate of interaction of the whole spanning protein with the T-tubule will be enhanced. Once the bond is formed, it is likely to be stable because multiple binding sites are involved in the association. This can account for our earlier observation that the junctions formed in the presence of cacodylate are stable even after cacodylate removal (16). We can therefore picture the effects of cacodylate in a consistent manner through its single specific action in causing a membrane association of a junctional protein. Cacodylate is clearly an artificial initiator, and we have therefore explored the properties that give rise to its action and the physiological correlates in other work (17).

The effect of cacodylate in preserving protein from dissolution by Triton is similar both in T-tubules and TC to the extent that both organelles are fully dissolved by Triton X-100 plus K gluconate while Triton X-100 plus cacodylate is associated with protection of part of the structure. Both organelles contain a protein of M_r = 80,000 and in both organelles this protein is fully preserved from dissolution by cacodylate while it is completely dissolved by Triton X-100 plus gluconate. Although our data are not conclusive, this consistent pattern of response of the 80,000 M_r protein supports the view that it is the same molecule in T-tubules and in TC. In T-tubules the only other protein preserved by Triton X-100 is one of M_r = 100,000 and this protein appears to be preserved only to a very limited extent. Moreover the amount retained in the fragment is a small fraction of the 80,000 M_r protein. In TC several proteins are partially or fully preserved by cacodylate from dissolution. However, if it is presumed that the primary preservation of one protein serves as the nucleus for the binding and preservation of the other, then the primary protein is likely to be the 80,000 M_r protein. This protein is clearly the primary preserved protein of T-tubules and the similar response of TC to cacodylate suggests that it is the primary preserved protein in that organelle also.

Proposed Organization of Triad Junction

Our earlier observation that, upon mechanical fracture of the triad junction, the spanning protein, although predominantly associated with TC could also associate with T-tubules, suggested that the protein spanned the gap but was not deeply embedded in the membrane (4). Moreover, electron microscopy has revealed no signs of disturbance of the membrane of either organelle by the mechanical disruption of the junction suggesting that the spanning protein is contiguous with the membrane but is not a part of it (6, 10). We therefore propose that the spanning protein associates with anchoring proteins in each organelle. We have previously demonstrated that the junction formation promoted by cacodylate is specific for TC and T-tubules (16). Therefore we are not observing a general membrane aggregation, and it is unlikely that nonspecific membrane constituents such as lipids provide the specificity of recognition to form a junction. In the T-tubules and TC the major protein preserved after Triton X-100 plus cacodylate treatment is the 80,000 M_r protein implying that this protein is the anchoring protein of both T-tubules and TC. Other proteins are preserved in the TC including calsequestrin and the spanning protein. However, calsequestrin is known to be a luminal protein (18) and is unlikely to serve as an anchor. There is a teleologic rationale for the same protein to serve as an anchor in both organelles in that, if it is organized in an array, the recognition sites for the spanning protein can be identically spaced in each organelle. This will favor, on the one hand, an organized array of the junctional feet such as is found in the muscle triad (19). On the other hand, the two junctional organelles are not necessarily identical because the anchoring protein may be immersed in a different lipid and/or protein environment and may be oriented either symmetrically or antisymmetrically on either side of the junction.

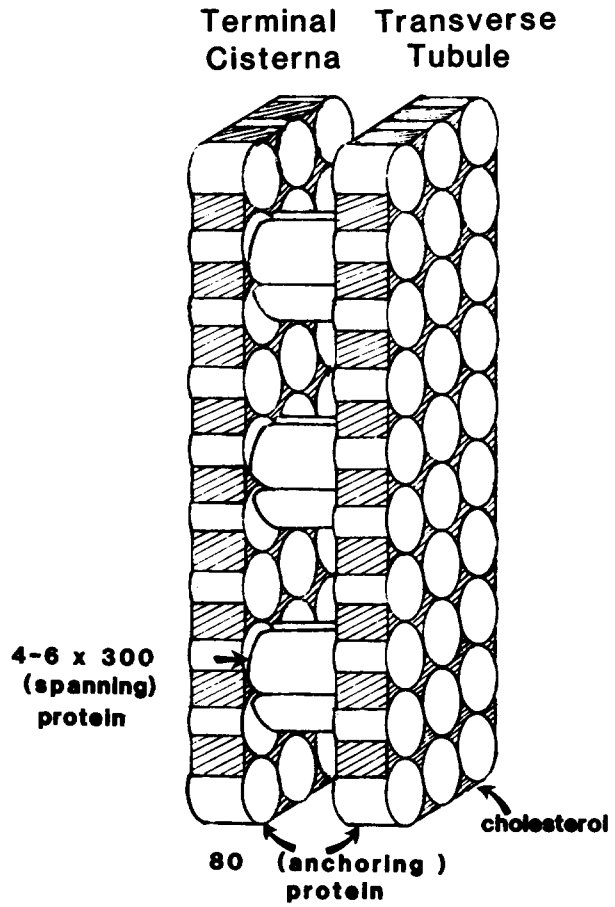

FIGURE 9 A model for the triad junction. We propose an array of membrane embedded protein subunit M_r = 80,000 to serve as the anchoring protein of both T-tubules and terminal cisternae. The spanning protein is considered to be a polymeric unit with subunit M_r = ~300,000. Cholesterol is present in the T-tubule junctional region, but not in that of TC. The model does not presume a symmetrical organization. Values are the molecular weights ($\times 10^{-3}$).

Role of Lipids in Membrane Preservation

The dramatic difference between the physical appearance of the T-tubule and the TC fragments after Triton X-100 plus cacodylate treatment may be explained by the differences in the lipids. Both organelles contain a full range of phospholipids with some slight quantitative differences in their content except that TC do not contain sphingomyelin (11). It is therefore not likely that differences in phospholipid content play the most important role in determining the degree of membrane preservation. The very low content of cholesterol in TC together with the high degree of retention of cholesterol in the T-tubule fragments may account for the differences in their preservation after Triton X-100 plus cacodylate treatment. The dominant picture that emerges is the strong association of cholesterol with the 80,000 M_r anchoring protein when this lipid is present. This does not preclude the possibility of the protein being immersed in a phospholipid bilayer in the intact vesicle especially in the TC.

We have not evaluated whether the intact T-tubule contains domains of 80,000 M_r protein or whether the protein is dispersed. Freeze fracture of intact T-tubules shows areas of the low-profile, intercalated particles which may represent domains (11), but the low contrast of the replicas of the particles and the high curvature of the vesicles make an effective examination difficult.

A Model

Fig. 9 is a molecular model, albeit preliminary, of the triad junction. We consider that the most likely disposition of the proteins is that an array of anchoring proteins holds the polymeric spanning protein in a specified array to form the junctional feet. We do not have any information concerning the relative orientation of the constituents.

The spanning protein of the triad has a more restricted distribution among the organelles than does the 80,000 M_r protein. The former is confined to the HTC whereas the latter is present in LTC as well as HTC and T-tubules. This more permissive distribution is not implausible. Clearly the feet processes have to be confined to the junction. However the 80,000 M_r protein is an integral membrane protein and may be fluid in the membrane matrix. Moreover, the distribution of this protein may be modified after muscle homogenization. We are currently exploring immunocytological approaches towards identifying the locus of the junctional proteins in intact muscle as well as isolated organelles.

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