Ultrastructural Observations of Isolated Intact and Fragmented Junctions of Skeletal Muscle by Use of Tannic Acid Mordanting

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ABSTRACT Tannic acid mordanting during fixation of isolated vesicles from skeletal muscle enhanced the resolution of the images. Isolated triadic junctions displayed two characteristic features not previously described: (*a*) a clear gap separated terminal cisternae from transverse tubules; (*b*) this gap was bridged by a separating array of structures which resembled the "feet" of intact muscle. When the triad was broken in a French press and subsequently reassembled by joining the two organelles, a similar gap was seen but the structure of the feet was less well defined. When the membrane of the triad was extracted by Triton X-100, the junctional region was retained and a similar gap between the two organelles could be discerned. The terminal cisternae characteristically displayed a thickening of the cytoplasmic leaflet of the membrane in select areas in which electron-dense material was apposed on the luminal leaflet. This thickened membrane was not observed in longitudinal reticulum or in terminal cisternae regions distal to the electron-dense matter. This thickened leaflet was not invariably associated with the junction, and some junctional regions did not display discernible thickening of the membrane. When the triad was treated with KCl, the electron-dense aggregate was dispersed and the thickened leaflet of the terminal cisternae dissipated, whereas the triadic junctional region with its feet remained unchanged. KCl treatment caused dissolution of three proteins of M_r = 77,000, 43,000, and 38,000. Treatment of Triton-resistant vesicles with KCl caused the loss of electron-dense aggregate but did not otherwise influence the appearance of the junction. A good degree of correlation both qualitatively and in quantitative parameters between the isolated vesicles and the intact muscle was observed.

Since the introduction of tannic acid mordanting for electron microscopy by Mizuhira and Futaesaku (19, 20), the method has been employed to increase electron density and contrast in a diversity of cellular structures when used in conjunction with glutaraldehyde and osmium tetroxide. The mechanism of tannic acid for enhancing the contrast of membranes is not clear. Simionescu and Simionescu (22, 23) have argued that low molecular weight constituents which are galloylglucoses act as mordants by reason of their polyphenolic structures. Their data showed a facilitation of lead binding by tannic acid, and they suggested that the compound reacts with bound osmium in membranes. Kalina and Pease (12), while also proposing a mordant role for tannic acid, have demonstrated a direct reaction of the compound with the choline residues of lipid vesicles rather than a reaction with osmium tetroxide. Nevertheless, in the more complex systems of biological membranes the site of reaction is not so well understood.

The technique has been used on skeletal muscle *in situ*. Bonilla (1) observed a preferential staining of the region of the transverse tubular junction, suggesting that a special pool of proteins may be present at that site. Somlyo (24) reported finding electron-dense bridges with a translucent core spanning the junctional gap of the triad of skeletal muscle. The core was apparently continuous with the electron-translucent central portion of the lipid bilayer of the terminal cisternae (TC). She proposed that the junction consisted of a bilayer of lipids continuous with the lipids of the transverse tubule (T-tubule) and the TC.

Saito et al. (21) have shown asymmetry of the membranes of isolated sarcoplasmic reticulum (SR) vesicles as well as in thin sections of muscle. Trypsin digestion of membrane proteins caused thinning of the cytoplasmic leaflet. Vesicles reconsti-

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tuted following solubilization in cholate have symmetrical halves of the membrane. Wang et al. (26) have further demonstrated that the thickened appearance of the SR required the presence of proteins in the membrane. Campbell et al. (4) have also used tannic acid mordanting to enhance the images of isolated sarcoplasmic reticulum. They reported structures protruding from isolated terminal cisternae which disappeared after the vesicles were washed with 0.6 M KCl. They have argued that these structures are residual junctional "feet."

In this paper we have used tannic acid in combination with glutaraldehyde and postfixation with OsO_4 to study the morphology of the junctional region in native and rejoined triads, TC, T-tubules, longitudinal reticulum (LR), and a Triton-resistant fraction from TC/triads. Our intent has been to describe more fully the morphology of isolated vesicles and to correlate our observations with those of intact muscle. In the sequential dismantling of the original muscle structure during isolation of the components of the triad, morphological criteria remain among the few approaches currently available to determine the integrity and possible viability of these components.

MATERIALS AND METHODS

Preparation of Organelles

Purified organelles were obtained from the sacrospinalis muscles of rabbits by methods developed in our laboratory (2, 6, 16). A brief description is as follows:

TC/triads and LR were obtained by fractionation of skeletal muscle microsomes on continuous sucrose density gradients. The injection and incubation of the muscle, homogenization, and differential centrifugation were carried out as described by Caswell et al. (6). Homogenization was effected in 10 vol of 250 mM sucrose, 0.5 mM EDTA, pH 7.4, and the microsomes were washed once by centrifugation in 250 mM sucrose, 2 mM histidine, pH 7.3 (sucrose-histidine). The microsomes were fractionated on a continuous gradient. Gradient formation in the Sorvall TZ-28 zonal rotor (DuPont Instruments-Sorvall Biomedical Div., Newtown, CT) and centrifugation conditions were performed as described previously (2). TC/triads and LR were obtained from the bands at 40 and 30% sucrose, respectively. Vesicles were concentrated by dilution with one volume of water followed by centrifugation at 125,000 *g* for 45 min. Pellets were resuspended in a small volume of sucrose-histidine.

T-tubules and heavy TC were prepared by disruption of TC/triads as described by Lau et al. (16). TC/triads were passed through the French press at 5,000 psi, and the extrudate was then fractionated on continuous sucrose gradients by centrifugation at 141,000 *g* for 5 h in a SW 27 rotor (Beckman Instruments, Inc., Palo Alto, CA). T-tubules were collected as a distinct band from the 22–28% sucrose region and heavy TC from the 38–42% sucrose region. Vesicles were concentrated by dilution with 1 volume of water followed by centrifugation at 125,000 *g* for 45 min and resuspended in a small volume of sucrose-histidine.

Rejoined triads were prepared as described by Caswell et al. (7). TC/triads were disrupted in the French press at 8000 psi and diluted with an equal volume of 0.6 M K cacodylate, pH 7.2. This material was then fractionated on continuous sucrose gradients. Rejoined triads appeared as a dense, sharp band in the 40% sucrose region of the gradient. Free T-tubules were absent. The rejoined triad band was collected and concentrated as described for TC/triads.

The Triton-resistant vesicles were prepared from TC/triads by adding Triton X-100, 1% final concentration, to 3 ml of vesicle suspension. This was overlaid on a tube of a Sorvall TV-850 vertical rotor containing a continuous sucrose density gradient. A linear density gradient was employed between 10 and 55% wt/wt sucrose which was centrifuged at 150,000 *g* for 1½ h. A visible band of vesicular material with isopycnic point at approximately 45–50% wt/wt sucrose was withdrawn, diluted with an equal volume of water, and centrifuged at 150,000 *g* for 1 h. The pellet was resuspended in 3 ml of sucrose-histidine.

Electron Microscopy

All organelles were fixed for electron microscopy on the day of isolation. Vesicles were diluted in sucrose-histidine or in 0.5 M KCl where indicated and centrifuged at 77,000 *g* for 25 min in the Beckman SW 27 rotor. The tubes were drained and the pellet was immediately overlaid with 2.5% glutaraldehyde, 3% sucrose, 0.1 M Na cacodylate buffer containing from 0 to 8% tannic acid (Fisher Scientific Co., Fair Lawn, NJ), and sufficient NaOH to adjust the pH to 7.2. After tannic acid–glutaraldehyde fixation at 4°C for 12–24 h, samples were briefly rinsed three times in 0.1 M Na cacodylate buffer, cut into smaller pieces, and postfixed in 1% OsO_4 in 0.05 M Na cacodylate buffer for 2 h. The pieces of pellets were dehydrated in a graded ethanol and acetone series and embedded in Araldite. Thin sections (silver to gray) were cut with glass knives in a Sorvall Porter-Blum MT-2B ultramicrotome. Blocks were oriented in such a manner as to encompass the whole depth of the original pellet. Sections were stained with saturated uranyl acetate in 50% ethanol, counter-stained with lead citrate, and then examined in a Philips 300 electron microscope at 80 kV.

Measurements of parameters of the vesicles and membranes were made directly from prints using an ×7 magnifier containing a micrometer ruled in 0.1-mm divisions. Total magnification of the prints ranged between × 80,000 and 165,000. A minimum of 10 measurements was made for each parameter. In order to enhance the accuracy of the measurements, only micrographs in which the membranes were well defined were employed. Samples for determination of parameters of the junction also displayed visible connecting processes between the organelles.

Biochemical Assays

Protein was assayed by the method of Wang and Smith (25). Triton was assayed by the method of Garewal (11) with slight modification. Ammonium cobaltothiocyanate was prepared by mixing 17.4 g of ammonium thiocyanate and 2.8 g of cobalt nitrate hexahydrate with 100 ml of water. Samples from the gradient (0.2 ml) were mixed with 0.3 ml of ethanol and 1.0 ml of ammonium cobaltothiocyanate. This was shaken vigorously with 3 ml of ethylene dichloride and allowed to stand overnight. The lower layer was extracted and assayed in a spectrophotometer at wavelengths of 622 and 689 nm. The difference in absorbance of these two wavelengths was employed to estimate the Triton content.

When [^{3}H]ouabain was employed to track the T-tubules, the Ringer's solution employed to inject the intact muscle also contained 25 μCi of the label. Radioisotope was assayed by sampling 0.5 ml from the gradient and counting in a scintillation counter in the presence of Triton X-100.

For preparation of the gels, the samples from the gradients were collected, diluted with an equal volume of water, and centrifuged in order to concentrate them. When the vesicles were treated with KCl, they were suspended in 0.5 M KCl and layered on 20% sucrose in a Beckman SW 56, and centrifuged at 120,000 *g* for 1 h. Samples were placed in a standard 8% acrylamide Laemmli gel (15). The standards contained myosin, β-galactosidase, phosphorylase, and ovalbumin.

RESULTS

In order to assure obtaining the best electron-contrast enhancement, we examined the images after mordanting with different percentages of tannic acid (TA) in the presence of constant glutaraldehyde and found that the 4% TA-glutaraldehyde combination showed the best results. Therefore in all the electron micrographs shown, unless stated otherwise, that concentration was used.

Isolated TC/Triad Vesicles

Fig. 1 *A–E* shows selected examples of native triads fixed by the TA-glutaraldehyde method, whereas in Fig. 1 *F* no TA was employed. In most cases the narrow T-tubule can be readily discerned apposed by TC on both sides, although in a few cases the section shows apposition on one side only (Fig. 1 *B*). Each vesicle is composed of a continuous membrane without apparent breaks. In conventional fixation procedures of the triadic junctional region without TA, the gap (clear space) between the T-tubule and TC is hard to visualize and the definition of individual feet is poor (Fig. 1 *F*).

Both fixation procedures reveal the presence of an internal electron-dense aggregate in the TC vesicles. Several investigators (4, 5, 16, 18) have presented evidence that this electron-dense material contains the Ca^{2+} binding protein, calsequestrin. In the majority of triads the internal electron-dense matter is in juxtaposition to the junctional region between T-tubules and TC, but the electron-dense material is not apparently associated with the junctional region in Fig. 1 *D* and *E*. The TA mordanting procedure reveals a clear distinction between the TC membrane and the internal aggregate (short arrows). The middle portion of the trilaminar membrane, translucent in

appearance, and a cytoplasmic leaflet (towards the T-tubule), opaque in density, show clearly. In many instances portions of the outer leaflet of the membrane opposite the internal aggregate are considerably thickened in appearance (long arrow). We do not find this thickening in membranes that do not have apposed internal material. There is not a clear correlation between this thickened membrane and the junctional region in that some junctions do not show thickening of the TC mem-

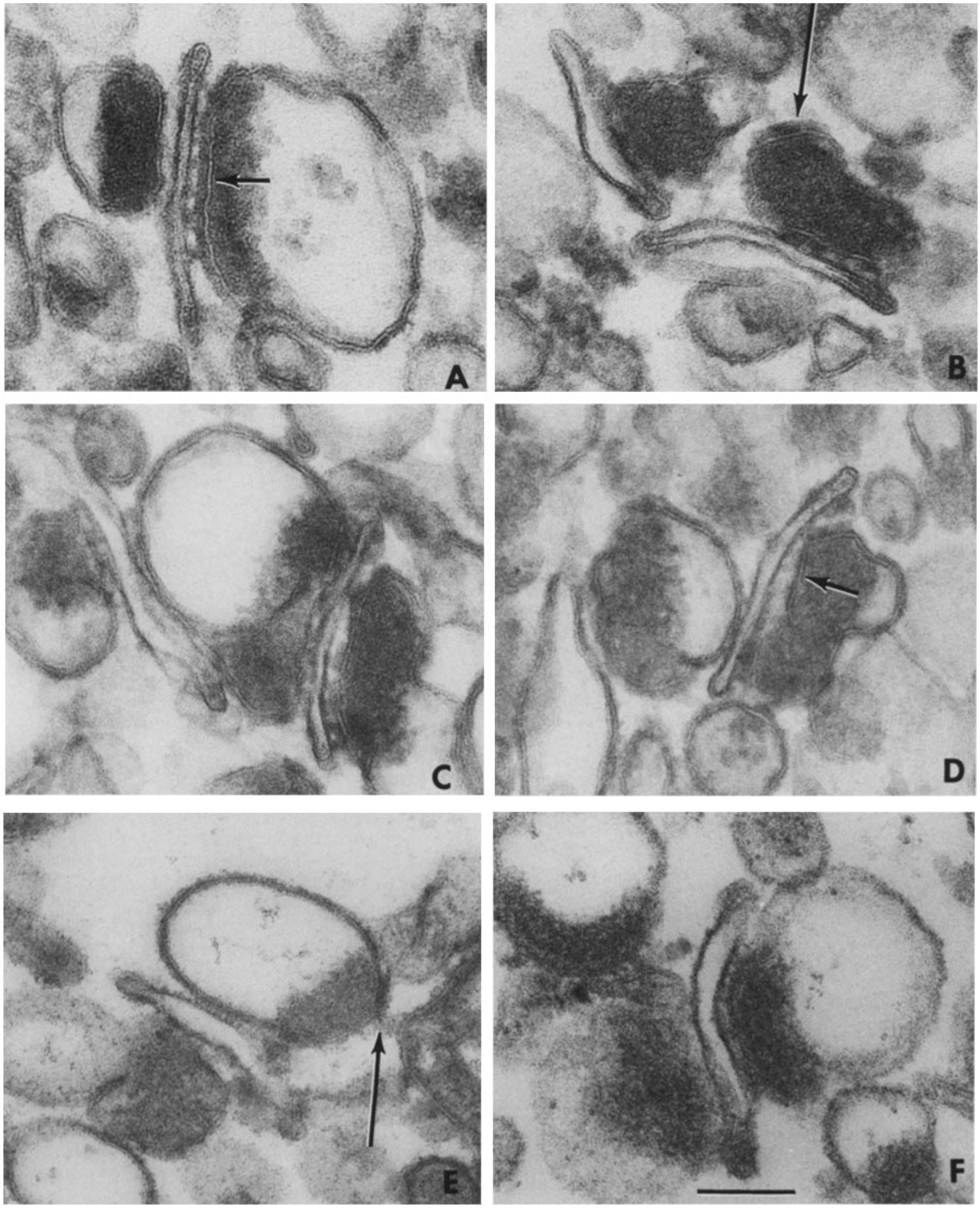


FIGURE 1 Native TC/triads obtained from the 38–42% band after fractionation on a sucrose gradient. *A–E* show selected organelles from that band fixed by the TA-glutaraldehyde mordanting method, whereas in *F* no TA was employed. Short arrows indicate regions where the TA mordanting reveals a clear distinction between the TC membrane and the internal aggregate. In many instances portions of the outer leaflet of the TC membrane opposite the internal aggregate are thickened in appearance (long arrows). The bar line in this and subsequent electron micrographs represents 1,000 Å.

brane, while in some nonjunctional areas a thickening occurs.

The most prominent feature exposed by the tannic acid is the visualization of the individual feet that span the gap between the TC and the T-tubules. Fig. 1 *A* and *B* clearly reveals a periodicity to the separation of the feet. Comparisons of this separation with that of intact rabbit sacrospinalis muscle (6) show a good correlation (280 Å in intact muscle, 280 Å in isolated triads). These feet or pillars are seen to traverse the full width of the junctional gap in making contact with the two organelles. This gives confirmatory evidence of our earlier observation that the T-tubules and TC are physically associated (6).

Fig. 2 *A* and *B* are TC/triads which have been incubated with 0.5 M KCl immediately before fixation in TA-glutaraldehyde. The KCl treatment causes two readily discernible effects: (*a*) The condensed appearance of the internal electron-dense matter of the TC has become more diffuse and of lighter density, suggesting that the calsequestrin is dispersed and separated from the membrane. This is in accord with observations (17) that KCl dissolves calsequestrin. (*b*) The thickening of the TC membrane opposite the calsequestrin is substantially or completely lost. On the other hand, the contact between the T-tubule and TC is still intact. Feet are still apparent, and we have found by comparison of triad junctions from the same preparation that KCl treatment does not significantly alter the integrity or number of junctions. We therefore conclude that the thickened membrane opposite the internal electron-dense matter is not an essential feature of the junction but appears to be associated with the region of anchoring the calsequestrin to the membrane.

Isolated TC, LR, and T-Tubule Vesicles

Fig. 3 *A* shows a typical field of isolated TC after tannic acid treatment. Thickening of the outer layer of the membrane was seen in direct apposition to the areas of internal electron-dense matter resembling the observations in the intact triad (arrow). This thickening of the membrane is not uniform but occurred as patches of variable lengths. We were not able to resolve this thickening into individual protrusions corresponding to the junctional feet as described by Campbell et al. (4), and we believe that this structure is more closely related to the anchoring of calsequestrin than to the junctional feet.

Fig. 3 *B* shows a representative field of LR vesicles after tannic acid mordanting. The vesicles are seen to have the intact membranes with well-defined trilaminar structure and asymmetry as described by Saito et al. (21). The interior of the vesicles shows little electron-dense material, and dense aggregates are not seen.

Fig. 3 *C* represents a typical field of isolated T-tubules. The middle (translucent) portion of their trilaminar membrane could be resolved in some sections, although the extreme curvature of the membrane frequently causes loss of resolution. Some diffuse electron-opaque material could also be seen protruding from the outer leaflet of their membrane (arrows).

Rejoined Triad Vesicles

Rejoined triads constitute a dense, sharp band obtained in the 40% sucrose region after the TC/triads are disrupted in the French press, diluted with an equal volume of 0.6 M K cacodylate (pH 7.2), and then fractionated on continuous sucrose gradients (7). Fig. 4 *A–E* is a composite of the rejoined triads which have been fixed in TA-glutaraldehyde. All the morphological features that were enhanced by the TA in the native triads described above are also seen in the rejoined triads, except that the feet between TC and the T-tubule are not so clearly defined. The rejoined triads display large regions of contact between the T-tubule and TC. The asymmetry of the trilaminar membrane and the thickening of the external leaflet of the TC membrane is clearly exposed by TA mordanting (arrow). In Fig. 4 *C*, one of the TC vesicles attached to the T-tubules lacks electron-dense matter in the region of the junction. When TA was not present in the fixative, the details of the membrane outline are not well resolved (Fig. 4 *F*). The figure reveals contact between TC and T-tubules such that the membranes of the two organelles are separated but electron-dense material spans the gap. In this sense the rejoined triads resemble intact triads (Fig. 1). However, the individual feet or pillars and the periodicity of their organization are ill defined. In Fig. 4 *A*, *C*, and *F* there is a suggestion of individual feet though the structure is more amorphous than in the intact triad. The appearance of the reconstituted triads was therefore broadly similar to that of the intact triads, but resolution of the individual feet was diminished.

Triton-Resistant TC/Triad Vesicles

The nonionic detergent Triton X-100 has been employed extensively to solubilize membrane proteins and to fractionate vesicular material. We found that 0.1% Triton effectively solubilized approximately 50% of protein from TC/triads, but that the rest of the protein was resistant to concentrations of Triton up to 2%. In subsequent experiments 1% Triton X-100 was employed. Fig. 5 shows the distribution of Triton X-100, protein, and [^{3}H]ouabain label on a linear density gradient after isopycnic centrifugation of a preparation of TC/triads which had been treated with 1% Triton X-100. Triton is found predominantly at the top of the gradient, indicating that little or no movement of the detergent into the gradient has been induced by membrane binding. The protein pattern shows three bands. The upper band presumably represents protein which has been effectively solubilized by the detergent and which does not sediment under the forces of the centrifugation. The band of highest isopycnic point at 45% wt/wt sucrose is present as an opaque band of vesicular material. Little if any of this band represents protein that has been dissolved and subsequently reconstituted by sedimentation out of the Triton inasmuch as a similar pattern is seen when the gradient itself contains detergent. Therefore most of this material represents protein which is resistant to solubilization by Triton. The [^{3}H]ouabain pattern from muscles which have been injected with the label has been employed by us in the past to track T-tubules. In Fig. 5 the [^{3}H]ouabain distributes as two bands, one of which is at the top of the gradient and the other of which has an isopycnic point identical to that of the third protein band (45% wt/wt sucrose). This indicates that a portion of the T-tubular protein is present in the dense band. Either the T-tubular membrane is not fully dissolved by Triton or the Na,K-ATPase has been reconstituted subsequent to detergent treatment. The isopycnic point of the visible band is 45% wt/wt sucrose; that of the intact TC/triads is 40% sucrose. This indicates a loss from the preparation of low density material which is probably lipid.

Fig. 6 is a representative field of Triton-resistant vesicles with isopycnic point at 45% sucrose after TA fixation. Vesicular remnants are heavily packed with electron-dense matter which presumably is identical to the material found within intact TC vesicles. This is confirmed by our finding of calsequestrin in

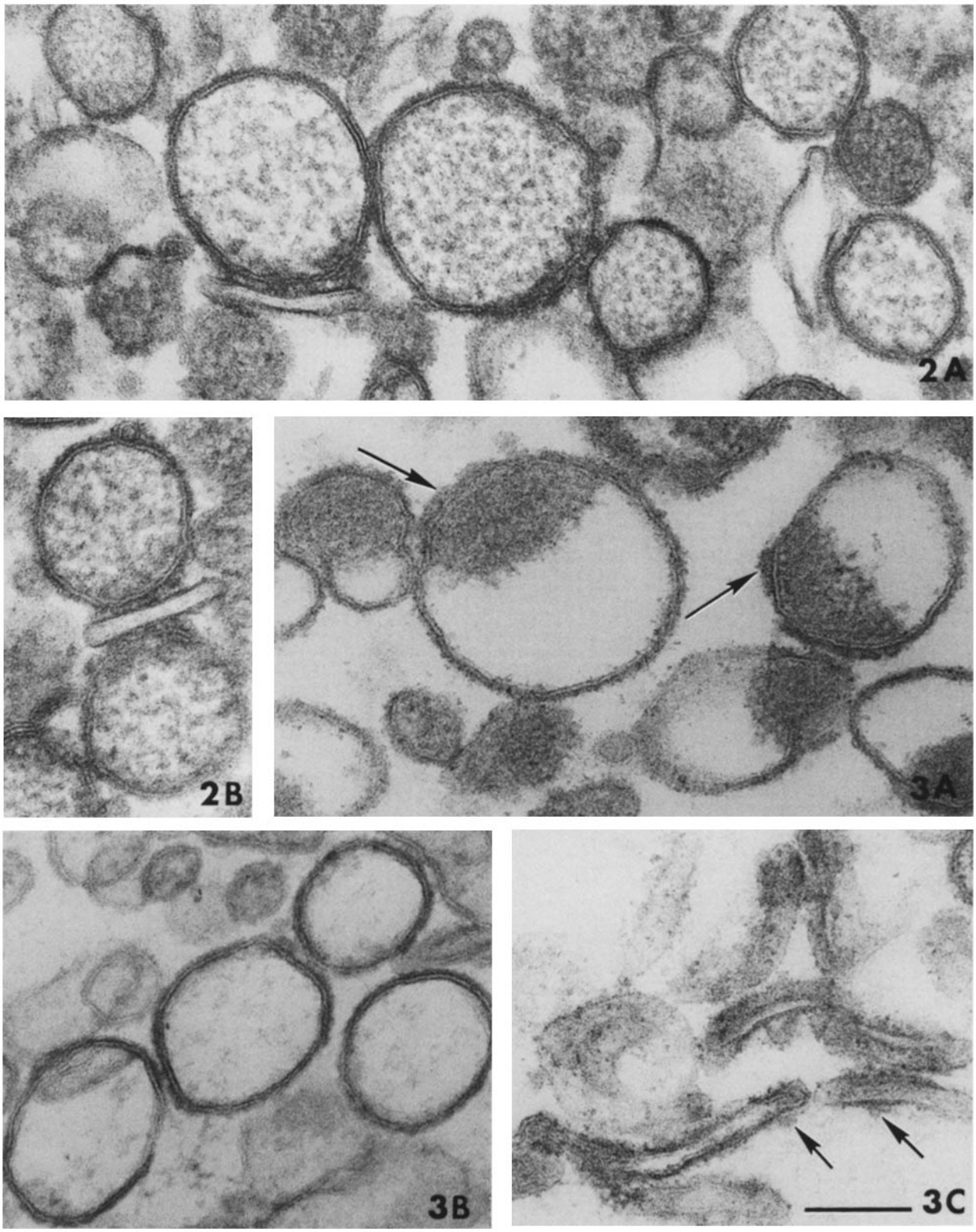


FIGURE 2 TC/triads treated with KCl before fixation with TA-glutaraldehyde. Note that the internal electron-dense matter and the thickening of the outer leaflet of the TC are substantially lost. On the other hand, the contact between T-tubule and TC is still intact.

FIGURE 3 Typical fields of isolated organelles fixed by the TA mordanting procedure. *A* represents a field of heavy TC. Thickening of the outer layer of the membrane is seen in direct apposition to the areas of internal dense matter. This occurred as patches of variable lengths (arrows). *B* represents a field of LR vesicles. *C* shows a field of T-tubules. Some diffuse electron-opaque material can be seen protruding from the outer leaflet of their membrane (arrows).

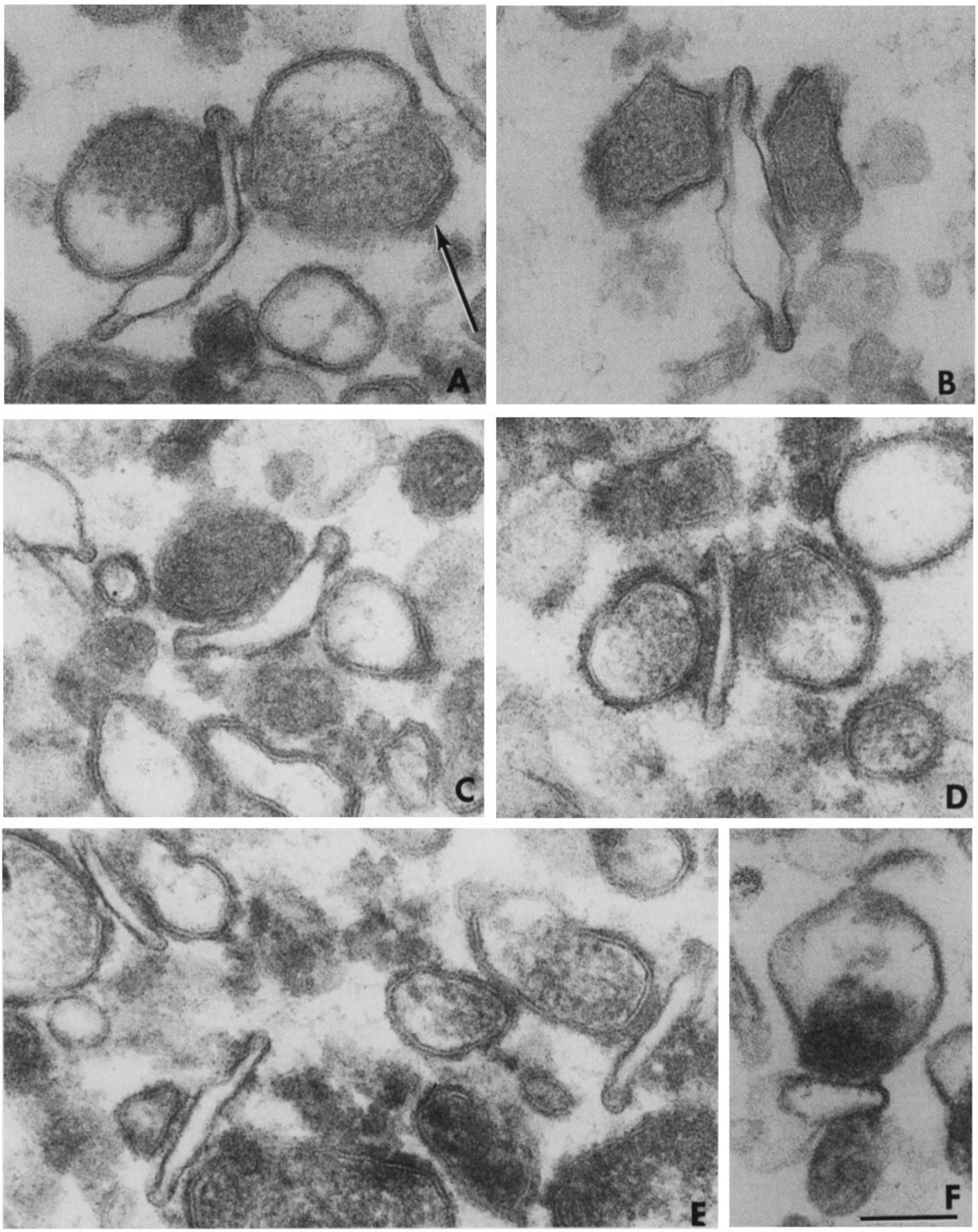


FIGURE 4 Composites of reconstituted triads obtained by rejoining free T-tubules and TC. *A–E* show the organelles fixed by the TA mordanting procedure using 4% TA, except *D*, in which 6% TA was used. In *F*, no TA was present in the fixative. The asymmetry of the trilaminar membrane and the thickening of the external leaflet of the TC membrane are well visualized as patches along the TC membrane (arrow).

the Triton-resistant vesicles (Fig. 8). In contrast to that of the intact triad, the membrane of the TC is absent except in immediate proximity to the electron-dense aggregate. This loss of membrane correlates with the diminution in relative content of Ca ATPase described in Fig. 8. The area of membrane that is preserved (long arrow) bears a striking resemblance to the thickened patches of membrane seen in intact TC (Figs. 1 and 3 A). Thus the electron-translucent middle of the trilaminar

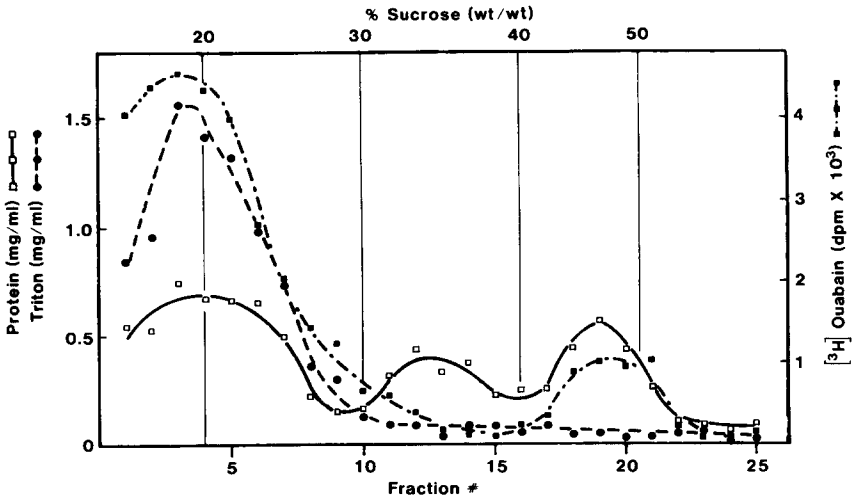


FIGURE 5 Density gradient profile of TC/triad vesicles after treatment with Triton X-100. The preparation of organelles, treatment with Triton, and biochemical assays are described in Materials and Methods.

structure is seen together with the broad outer leaflet.

The most interesting structural feature of the Triton-resistant fraction is the presence of TC vesicles which make contact with external intact or residual vesicular membrane. The overall morphology seen in a number of vesicles is a thickened TC membrane in contact with, but separated from, a membrane fragment. The gap between the two membranes is approximately 114 Å (short arrow). Some amorphous electron-dense matter is seen in this gap. In some cases the membrane to which the TC is joined is a fully formed elongated vesicle which resembles an intact T-tubule (thick arrow). We believe that the membrane fragments to which the TC is joined represent the junctional portion of the T-tubule membrane, and that many features of the junctional region of the triad are retained after treatment of the TC/triads with Triton X-100.

Fig. 7 shows the precipitated material from the Triton-resistant fraction which has been subsequently treated with 0.5 M KCl. Some sharply defined trilaminar membranes are seen (arrowheads) which closely resemble the T-tubule membrane fragments in Fig. 6. The condensed appearance of calsequestrin seen in Fig. 6 is substantially lost or dispersed. However, residual membrane fragments which resemble the TC membrane in Fig. 6 are still observed (curved arrows), frequently as short segments. These show thickened membranes with some electron density on both sides of the translucent central leaflet. Although intact junctions are no longer easily identified, the sharply defined membrane that is probably T-tubular may still be seen to be in close proximity to the remnants of TC vesicles, suggesting that the triadic junction is still intact though morphologically altered.

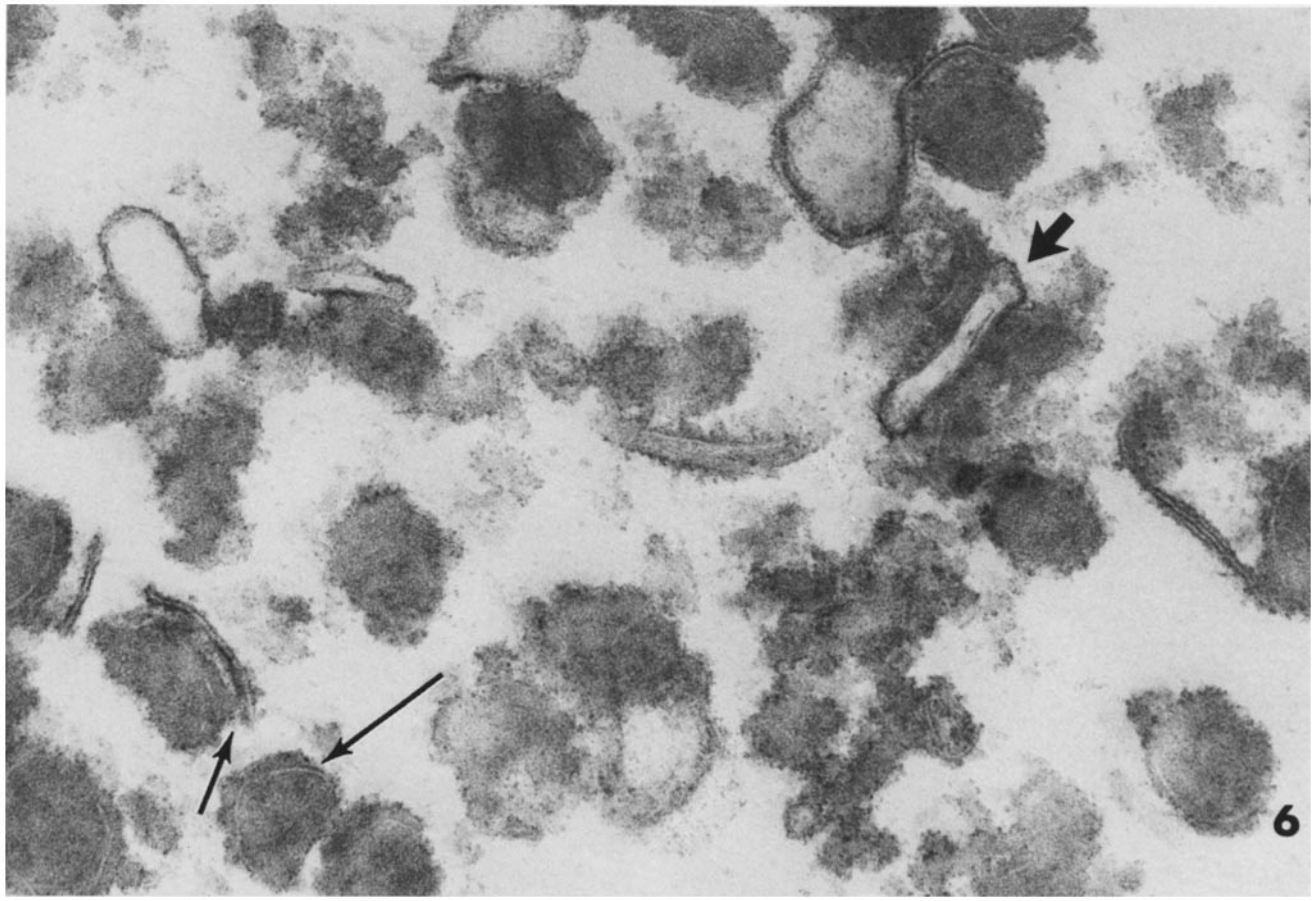
The pattern of solubilization by Triton X-100 and KCl is illustrated by SDS gel electrophoresis in Fig. 8. The intact TC/triads (lane *1*) display a normal pattern of proteins, with the Ca pump and calsequestrin predominating. A protein doublet of high molecular weight (designated HMW) is also present which has been identified by Cadwell and Caswell (3 and following article) as a constituent of the junctional complex. The Triton-resistant fraction from TC/triads treated with Triton X-100 differs from that of the intact TC/triads in that the Ca pump is markedly diminished in intensity (lane *3*) whereas the supernate from Triton treatment contains predominantly the Ca pump (lane *2*). Those proteins that are not dissolved by Triton treatment appear in the gel with enhanced intensity in that the amount of total protein added to each gel was the same. A number of proteins are therefore seen as more intense bands including calsequestrin and the high molecular weight doublet. Treatment of TC/triads with 0.5 M KCl causes partial or complete solubilization and separation of proteins of M_r 77,000, 43,000, and 38,000 (lane *4*). Little, if any, calsequestrin is separated by this protocol, probably indicating that, although calsequestrin may be dissolved by KCl, it cannot escape from the interior of the vesicle. Lane *5* indicates that the sediment after KCl extraction still contains Ca pump, calsequestrin, and the junctional doublet (HMW). KCl treatment of the Triton-resistant fraction differs from that of intact triads in that calsequestrin is significantly separated from the sediment, while the protein of M_r 77,000 is not substantially removed (lane *6*). The sediment shows substantial enhancement of high molecular weight proteins (including the junctional doublet) compared with TC/triads (lane *7*).

Morphometric estimates of our intact and isolated skeletal muscle organelles show that a high degree of correlation between these systems is observed. At the junctional region the gap between TC and T-tubule ranges from 90 ± 17 to 108 Å ± 34, showing considerable consistency from intact muscle through isolated triads and reconstituted triads. Other observers (8, 10, 13, 24) have measured the triadic junctional gap in nonmammalian skeletal muscle with a range from 110 to 180 Å. The feet width and periodicity also show a good consistency between intact and isolated material. These values do not differ significantly from those reported by others showing feet width of 100–300 Å and periodicity of 250–300 Å (8, 10, 13, 24).

DISCUSSION

The morphological parameters of the triadic junctional region *in situ* have been extensively investigated in order to elucidate the physiological and biochemical cascade that arises after excitation of the muscle (9, 10, 13, 14). In this paper we have investigated the morphology of isolated vesicles and enriched fragments that constitute the junction between T-tubules and TC with a view to (*a*) determine the degree of correlation with intact muscle and (*b*) delineate the structures in the membrane that are truly associated with the junction from those that are involved in other morphological features. We have used tannic acid to highlight the fine detail of the ultrastructure of our isolated and rejoined organelles. Tannic acid mordanting has permitted us to resolve in many cases the individual feet that form the connections of the triad junction and has permitted the delineation of the terminal cisterna membrane from the attached internal electron-dense material.

The preparation of isolated TC/triads has been our starting



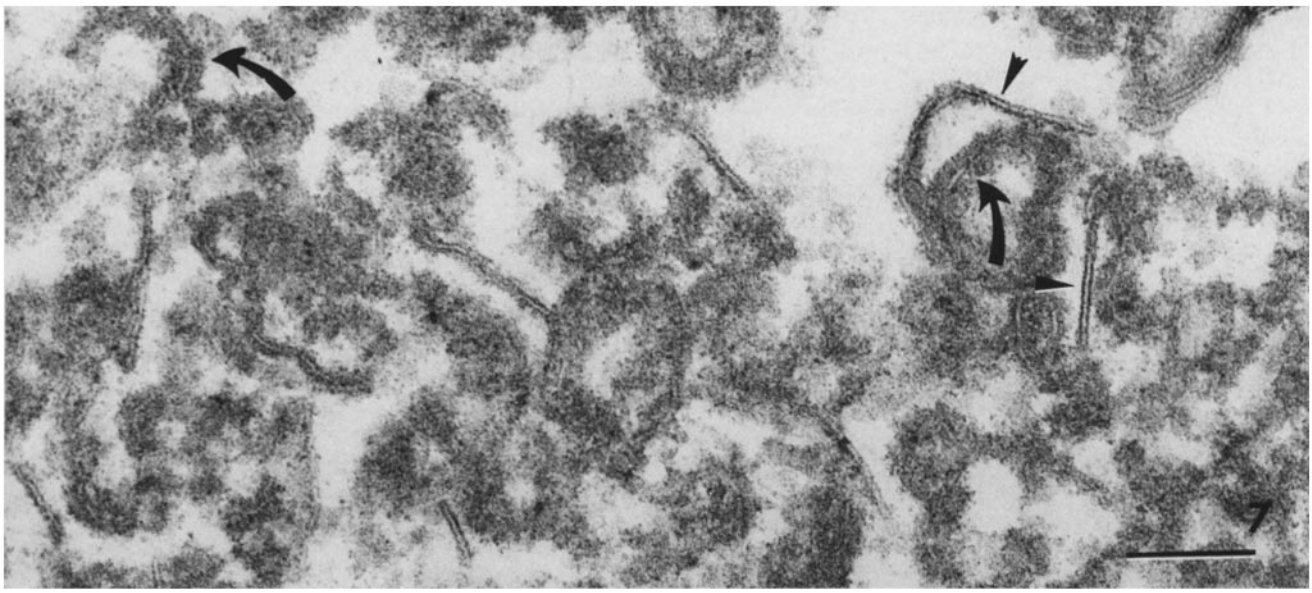


FIGURE 6 Triton-resistant TC/triad vesicles with isopycnic point at 45% sucrose fixed with TA. Some areas of the Triton-resistant vesicles show preservation of their trilaminar membrane with thickening of the outer leaflet (long arrow). Short arrow indicates a TC vesicle that is in contact with, but separated from, a membrane fragment. In other instances the membrane to which the TC vesicle is joined is an elongated vesicle resembling an intact T-tubule (thick arrow).

FIGURE 7 Precipitated material from the Triton-resistant fraction that was treated with KCl and fixed with TA-glutaraldehyde. Trilaminar membranes resembling T-tubule membrane fragments (arrowheads) and membrane fragments resembling TC membrane (curved arrows) as seen in Fig. 6 are still observed. Much of the electron-dense aggregate of Fig. 6 is lost.

point for further fractionation of the organelles that contain the junction. We have been concerned at all stages as to whether our preparation is biochemically and physiologically intact. However, we still have limited knowledge of the biochemical properties of the junction and have therefore employed morphological criteria for the integrity of the components of this complex.

Our data indicate that a discrete portion of the TC/triad

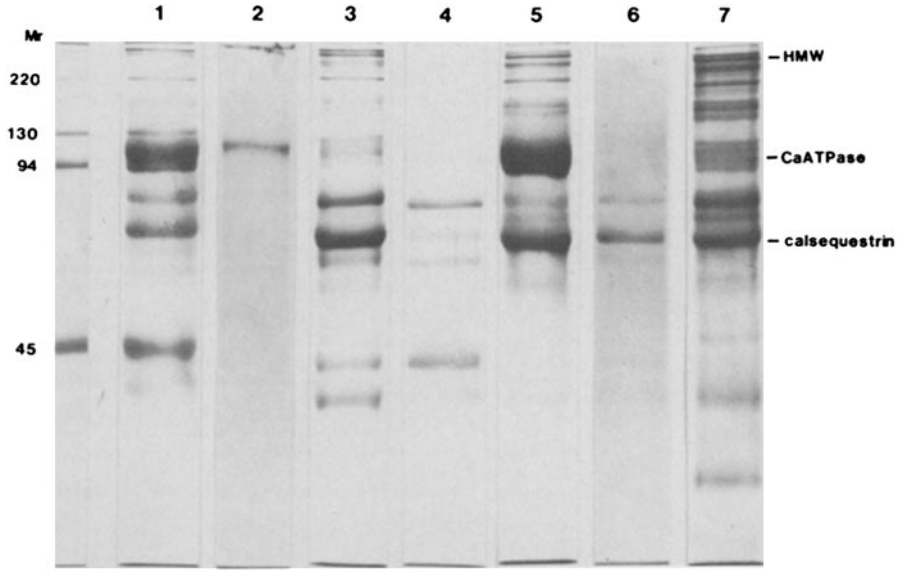


FIGURE 8 Lane *1* is intact TC/triads (20 μg). Lane *2* is supernatant from Triton X-100 extraction of TC/triads (7 μg). Lane *3* is Triton-resistant vesicles (20 μg). Lane *4* is supernatant from KCl extraction of TC/triads (7 μg). Lane *5* is sediment from KCl extraction (20 μg). Lane *6* is supernatant from KCl extraction of Triton-resistant fraction (7 μg). Lane *7* is sediment from KCl extraction of Triton-resistant fraction (20 μg). The unmarked lane shows M_r standards ($\times 10^{-3}$).

preparation is dissolved neither by Triton nor by prior or subsequent treatment with hypertonic KCl. The gel data, the [^{3}H]ouabain assay, and the electron microscopy all support the view that the resistant portion is a fraction of the TC/triads which is distinct from the general SR membrane. The moiety contains the extrinsic protein, calsequestrin, the TC membrane that is in contact with the electron-dense matter, and portions of the T-tubule membrane which are attached to the TC.

We operationally define the junctional complex as constituting the spanning material that holds the membranes of the two organelles together and any anchoring material in the membranes which is responsible for (*a*) attaching to spanning material, (*b*) maintaining the spatial properties of the junction both perpendicular and parallel to the membranes, and (*c*) holding any other material in a special configuration with respect to the junction. Using this definition, we argue that the Triton-resistant fraction is enriched in junctional complex. T-tubular membrane is held in apposition to the TC separated by a gap of 108 Å which is similar to the value of 90 Å observed in isolated triads. The TC membrane in this region retains its morphology in that the trilaminar leaflet is visible. On the other hand, Ca ATPase, the major intrinsic protein of the SR, is greatly diminished and the general SR membrane is dissolved. The Ca pump is known to be the major protein of LR and may be occupying the bulk of the nonjunctional membrane of TC, since this is continuous with the LR. The retention of a visible membrane structure in the TC and T-tubules after treatment with Triton suggests that this structure in the specialized region of the junction is held together substantially by protein-protein interactions. Other specialized membrane regions such as the gap junction also survive mild detergent treatment. These organized arrays appear to be less dependent than general membranes on the lipid bilayer for their associative forces.

Our data here indicate that electron-dense matter remains as an aggregate in Triton-resistant vesicles. Because the material is known to contain calsequestrin (18), we believe that this protein exists in the vesicle as an aggregate which may be either crystalline or in the form of a gel. This aggregation is not an artifact of fixation procedures because disruption of the TC membrane does not dissolve the protein, whereas if this protein were in solution, it would remain in the supernatant after treatment with Triton. The TA mordanting reveals a close association between calsequestrin and portions of the TC membrane. In intact and detergent-treated vesicles the electron-dense matter appears continuous with the inner leaflet of the trilaminar membrane. Moreover, a thickening of the outer leaflet is also observed in TC membrane areas apposed to electron-dense matter. Although this thickening is not continuous, we do not find thickening in membrane regions devoid of attached electron-dense matter. This morphology suggests that calsequestrin is anchored to the luminal face of the TC membrane, and that a transmembrane structure exists such that the cytoplasmic surface is also part of the organization.

The thickened TC membrane has been described by Campbell et al. (4) as projecting feet. Three observations permit us to distinguish this thickened membrane from the structures that join T-tubules to TC: (*a*) In some cases we observe both the thickened membrane and the junctional connections in the same area (Fig. 1*A*). In this case the thickened membrane is distinct from the projections. (*b*) In many cases the junctional connection occurs without evident thickening of the TC membrane. Thus, the thickening of the TC does not correlate with the junctional region. (*c*) We find in common with Campbell et al. that KCl removes the thickening of the membrane from the TC, but that KCl has no evident effect on the number or appearance of triad junctions. On the other hand, this thickened membrane is invariably associated with the anchoring of calsequestrin to the membrane. Whether the thickening is a necessary and causative part of the association between the electron-dense matter and the membrane or whether it is incidental is not yet clear. Treatment with KCl causes both the disappearance of the thickening and dispersion of the electron density. However, since it is known that KCl dissolves calsequestrin, we cannot evaluate whether KCl has dispersed calsequestrin by direct dissolution or by removing a necessary membrane component which is responsible for the anchoring.

Treatment of the intact triads with KCl causes loss of membrane thickening. This treatment causes dissolution and separation of several proteins including ones of M_r 75,000, 41,000, and 37,000. Treatment of the Triton-resistant fraction with KCl appears not to influence substantially the thickness of the

terminal cisterna membrane. This KCl treatment causes removal of much calsequestrin but apparently does not substantially release the three proteins cited above. We believe that one or more of the above extrinsic proteins is responsible for the membrane thickening in apposition to the internal electron-dense matter.

We have observed loss of some morphological characteristics as the triad is dissected. Thus, the individual junctional feet in reconstituted triads or in the detergent-resistant band are not well resolved. The existence of a bridging structure between the organelles is apparent but its organization in arrays has deteriorated. We do not know whether the molecular constituents have been altered or whether the pattern of association has been modified. Considering the extensive treatments necessary to prepare enriched or reconstituted triads, this small degree of disorganization is not surprising. In the course of enriching the triad junction by successive treatment of TC/ triads with Triton X-100 and KCl, we have diminished the content of proteins such as Ca pump and calsequestrin as well as other minor proteins but have enhanced the intensity of others including the high molecular weight doublet described in the accompanying paper as the spanning protein of the junction. The correlation of protein constituents with electron microscopically identified membrane communication of the triad therefore offers an approach to understanding the architecture of the junction. Many of the structures of the triad are retained, and this offers promise of our being able to extract and reassemble functional constituents.

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