

STUDIES OF THE TRIAD

I. Structure of the Junction in Frog Twitch Fibers

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ABSTRACT

The structure of the junction between sarcoplasmic reticulum (SR) and transverse tubular (T) system at the triad has been studied in twitch fibers of the frog. The junction is formed by flattened surfaces of the SR lateral sacs and the T-system tubule, which face each other at a distance of 120–140 Å. At periodic intervals of about 300 Å, the SR membrane forms small projections, whose tips are joined to the T system membrane by some amorphous material. The SR projections and the amorphous material are here called SR feet. The feet are disposed in two parallel rows, two such rows being present on either side of the T-system tubule. The junctional area between the feet is apparently empty. The feet cover no more than 30% of the T system surface area and 3% of the total SR area. The functional significance of this interpretation of the junctional structure is discussed.

INTRODUCTION

In the triads of vertebrate muscle fibers, one tubule of the transverse tubular (T) system and two elements of the sarcoplasmic reticulum (SR) come to apposition. Pentads (Revel, 1962), dyads (Smith, 1961; Rosenbluth, 1969), and couplings (Johnson and Sommer, 1967) are the corresponding structures in a variety of muscles. These are characterized, as indicated by the name, by a different number of composing elements. There is now general agreement that, at the level of the triads or equivalent structures, events occur which link the depolarization of the T system with the release of calcium from the adjacent sacs of the SR (see Ebashi and Endo, 1968).

It has been tempting to assume that SR and T system are electrically coupled at the triad and, on that assumption, morphological details of the triadic junction structure have been related to those of known loci of electrical coupling between cells (Fahrenbach, 1965; Peachey, 1965; Walker

and Schrodt, 1965; D. E. Kelly, 1967). The results of this study indicate that the structure of the triad in frog fibers is distinct from that of any known low-resistance junctions and also that the triad is badly designed to function as a "capacitative" coupling.

MATERIALS AND METHODS

Sartorius muscles of *Rana pipiens* were fixed in paraformaldehyde-glutaraldehyde (Karnovsky, 1965). Superficial bundles of fibers were dissected after 2 hr fixation, washed overnight in 0.1 M cacodylate buffer containing 10% sucrose, post-fixed in 1% OsO₄ in 0.1 M cacodylate buffer for 2 hr, and, following dehydration, immersed in a saturated solution of uranyl acetate in absolute alcohol for ½ hr. Embedding was done in Maraglas (Polysciences, Inc., Rydal, Pa.) Sections were cut on a Cambridge (A. F. Huxley pattern) microtome and stained in lead citrate (Venable and Coggeshall, 1965), either alone or preceded by uranyl acetate. Micrographs were taken in an AEI 6B microscope, which was cali-

brated at intervals by using a diffraction grating replica.

RESULTS

The T-System Openings

Experiments with electron-opaque tracers and fluorescent dyes leave no doubt that the T system of frog fibers, twitch and slow, is open to the outside (Endo, 1964; H. E. Huxley, 1964; Page, 1965; Eisenberg and Eisenberg, 1968; Peachey and Schild, 1968). However, a direct visualization of the continuity of the T-system membrane with the sarcolemma has proved to be very difficult, despite the widespread use of glutaraldehyde, a fixative which is known to preserve the continuity of the T system in fibers from other animals (Franzini-Armstrong and Porter, 1964). It seems worthwhile to present here one of the two unequivocal examples of T-system openings in frog fibers that I have encountered in the course of this study, particularly since this example sheds some light on why it is so difficult to obtain such an image.

Fig. 1 illustrates the peripheral portion of a fiber, cut longitudinally. As it approaches the sarcolemma, the T system is typically enlarged and often convoluted (see Peachey, 1965). The lateral sacs of the triad are apposed to it up to the very edge of the fiber, as shown in the two triads which have been cut tangentially just under the sarcolemma. In one of the two triads the T-system opening is visible; an enlargement of the same area is shown in Fig. 2. The final segment of the T system, once it leaves the lateral sacs of the triad, becomes narrower and bends, so as to run almost parallel to, rather than perpendicular to the sarcolemma.

In the particular case here illustrated, the twisting peripheral segment of the T-system tubule is 210–240 Å wide and, assuming that it has a circular cross-section, one expects the wall of the tubule to be included in the plane of the section. The gray material apparently filling the tubule may thus be interpreted as a grazing view of its limiting membrane. The same is true at the mouth, where the diameter of the T tubule is 130 Å: the faint line apparently crossing the mouth is most probably its perimeter.

The Triad

Once it penetrates into the fiber, the T system is followed in its course by two enlarged cisternae

of the sarcoplasmic reticulum: the lateral sacs of the triad (Porter and Palade, 1957). In frog twitch fibers, the lateral sacs of the triad are continuous across the fiber, with few interruptions, as can be estimated from the fact that one seldom notices triads in which a lateral sac is missing (Fig. 7). The whole T system, thus, participates in the formation of triads, and most of the T-system membrane (about 80%, as calculated by Peachey, 1965) faces the SR membrane. The following description focuses on the structural details of the triadic junction: i.e., the space that separates the apposed SR and T system membranes.

It is convenient to define a long axis of the triad: this coincides with the long axis of the T-system tubule, therefore, it is transverse to the long axis of the fiber. In longitudinal sections of the fiber, the plane of sectioning may be either: (a) parallel to the long axis of the triad, so that an extended view of the junction is provided (Fig. 3), or (b) perpendicular to the long axis of the triad, so that T system and lateral sacs are cut crosswise (Fig. 7). Owing to the irregularly polygonal shape of the fibril, planes a and b are found mixed at random in any longitudinal section and thus often conveniently side by side in the same micrograph (Figs. 1, 3). Cross-sections of the fiber (plane c), provide a third view of the triad, which is at right angle to the two previously mentioned.

In plane a, the space between SR and T system may have three appearances: (a) SR and T-system membranes run parallel at a distance of 100–130 Å. Periodically repeating small densities, apparently joining the facing membranes, occupy the junction: these will be referred to as SR feet. The feet repeat at intervals of 280–300 Å, and they are 150–180 Å wide and are separated by less dense areas, 120–140 Å wide. Rows of feet may be followed for up to 1 μ , when the section is favorably oriented (Figs. 3, 4). (b) The SR and T-system membranes run parallel and are separated by an empty gap. This usually occurs where SR and T system are about to leave the plane of sectioning (arrows, Figs. 3, 4). (c) The separation of SR and T-system membranes is not uniform. Instead, the SR membrane is scalloped, with the period of the scallops coinciding with the position of the feet; i.e. the SR membrane gets closer to the T system, wherever a foot is present (S, Figs. 3, 4 and Figs. 5, 6). The apparent depth of the scallops is variable, even in the same image. In some

cases, particularly in thin sections (Figs. 5, 6), the SR scallops cross most of the junctional gap. Actual contact of the tips of the scallops with the T-system membrane was never noticed in these preparations. As measured from Figs. 5 and 6 (at *S*), the remaining gap is at least 40–60 Å. A more accurate measurement of the exact separation of SR and T-system membranes is not possible, owing to uncertainty in determining the exact boundaries of the SR membrane where it is curved and accompanied by the density of the amorphous material forming the feet. Material stained in lead only, in which this material is less contrasty (see below), cannot be used for these measurements, because the two leaflets of the SR membrane are not resolved.

Most frequently, the SR membrane scalloping is only slight, and something intermediate between appearances described under (*a*) and (*c*) results.

The three types of images can be interpreted as resulting from sections along a row of feet, with the plane of section being oriented almost perfectly parallel to the row in all cases, but at different levels relative to it. Thus, image (*c*) arises when the plane of section coincides with the center of the row; image (*a*) arises when the plane is off center, and image (*b*) when the row of feet is not included in the plane of sectioning. Each foot is made up of an approximately hemispherical projection of the SR membrane and of some amorphous material, which joins a distal segment of the hemisphere to the T-system membrane.

In plane *b*, the T tubule is cut exactly in cross-section and it has, in aldehyde-fixed material, an oval shape, the longer diameter being directed across the fiber and the two flat faces facing the SR (Fig. 7). The lateral sacs of the triad, also cut cross-wise relative to their long axis, have a flattened face against the T system. In the space between SR and T system are two spots (Figs. 7, 9, 10) which are identified as two of the feet visible in plane *a*. The center-to-center distance between the feet is variable between 400 and 550 Å. On either side of the feet, the SR and T-system membranes run approximately parallel for a

short distance and then separate. The two feet are obviously discrete and separated by a less dense area (Figs. 9, 10). The SR membrane is usually slightly scalloped in correspondence with the two feet.

The view derived from planes *a* and *b* are interpreted as indicating that the feet are disposed in two rows.

Plane *c*, obtained when the fiber is cut in cross-section, shows the two rows of feet as two dense lines, running parallel between SR and T system, at a center-to-center distance of approximately 500 Å (Figs. 11, 12). The over-all density of the junctional area in plane *c* is due to the superimposition in the image of tangentially cut SR and T-system membranes, as well as, in most cases, of some of the dense material filling the lateral sacs of the triad. As a result, the two rows of feet are visible, but the details are none too clear. Only occasionally one can distinguish the individual feet forming the rows as separate densities (arrows, Figs. 11, 12) whose center-to-center distance along the row is the same as that separating the feet in plane *a* of sectioning. A more detailed picture of the feet has been obtained by Kelly and Cahill (1969) using very thin sections.

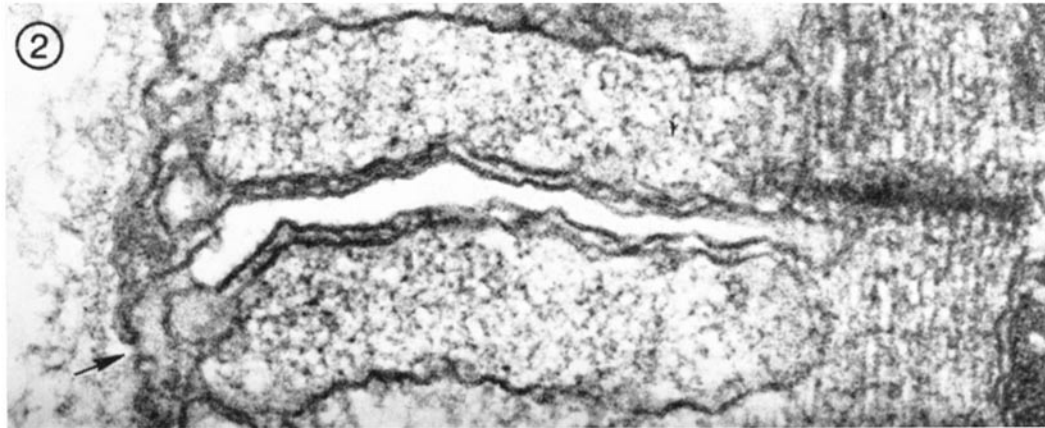
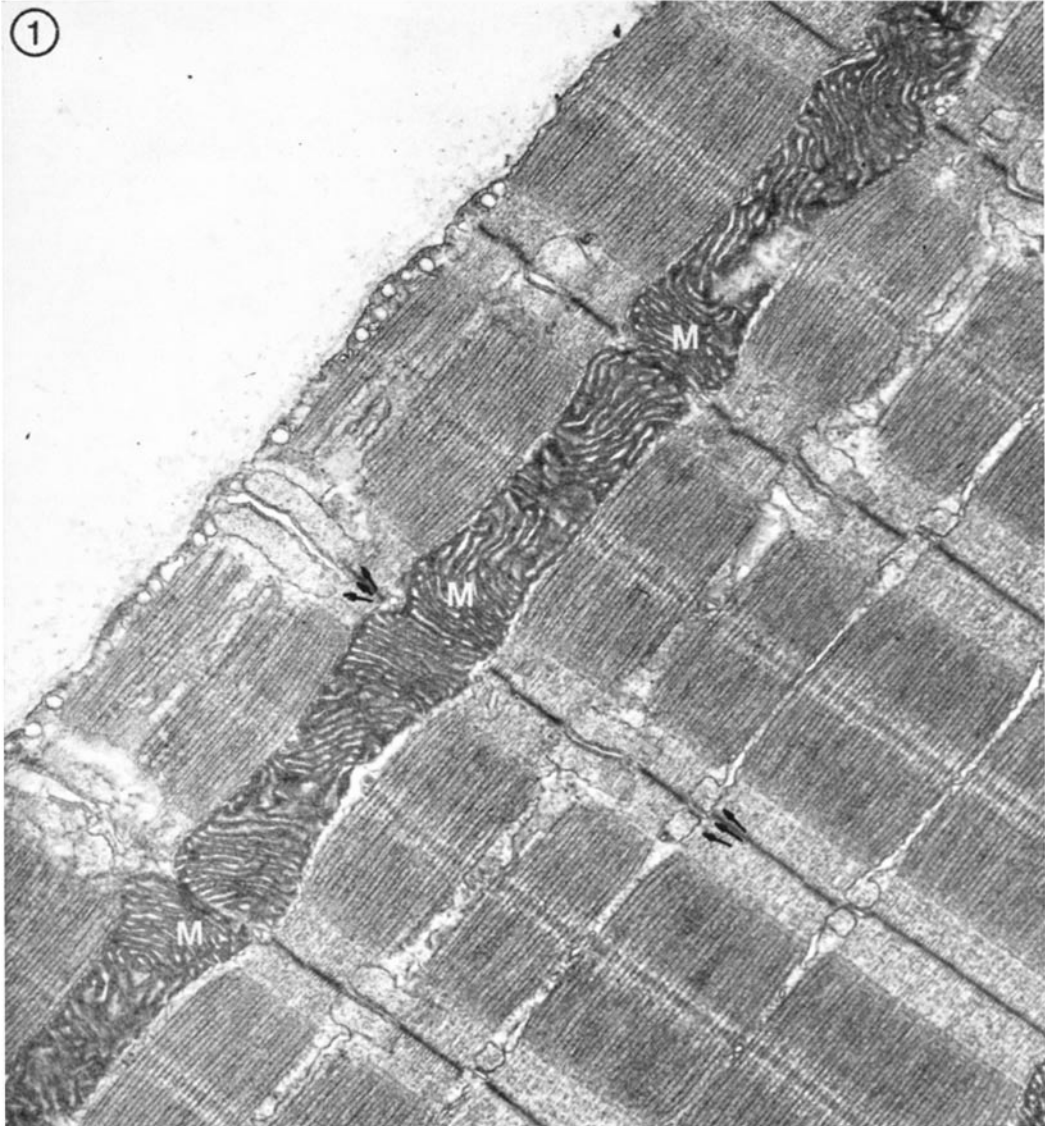
All micrographs in this paper are of material in which the contrast has been enhanced by lead and uranium salts (see Methods), except for Fig. 8. This figure is of material exposed to lead salts only, and the appearance of the triadic junction is different in one significant detail. Namely, whereas SR and T-system membrane have a good contrast, and a slight scalloping of the SR membrane is noticeable, the rest of the feet are barely visible. Thus, the material forming the last portion of the feet differs in its staining properties from the SR and T-system membranes. The importance of this detail will be considered in the discussion.

The Lateral Sacs of the Triad

Prefixation in paraformaldehyde and glutaraldehyde, as opposed to either prefixation in glutaraldehyde alone or direct fixation in osmium

FIGURE 1 Longitudinal section at the periphery of a twitch fiber. Under the sarcolemma are numerous caveolae. Two triads (three arrows) can be followed to the periphery of the fiber. *M*, mitochondria. $\times 26,000$.

FIGURE 2 Detail of one of the triads shown in Fig. 1. An arrow points to the T-system opening. $\times 120,000$.



tetroxide, is particularly promising in the study of the structure of the content of the lateral sacs of the triad. It has long been known that, unlike the rest of the sarcoplasmic reticulum, the lateral sacs of the triad are filled by some not well defined material. This has been described as: "a fine meshwork" (Revel, 1962), "granular content" (Peachey, 1965), "membrane-like structures" (Walker and Schrodt, 1966), "rather dense content" (Pellegrino and Franzini-Armstrong, 1969). Obviously, the structure has not yet been resolved, and this seems to be a problem of fixation, and in most preparations, the material filling the lateral sacs of the triad is variable in appearance (being more or less coarse and granular), and often it is in the form of a coarse precipitate, which does not completely fill the sacs, as if it had retracted. In the material presented here, on the other hand, the content of the lateral sacs is in the form of a delicate meshwork, which completely fills the lateral sacs. Occasionally, small hexagons can be distinguished (Fig. 4, asterisk). It is hoped that with even better preservation and the use of stereoscopic images the details of the structure of the meshwork of the lateral sacs may soon be worked out.

DISCUSSION

The T System

In a number of fibers from higher vertebrates, the final segment of the T system is narrow and tortuous, so that it is rarely included in the plane of the sections for any distance, except in rare cases (Walker and Schrodt, 1966; Rayns, Simpson, and Bertaud, 1968). Also, the T-system mouth is small and undistinguishable from the openings of the numerous "caveolae," which line the sarcolemma (Ezerman and Ishikawa, 1967; Rayns et al., 1968; Schiaffino and Margreth, 1968). My observation on the T-system opening in frog fibers, to which similar unpublished observations by B. Eisenberg and L. D. Peachey should be added,

indicates that the same is true in frog fibers. The twisting of the last portions of the T system is, in itself, sufficient to explain why, even though triads can be followed to the edge of the fiber with relative frequency, the T-system mouth is so rarely detectable. Perhaps a second factor is at play: in the local stimulation experiments of A. F. Huxley and Taylor (1958), it was noticed that in frog fibers the sensitive spots, i.e., probable sites of T-system opening, were present only at average circumferential intervals of about 5 μ . Thus, at any time the T system may be open only once every few fibrils around the circumference. Unfortunately, as indicated above, electron microscopy of thin sections cannot provide an indication on the number of T-system openings per unit surface area.

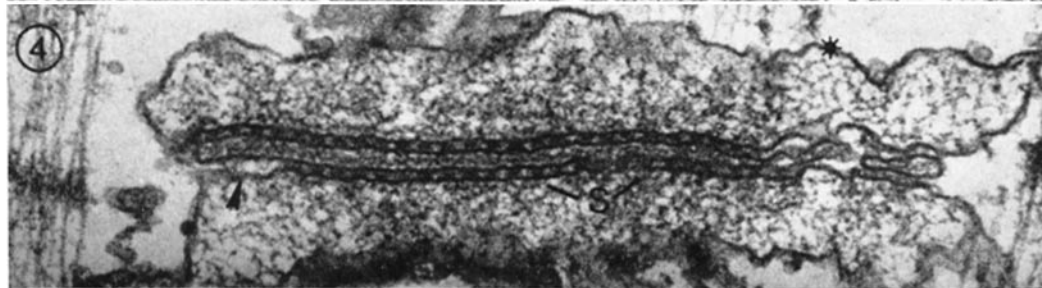
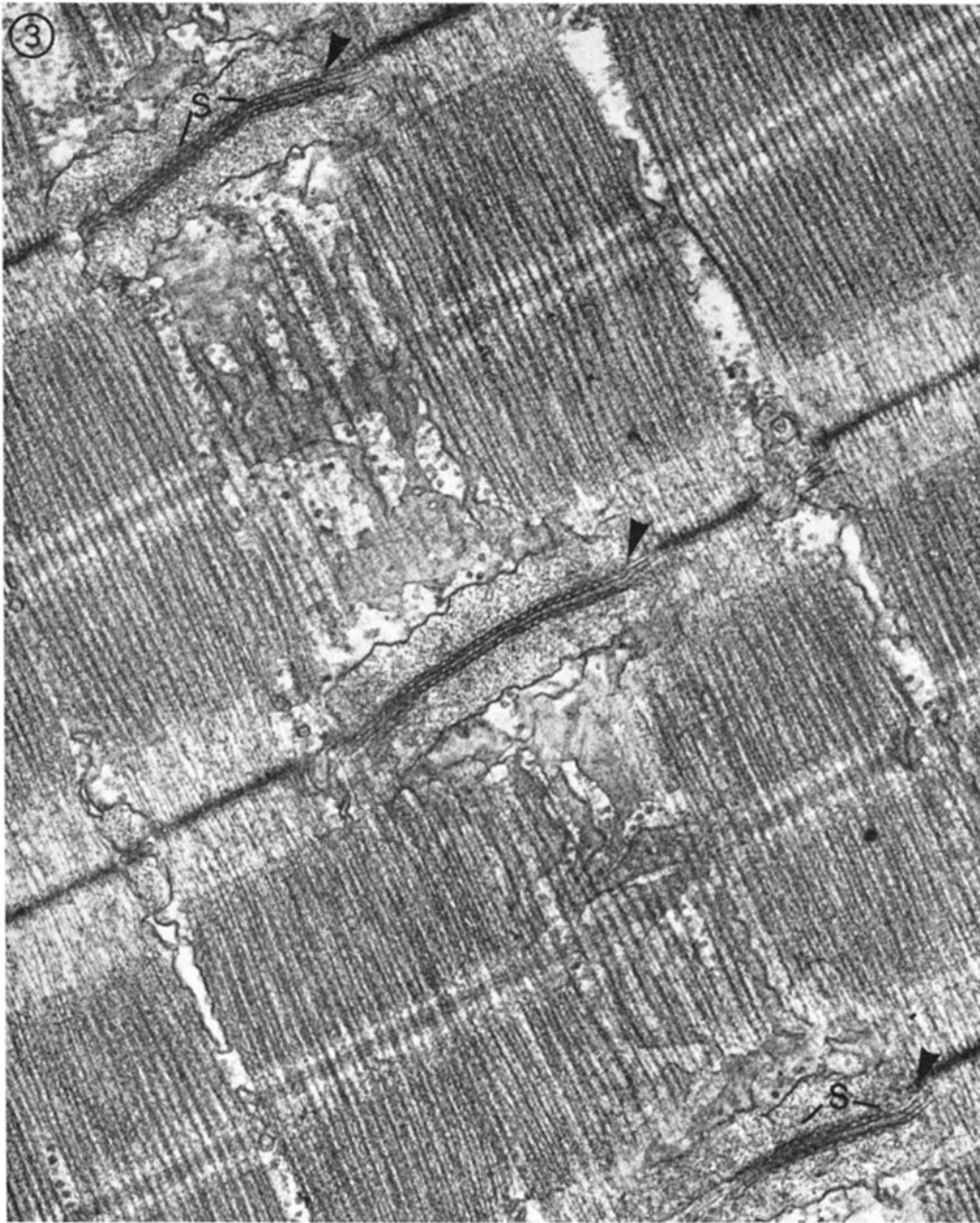
The final portion of the T system, immediately beneath the sarcolemma, is clearly distinguishable in its morphological characteristics from the T system within the triad (see also A. M. Kelly, 1969). It is interesting to notice that, even when the T system of frog fibers has been made to swell to a considerable extent, the opening is still not easy to visualize (H. E. Huxley, Page, and Wilkie, 1963; Freygang, Goldstein, Hellam, and Peachey, 1964; Foulks, Pacey, and Perry, 1965; Rapoport, Peachey, and Goldstein, 1968): this would indicate that the final portion of the system does not swell with the rest of it.

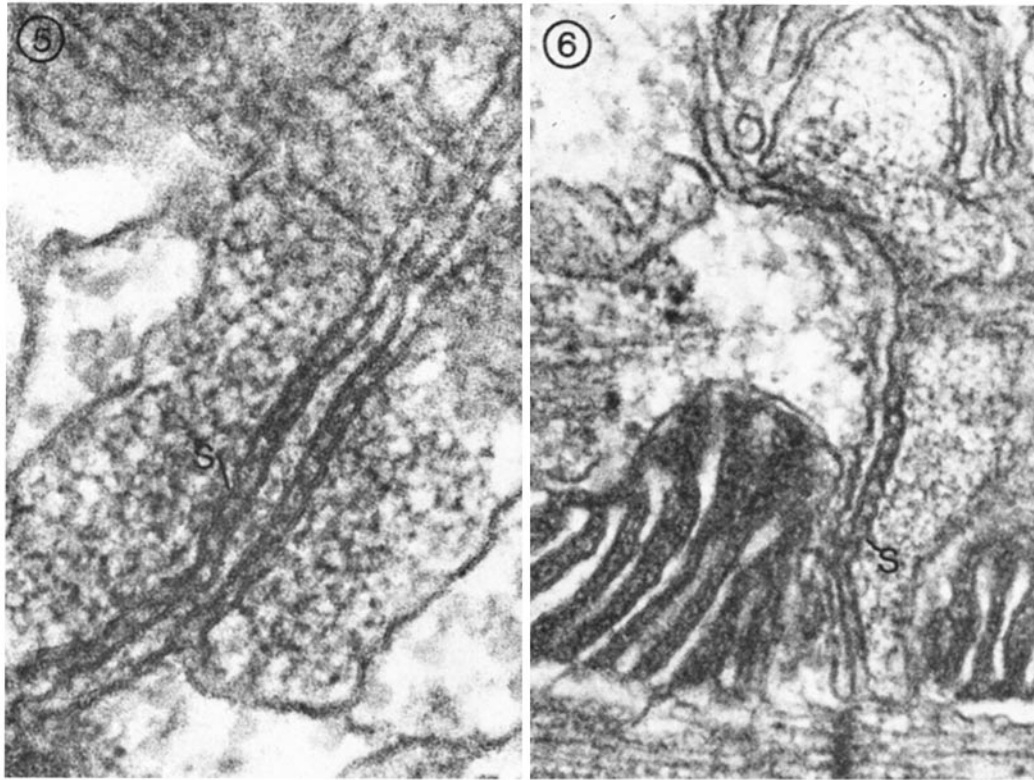
The Triad

The following is a tridimensional reconstruction of the structure of the triadic junction, based on the micrographs described in the Results section. The facing membranes of SR and T system are separated by a 120 A wide gap. The SR membrane abuts in two parallel rows of evenly spaced projections (the "dimples" of D. E. Kelly, 1967 and D. E. Kelly and Cahill, 1969). The tips of the projections do not reach the T system, but are separated from it by a gap, at least 50 A wide. This gap is crossed by small tufts of amorphous

FIGURE 3 Tangent view of three triads (plane a). Arrows indicate point where SR and T system run parallel. S indicates scalloped regions of the SR. $\times 45,000$.

FIGURE 4 A single triad in tangent view. The periodically repeating feet are well visible. S indicates a region in which SR scalloping is visible. The structure of the SR lateral sacs content is particularly regular at asterisk. $\times 100,000$.





FIGURES 5 and 6 Some of the SR scallops in these two triads are cut very close to the center. However, even the deepest scallops (*S*) are separated by a visible gap from the T-system membrane. Fig. 5, $\times 150,000$; Fig. 6, $\times 90,000$.

material, which is only lightly stained by lead but intensely stained by a combination of lead and uranium. The SR projections and the amorphous material constitute the feet. The area of T system covered by the feet, that is, the area effectively participating in the junction, is at most 30%. Since the SR surface area is approximately ten times as large as the T-system area (Peachey, 1965), no more than 3% of the SR membrane participates in the triadic junction.

The SR-T-system separation is not affected by either the use of different fixatives or changes in shape of SR and T system. Thus, dilation of the T system (Rapoport et al., 1969), as well as of the SR (unpublished observations), does not produce alterations in the junctional complex. An approximately 100 Å gap is visible in triads fixed in osmium tetroxide (e.g., see Porter and Palade, 1957; Revel, 1962), and in glutaraldehyde (e.g., see Peachey, 1965).

This may be taken to indicate that the approxi-

mately 120 Å junctional gap observed in fixed preparations is close to the *in vivo* distance between SR and T system. In addition, the junction is mechanically strong, since it withstands the stresses imposed on it by the quick movements of the fibrils during contraction. With some precautions, intact triads have been isolated from the fiber (Hasselbach and Elfvén-Lars, 1967).

The depth of the projections, on the other hand, varies considerably in different preparations. The cause of this variability is not clear: it is possible that shrinkage and dilation of the fiber during preparative procedures have an influence on the SR scalloping in the final preparations. Possibly, the waviness of the SR is secondary to the attachment of the amorphous material of the feet to the SR membrane. The fixative used is not a determinant factor: SR scallops have been described in osmium tetroxide-fixed preparations (Revel, 1962; D. E. Kelly, 1969), in glutaraldehyde-fixed material (Franzini-Armstrong and Porter, 1964;

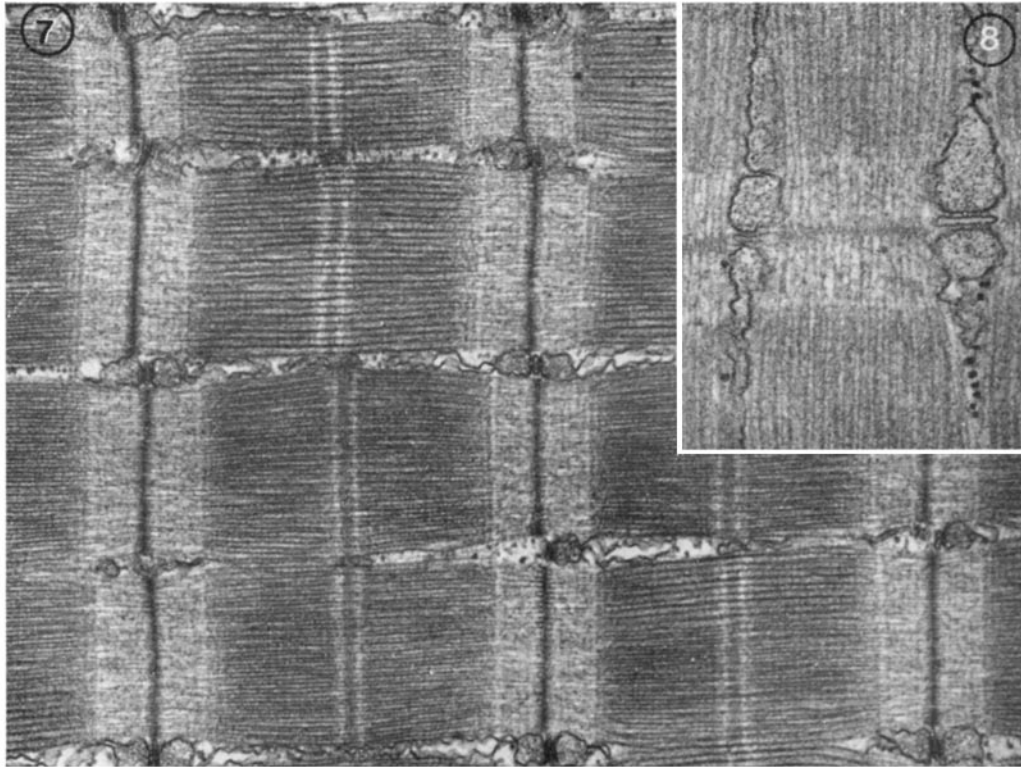


FIGURE 7 In plane b of sectioning, the triads are cut cross-wise. Notice the dense appearance of the junctional area in this section stained with lead and uranyl acetate. The two feet forming the junction are visible in some triads. $\times 22,500$.

FIGURE 8 This section was stained in lead only. Notice the low density of junctional area relative to SR and T system membranes. The feet are barely visible. $\times 38,000$.

Walker and Schrodt, 1965), as well as in paraformaldehyde-glutaraldehyde-fixed muscles (this paper). On the other hand, in frozen-etched preparations of a fish muscle (Bertaud, Rayns, and Simpson, 1970), which had been previously shown to present SR scallops in thin sections (Franzini-Armstrong and Porter, 1964), the edge of the SR appears flat and some elongated particles, probably the feet, are seen to join SR and T system across the junctional gap.

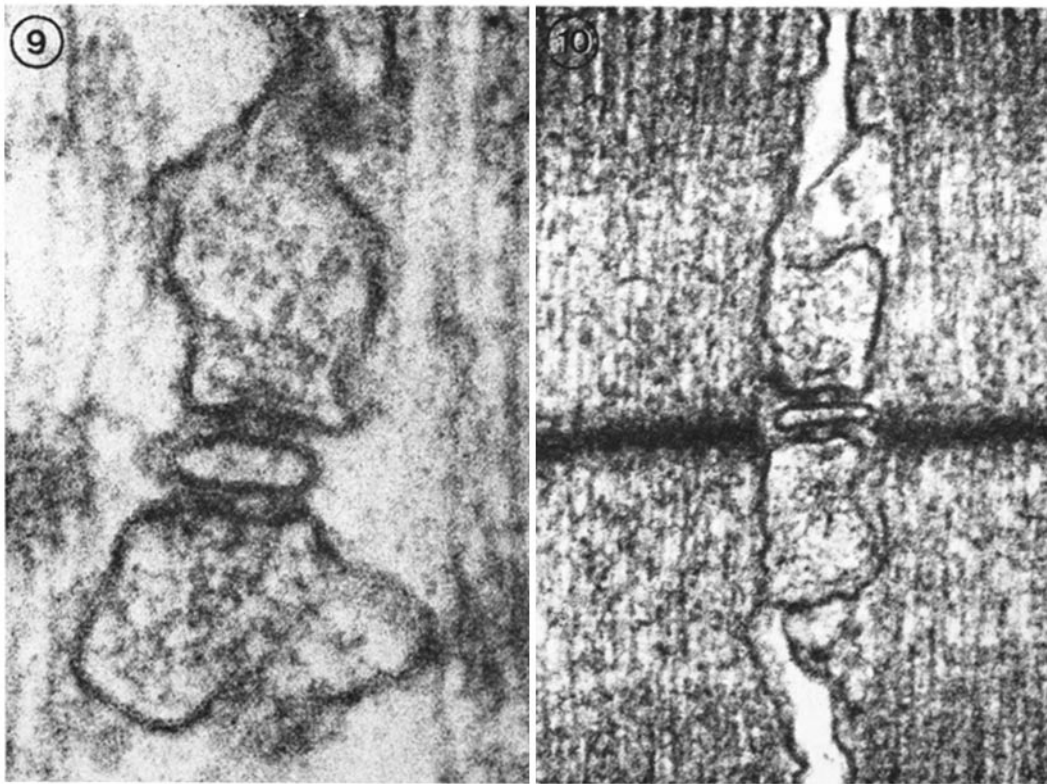
In some muscles from invertebrates (Hoyle, 1965; Rosenbluth, 1969) the SR membrane is also flat in the junctional area, and some material is present, at periodically repeating intervals, between the two. Rosenbluth (1969) clearly demonstrates that this material has staining characteristics different from those of SR and T system membranes.

My interpretation of the triadic junction struc-

ture is at variance in several respects with others, and it is worth discussing in detail the major points of difference.

It has been proposed (Fahrenbach, 1965) that the triad is a tight junction. This was subsequently shown to be unfounded, since a gap exists between SR and T-system membrane.

The major characteristic of D. E. Kelly's (1967, 1969) and D. E. Kelly and Cahill's (1969) tridimensional reconstruction of the structure of the triad is that the SR scallops reach the T-system membrane with which they form localized areas of either tight, or very close junction. Although it is clear from their elegant pictures that in their preparations the SR scalloping is very deep, the exact separation of the SR membrane from the T system should be assessed, as done here, by using a differential staining for the membranes and the rest of the feet. A very interesting observa-



FIGURES 9 and 10 View b of two triads at higher magnification. The two feet are well visible. Fig. 9, $\times 180,000$; Fig. 10, $\times 120,000$.

tion by the same authors is that in some faster acting muscles the feet (or dimples, in their terminology) are in four rather than two parallel rows.

The demonstration that one portion of the feet has staining characteristics different from those of the SR and T-system membrane is contradictory to Walker and Schrodt's (1965) interpretation that the feet are "membrane-like" structures.

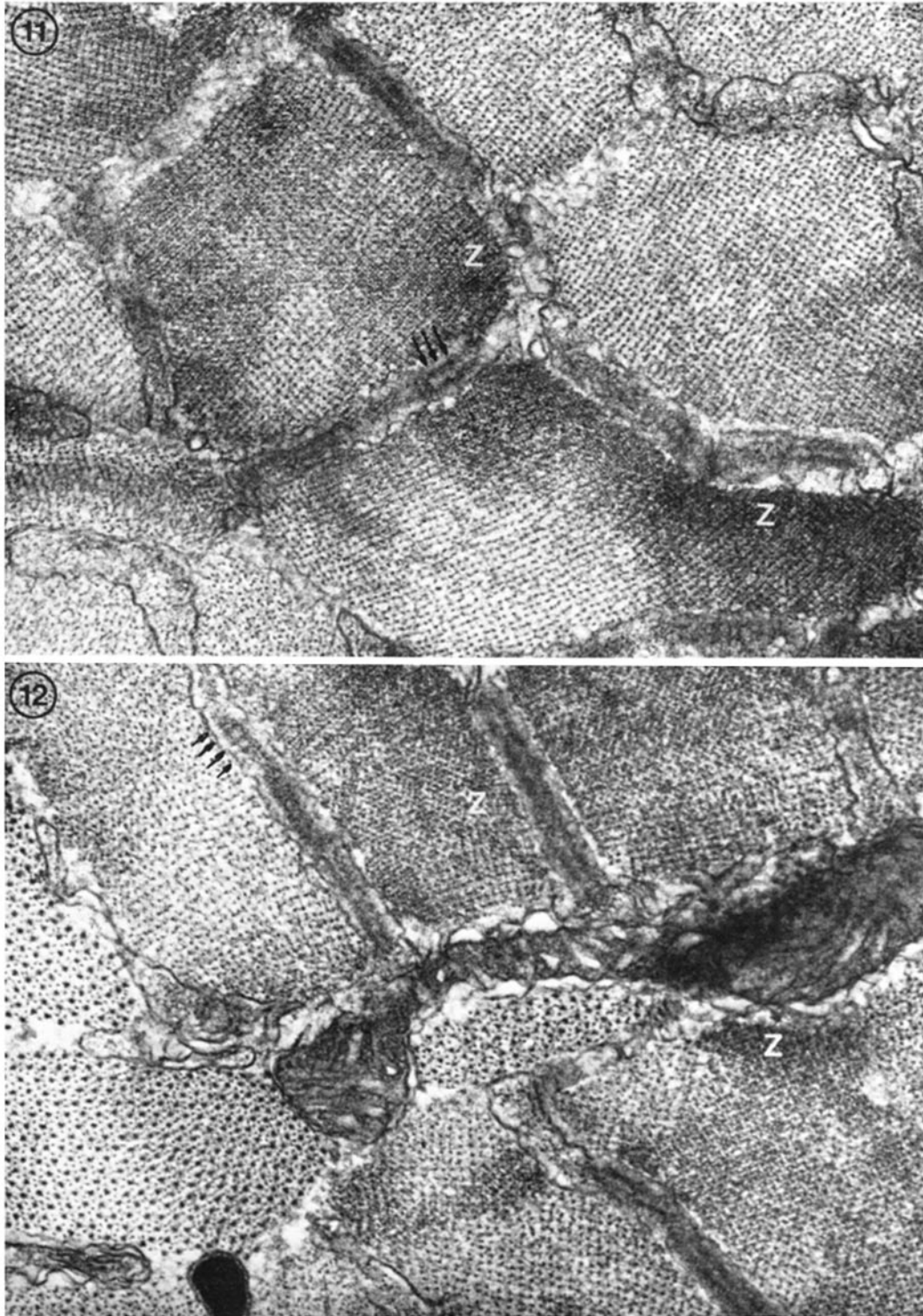
Both Walker and Schrodt (1965) and D. E. Kelly (1967, 1969) propose that some substance fills the space between the feet, thus sealing it off from the rest of the sarcoplasm. In conventional fixations, the space between the feet appears devoid of any structure. It is noticeable, also, that in frozen-etched preparations (Bertaud, Rayns, and Simpson, 1970) this same space is deeply etched, thus indicating either that it contains no structure or that, if any material is present there, this material is in a highly hydrated state.

The question of whether a functional barrier

exists between the junctional area and the rest of the sarcoplasm is amenable to experimental investigation. The results of attempts in this direction (Franzini-Armstrong, 1969) will be fully presented in a later report. The following discussion is based on the assumption that the feet are the only structure forming a junction between SR and T system.

One commonly formulated hypothesis on excitation-contraction coupling is that calcium release from the sarcoplasmic reticulum is due to an initial depolarization of the SR membrane, which is brought about by electrical coupling of the T-system tubule with the SR. It is assumed that the triadic junction is a likely candidate for such coupling.

There are two specific hypotheses of electrical coupling, and these are discussed below. Before doing so, it is worthwhile to point out that morphological data already available cast doubt on the possibility of electrical coupling, whatever the



FIGURES 11 and 12 Cross-sections at the level of the Z line (Z) and triadic junctions (view c). The two dense lines in the junctional area are the two rows of feet. Small arrows indicate distinguishable feet, $\times 60,000$.

details of the mechanism. If the T-system tubules cannot sustain an action potential, all of the current for depolarizing the SR would have to be supplied by the sarcolemma. Since the sarcolemma surface area is approximately 90 times smaller than the SR area (Peachey, 1965), this results in an impedance mismatch of the type which brought Katz (1961) to the conclusion that the neuromuscular junction is "poorly designed" to transmit an electrical signal. If an action potential is propagated along the T-system tubule, then the mismatch is less serious, although still present.

The two possible mechanisms which have been proposed for electrical coupling at the triad are: (a) A low-resistance pathway, through which direct ion flow may occur. (b) It has also been proposed, perhaps none too seriously, that a capacitance coupling exists between SR and T-system lumina (Eisenberg and Gage, 1969).

As regards the first hypothesis, it is informative to compare the structure of the triadic junction with that of junctions between cells, where a low-resistance pathway has been experimentally established. Rather than put into evidence the possible similarities, I would like to emphasize the differences.

The structure of areas of cell junction to which low resistance has been attributed can, on morphological grounds, be classified into the two following categories: (a) Junctions in which the membranes of adjacent cells are separated by a very small (about 30 Å) gap (gap junctions) or in which the outer leaflets of the apposed membranes fuse together (tight junction). Numerous supposed tight junctions have been, in the course of time, demonstrated to be gap junctions. I have thus pooled the references for the two types of junctions together (Tsubo and Brandt, 1962; Choi, 1963; Robertson, 1963; Barr, Dewey, and Berger, 1965; Revel and Karnovsky, 1967; Sommer and Johnson, 1968; Barr, Berger, and Dewey, 1968). Usually, the junctional area is in plaques no less than $0.25 \mu^2$. The triad is obviously different from this type of junction because the distance between SR and T-system membrane is relatively large (120 Å) over most of the junctional area, and closer proximity (to no less than 40 Å) occurs over only a small fraction of the junctional area. (b) "Septate junctions" or "septate desmosomes," which characterize the junction between some epithelial cells (Locke, 1965; Wiener, Spiro, and Lowenstein, 1964; Gilula and Satir, 1969). The

following points of dissimilarity exist between triads and septate junctions: (i) In septate junctions the two cell membranes run parallel (no scallops). (ii) The distance between the facing membranes is 150 Å (relative to 120–130 Å for the triad). (iii) The distance between joining spots in the septate desmosomes is 100–150 Å, versus 300 Å between the feet. (iv) In tangential section, the septate junctions show a continuous hexagonal honeycomb-like pattern, so that one may postulate that the junctional area is sealed off from the rest of the extracellular fluid. In the triad, on the other hand, the feet are discrete and no apparent morphological barrier exists between the junctional area and the rest of the sarcoplasm. (v) Finally, the septate desmosome is a junction between two outer surfaces of the plasmalemma, while at the triad a junction between cytoplasmic surfaces of a plasmalemmal derivative and of an intracellular membrane is formed. Points i and ii are probably secondary, but points iii, iv, and v indicate that in their morphology the two types of junctions have very little in common.

On morphological grounds alone, it is not possible to state that the triad is a likely low-resistance junction (see also Forssmann and Girardier, 1970). However, a low-resistance junction cannot be ruled out solely on the basis of the results presented here.

The triadic junction is also badly designed for a capacitance coupling, which, to be effective, requires that the two conductors (in this case SR and T-system lumina) be close together over a large area. Neither requirement is met by the junction. The two conductors in the triad are separated by two layers of membranes (about 140 Å total thickness) and an intervening space, which over most of the junction is 100 Å wide. Closer proximity occurs over only at most 30% of the T-system surface area (at the feet), and, even though the capacitance of the junction would be increased if the feet were composed of a high dielectric constant substance, the coupling would still be not so good as in the case of close proximity or contact of SR and T-system membranes over a large area.

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