THE SARCOPLASMIC RETICULUM AND TRANSVERSE TUBULES OF THE FROG'S SARTORIUS

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ABSTRACT

The sarcoplasmic reticulum of the frog's sartorius muscle was examined by electron microscopy following sequential fixation in glutaraldehyde and osmium tetroxide and embedding in Epon. The earlier results of Porter and Palade on *Ambystoma* muscle were confirmed in the sartorius. In addition, the transverse tubules were observed to be continuous across the width of the fiber, a set of flat intermediate cisternae was seen to connect the terminal cisternae to the longitudinal tubules in the A band, and the continuous reticulum collar at the center of the A band was found to be perforated by circular and elongated pores (the fenestrated collar). The transverse tubules have a volume about 0.3 per cent of the fiber volume, and a surface area about 7 times the outer cylindrical surface area for a fiber 100 μ in diameter. The terminal cisternae, the intermediate cisternae, and the longitudinal tubules together with the fenestrated collar each have a volume of 4 to 5 per cent of the fiber volume and a surface area 40 to 50 times the outer surface area of a fiber 100 μ in diameter. Some evidence for continuity of the transverse tubules with the fiber surface is presented, but this is thought to be not so convincing as evidence presented by others. The results are discussed in terms of a possible mechanism for a role of the transverse tubules and sarcoplasmic reticulum in excitation-contraction coupling, as suggested by their morphology and a variety of physiological studies. In this scheme, the transverse tubules are thought to be electrically coupled to the terminal cisternae, so that depolarization of the fiber surface spreads inward along the transverse tubules and to the terminal cisternae, initiating the release of a contraction-activating substance.

INTRODUCTION

In 1957 Porter and Palade described the fine structure of the sarcoplasmic reticulum of three types of vertebrate striated muscles, including caudal myotomes of amphibian larvae. Their results and the results of the local stimulation experiments of A. F. Huxley and Taylor (1958) on adult frog muscles suggested that the structures that Porter and Palace called "triads" are involved in the coupling of surface excitation to contraction in striated muscle cells. The development of this hypothesis has been reviewed several times in recent years (see A. F. Huxley, 1959; Porter, 1961). By now the evidence supporting this idea has become quite strong, and evidence on the mechanism of the coupling is beginning to accumulate. In order that sound morphological information may be available for the formulation of physiological models of the triads or the rest of

the sarcoplasmic reticulum, it seemed worth while to examine the structure of these systems in the particular muscles that are commonly used for physiological experiments and to take advantage of the improved methods of electron microscopy that have become available since Porter and Palade's 1957 study. In particular, it seemed of interest to get quantitative information on surface area and volumes of various membrane-limited compartments so that these could be related to surface and volume estimates for special compartments based on physiological studies (Hodgkin and Horowicz, 1960*a*; Adrian and Freygang, 1962; Freygang *et al.*, 1964*a*, 1964*b*).

MATERIALS AND METHODS

Frogs, *Rana pipiens* from Vermont, were used throughout this study. Several fixation procedures were used, but the best results were obtained using a double fixation consisting of 1 hour in a 6.2 per cent solution of glutaraldehyde (0.1 M sodium cacodylate + 2 mM calcium chloride, pH 7.0 to 7.2), followed by 1 to 3 hours' rinsing in a 10 per cent sucrose solution (0.1 M sodium cacodylate, pH 7.0 to 7.2) and 1 to 2 hours' second fixation in a 1 per cent osmium tetroxide solution (0.1 M sodium phosphate + 2 mM calcium chloride, pH 7.0 to 7.2). The osmolality of the glutaraldehyde fixative was between 950 and 1000 mOsM per kg water; that of the buffer rinse, from 460 to 480 mOsM per kg water; that of the osmium tetroxide fixative, from 250 to 260 mOsM per kg water. Since calcium phosphate precipitates from the osmium tetroxide fixative, the calcium chloride was not added until just before the fixative was used. No comparative studies were made to test whether the presence of calcium improves the quality of fixation in this procedure, although earlier comparative studies in which osmium tetroxide was used alone did show more continuity of membrane structure when calcium was present.

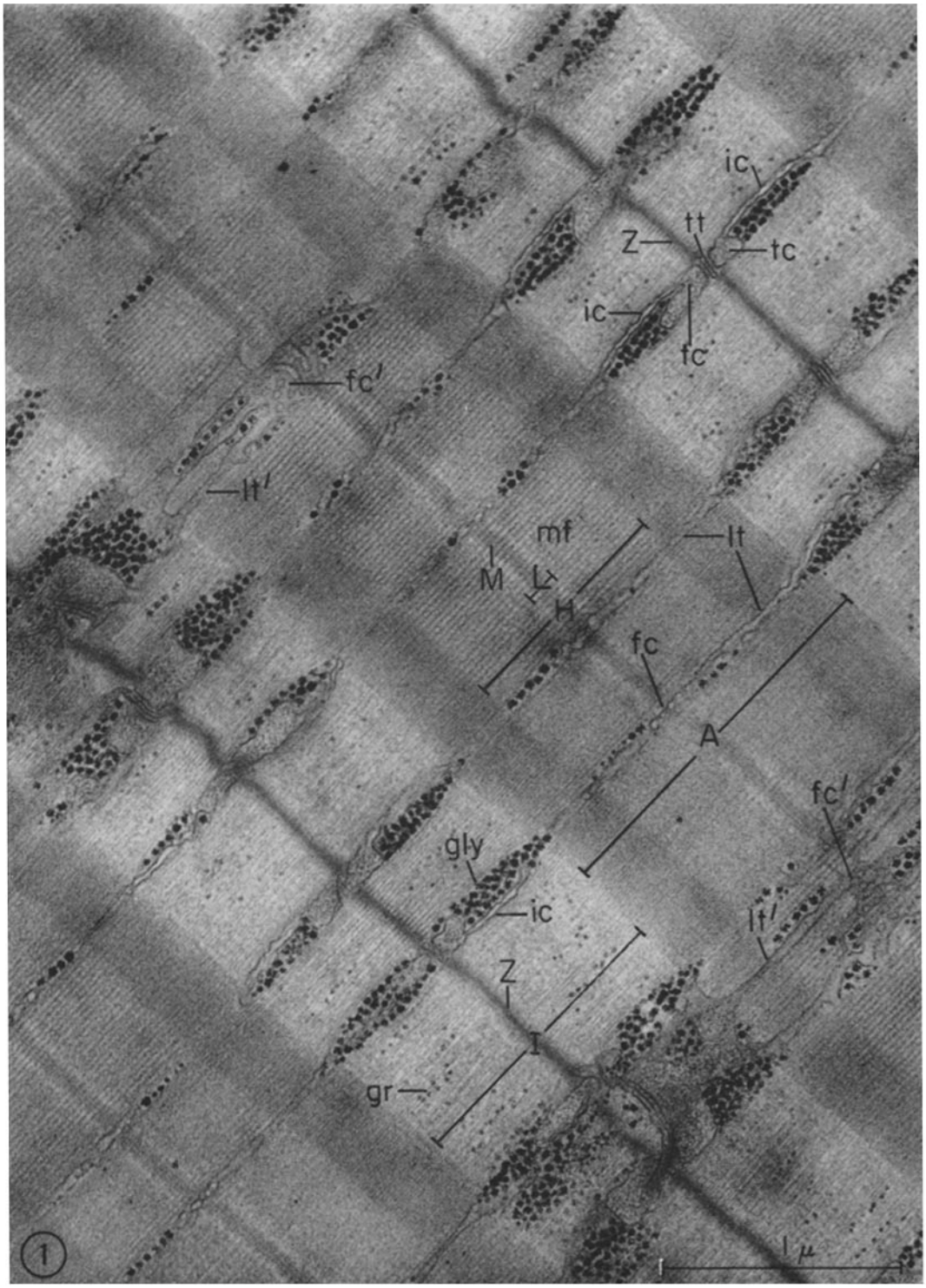
The muscles were held isometrically at 120 to 150 per cent of their slack lengths until the end of the buffer rinse, after which they did not appreciably change length when released. The glutaraldehyde fixation and the buffer rinse were done at room temperature (22 ± 2°C). Just before the second fixation, bundles 1 to 2 mm long of about 30 fibers were cut from a portion of the muscle midway between the proximal and distal ends. The second fixation was at 0°C. Dehydration was done in a graded series of ethanol-water mixtures, and embedding was done in an Epon mixture consisting of Epon 812, 47 per cent *v/v*; methyl nadic anhydride, 29.5 per cent; dodecanyl succinic anhydride, 22 per cent; and benzyl dimethylamine, 1.5 per cent. Sections were cut on Cambridge-Huxley and Sorvall–Porter-Blum microtomes using glass and diamond knives. Sections were mounted on uncoated 200-mesh screens, stained for 3 to 5 minutes in lead citrate (Reynolds, 1963), and examined in an RCA EMU 3F operated at 50 kv using a 25 μ diameter, rear focal plane objective aperture.

RESULTS

In *Ambystoma* muscle, according to Porter and Palade (1957), the sarcoplasmic reticulum consists of three-part structures (triads) located adjacent to the Z lines of the myofibrils, and a system of tubules which join into continuous bands around the myofibrils at the centers of A bands. The larger and outer elements of the triads are the *terminal cisternae*, in Porter and Palade's terminology, and the central elements of the triads, which appeared as rows of vesicles included in the narrow spaces between the terminal cisternae, were referred to as the *intermediary vesicles*.

FIGURE 1 Longitudinal section, oriented with the long axis of the fiber extending from lower left to upper right in the figure. The myofibrils (*mf*) show a typical transverse banding pattern, with dense Z lines (*Z*) spaced about 3 μ apart at the centers of the light I bands (*I*). The A bands (*A*) have a central light H zone (*H*) and denser outer regions where primary and secondary filaments overlap. At the center of each A band is a light L zone (*L*) with a dark M line (*M*) down its middle.

Triads consisting of two terminal cisternae (*tc*) and a centrally located transverse tubule (*tt*) are seen adjacent to the Z lines (*Z*) of the myofibrils. Connecting to the terminal cisternae are the intermediate cisternae (*ic*). Adjacent to the myofibrillar A bands, elongated profiles of the longitudinal tubules (*lt*) and oval profiles of the fenestrated collar (*fc*) are seen. These latter structures are also seen overlying the myofibrils at two places where the section grazes the surface of the myofibril (*fc'* and *lt'*). The large, densely stained granules are glycogen (*gly*). Smaller granules (*gr*) are found within the I bands. × 36,000.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The sarcoplasmic reticulum of the frog's sartorius is seen, in the present study, to have a similar but not identical morphology. The central element of the triad is found to be a continuous element across the width of the fiber, the continuous collar around the fibrils at the center of the A band is found to be perforated by a number of holes, and the tubules of the A band are seen to connect to the terminal cisternae through flattened, longitudinally oriented cisternae which join the terminal cisternae along lines that are often near the Z lines.

Each of these structures can be seen in a longitudinal section, such as the one shown in Fig. 1, but a more detailed analysis of both longitudinal and transverse sections is necessary to establish all the morphological features of this membrane system.

The Triads

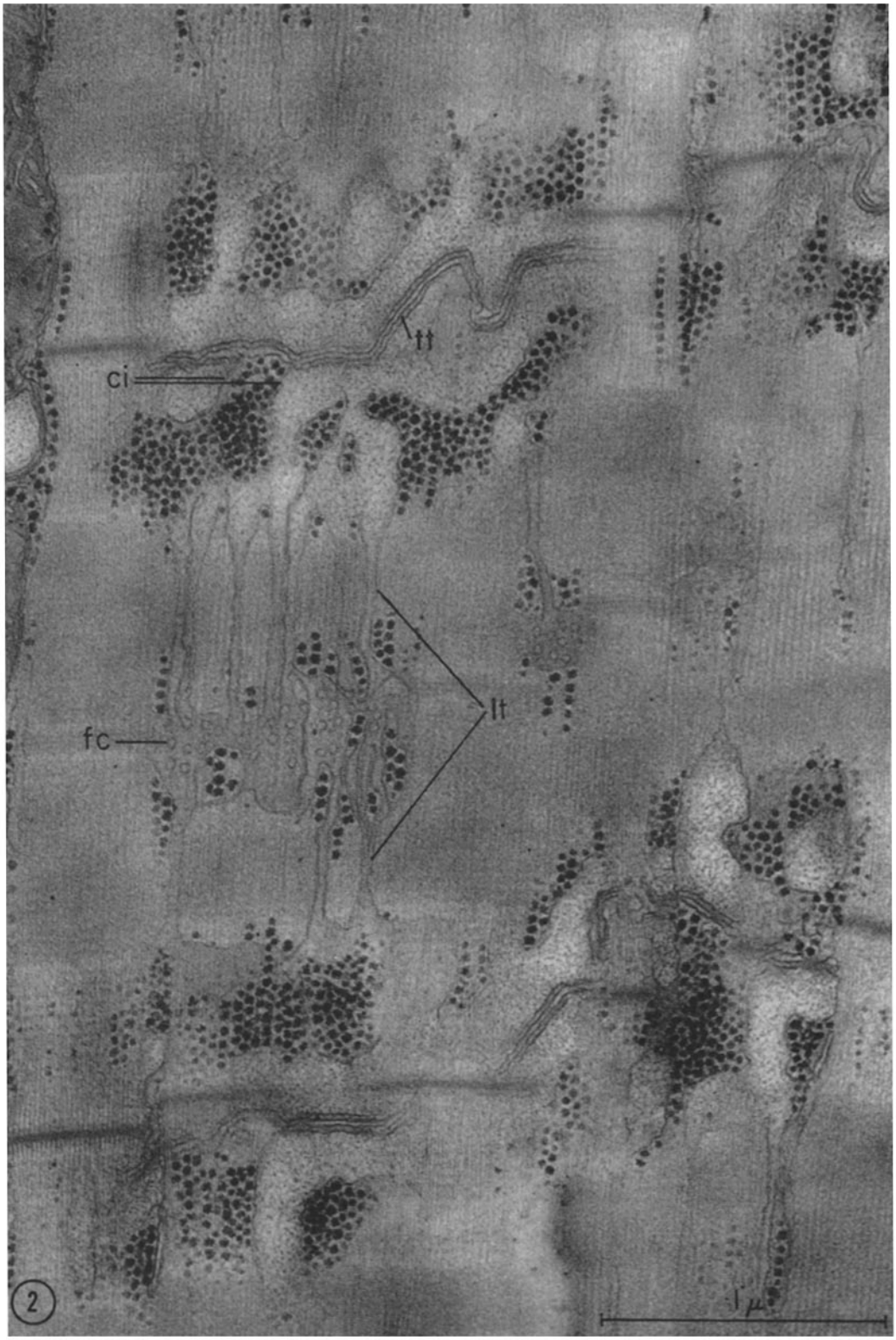
The triads are located in the sarcoplasm adjacent to the Z lines of the myofibrils. In longitudinal sections, the triads are shown in various orientations depending on the way the plane of section passes through them and through the adjacent myofibrils. When the section passes through the median parts of the myofibrils, the triads are cut across their narrow dimensions and appear as shown near the upper right corner of Fig. 1. The central element (*tt*) of each triad usually appears as a single tubular element flattened in the transverse plane of the fiber and having the same width (perpendicular to the fiber axis) as the outer elements (*tc*) of the triads. This dimension is about 0.08 μ. The average dimension of the central element parallel to the fiber axis has been measured previously, and is 260 A (Freygang *et al.*, 1964*b*). Following the terminology of Andersson-Cedergren (1959), the central element of the triad will be referred to as the *transverse tubule* or *T system*, because of its orientation perpendicular to the longitudinal axis of the fiber.

The outer elements of the triads, the *terminal cisternae*, are larger, dilated sacs whose surfaces are flattened where they face the transverse tubules. The terminal cisternae are about 200 A distant from the transverse tubules and extend for about 0.2 μ in the longitudinal direction. They are filled with a diffuse granular material containing some very dense granules 50 to 100 A in diameter (Figs. 1 and 3 *a*).

When the plane of section coincides with the surface of one or more myofibrils, the sarcoplasmic reticulum is caught in "face view," as shown in Fig. 2. The transverse tubules are found to be continuous for as far as the triad stays in the plane of the section, a distance of about 2 μ at the top of Fig. 2. Since a continuous tubule is seen whenever the plane of section is favorable, it is concluded that the transverse tubules are continuous most or all of the way across the fiber at each Z line level. Continuity of transverse tubules has already been demonstrated in osmium tetroxide–fixed mouse skeletal muscle by Andersson-Cedergren (1959) using serial sections. It has also been demonstrated in osmium tetroxide–fixed fish swim bladder muscle (Fawcett and Revel, 1961) and bat striated muscle (Revel, 1962), and in fish myotomes fixed in glutaraldehyde and osmium tetroxide (Franzini-Armstrong and Porter, 1964). Interruptions are occasionally seen in the terminal cisternae (Figs. 2 and 4), so that it is possible that these elements, though found in virtually every interfibrillar space, may not have complete lateral continuity.

Dense structures connect the flat faces of the terminal cisternae to the transverse tubules, giving this space a beaded or serrate appearance, as seen earlier in bat striated muscle by Revel (1962). These bridging structures are spaced about 200 A

FIGURE 2 Longitudinal section showing an extensive "face view" of the sarcoplasmic reticulum. Near the top of the figure, the transverse tubule (*tt*) can be followed for almost 2 μ as a continuous structure. A discontinuity in a terminal cisterna (*ci*) is indicated. Note the serrate appearance of the 200 A spaces between the transverse tubules and the terminal cisternae. This appearance results from the presence of dense lines running between these structures and spaced about 200 A apart. Other elements of the sarcoplasmic reticulum are also visible and marked for identification: *lt*, longitudinal tubules; *fc*, fenestrated collar. × 44,000.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apart, and are seen most clearly in face views such as are shown in Figs. 2 and 5.

Occasionally an arrangement of terminal cisternae and transverse tubules different from the usual triad arrangement is observed. Fig. 3 shows the usual arrangement (Fig. 3 *a*) and two variations, one with an extra transverse tubule (Fig. 3 *b*) and one with an extra transverse tubule and an additional, central cisterna (Fig. 3 *c*).

The Intermediate Cisternae

In longitudinal sections such as are shown in Fig. 1 and at higher magnification in Fig. 3 *a*, one observes long, narrow profiles (*ic*) connecting to the terminal cisternae and extending to the edge of or into the A band. The connection of these structures to the terminal cisternae is often made through a narrow junction at a point quite close to the Z line, so that the profile runs as an independent unit alongside the terminal cisterna before joining it. In transverse sections through the I band, as seen in the upper part of Fig. 4, one also observes elongated profiles (*ic*) with clear contents. Since these membrane elements in the I band appear elongated in both transverse and longitudinal sections, they must be flattened cisternae three-dimensionally. They will be called the *intermediate cisternae* to emphasize their position between the terminal cisternae and the longitudinal tubules. Commonly, the granular content of the terminal cisternae does not extend into the intermediate cisternae (Fig. 3 *a*), further emphasizing their separate identity.

As seen in Fig. 4 (arrows), sometimes there are two intermediate cisternae in the space between one pair of myofibrils. These cisternae are not continuous around the fibrils, but individual cisternae often are as wide as a myofibril, or about 0.5 to 1.0 μ. Taken together, the intermediate cisternae around a particular fibril are roughly equivalent in quantity to a complete single-layered collar of cisternae.

The Longitudinal Tubules

The elongated profiles seen in I band regions of longitudinal sections often penetrate into the regions between the A bands of the adjacent myofibrils (*lt*, Figs. 1 and 3), but only oval profiles are seen in the A band regions in transverse sections (*lt*, Fig. 4). Thus, the intermediate cisternae must connect, approximately at the edge of the A band in these preparations, to longitudinally oriented tubular elements which extend into the A band region. Actually this connection is seen clearly at the lower right in Fig. 1, and above and below center in Figs. 2 and 5, but these views alone could not prove this tubular appearance not to be an artifact of the plane of section. These tubules will be called *longitudinal tubules.*

The Fenestrated Collar

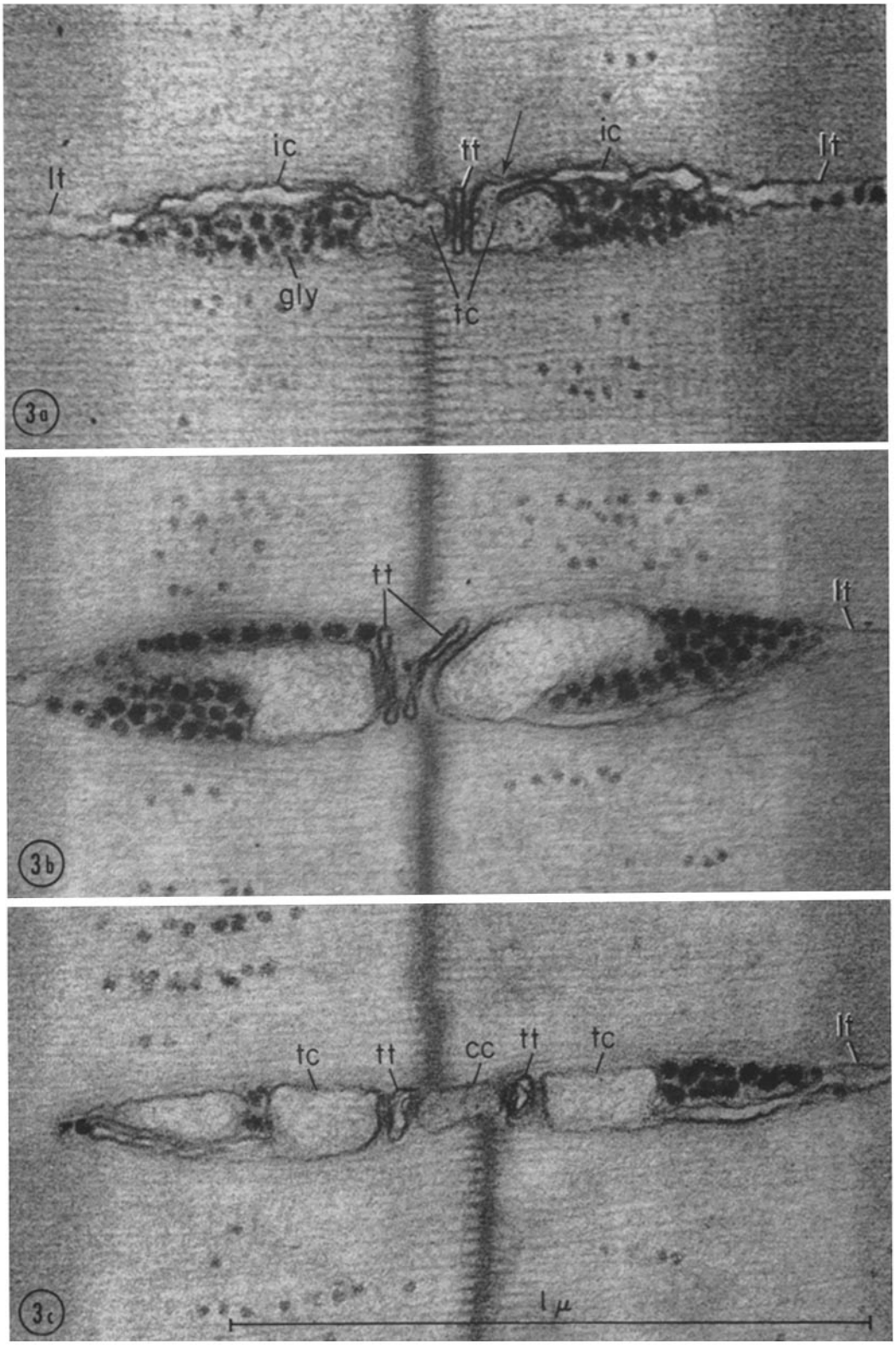
Near the center of the A band the longitudinal tubules fuse to form continuous collars around the myofibrils, as is best shown (*fc*) in face views of the sarcoplasmic reticulum (Figs. 2 and 5). These collars are penetrated by a number of fenestrations which are circular or elongated in face view. The fenestrations are seen particularly clearly in extremely thin sections (probably less than 300 A), such as the one shown in Fig. 5, where the section includes only the sarcoplasmic reticulum and none of either the underlying or the overlying myofibril

FIGURE 3 Higher magnification of longitudinal sections through triads and intermediate cisternae.

Fig. 3 *a* shows the most common triad morphology, with a single transverse tubule (*tt*) between the flat faces of opposing terminal cisternae (*tc*). The intermediate cisternae (*ic*) connect to the terminal cisternae at points which are sometimes near to the centers of the triads (arrow), and pass alongside the terminal cisternae and glycogen masses (*gly*) toward the A bands, where they join to the longitudinal tubules (*lt*). The granular material within the terminal cisternae is not present in the intermediate cisternae. × 100,000.

Fig. 3 *b*. A pair of transverse tubules (*tt*) in a single "triad," an image encountered only infrequently. A longitudinal tubule is marked (*lt*). × 100,000.

Fig. 3 *c*. A "pentad" consisting of two terminal cisternae (*tc*), two transverse tubules (*tt*), and an additional central cisterna (*cc*) also encountered only infrequently. *lt*, longitudinal tubule. × 100,000.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in this area. Some of the elongated openings in this fenestrated collar are continuations of the spaces between the longitudinal tubules and some are isolated openings. The circular openings are 300 to 400 A in diameter and are disposed in a moderately regular hexagonal pattern in which the center-to-center spacing is about 700 A. The ends of the elongated openings seem to coincide with points in the hexagonal array of circular openings (see Fig. 5), as if the elongated openings were formed by a confluence of two or more circular openings.

Porter and Palade saw these fenestrations in *Ambystoma* muscle, and referred to them as "pores or thin places in the membrane." More recently Franzini-Armstrong (1963) has concluded that they represent perforations in one of the two membranes of the sarcoplasmic reticulum collar and thus are places where the internal content of the reticulum is in continuity with the sarcoplasm. The results of the present study are not consistent with this view and indicate that the two membranes of the collar are fused around a circular or elongated area which is the pore. Thus the inside of the sarcoplasmic reticulum is not continuous with the sarcoplasm, but rather the sarcoplasm adjacent to one myofibril is continuous through the pore with the sarcoplasm adjacent to the next myofibril. The types of images on which this interpretation is based are shown in Figs. 6 and 7. In both of these views, one longitudinal and one transverse, the fenestrated collar (*fc*) appears as a row of separated vesicular profiles between which are channels (*p*) of sarcoplasm.

The reader may note in Fig. 7 the frequent appearance of a relatively dense line (*pd*) connecting adjacent vesicular profiles. This density might be interpreted as a diaphragm across the pore, but since the section thickness in this case is roughly equal to the diameter of the circular pores, the density is better interpreted as a portion of the edge of the pore included in the section. The places where no such density appears between adjacent profiles are likely to be sections through the elongated openings.

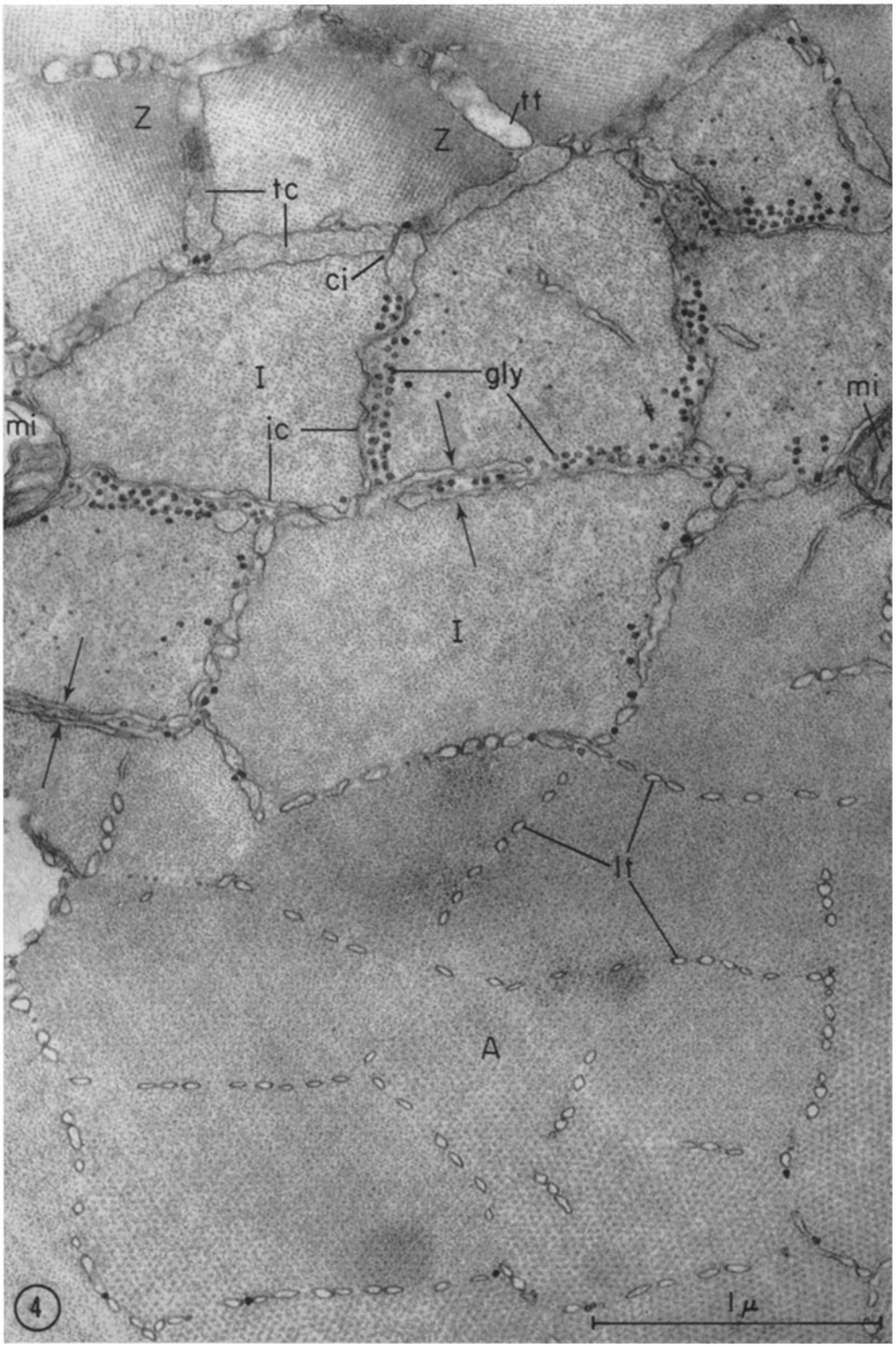
At the points where three myofibrils come together (see arrows, Fig. 7), the fenestrated collar branches and forms, in effect, a three-dimensional network continuous across the fiber. This continuity was detected by Porter and Palade (1957), who observed that membrane elements seemed always to be present opposite the H bands in longitudinal sections.

The results so far describe the typical appearance of the sarcoplasmic reticulum of the frog's sartorius and are summarized in the diagram shown in Fig. 8. Some special features have also been found, but are not included in the diagram either because they are only infrequently found or because they are associated with special regions of the fiber, *e.g.* near the nucleus or near the surface of the fiber. These special features will now be presented.

Connections between Opposing Terminal Cisternae

Porter and Palade (1957) occasionally found tubular connections between the two terminal

FIGURE 4 Very thin, slightly oblique transverse section showing a Z line (*Z*) as dark patches with a square pattern at the upper left corner of the figure. The I band (*I*) appears as the lighter areas around and below the Z line patches. The A band (*A*) is at the bottom of the figure. Near the Z line and for about one-third of the I band the fibrils are completely surrounded by wide membrane-bounded elements of the triads. Dark patches within these elements are interpreted as regions where the membranes of the central portion of the triad (transverse tubule and flattened ends of terminal cisternae) lie within the section. The triad elements that are filled with granular material are the terminal cisternae (*tc*), and the very light patches (*tt*) are interpreted as areas where only the transverse tubule lies within the section, implying that the section thickness in this region is somewhat less than 300 A. One interruption in lateral continuity of the terminal cisternae is indicated (*ci*). Below these elements in the figure, but still within the I bands, are found the narrower profiles of the intermediate cisternae (*ic*). These are found alongside, and sometimes flanking both sides, of the glycogen (*gly*) masses (see arrows). The intermediate cisternae extend as far as the edge of the I band. Beyond this, within the A band, roughly circular profiles of the longitudinal tubules (*lt*) are seen. Parts of two mitochondria (*mi*) appear in this figure. × 45,000.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cisternae of the same triad. Similar connections have been found in the present study. One such connection is shown in Fig. 9. At this place, no transverse tubule is seen, but a tubular connection passing the Z line level is clear. In other places, both the transverse tubule and the cisternal cross-connection are present, the cross-connection forming a sort of cup around the transverse tubule. Since the terminal cisternae are continuous for considerable distances across the fiber, each such longitudinal connection between them would serve to unite the reticulum in a large portion of one sarcomere with that in a large portion of the next sarcomere. The frequency with which such longitudinal connections are found is sufficiently high to make it seem possible that the entire sarcoplasmic reticulum may be connected in this way into a single network.

Connections with the Nuclear Envelope

The terminal cisternae are connected to the nuclear envelope, and indeed one or both of the terminal cisternae of some triads are dilations of the outer membrane of the nuclear envelope (Figs. 10 and 11). No connection has been seen between transverse tubules and the nuclear envelope. Since in other types of cells (Watson, 1955) the endoplasmic reticulum is connected to the outer nuclear membrane, the present result supports the idea that the sarcoplasmic reticulum and the endoplasmic reticulum are homologous structures, as suggested earlier by other authors (Edwards *et al.*, 1956; Porter and Palade, 1957).

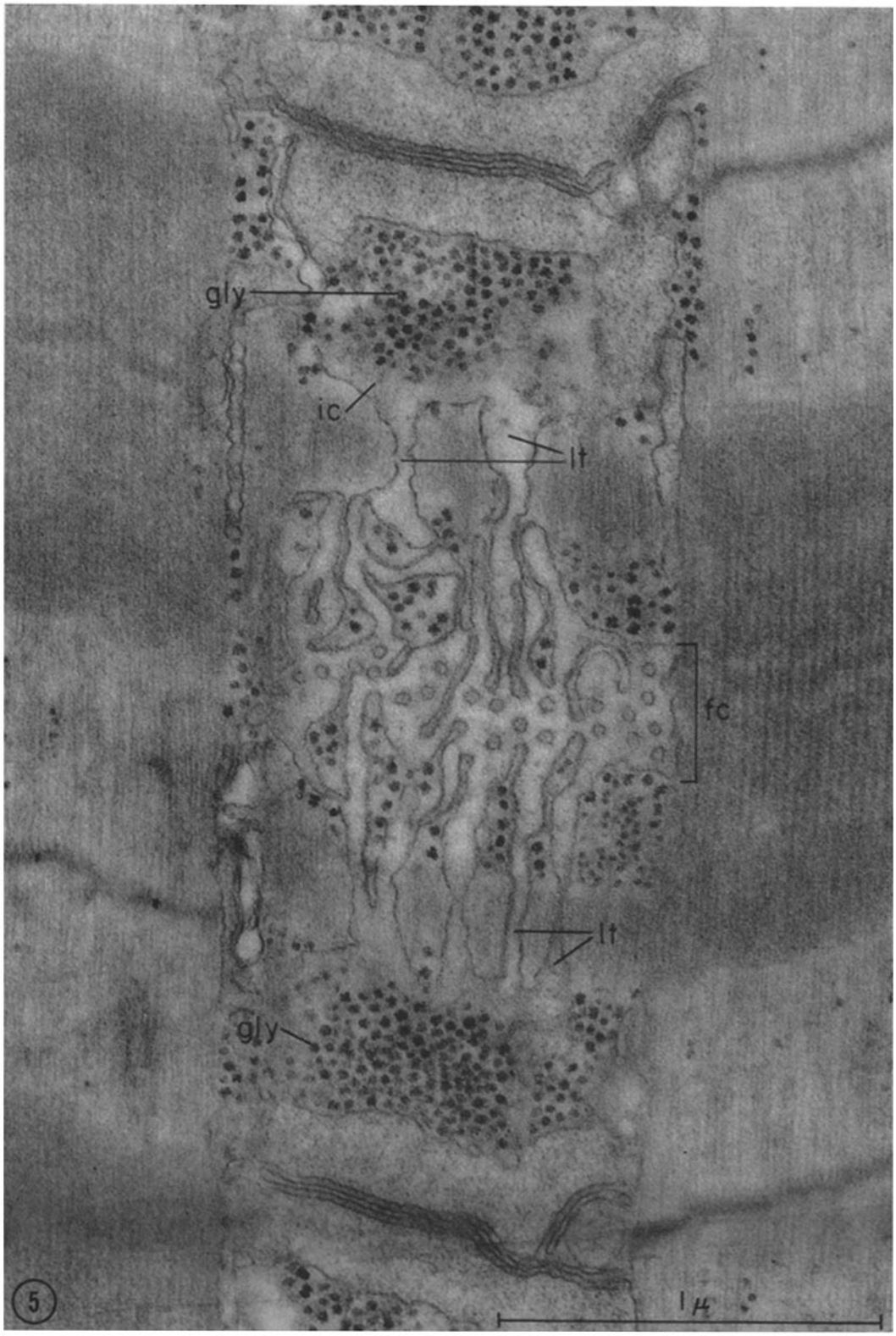
Association with the Fiber Surface

Near the fiber surface and extending to a depth of about 2 μ, the transverse tubules are enlarged and rather irregular in form (Figs. 12 to 14). The longitudinal dimension of the transverse tubules increases from 260 A in the interior of the fiber to as much as 750 A in this surface region. Involutions of the transverse tubules are sometimes seen in this region (Fig. 14). These involutions result in complex images which are difficult to interpret in three dimensions and which would make it difficult to detect any connections that might exist between the membranes of the transverse tubules and the surface membrane. A possible connection of this type is shown in Fig. 15. No suggestion of connection of any part of the sarcoplasmic reticulum to the surface membrane has been seen.

Branching and Longitudinal Extensions of Transverse Tubules

Occasionally one observes a branched transverse tubule, as in the lower triad in Fig. 5. These branched transverse tubules with additional terminal cisternae between the branches form double triads or pentads as shown here in Fig. 3 *c* and as seen in a bat muscle by Revel (1962). Furthermore, the orientation of transverse tubules in the longitudinal direction leads to an occasional observation of a triad in a transverse section (Fig. 16). It is not clear how common these longitudinal extensions of the transverse tubules are and how far they can extend in the longitudinal direction, because it is difficult to distinguish them from intermediate cisternae and longitudinal tubules except when a direct connection with an identified transverse tubule can be seen. More extensive longitudinal extensions of transverse tubules were seen in frog muscle by H. E. Huxley (1964), who identified them by their content of ferritin that had entered from the external medium, and in bat muscle by Revel (1962).

FIGURE 5 Very thin (probably less than 300 A thick) longitudinal section. The central, vertical strip of the area shown is almost entirely confined to the sarcoplasm between two myofibrils. Small parts of the underlying myofibril are seen between the longitudinal tubules (*lt*) just above and below the center of the figure. This underlying myofibril is in register with the myofibril at the right side of the figure, which thus can be used for reference to myofibril bands. The myofibril at the left is shifted upward about 0.8 μ. Triads appear at the top and bottom of the figure, opposite Z lines. The intermediate cisternae (*ic*) are largely out of the section, which passes through the glycogen masses (*gly*) adjacent to them. The longitudinal tubules (*lt*) and the fenestrated collar (*fc*) are shown near the center of the figure. Their location corresponds quite precisely to that of the A band of the adjacent myofibril. Note, in the fenestrated collar, the roughly hexagonal pattern of perforations, some of which are connected together to form elongated openings. × 60,000.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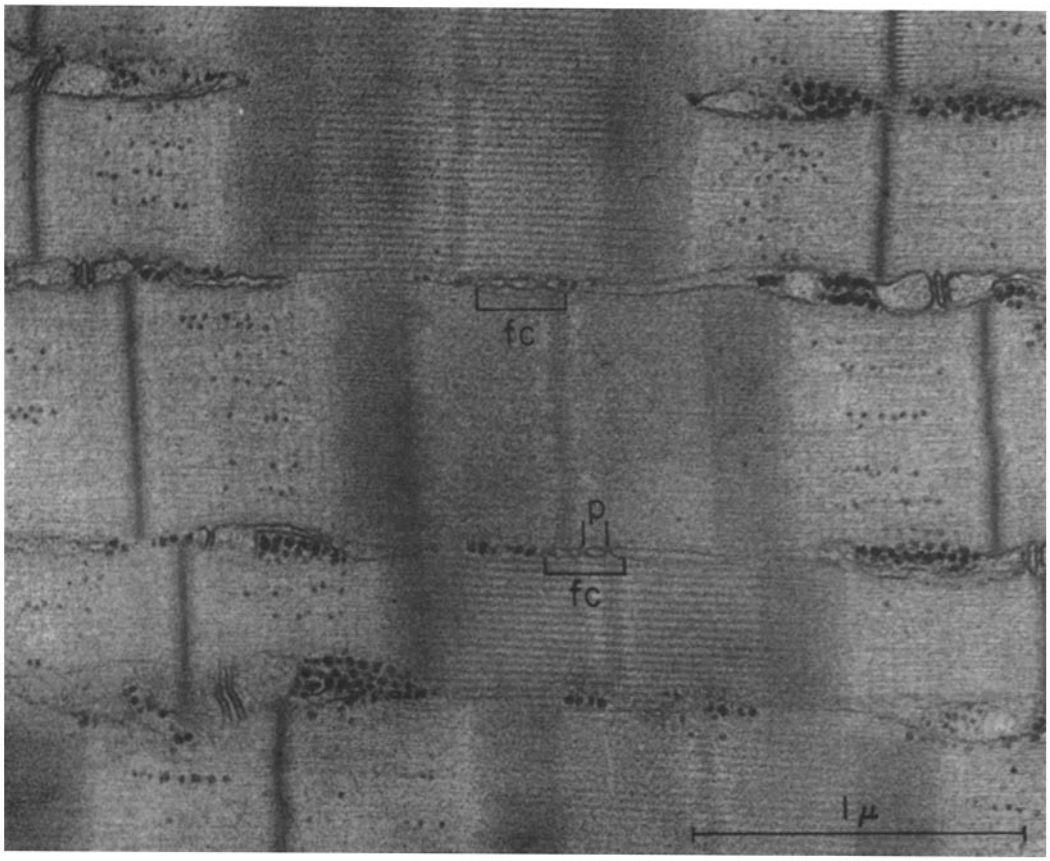


FIGURE 6 Longitudinal section with the long axis of the fiber oriented horizontally in the figure. This micrograph demonstrates the continuity of pores (*p*) through the fenestrated collar (*fc*). × 44,000.

Volumes of Transverse Tubules and Sarcoplasmic Reticulum

The volumes of the various portions of the sarcoplasmic reticulum in these preparations were estimated from measurements on electron micrographs and were expressed as fractions of the fiber volume (excluding large organelles such as nuclei and mitochondria, which occupy less than 1 per cent of the fiber volume in the sartorius). The cross-sectional area occupied by each compartment was measured in transverse sections and expressed as a fraction of the total transverse area. This fractional transverse area was multiplied by the length of the compartment in each sarcomere as a fraction of the sarcomere length, to get the volume of the compartment as a fraction of the total volume of the fiber. The calculations are summarized in Table I.

The transverse tubules and terminal cisternae occupy the entire space between fibrils near the Z lines and have a mean transverse area of about 0.12 μ^2 per myofibril. Since the mean myofibril area in this region is 0.30 μ^2, the fractional transverse area of the transverse tubules and terminal cisternae is 0.30. The average longitudinal dimension of the transverse tubules is 0.026 μ (neglecting the enlargement near the fiber surface) and that of the terminal cisternae is 0.23 μ. Since there are two terminal cisternae per sarcomere, a value of 0.46 μ was used for the longitudinal dimension of this element.

The longitudinal tubules and fenestrated collar occupy about one-half of the sarcoplasmic space between the myofibrils at the level of the A band, where the myofibrils have an average cross-sectional area of 0.36 μ^2. Thus this system has a mean cross-sectional area per myofibril of 0.03 μ^2 and a fractional transverse area of 0.07. It occupies the entire length of the A band, which is 1.5 μ.

As was shown above, in the I band the longitudinal

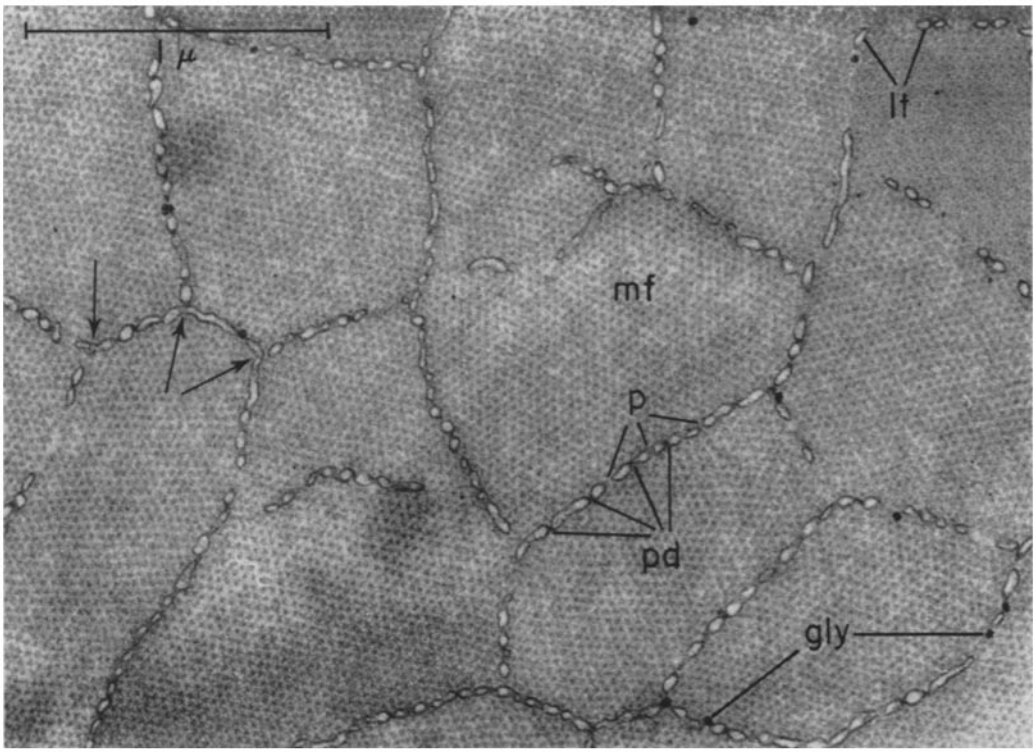


FIGURE 7 Transverse section through the A bands of several myofibrils (*mf*). The section passes through the H (non-overlap) zone in all of this figure except for the fibril at the upper right corner, where both thick and thin filaments are seen. Only a few tubules (*lt*) are seen around this fibril. These are the longitudinal tubules found at the ends of the A band. Throughout the rest of the figure, where no thin filaments are seen, a more continuous grouping of vesicular profiles surrounds each myofibril as a single layer. These are profiles of the fenestrated collar elements. The arrows indicate places where profiles of the fenestrated collar are continuous at the junction of three interfibrillar sarcoplasmic spaces. As in Fig. 6, it is clear that the perforations (*p*) extend entirely through the collar. Those pores (*pd*) that appear filled with a dense material (other than glycogen granules, *gly*) are most likely circular pores cut so that part of the rim of the pore is included in the section, which is only slightly thinner than the pore diameter. × 40,000.

tubules fuse to form the intermediate cisternae, which are the equivalent of a continuous cisterna around each myofibril, although in some places two cisternae are found between myofibrils and in other places none is found. The average thickness of these cisternae is 0.02 μ and their area per myofibril is 0.05 μ^2, giving a fractional transverse area of 0.12. These sheets measure 0.44 μ, on the average, in the longitudinal direction, and there are two of them per sarcomere.

As seen in Table I, the terminal cisternae, the intermediate cisternae, and the longitudinal tubule–fenestrated collar system each occupy about 4 or 5 per cent of the fiber volume. The transverse tubular system is considerably smaller, being about 0.3 per cent of the fiber volume. The total volume of the membrane-limited compartments of the sarcoplasmic reticulum and the transverse tubules is about 13 per cent of the fiber volume.

Surface Area of the Transverse Tubules

Since the transverse tubules are probably connected to the plasma membrane at the surface of the fiber, it is of interest to calculate how much membrane area they could contribute to the total surface area of the fiber.

The surfaces of the transverse tubular system oriented in the transverse plane, that is, the surfaces

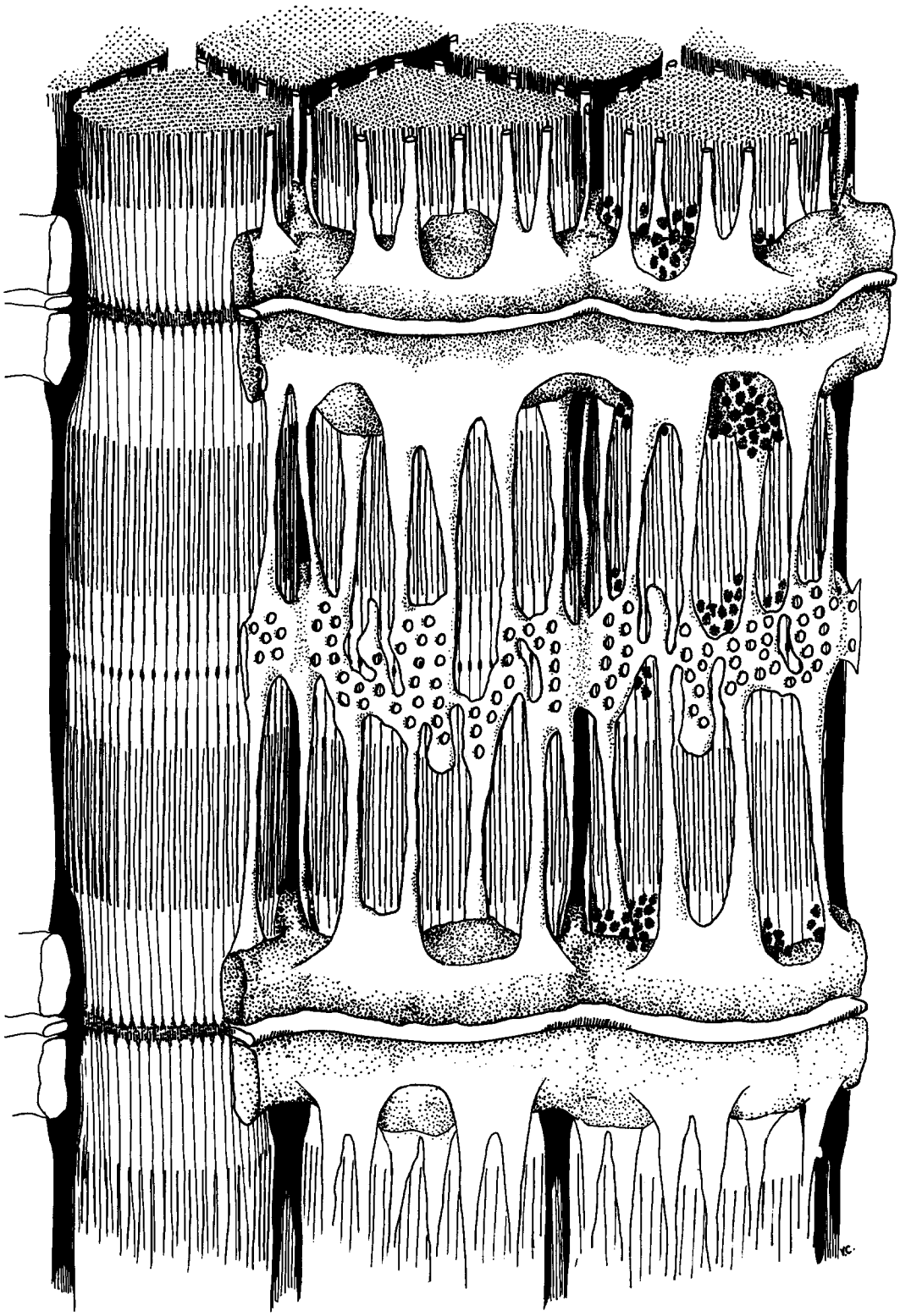


FIGURE 8 Three-dimensional reconstruction of the sarcoplasmic reticulum associated with several myofibrils. The major features present in this drawing (and absent from the one of *Ambystoma* muscle presented by Porter and Palade, 1957) are the continuity of the transverse tubules, the presence of intermediate cisternae, and the fenestrations of the collar at the center of the A band. × approximately 40,000.

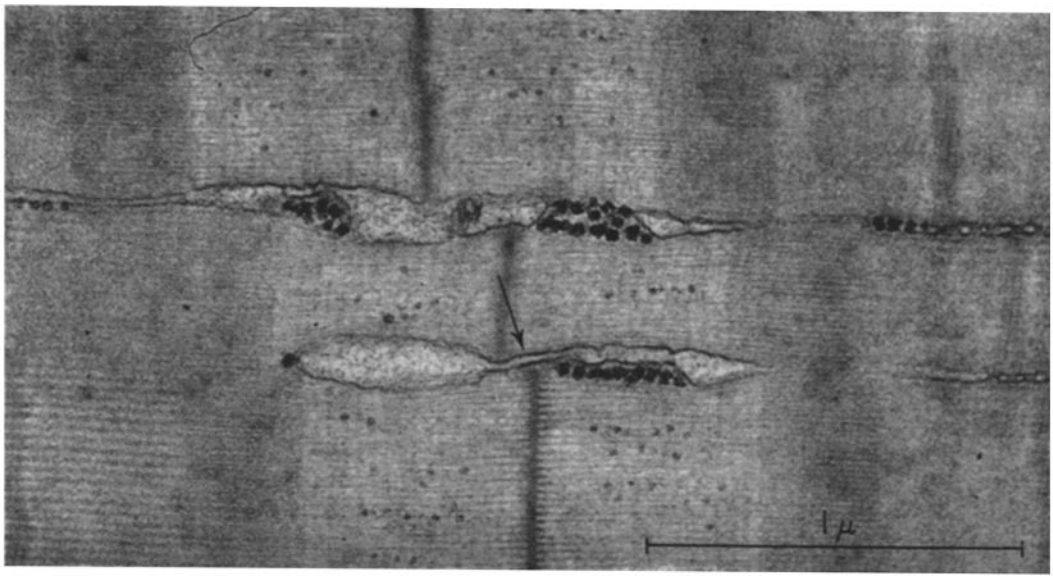


FIGURE 9 A longitudinal connection (arrow) between terminal cisternae on opposite sides of a Z line. × 50,000.

abutting on the terminal cisternae, are 0.3 times the cross-sectional area of the fiber times 2 because the T system has two such surfaces. For each centimeter of length of fiber, there are 3800 sarcomeres 2.6 μ in length, and an equal number of transverse tubular systems. The transversely oriented surface area of the transverse tubules in one cm length of a fiber with a radius of a (cm) is

$$(0.6\ \pi a^2)(3800) = 2.3 \times 10^3\ \pi a^2\ \text{cm}^2.$$

Dividing by the outer surface area of 1 cm length of fiber, this becomes $1.1 \times 10^3 a$ cm^2 per cm^2 of outer surface area. This figure does not include the area of the longitudinal membranes of the transverse tubules. These membranes, which face myofibrils rather than terminal cisternae, have an area per myofibril equal to the fibril perimeter times the longitudinal dimension of the transverse tubules, or $2.5\ \mu \times 0.026\ \mu = 0.065\ \mu^2$, where 2.5 μ is the perimeter of a myofibril of average area measured at the Z line. This is equal to 27 per cent of the transverse area of the transverse tubules per myofibril ($0.24\ \mu^2$), so the total area of the transverse tubules is given by $(1.1 \times 10^3 a \times 1.27 = 1.4 \times 10^3 a$ cm^2 per cm^2 of outer surface area.

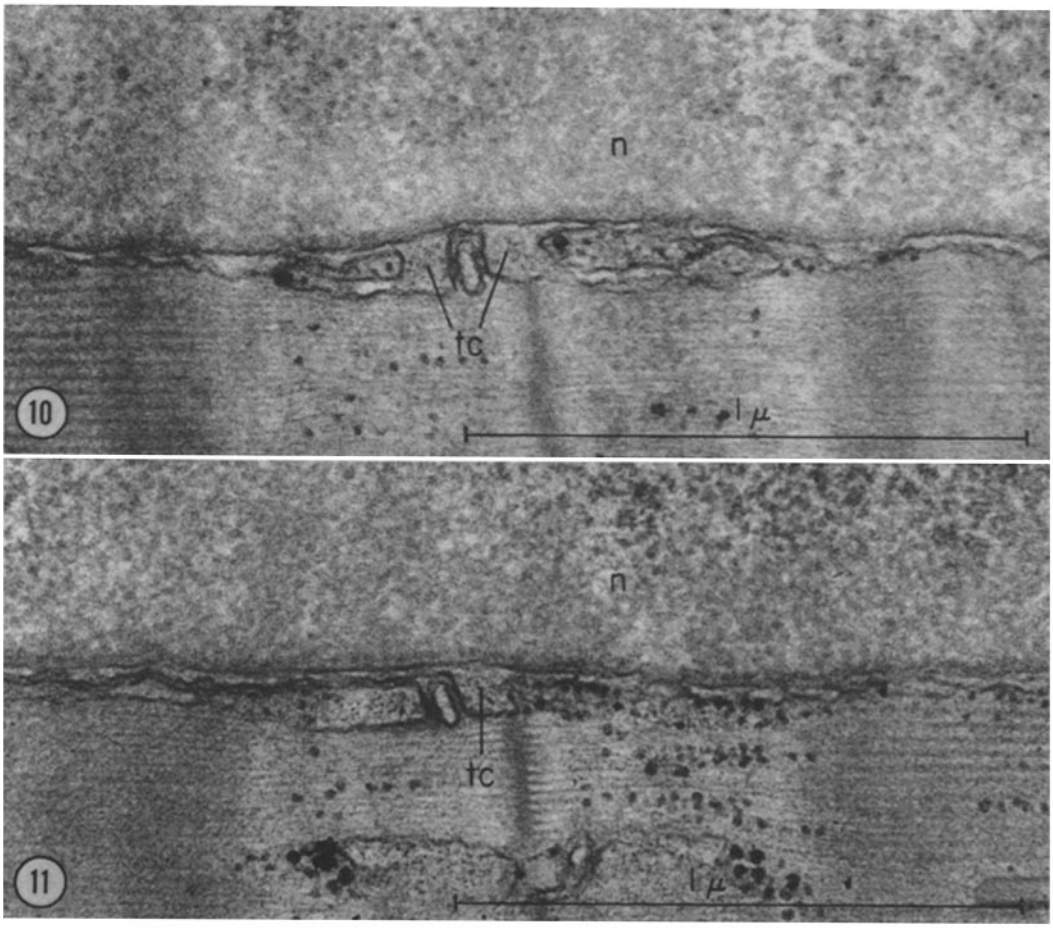
For a fiber 100 μ in diameter, the latter figure calculated above gives a value of 7 cm^2 of transverse tubule area per cm^2 of outer surface area. A similar ratio of 5 for 100 μ diameter sartorius fibers of *Rana temporaria* was estimated by Falk and Fatt (1964).

Surface Area of the Sarcoplasmic Reticulum

It is less easy to estimate the surface area of the sarcoplasmic reticulum because of its complex structural form.

Assuming the terminal cisternae to completely surround each myofibril and each to extend 0.23 μ in the longitudinal direction, one obtains a value of $7 \times 10^3 a$ cm^2 per cm^2 of outer surface area. This includes the surfaces facing toward the A bands, but excludes the flattened surfaces facing the transverse tubules. The intermediate cisternae are each 0.44 μ long, which gives a surface area of $10^4 a$ cm^2 per cm^2 of outer surface area. The longitudinal tubules and fenestrated collar can be approximated by a set of straight tubules for purposes of estimating their surface area. A diameter of 0.03 μ, a length of 1.5 μ, and a population of 30 around each myofibril seem to be reasonable choices for the dimensions and numbers of these tubules. This gives a surface area of $10^4 a$ cm^2 per cm^2 of outer surface area.

For a fiber 100 μ in diameter, these results (Table I) give surface areas of about 40 cm^2 per cm^2 of outer surface area for the terminal cisternae, and 50 cm^2 per cm^2 of outer surface area both for the intermediate cisternae and for the longitudinal tubules combined with the fenestrated collar. These estimates could be consider-



FIGURES 10 AND 11 Two examples of terminal cisternae (*tc*) continuous with the nuclear envelope. In each figure, the nucleus (*n*) is at the top and the nuclear envelope stretches across the figure near the center. In Fig. 10, both terminal cisternae connect to the outer nuclear membrane. In Fig. 11, only one cisterna is connected. This continuity indicates that the sarcoplasmic reticulum (but not the transverse tubules) and the endoplasmic reticulum of other types of cells are homologous structures. × 75,000.

ably in error, perhaps by a factor of 2 or 3, because of the irregular form of these structures and the assumptions made in the calculations.

DISCUSSION

The description of the internal membranes of the frog's sartorius presented here is thought to depict the form of this part of the amphibian muscle fiber more completely and more accurately than earlier studies through the use of improved preparation methods. Particularly important is the preservation of membrane continuity through the use of glutaraldehyde for fixation and epoxy resins for embedding. Although there is no sure way to distinguish between good and poor preservation of fine structure, one reasonable argument can be invoked in this case: in the living state, the transverse tubules are probably continuous as seen here, rather than vesiculated as seen when osmium tetroxide is used as the only fixative, because it seems more likely that a tubular system will break up into vesicles during preparation than that a system initially vesicular will artificially fuse in a specific way.

The evidence presented here for a direct connection of the transverse tubules to the plasma membrane of the fiber is not very convincing. However, good electron microscope evidence is

available from another vertebrate muscle (fish myotomes) for a direct membrane continuity such that the content of the tubules is continuous with the extracellular fluid (Franzini-Armstrong and Porter, 1964; confirmed by the present author, unpublished data). Also, H. E. Huxley (1964) has recently used electron microscopy to demonstrate the entrance of ferritin from the external medium into transverse tubules of frog muscle, and Endo (1964) has seen by light microscopy the entrance of a fluorescent dye into the I band regions of an isolated frog muscle fiber, presumably into the transverse tubules.

Thus the transverse tubules should be considered to be part of the plasma membrane of the muscle cell, not part of the sarcoplasmic reticulum. Specific association between these tubules and the reticulum is seen, however, in the triad, where the transverse tubules projecting inward from the surface meet the dilated cisternae of the sarcoplasmic reticulum. A similar situation has been seen in insect (Smith, 1961*a*, 1961*b*, 1962) and crustacean (Reger, 1962; Fahrenbach, 1963; Girardier *et al.*, 1963; Peachey and Huxley, 1964) muscles.

A different type of evidence for continuity of transverse tubules with the plasma membrane of frog muscle fibers comes from estimates of membrane capacitance based on the time course of the voltage response to a step-function current. The values obtained (5 μF/cm^2, Katz, 1948; 8 μF/cm^2, Fatt and Katz, 1951) are high relative to the values (about 1 μF/cm^2) obtained for nerve cells (Curtis and Cole, 1938; Hodgkin and Rushton, 1946; Hodgkin, Huxley, and Katz, 1952). It has been suggested that a major part of this capacitance resides in the tubule membranes and is in parallel with the true surface capacity (Falk and Fatt, 1964), which requires that the membranes be connected in some way. This suggestion is supported by the observation that the slow striated muscle fibers of the iliofibularis muscle of the frog, which have few if any transverse tubular structures (Peachey and Huxley, 1962), have a membrane capacity estimated in the same way to be only 2.5 μF/cm^2 (Adrian and Peachey, 1965). In addition, crab muscles, which have extensive infolding of the plasma membrane (Peachey and Huxley, 1964), have a very high capacitance of about 40 μF/cm^2 (Fatt and Katz, 1953).

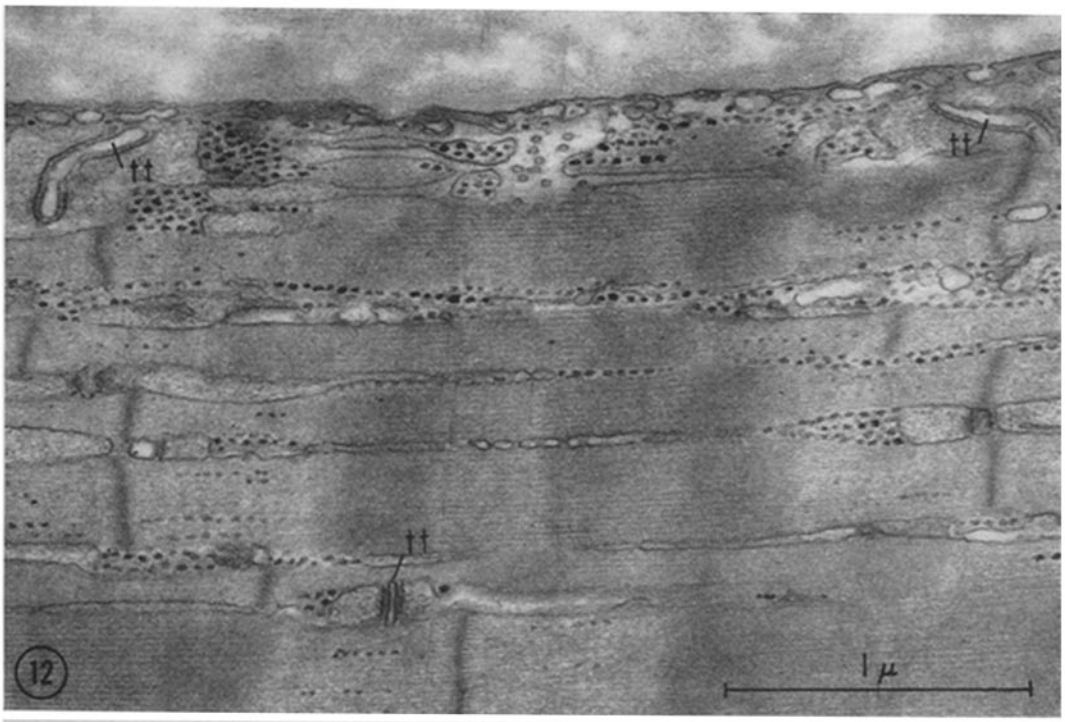
The surface of the transverse tubules of a fiber 100 μ in diameter was estimated here to be 7 times the plasma membrane area. This is similar to the value of 5 estimated by Falk and Fatt (1964) for a fiber of similar diameter and sarcomere length, and found by them to fit satisfactorily into their model in which the fiber capacitance is distributed between external and internal membranes.

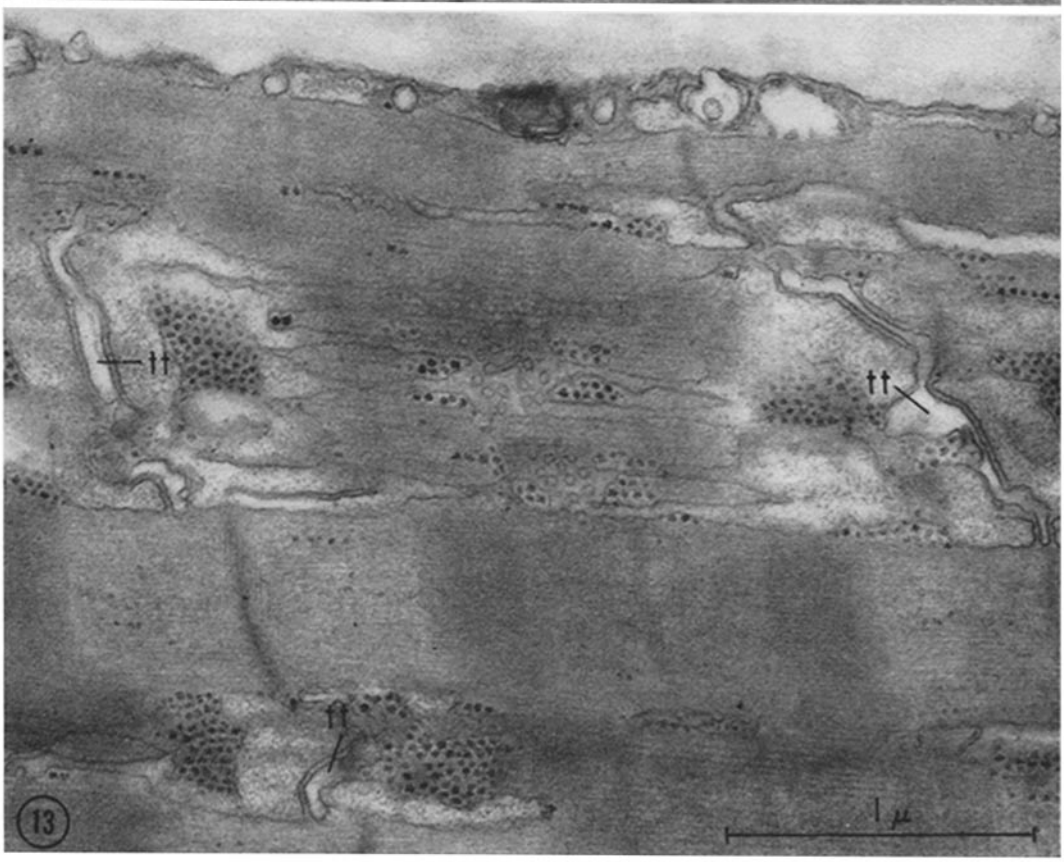
The estimated volumes presented here can be compared with estimates of volumes of compartments in frog muscle based on physiological experiments. It should be noted, in making these comparisons, that the volumes were estimated here from measurements on fixed and embedded specimens, and the possibility of volume change during preparation cannot be ruled out. The absence of gross distortion in the preparations, however, suggests that there have not been large changes in volume and that it is worth while to make comparisons with physiological data.

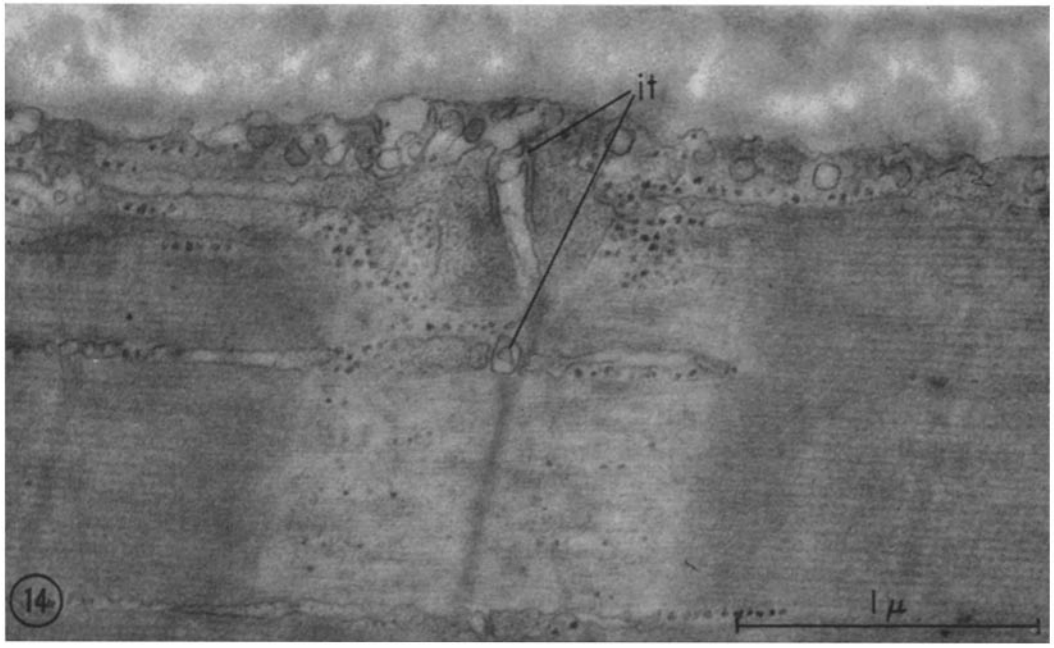
Hodgkin and Horowicz (1960*a*) estimated a volume of 0.2 to 0.5 per cent of the fiber volume for a "special region" in which potassium ions were retained after the potassium concentration of the external medium was suddenly reduced. The depolarizing effect of these retained potassium ions suggested that they were located outside the membrane across which the membrane potential was maintained, but perhaps within some tubules that were electrically continuous with the external fluid.

Examination of Table I reveals only one compartment whose volume is within an order of magnitude of the mean volume of the special region estimated by Hodgkin and Horowicz. This is the transverse tubule compartment, whose volume was estimated here to be approximately 0.3 per cent of the fiber volume.

Further support for the identification of the transverse tubules as the special region of Hodgkin and Horowicz has come from some extensions of their original ideas. Adrian and Freygang (1962) developed a model of ion movements in a muscle fiber that included a special region (their compartment 2) separated from the sarcoplasm by a membrane with a relatively high potassium permeability. The assignment to this compartment of a volume (for a fiber 80 μ in diameter) equal to about 0.2 per cent of the fiber volume, again similar to the estimated volume of the transverse tubules, allowed those authors to fit their model satisfactorily to the changes in potassium conductance that they observed during the passage of constant hyperpolarizing currents and constant


12
1 μ


13
1 μ



FIGURES 12 TO 14. Longitudinal sections showing alteration of the form of the transverse tubules (*tt*, Figs. 12 and 13) that occurs near the fiber surface. The lumens of the tubules are two to three times larger here than they are deeper in the fiber, and they often show complex forms which sometimes appear to be involutions (*it*, Fig. 14) of the tubule membrane into the tubule lumen. × 40,000.

depolarizing currents not large enough to cause contracture. Freygang *et al.* (1964*a*) have used this model to explain a slowly decaying residual depolarization that follows a train of action potentials in frog sartorius fibers, using the same value for the volume of a special region in which potassium accumulates during the action potentials. Furthermore, Freygang *et al.* (1964*b*) have shown that, under certain experimental conditions, the time course of this late after potential is lengthened, and that the changes observed can be explained according to the model if it is assumed that the volume of the special region is increased. Electron microscope measurements on the transverse tubules of these experimental muscles show volume increases that agree satisfactorily with the changes demanded by the electrical measurements and the model.

These results, taken together, suggest that when an action potential passes over the surface of a frog twitch muscle fiber a depolarization spreads passively into the fiber along the membranes of the transverse tubules, and that potassium ions enter the tubules from somewhere in the muscle cell. This effect, which occurs in the vicinity of the myofibrils, is in essence the solution of the first step in the excitation-contraction coupling problem first stated by Hill (1948, 1949). What remains to be discovered is how this effect is linked to myofibrillar contraction. Presumably calcium release from the transverse tubules or from some part of the sarcoplasmic reticulum is an important part of this step (Hodgkin and Horowicz, 1960*b*, 1960*c*). As has been pointed out by H. E. Huxley (1964), release from the transverse tubules is unlikely because the amounts of calcium that must be released seem to be too large to reside in the transverse tubules.

Autoradiographic experiments done by Winegrad (1965) indicate that, during activation of frog muscle, calcium moves from the central portion of the I band to the A band. Recent experiments of Hasselbach (1964) and of Constantin *et al.* (1964) suggest that calcium accumulated by the sarcoplasmic reticulum during relaxation concentrates in the terminal cisternae. Thus one looks naturally to the terminal cisternae as possible sites of calcium release. Their volume (5 per cent of the fiber volume) is such that if they contained calcium ions at the same concentration as Ringer's

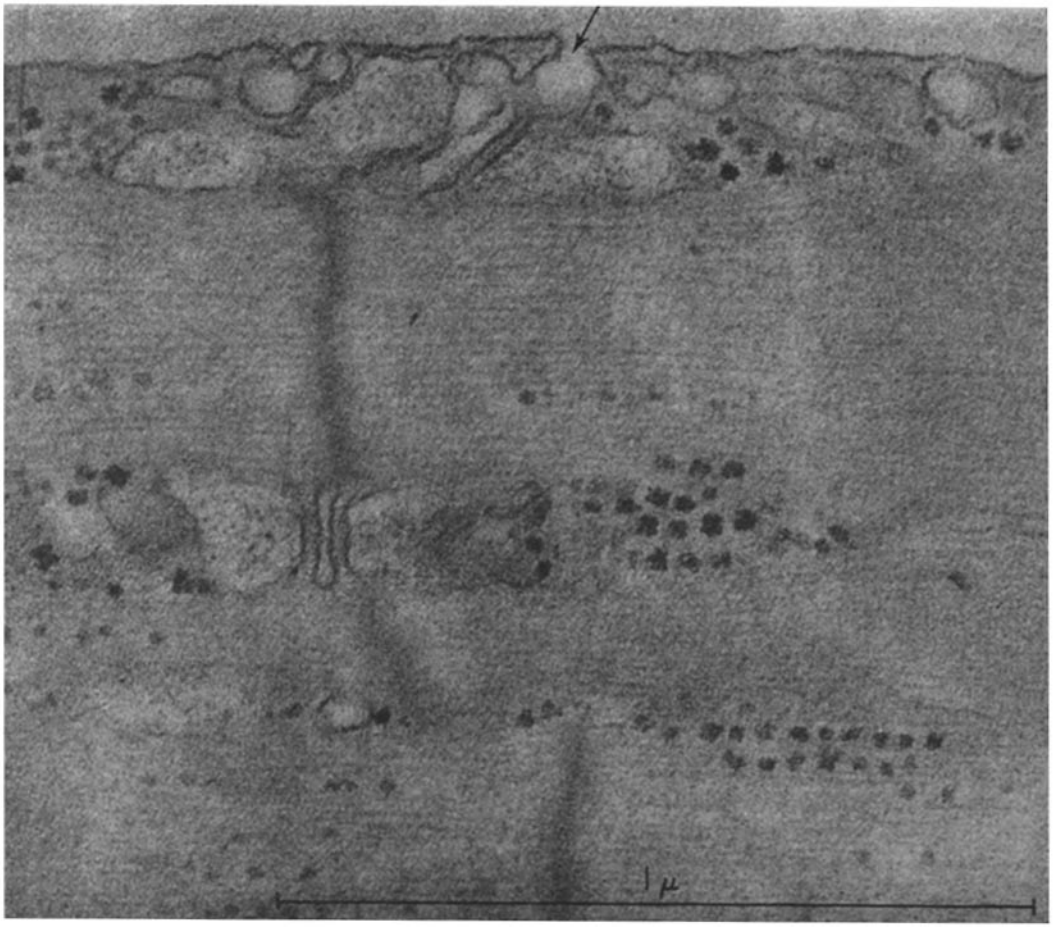


FIGURE 15. Longitudinal section at the surface of a fiber, showing what might be a connection (arrow) of the transverse tubule to the surface membrane of the fiber. × 100,000.

solution (about 2 mM), and if this calcium were equilibrated with the sarcoplasm during activation, the concentration reached (0.1 mM) would be just that thought to be required for contraction (H. E. Huxley, 1964).

We might next ask now calcium release from the terminal cisternae is coupled to depolarization of the transverse tubules. The morphology of these structures seems to suggest some answers. About 80 per cent of the transverse tubular surface is covered by the flattened surfaces of the terminal cisternae, leaving only 20 per cent of the tubular surface in direct contact with the sarcoplasm. Thus it is plausible to suppose that some if not most of the tubular ionic current passes into the terminal cisternae and then to the sarcoplasm rather than going directly from the transverse tubules to the sarcoplasm. The similarity of the serrate appearance of the junctions between transverse tubules and terminal cisternae to that of the septate junctions between epithelial cells (Wood, 1959; Wiener *et al.*, 1964), which have been implicated as low resistance pathways for ions (Kanno and Loewenstein, 1964; Loewenstein and Kanno, 1964), suggests that an even larger fraction of the tubular current might pass directly to the terminal cisternae.

If such a direct ionic conduction pathway does exist between the transverse tubules and the terminal cisternae, then the resting potential of the fiber would be divided between this junction and the membranes between the sarcoplasmic reticulum and the sarcoplasm, the division depending on the relative permeabilities of these surfaces to

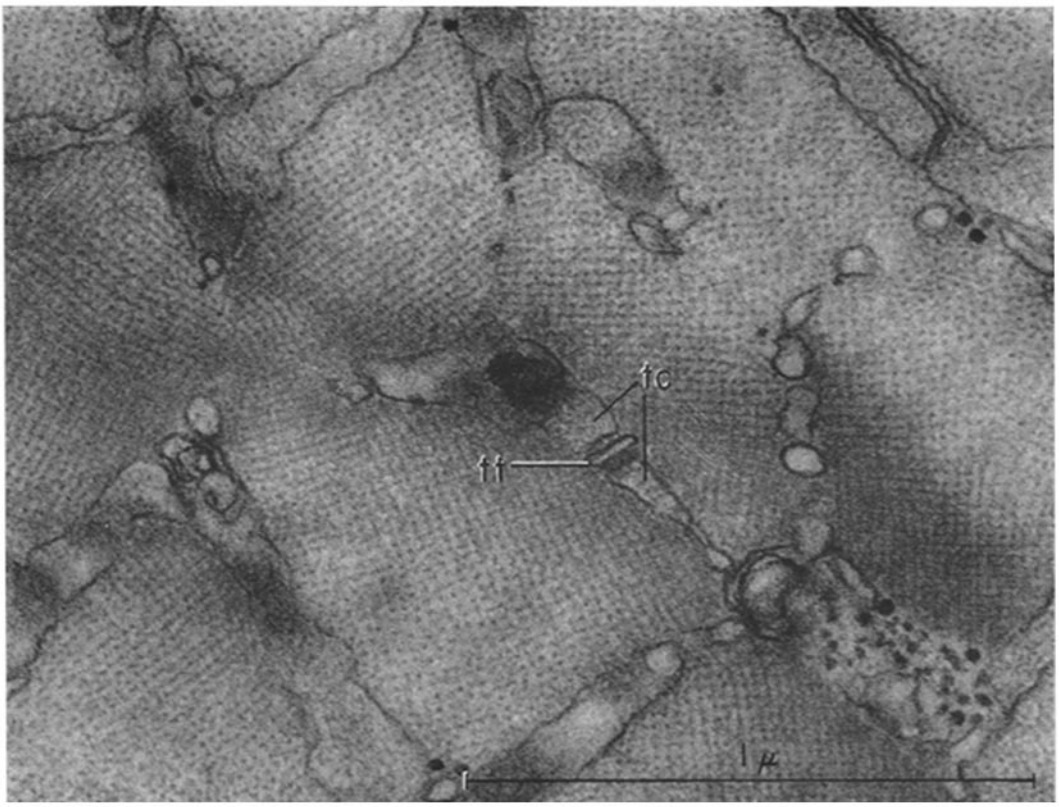


FIGURE 16 Transverse section through the I band. The central element (*tt*) of a triadlike structure (*tt, tc*), seen near the Z lines of the myofibrils, is interpreted as a longitudinally oriented transverse tubule such as has also been observed in longitudinal sections (Fig. 5). × 75,000.

TABLE I

Volumes and Surface Areas of Sarcoplasmic Reticulum and Transverse Tubules

Compartment	Transverse area per 0.42 μ^2 fibril	Fractional transverse area	Length per 2.6 μ sarcomere	Fractional fiber volume	Surface area for fiber of radius a (cm) per cm^2 fiber outer surface
	μ^2		μ		cm^2
Transverse tubules	0.12	0.30	0.026	0.003	$1.4 \times 10^3 a$
Terminal cisternae	0.12	0.30	0.23×2	0.05	$7 \times 10^3 a$
Intermediate cisternae	0.05	0.12	0.44×2	0.04	$1 \times 10^4 a$
Longitudinal tubules plus fenestrated collar	0.03	0.07	1.5	0.04	$1 \times 10^4 a$
Total				0.13	$3 \times 10^4 a$

various ions and the ionic concentrations in the various compartments. Ionic currents flowing across the fiber surface and the walls of the transverse tubules, such as occur during an action potential, would produce potential changes across the membranes of the sarcoplasmic reticulum, according to this scheme, and could lead to the release of an activator of contraction from the sarcoplasmic reticulum. What part of the sarcoplasmic reticulum releases this activator is not known, but

it is clear from Huxley and Taylor's (1958) local stimulation experiments that activation does not spread past the center of the A band to the next half sarcomere. This could be explained if only the terminal cisternae carried currents and were involved in the release of activator, and if the activator did not diffuse past the M lines of the myofibrils. The failure of lissamine rhodamine B200 to penetrate into the A bands of frog muscle fibers in the experiments of Endo (1964) could also be explained if this dye entered only the transverse tubules and terminal cisternae, or the transverse tubules alone. The volume of distribution of this dye (1 per cent), estimated by Endo, lies between the volume of the transverse tubules (0.3 per cent) and the combined volumes of the transverse tubules and terminal cisternae (5 per cent) estimated here. Furthermore, the presence of granular material in the terminal cisternae but not in the intermediate cisternae suggests the presence of some form of barrier between these structures, perhaps at the narrow channel observed at their common junction. This morphological distinction between these elements might reflect an underlying functional difference.

Evidence that apparently argues against a large ionic conductance between the transverse tubules and the terminal cisternae is found in the rather good agreement of physiological estimates of the volume of the special space of Hodgkin and Horowicz (1960*a*) and of the surface area of the internal membranes of Falk and Fatt (1964) with morphological estimates of the same parameters of the transverse tubules. The agreement is not nearly so good if one considers the special space and internal membranes to include the terminal cisternae. While this evidence seems to rule out a large conductance across the serrate junction between these structures, it is not impossible that ionic currents could produce potential changes across this junction that would stimulate the terminal cisternae to release calcium.

We have no basis on which to surmise what the function of other parts of the sarcoplasmic reticulum might be, but it would be consistent with the idea expressed above to assume that they recapture released calcium during relaxation and return it to the terminal cisternae. Connections across the triads between terminal cisternae might act to equilibrate activator concentrations in the longitudinal direction, thus aiding the maintenance of equal contractile force along the fibrils. It may be hoped that studies of the variation of the form of the sarcoplasmic reticulum with sarcomere length or with contractile state of the muscle fiber will shed further light on these problems.

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