SARCOLEMMAL INVAGINATIONS CONSTITUTING THE T SYSTEM IN FISH MUSCLE FIBERS

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ABSTRACT

Striated muscle fibers from the body and tail myotomes of a fish, the black Mollie, have been examined with particular attention to the sarcoplasmic reticulum (SR) and transverse tubular (or T) system. The material was fixed in osmium tetroxide and in glutaraldehyde, and the images provided by the two kinds of fixatives were compared. Glutaraldehyde fixes a fine structure that is broadly comparable with that preserved by osmium tetroxide alone but differs in some significant details. Especially significant improvements were obtained in the preservation of the T system, that is, the system of small tubules that pervades the fiber at every Z line or A-I junction level. As a result of this improved glutaraldehyde fixation, the T system is now clearly defined as an entity of fine structure distinct from the SR but uniquely associated with the SR and myofibrils. Glutaraldehyde fixation also reveals that the T system is a sarcolemmal derivative that retains its continuity with the sarcolemma and limits a space that is in direct communication with the extracellular environment. These structural features favor the conclusion that the T system plays a prominent role in the fast intracellular conduction of the excitatory impulse. The preservation of other elements of muscle fine structure, including the myofibrils, seems for reasons discussed, to be substantially improved by glutaraldehyde.

INTRODUCTION

The sarcoplasmic reticulum (or SR) in vertebrate skeletal muscle fibers is a labyrinthine system of vesicles structurally continuous within the limits of each sarcomere (3, 4, 13, 46, 48, 50). At every Z line, and in some muscles also at every A-I junction, the longitudinal continuity of the system is interrupted along a plane normal to the long axis of the fiber by the presence of a second membranous system. The elements of the second system are in the form of short tubules or vesicles and appear sandwiched between two terminal sacs or cisternae of the SR. Together these make up a three-part structure that has been called a triad (46). The central element of the triad is recognizably different from the other two vesicles belonging to the sarcoplasmic reticulum; its dimensions are smaller, its limiting membrane is more prominent, and its content is never continuous with the content of the SR. It thus appears as a distinct and separate element among the intracellular membranous components of the fiber. On the basis of observations by Andersson-Cedergren (3) and subsequently by Revel (50), this intermediate element of the triad is generally considered to be part of a continuous vesicular or tubular system which runs transversely across the fiber and has come to be referred to as the T system of the muscle fiber.

This interpretation of the T system has aroused

some interest, especially among physiologists, because it suggests a mechanism for transmitting excitation transversely in the fiber. In performing such a role the T system would provide a solution to one of the more puzzling problems in excitation-contraction coupling: this is the remarkable fact that, within 40 msec from the time of excitation, the whole cross-section of the fiber enters into a full state of activity. In the frog sartorius, then, as shown by the experiments of Hill (20), it takes less than 40 msec for the excitation to spread to the center of the fiber 50 μ from the sarcolemma. Hill (19) makes the point that any substance involved in triggering the contractile mechanism would take much longer than this to reach the center of the fiber by diffusion. Even more impressive in this regard is the case of really fast muscles such as the internal rectus of the kitten where, though the radius of the fiber is reduced to 25 μ, the time required for the excitation of central myofibrils is only about 4 msec.

It follows that a definite structural pathway for supporting and conducting the excitation has to be postulated, and it has been proposed that the Z membrane, thought to be continuous across the fiber, might perform this role (24). Subsequently Huxley and coworkers (22, 23, 25) discovered that a transverse conduction of excitation could be demonstrated when a subthreshold stimulus was applied opposite the triad, whether at the level of the Z line or A-I junction. This suggested that some part of the SR at the level of the triad might be involved in the conduction of the excitation (41). Later still the T system was proposed as the appropriate element for this role (44). It was recognized that to meet the requirements of such a role, the system should fulfill two conditions: (*a*) it should be continuous across the fiber, or at least some part of the fiber, and (*b*) it should be an extension of the sarcolemma so that an action potential might flow from one to the other.

The first condition has been demonstrated to be apparently true in a few cases (3, 13, 50), and we shall here provide evidence for it in yet another form. But a direct connection of the T system with the sarcolemma in vertebrate skeletal muscles has not been clearly shown, following the use of standard fixation procedures, despite numerous efforts to trace one (3, 44, 46, 48, 50). It has therefore been concluded that connections, if natively existing, are either very rare and possibly continuously shifting in position, or are perhaps labile and disrupted by the action of the fixative. With regard to the latter hypothesis, some results reported by Rosenbluth (53) are significant. He compared the fine structure of osmium tetroxide–fixed *versus* permanganate-fixed toad spinal ganglia and noticed that the form of certain invaginations of the plasmalemma into the cell were preserved as continuous tubules by permanganate, whereas following osmium tetroxide the "tubules" appeared as a row of discrete vesicles. Thus we had reason to wonder whether any native continuity which might exist between the T system and the sarcolemma would indeed be preserved by osmium tetroxide as ordinarily used in fixation.

Prompted by this thought, we have experimented with glutaraldehyde which has been recently introduced for the preservation of tissues for electron microscopy (54). We also chose to use a small animal, the aquarium fish black Mollie, the segmental muscles of which can be fixed instantly by immersion of the animal in the fixative, thus avoiding possible alterations in the activity and morphology of the fiber that might result from the use of anesthesia and dissection.

The purpose of this report is to illustrate the form and disposition of the elements of the SR and T system in the red and white fibers of this small fish. Particular attention will be given to the structure of the latter after preservation in OsO_4 alone and in glutaraldehyde followed by OsO_4. As a result of this study, it has been possible to distinguish definitely between the elements of the T system and the SR and to demonstrate that the former is an extension of the sarcolemma which penetrates deeply into the fiber. The implications of these observations are discussed.

MATERIALS AND METHODS

Young black Mollies (*Mollienesia* sp.), 2 to 3 cm long, were fixed for 1.5 hours in glutaraldehyde (buffered with cacodylate at pH 7.2, essentially according to Sabatini *et al.*, reference 54) and, after several hours of washing in buffer, they were post-fixed in 1 per cent OsO_4 in water for 1 hour. Other animals were fixed for 1.5 hours in the conventional Veronal-acetate buffered osmium tetroxide (37). All fixations and the dehydration were at 0°C. After dehydration in alcohol followed by propylene oxide, the material was embedded in Epon 812 (32). Sections were cut on a Porter-Blum microtome, and stained with lead hydroxide (30) or lead citrate (51), and thereafter examined with a Siemens Elmiskop I or a Philips 200.

OBSERVATIONS

We shall first describe the morphology of the muscle following glutaraldehyde plus osmium tetroxide fixation and then compare this with the more conventional image obtained following osmium tetroxide fixation alone.

The animals used were approximately one-third of the adult size, consequently the dimensions of the fiber were probably smaller than in the adult. This fact notwithstanding, the majority of the fibers appeared completely differentiated. Only in a few instances were peripheral zones of the fiber rich in ribosomes and glycogen and probably still undergoing differentiation. Even in these the rest of the fiber was fully occupied by fibrils, sarcoplasmic reticulum, glycogen, and mitochondria, just as in any adult fiber.

1. Size and distribution of the Fibers

The black Mollie tail and body metameres (segmental muscles) are composed mostly of "white" fibers. These constitute the bulk of both the hypo- and epi-axial regions into which the myomere is divided by a septum running along the midline of the body. Coincident with the lateral line there is a separate group of fibers disposed to form what, in a transverse section of the animal, appears like an equilateral triangle with its base against the skin. In this and other teleosts, this richly vascularized muscle shows a deep red color. We shall refer to the fibers of this lateral muscle as "red" fibers.

In red fibers (Fig. 1), which have a diameter of 4 to 8 μ, the peripheral sarcoplasm contains large numbers of mitochondria and rich deposits of glycogen. These broad marginal zones, devoid of contractile material, do not, however, extend continuously around the fiber but are interrupted at a few places where the sarcolemma is relatively close to the peripheral fibrils. In white fibers (Fig. 2), on the other hand, the diameters of which range from 6 to 12 μ, this homologous marginal zone contains only a little sarcoplasm around the fibrils and only occasionally is thick enough to accommodate a few mitochondria and some glycogen. The red fibers are, moreover, always richer in glycogen, which is distributed in the sarcoplasmic spaces (or rays) between the fibrils as well as under the sarcolemma.

2. The Fibrils

As in other fish muscles (13, 35, 60), both kinds of fibers contain ribbon-like fibrils which extend from the center to the periphery of the fiber and often branch (Figs. 1 and 2). Thus the fibrils have one relatively large dimension in a radial direction in the fiber and a relatively thin dimension in the other or tangential direction. In the center of some of the larger fibers one finds in addition a few cross-sections of irregularly polygonal small fibrils surrounded by sarcoplasm and its constituents.

The sarcomeres of fibrils as observed in this material are between 1.4 and 1.6 μ in length whether fixed in glutaraldehyde or in osmium tetroxide.[1] The M band is unusually dense and occupies most of the width of what is evident as the H band (Figs. 6, 8, and 13). The filaments are closely packed after glutaraldehyde fixation and appear superimposed in a longitudinal section unless the section is extraordinarily thin. The Z line, after each fixation, shows the zig-zag pattern (for example, Fig. 8) described by Knappeis and Carlsen (31) and Franzini-Armstrong and Porter (14).

Transverse sections clearly reveal the disposition of the myofilaments in the different bands of the sarcomere. In the A band each thick filament is surrounded by 6 thin filaments, disposed hexagonally (Fig. 3), and the thick filaments are arranged in a hexagonal pattern descriptive of close packing. Toward the center of the sarcomere the thin filaments are less dense, and in the middle of the H band (*i.e.* in the M band) they are distinctly present in a trigonal position with respect

[1] This sarcomere length is obviously shorter than that frequently found in skeletal muscle. The reasons may be several. First it should be noted that these are fibers of segmental muscles and that in the same muscles of amphibian larvae it was earlier noted that the I bands are unusually short and that the sarcomeres measure about the same as here; *i.e.*, 1.5 μ (46). This similarity notwithstanding, it may be that some shortening took place in these muscle fibers before fixation. This possibility is suggested by the relative diffuseness of the I band margins and the configuration of the H-M complex. Some further shortening of the sarcomere may be superimposed by the compressive action of sectioning which in this case was directed parallel to the long axis of the fibrils. However, since the plane of section closely parallels the long axis of the fibrils, none of the shorter length can be referred to any obliquity of section orientation.

to the thick filaments (asterisks in Fig. 3), though just barely visible. Since the extent of sarcomere shortening is undetermined, we cannot be sure whether these represent thin filament connections across the H-M zone (the S-filaments of the Huxley-Hanson model, reference 16), or thin filaments which have moved into the zone, according to the sliding filament theory of contraction. We favor the first interpretation because we feel that the shortening, if any, is minimal and because heretofore OsO_4, with its obvious destructive action, has been used as a fixative and has probably destroyed the thinner extensions of the thin filaments. In the M band, cross-bridges appear to join the thick filaments and to contribute to the density of this band (arrows, Fig. 3). This is the picture in glutaraldehyde-fixed preparations.

On the other hand, after OsO_4 alone, the morphology of the A, H, and M bands differs considerably (Fig. 4). For example, in the A bands the distance between the centers of the thick filaments, *i.e.* the dimension of the lattice, is the same as after glutaraldehyde, but the diameter of the filaments is considerably smaller (about 130 A as compared to 150 A). Also, in osmium tetroxide-fixed preparations, the edges of the H band are sharply set off from the adjacent A bands, apparently because at the H band the thin filaments suddenly disappear (as described by Huxley, 27, 28) and are not discernible all through the band. Also evident are the bridges which connect the thick filaments at the level of the M band, and which are similar to those described by Spiro (59) in heart muscle and by Huxley and Hanson (26) in insect muscles. They are present after both types of fixation. It is to be noted further that the thick filaments appear as fine hollow tubules, particularly at the level of the M band.

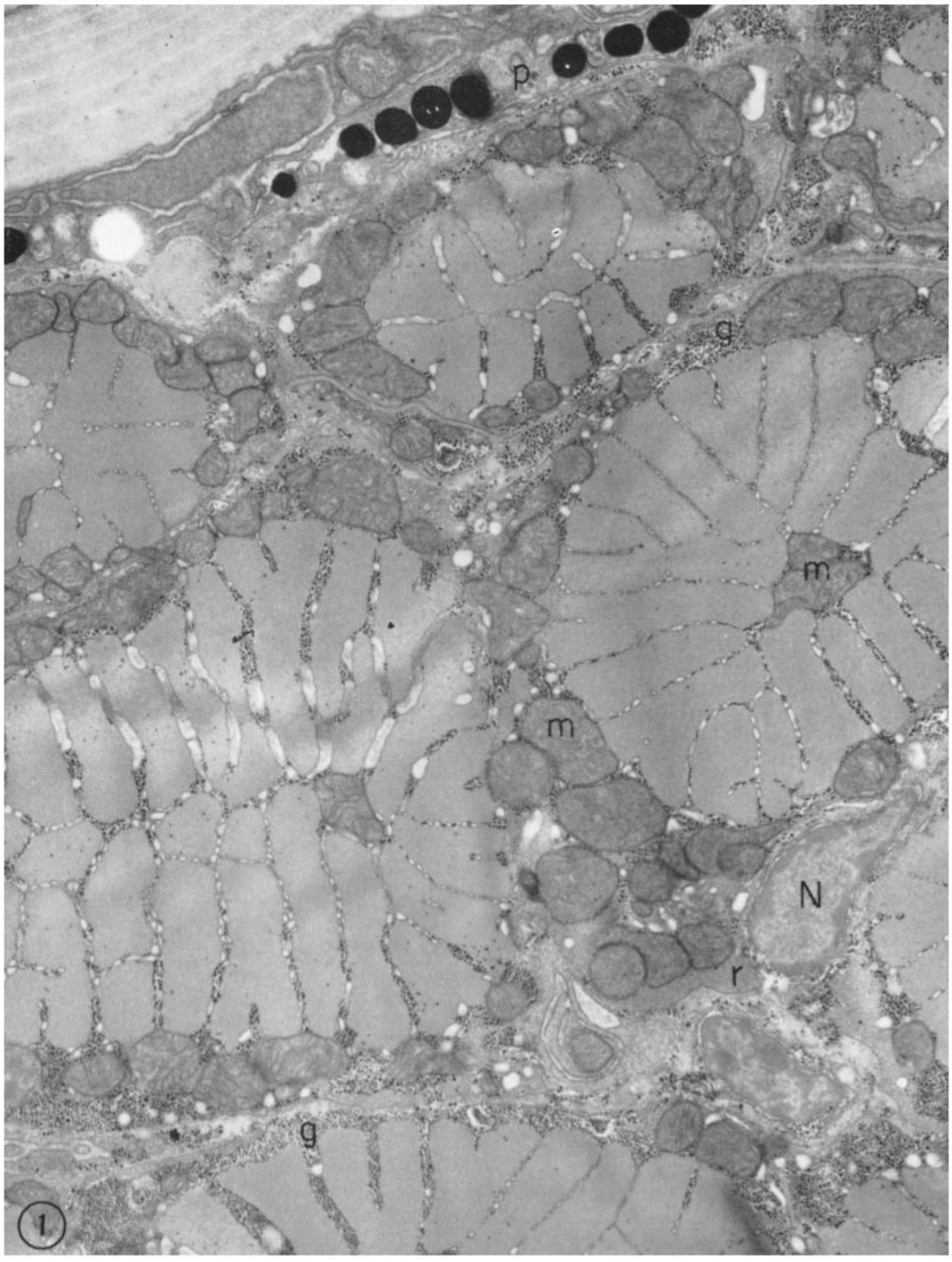
In the I band the regular hexagonal array of thick and thin filaments characteristic of the A band level is replaced by a rather irregularly disposed arrangement of thin filaments (Fig. 5). These do not run exactly parallel, since some of them appear as dense dots, and so are oriented exactly perpendicular to the plane of section, while others pass through the section obliquely and appear as less dense dots or as short rods. The thin filaments are not so closely packed as in the A band, because the thick filaments are here missing; there is room among them even for glycogen granules. Near the Z line level, however, the thin filaments follow a more organized course in a direction parallel to the long axis of the fibril. And in the zone immediately adjacent to the Z line, they are organized in a square pattern of considerable regularity, having apparently shifted from their previous hexagonal arrangement peculiar to the A band (31). At the Z line level the square pattern is maintained, though less readily discerned because of the presence of dense material constituting the Z membrane or septum (14).

In this limited study of the I and Z bands (compare Fig. 5 with Fig. 10), we find again some significant differences between the images after osmium tetroxide and glutaraldehyde fixations. In the former, the disorder of the I band filaments is much more pronounced, and the band appears much less dense, as though extracted. The Z line, on the other hand, shows a more sharply defined structure of filaments and dots in the osmium tetroxide-fixed preparations (possibly because of the partial extraction), whereas this structure is somewhat masked in the glutaraldehyde preparations by some intervening material.

3. The Sarcoplasmic Reticulum

The narrow partitions or sheets of sarcoplasm between the fibrils are occupied by a large number

FIGURE 1 Transverse section of body muscle fibers of a black Mollie from a region immediately under the skin and adjacent to the lateral line. The collagen layers of the dermis appear in the upper left-hand corner; beneath them there is a layer of pigment cells (*P*), and finally muscle cells of the myotomes. Several fibers appear in the field. Since this is a red muscle, the fibers are small and rich in mitochondria (*m*) and glycogen (*g*), in this case mostly located in large areas of sarcoplasm immediately adjacent to the sarcolemma. At the periphery of the fiber there are also groups of ribosomes (*r*). The fibrils are mostly ribbon-like and oriented as radii within the circular cross-sections of the fibers. Elements of the sarcoplasmic reticulum occupy the spaces between them. The nucleus (*N*) belongs to a connective tissue cell. Glutaraldehyde fixation. × 8500.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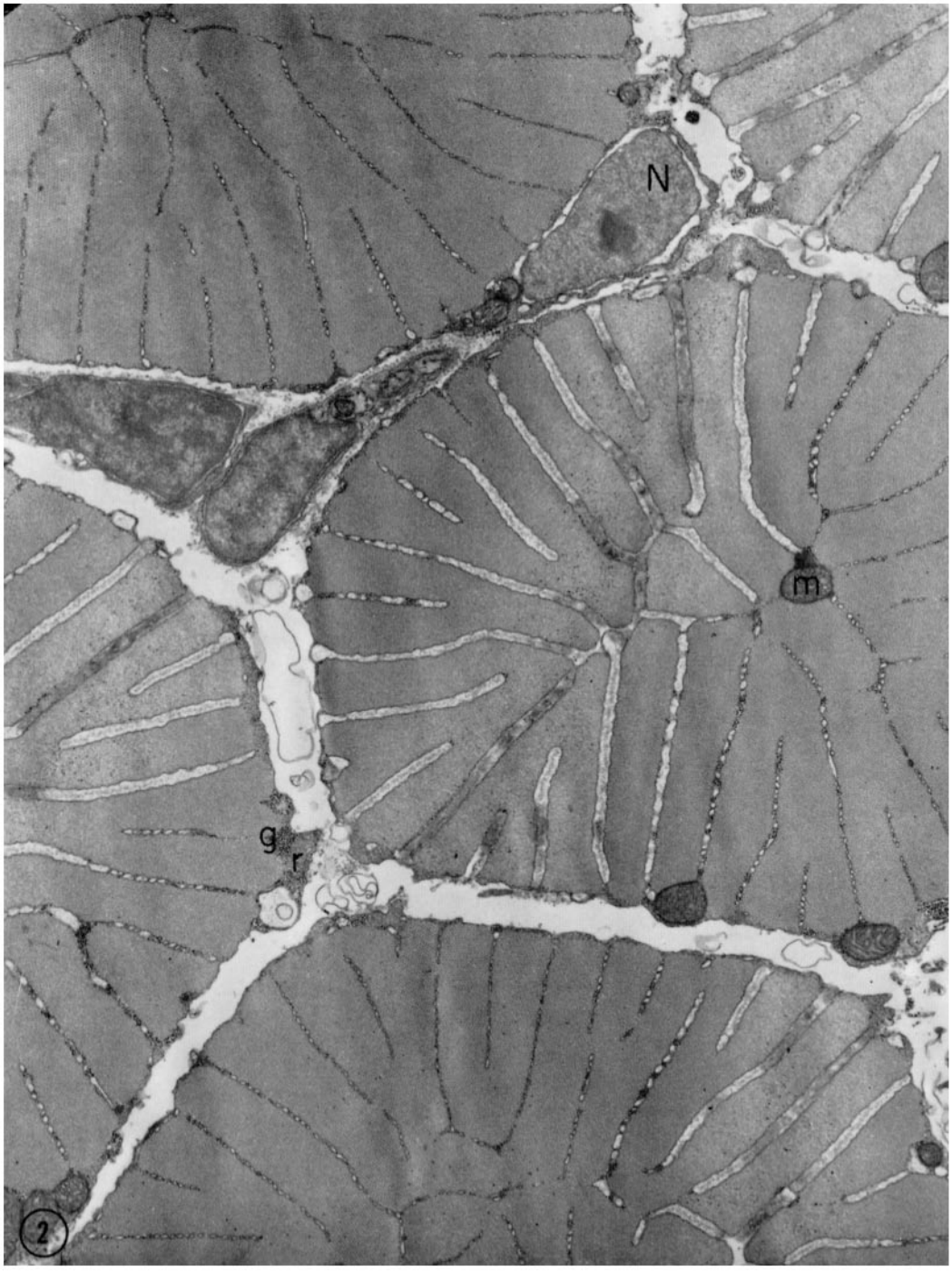
of tubules and vesicles belonging to the sarcoplasmic reticulum and the T system of these fibers. Since these are not substantially different in the red and white muscles, the following description applies to both.

The SR in these Mollie muscles (Fig. 6) appears in longitudinal sections in the repeating pattern typical of vertebrate skeletal muscle fibers. Its elements are very numerous and very regularly disposed. The study of the SR morphology is greatly facilitated by the shape of the fibrils, which are asymmetric in cross-section and possess broad, flat lateral faces. Longitudinal sections which pass obliquely across the short dimension of the fibrils provide lateral views of several of the interfibrillar sheets of sacroplasm as they enter and leave the section. In Fig. 6 there is a view of this kind which is suitable for demonstrating the regularity of the pattern formed by elements of the SR. These elements fill almost completely the interfibrillar spaces and appear as canals oriented longitudinally along the side of the fibrils. Upon reaching the I band at either end of the sarcomere, the profiles of the SR vesicles dilate noticeably and in this form (called terminal sacs) the SR of that sarcomere terminates opposite the I band. On the other side of the Z line, an identical terminal sac marks the beginning or ending of another SR segment, which stretches with identical characteristics along the next sarcomere. Between the two terminal sacs there is then a third small vesicular element representing the T system of the fiber. The three together form a typical triad, as described previously in several skeletal muscle fibers (Fig. 7, arrows). When the orientation of the plane of section changes with respect to the fibrils so that they are not cut obliquely through their short dimension but parallel to and including their broad face, some larger expanses of the reticulum are included in the section (shown to some extent in Fig. 6 and better in Fig. 7). One can then see that cisternae with long axes oriented either longitudinally or transversely to the long axis of the fiber contribute to the SR pattern. Longitudinally oriented tubular sacs are very numerous along the A band and run parallel and in a straight course over the length of the sarcomere. It is important to note here that the shape and dimensions of these thin sacs seem much better preserved in glutaraldehyde than in OsO_4 alone. After the latter the sacs appear to have varying dimensions, and their profiles show a wavy outline probably due in part to the fact that osmium tetroxide induces contraction of the fibers during its penetration into the tissue (see Discussion). At any rate, glutaraldehyde preserves, in this muscle, an amazing regularity in the disposition of the SR elements.

Transverse continuity of the SR is normally achieved by a confluence of the longitudinal A band sacs at the H and I band levels. The continuity at the H level is not so conspicuous here as in certain other types of muscle fibers (46). At the I band level, on the other hand, the terminal sacs are dilated, and because of their conspicuous appearance these terminal cisternae can be easily followed without interruption along the section and among the fibrils. They never fail to be present wherever the section includes sarcoplasm at the I band level. At the outer limits of the fiber the large terminal sacs can often be seen just under the sarcolemma (Fig. 6). From a section such as the one shown in Fig. 6 one gets the impression that, within the limits of one sarcomere, the SR is like a fence separating the fibrils from one side to the other of the fiber. The transversely oriented terminal sacs opposite the I bands comprise railings which are connected to one another by the smaller longitudinally oriented sacs opposite the A band. In this regard transverse sections such as shown in

FIGURE 2 Shows a transverse section through the muscle fibers making up the bulk of the myotome. The fibers are white; *i.e.*, larger, less rich in mitochondria (*m*) and glycogen (*g*) than are the red fibers (Fig. 1). Clusters of ribosomes (*r*) are present here as well as in the red fibers. The fiber in the center of the figure is cut in part at the I band level and illustrates the continuity of the terminal sacs of the SR across the fiber. Part of the fiber is cut also at the A band, at which level regularly spaced round profiles of the SR occupy the interfibrillar spaces. These are cross-sections of the longitudinally oriented "railings" of the sarcoplasmic reticulum "fence" which surrounds the fibrils. The nucleus marked (*N*) belongs to a muscle fiber. Fixation same as for Fig. 1. × 8500.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Figs. 2 and 11 are informative. In these the triad stays in the plane of section for a considerable depth owing to the fairly large dimensions of the two terminal sacs. Thus it can be followed from the periphery into the interior of the fiber as a continuous structure around the fibrils. When the section enters the level of the A band, it cuts transversely through the more slender, longitudinal sacs which appear as round profiles regularly disposed among the fibrils. One sarcoplasmic sheet and its content of SR profiles is shared by two adjacent fibrils (Fig. 2).

Within the limits then of one sarcomere the continuous internal phase of the SR, separated by a membrane from the surrounding sarcoplasm, provides for the possible transport of metabolites across the whole fiber and from the Z band level to the next longitudinally. Connections between the SR of one sarcomere with that of the next have not been observed in this material. It would seem, therefore, that the SR of the fiber is divided into independent segments which coincide with the sarcomeres. It is further noted that no direct connection of the SR cisternae with the sarcolemma has ever been observed, though as noted above they often come into close proximity to one another. The SR is, however, continuous with the nuclear envelope, just as the endoplasmic reticulum is in other cell types (43, 61). Actually one feature peculiar to the sarcoplasmic reticulum, its periodically repeating pattern, is represented to some extent in the structure of the nuclear envelope (Fig. 8). Like the SR, the envelope shows at every Z line level dilated regions which form triadic arrangements with intermediate vesicles belonging to the T system. This similarity in morphology is descriptive of the common origin of the SR and the nuclear envelope.

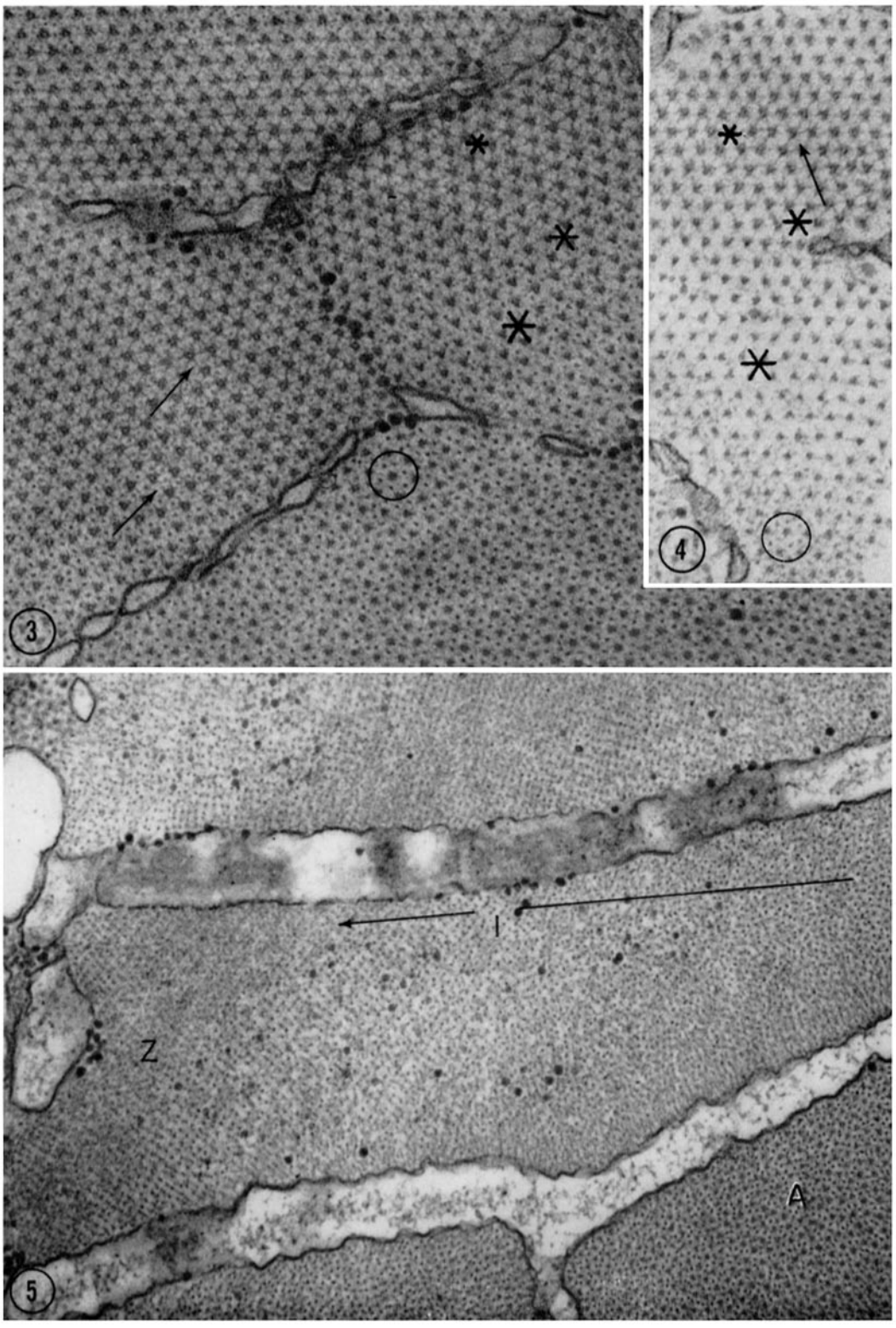
4. The T System

Between the two large terminal sacs of the SR, the T system, after OsO_4 fixation, appears in longitudinal sections of the fiber as a series of discrete vesicles or short tubules. After glutaraldehyde fixation, on the other hand, it appears as a long continuous profile (Fig. 7). It is this striking and very significant difference in the response of the T system to the two fixation procedures that constitutes the major observation of this study and justifies the detailed description that follows.

We shall first illustrate the morphology of the T system after osmium tetroxide fixation and then compare this more conventional appearance with that observed after glutaraldehyde fixation. Transverse sections (Fig. 9) are particularly informative because the T system lies in a plane transverse to the long axis of the fiber. Since the depth of this plane in which the T system resides is rather small (40 to 80 mμ), elements of it ordinarily included in any one section are apparent only over short distances. A suitable section will include the Z band of the myofibrils in the case of the black Mollie fibers, since the T system is localized at that level. In such a section (Figs. 9 and 10), a small tubule

FIGURES 3 and 4 Transverse section of, respectively, a glutaraldehyde- and an osmium tetroxide-fixed fiber. The circle marks in both figures an area of the A band where the hexagonal disposition of thin and thick filaments is evident. The thick filaments are separated by the same distance in both images, but they are obviously larger and more prominent in Fig. 3. The asterisks, in decreasing order of size, mark the H, margin between H and M, and M bands, respectively. In Fig. 3, thin filaments can be seen to persist all the way through H and M bands, whereas in Fig. 4 they disappear at the border between A and H. In both cases small bridges connect (arrows) the thick filaments at the M band level where the filaments appear hollow. × 90,000.

FIGURE 5 Transverse section of a glutaraldehyde-fixed preparation for the most part at the level of the Z line and I band. In the latter, the thick filaments are not present and the thin filaments are somewhat disordered. Differences in the sharpness of the filaments in the image indicate a different orientation with respect to the plane of section. Just at the border of the Z line (tip of the arrow) the thin filaments are organized in a very regular fashion at the corners of a square lattice. In the region of the Z line the square pattern is evident, but somewhat masked by some matrix material. The large dense granules between the thin filaments represent glycogen. × 60,000.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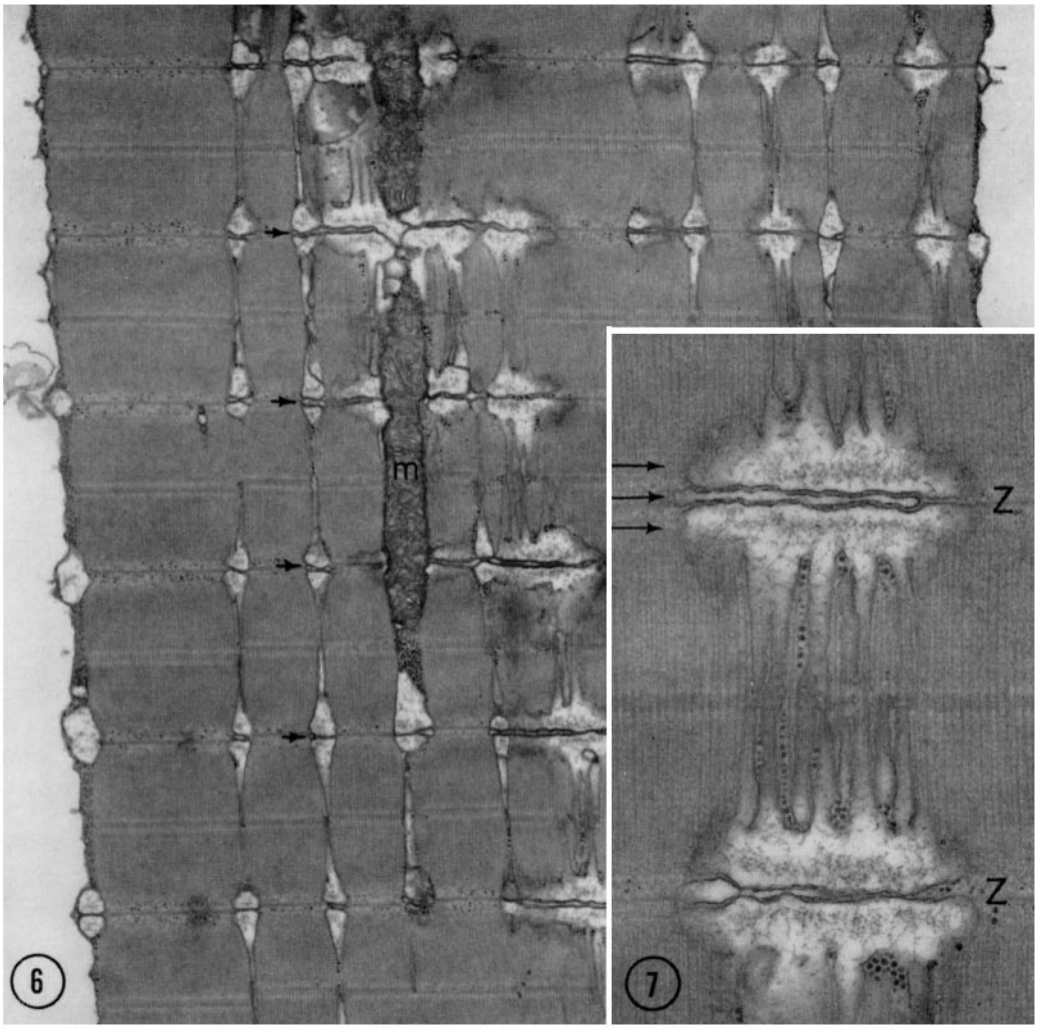
of dimensions varying between 15 and 30 mμ can be seen to run between the fibrils. The tubule appearing after OsO_4 fixation has a very sinuous course as it runs back and forth between the fibrils by which it is confined. Though it is easily lost from the plane of the section, it appears again whenever the section includes the Z band of the adjacent fibrils (identified by its higher density and typical square pattern). It is obvious in Fig. 10 that this contorted tubule is continuous across substantial stretches of the fiber and that at certain points it branches to surround the fibrils on all sides (Fig. 9). It follows that the T system at any one Z band level when thought of in isolation may be imaged as a net, composed of tortuous tubules and nodal points, through the openings of which run the fibrils. It is readily noted as well that the system is included in the sarcoplasmic spaces across the fiber and interrupts the longitudinal continuity of the SR at every Z line level. In sections longitudinal to the fibril, in this muscle as well as in others described in the literature (3, 4, 45), the T system appears as a row of discrete vesicles after osmium tetroxide fixation. The reason for this is easily discovered by looking at a transverse section such as that depicted in Fig. 10 and imagining the appearance of a longitudinal section cut along the black line. Clearly the tubule would be intersected several times by the section and the parts within the section would appear as a row of small profiles or short segments of tubules.

After glutaraldehyde fixation the T system at any one Z line level has a decidedly different morphology. From longitudinal sections (Figs. 6 and 7) it appears as a tubule that runs without interruption for long distances across the fiber and replaces the row of distinct vesicles appearing after OsO_4. The dimensions in this image are slightly larger than those of the osmium equivalent; *i.e.*, 20 to 40 mμ. In a transverse section (Fig. 11), it appears after glutaraldehyde fixation as a flattened cisterna that lies in a plane coinciding with the Z line and fully occupies the space between the fibrils. The limiting membrane in this image is straight or smooth and closely apposed to the contractile material. For the most part, the cavity of the system is expanded in a plane transverse to the fiber. Thus it has the form of a long, narrow, and somewhat flattened vesicle which runs among the fibrils. Again, it passes easily out of the plane of a cross-section (unless the section is very favorably oriented), but for as long as it stays in the section it is continuous. In isolation the T system would appear as a fenestrated structure with the

FIGURE 6 Low power of a longitudinal section through a glutaraldehyde-fixed white fiber. The regularly repeating pattern of the SR is characterized by large, transversely oriented terminal sacs at every I band level. Two terminal sacs from adjacent sarcomeres always face each other across a space at the Z line level and run parallel around the fibrils. Together with the intervening element, which resides in the space and belongs to the T system, the terminal sacs form a triad, opposite each Z line (arrows). Longitudinal elements of the SR run along the A band parallel to its long axis. Mitochondria (*m*) are arranged longitudinally in the narrow space between the fibrils. × 15,500.

FIGURE 7 This longitudinal section happens to include an expanse of sarcoplasm and the surface of the underlying fibril including a layer of sarcoplasmic reticulum. It can be seen that, from one Z line (*Z*) to the next, the SR is a continuous structure with transversely-(terminal sacs at I band level) as well as longitudinally-oriented elements (along the A band). At the Z line the transverse (T) system intrudes between the two terminal sacs in the form of a continuous cisterna. The three constitute a triad, indicated by three arrows. Glutaraldehyde-fixed white fiber. × 30,000.

FIGURE 8 Longitudinal section through the periphery of two adjacent white fibers. One of them shows a nucleus (*N*). The perinuclear cisterna (nuclear envelope) is slightly dilated and thus more readily visible for its low density content. At the Z line level, part of the nuclear envelope is in close structural relationship with elements of the T system to form a typical triad (at three arrows). In the adjacent fiber at the left, the T system appears in wide communication with the outside. Glutaraldehyde fixation. × 25,000.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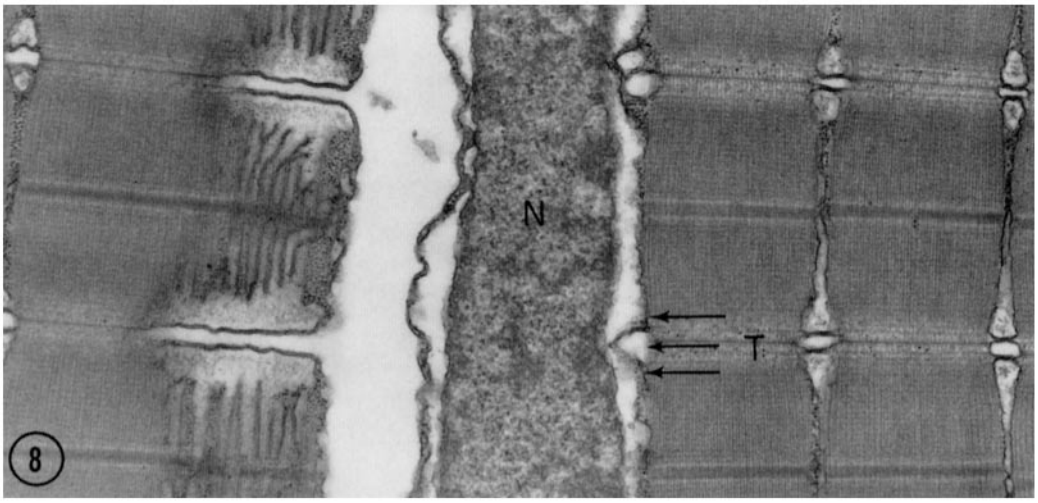
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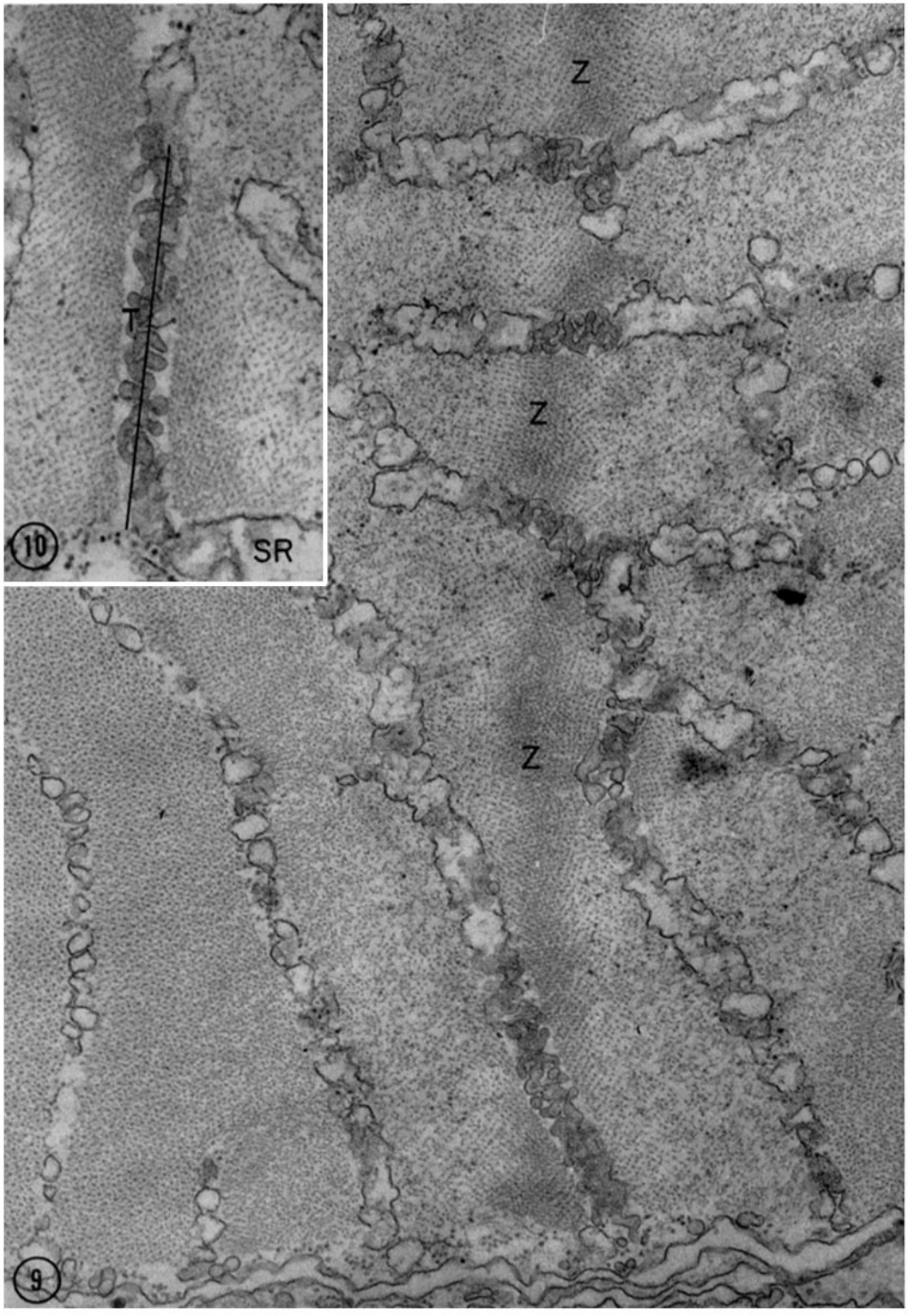
contractile material occupying the openings or fenestrae. From this image it becomes evident (*a*) that the T system exhibits a different response to the two fixatives, and (*b*) that the SR and T system may react differently in terms of structural changes to the same fixing reagent, since after the two fixations the sarcoplasmic reticulum appears essentially the same.

This demonstration of continuity in the T system after glutaraldehyde makes it seem more probable that any connection it might have with the sarcolemma would be preserved. Indeed when such a possibility is investigated in the case of the black Mollie by tracing the T system to the periphery of the fiber, we find that *its content is in wide communication with the outside space and that its membrane is continuous with the sarcolemma.* Therefore, at certain points around the fiber at each Z line level the sarcolemma is reflected into the interior of the fiber and penetrates between the two terminal sacs of the SR to form the limiting membrane of the T system (Fig. 8). Such invaginations of the membrane have been followed, in fortunate sections (Fig. 13) toward the center of the fiber, along the interfibrillar-sarcoplasmic spaces. Figs. 11 and 14, together with Figs. 8 and 13, provide images of openings of the T system to the outside in sections located in three planes taken at right angles to one another. In Fig. 11, for example, a section transverse to the long axis of the fiber shows how the sarcolemma invaginates deeply into the fiber in the form of a prominent canal between the fibrils (arrows). In Fig. 14 the image depicts a tangential section including the sarcolemma and fiber and the opening of the canal (or T system) to the exterior (asterisks). At the margin of the reflection of the sarcolemma into the fiber, the opening appears round or oblong in profile (Fig. 14). Both in longitudinal and in transverse sections it is evident that the openings are funnel-shaped, in other words, the mouth of the opening is slightly larger than the T system canal immediately beyond it. A comparison of these images with those provided by osmium tetroxide fixation (Fig. 12) demonstrates that the preservation of this wide communication of the T system to the outside is a reflection of glutaraldehyde fixation and not merely a peculiar feature of the black Mollie fibers.

It is possible now on the basis of the above observations to complete the picture of the T system. In a cross-section of the fiber, exactly at the Z line level, the sarcoplasm, divided by the fibrils into radially oriented sheets, is occupied by the T system all the way across the fiber. At the circumference of the fiber at each Z line, wherever the sarcolemma intersects one of these sarcoplasmic sheets it invaginates and forms the walls of a T system canal. These invaginations considered together form the fenestrated structure previously described. Whether the invaginations meet and fuse in the center has not been determined. Viewed from the outside, the intact fiber therefore shows

FIGURE 9 Transverse section through an osmium tetroxide-fixed white fiber showing the more conventional image of the T system. This is recognizable in the section as a winding, rather dense tubule, which is present whenever the section runs out of a terminal cisterna of the SR (*SR*) exactly at the level of the Z line (*Z*). The latter is distinguishable from the adjacent bands because of its higher density and square pattern. The T system tubule after osmium tetroxide fixation is always narrower than the elements of the SR and appears denser, because its entire limiting membrane is included in the section. × 32,000.

FIGURE 10 Higher magnification of a transverse section of osmium tetroxide-fixed red fiber. The T system (*T*) appears as a tubule which can be followed without interruption for fairly long distances in the section. At the top and bottom of the image the T system goes out of the section and so appears interrupted. The black line marks the orientation of a longitudinal section into and out of which the T system, due to its wavy course, would pass numerous times. Its image would thus appear in the form of round or tubular profiles. On the opposite sides of the T system, the section cuts across two adjacent fibrils at the Z-line level. One can notice, in comparison with Fig. 5, that in this osmium tetroxide-fixed preparation the square pattern of the Z line is much more distinct, possibly due to the fact that some masking material has been dissolved out. The I band too appears less dense. × 60,000.


T
SR
10
Z
Z
Z
9

at the Z line level a row of holes all around its circumference which represent the sites where the sarcolemma invades the fiber.

It is interesting that in the Mollie fibers the T system cisternae often appear occupied, both at the periphery and for some distance into the interior of the fiber, by strands of glycogen-rich cytoplasm limited by a membrane (Figs. 11 and 13). This finding was puzzling until we were able to trace some of these strands back to the surface of and into the adjacent muscle fiber. It then became evident that some fibers send from their surfaces finger-like projections which penetrate deeply into the T system of neighboring fibers.

As previously noted, the T system penetrates the fiber between two terminal sacs of the SR, to which it is closely contiguous. In the narrow space between the two contiguous systems there appears on occasion to be a dense material presumably binding the two together. This is particularly evident in glutaraldehyde-fixed preparations where the membranes of the two systems may run parallel for long distances. In such instances we have noticed also that the SR membrane facing the T system is sometimes scalloped in profile and that at regular intervals small densities traverse the gap between the two membranes (Fig. 15). Only in a few images, however, have we encountered a corresponding structure in the osmium tetroxide-fixed preparations where generally only a diffuse density can be seen between the membranes.

The structural relationship of the T system and sarcolemma described above has now been noticed in a variety of other skeletal muscles and so is not peculiar to fibers of the black Mollie.[2] Observations on these will be presented in subsequent reports.

DISCUSSION

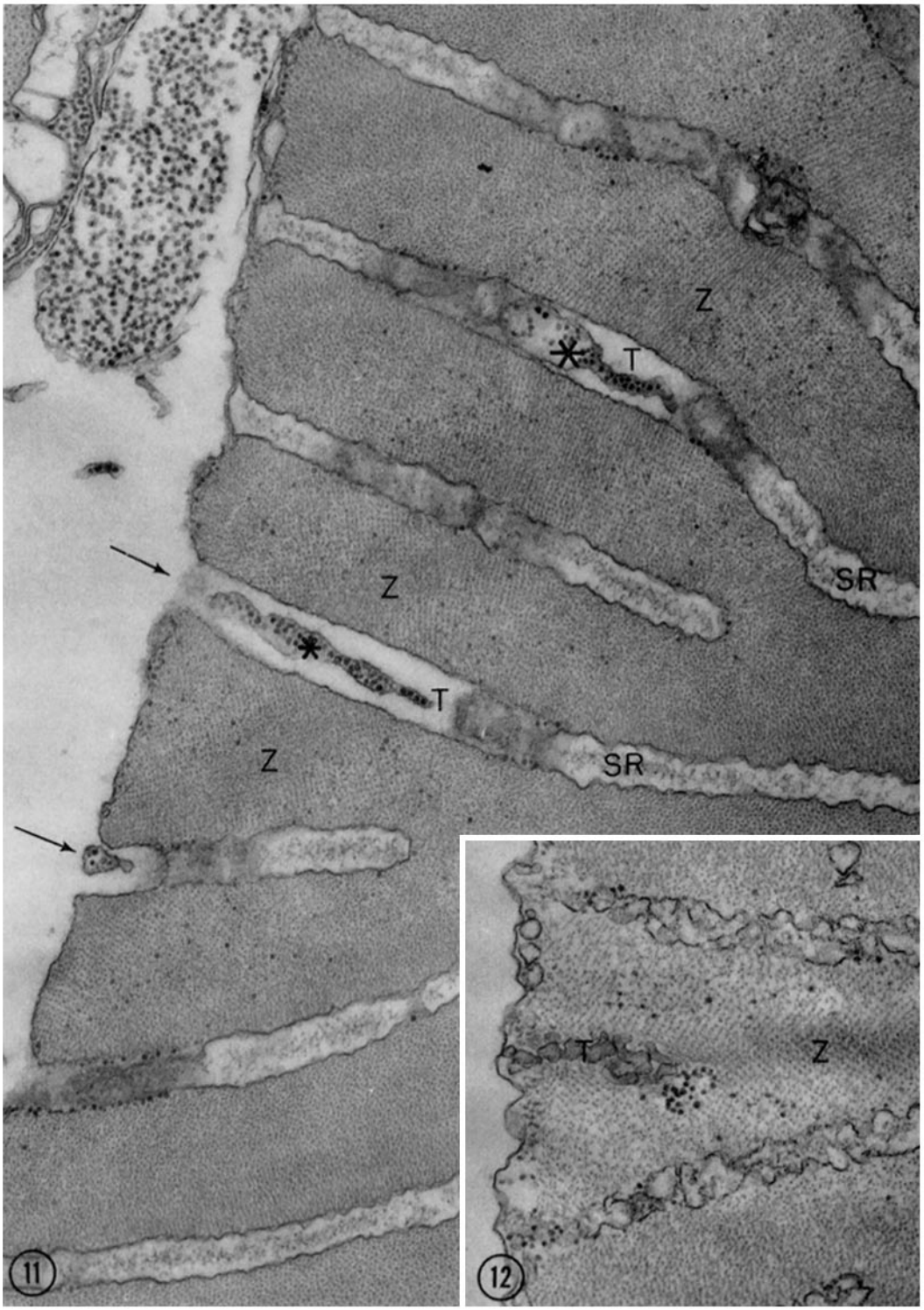
1. Comparison between Glutaraldehyde and Osmium Tetroxide Fixations

The most important points of information in these observations arise from the improved preservation of the myofibrils and the interfibrillar membranous structures (SR and T system) resulting from the use of glutaraldehyde as a fixative. Though the recognized improvements are slight compared with fixation after OsO_4 alone, they are very significant in the details they add to the present knowledge of the morphology of these structures and in the elucidation they provide for some problems in the correlation of structure and function. It seems important therefore to examine the evidence in some detail.

[2] Electron microscopy by Franzini-Armstrong on axial muscles of tadpole tails, *Rana pipiens*, and extensor muscles of the fins of *Fundulus heteroclitus* and *Opsansus tau*, following glutaraldehyde fixation has demonstrated similar continuities between the T system and sarcolemma. A personal communication from Vincent Kilarski, Krakow, including remarkable micrographs, describes identical invaginations in striated muscle fibers of the swim bladder of *Lota vulgaris*.

FIGURE 11 Transverse section of a glutaraldehyde-fixed white fiber to show the form and disposition of the T system. The section cuts across the Z line (*Z*) and so includes the middle of the T system cisterna. This latter occupies fully the space among the fibrils, and its membrane is closely apposed to the contractile material. We can imagine the T system as a long, flat cisterna. Its content has the same density as the external environment, with which it is in direct continuity. Openings or orifices of the T system (*T*) to the outside are shown (at arrows) wherever the section includes, exactly at their level, the surface of the fibers. Strands of sarcoplasm (asterisk) which penetrate into the T system from neighboring fibers can be followed for some distance into it. Where the section cuts at the level of the I band, the terminal sacs of the SR (*SR*) occupy the interfibrillar spaces. At the periphery of the fiber the sarcolemma and the SR membrane are closely apposed, but not in continuity. × 38,500.

FIGURE 12 This cross-section of a peripheral portion of an osmium tetroxide-fixed white fiber demonstrates that in this case the T system appears in the form of small dense tubules, whose connections with the sarcolemma and openings to the outside are disrupted, apparently, by the action of the fixative. × 48,000.


Z
T
Z
SR
T
Z
SR
11
Z
T
Z
12

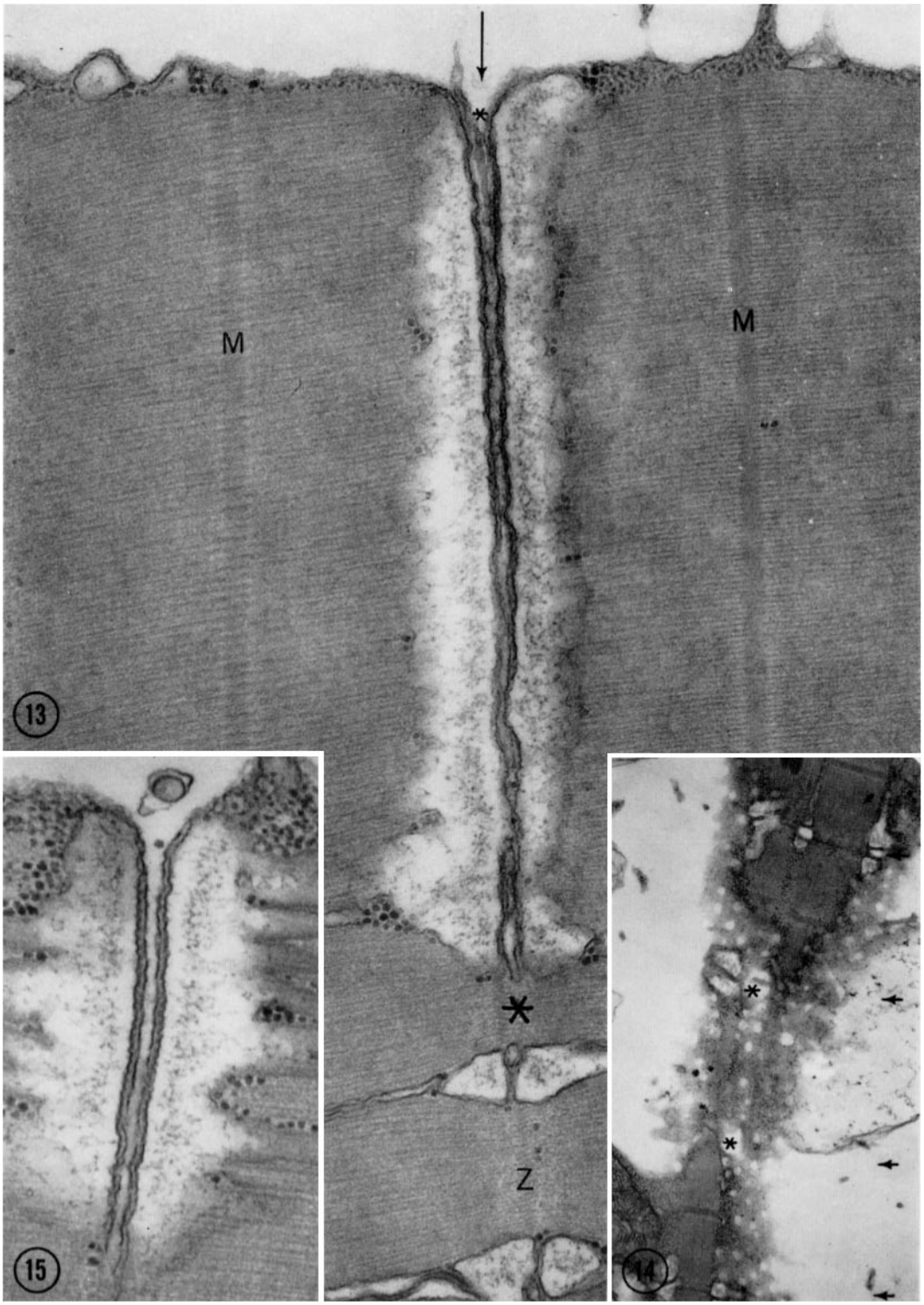
The Myofibrils

The glutaraldehyde-fixed muscle fiber is remarkable for the geometrical regularity displayed by the fine structure of the myofibrils. This alone indicates that a more faithful preservation is achieved than with OsO_4 which frequently produces some distortion and disarrangement of the finer elements and leaves them mobile to move about or change during dehydration and embedding. These differences in the effects of the two fixatives are particularly well demonstrated by the order and symmetry in the disposition of the I band filaments as well as the sacs and cisternae of the SR associated with each sarcomere. To a certain extent these differences are referable to the fact that OsO_4 excites contraction of the fiber during its penetration, whereas glutaraldehyde seems not to. But more especially it seems to be a product of the greater capacity of glutaraldehyde to render the various molecular species of the cell insoluble and to bind them together in a stable lattice. One is reminded as well of the fact, demonstrated some years ago (38, 45), that osmium tetroxide fixation in time has a dissolving action on the cell. Thus when we compare transverse sections at the Z band level, we see that the band is denser in glutaraldehyde than in osmium tetroxide; *i.e.* that some material has been retained by glutaraldehyde which is dissolved out in osmium tetroxide preparations. The same impression of more complete preservation is provided by the greater diameter of the filaments in the A band and the essential absence of any clear space between them. Likewise the greater prominence of the thin filaments in the H-M zone after glutaraldehyde may be referable to the more complete preservation achieved with this reagent. It appears therefore that the image obtained from preparations of glycerinated muscles fixed in osmium tetroxide (27, 28) is not a complete representation of the contractile material in the fibrils, though it probably represents rather faithfully the gross structural pattern in which the material is distributed. In this connection we should note that the diameters of isolated thick filaments (29)

FIGURE 13 This longitudinal section of a glutaraldehyde-fixed muscle preparation includes one of the elongated sarcoplasmic sheets which separate the flat faces of the ribbon-like fibrils. It thus provides a lateral view of the T system and terminal sacs of the SR for a long distance into the fiber. The longitudinal sacs in the SR are not included in the plane of the section. The sarcolemma (arrow) is obviously deflected into the fiber at the Z line level and forms the limits of the canal or cisterna of the T system. The profile of the canal disappears from the section (at the large asterisk), but appears again when the section cuts across an adjacent sarcoplasmic ray. A small asterisk, near the mouth of the T system tubule, indicates one of the sarcoplasmic strands which penetrate into the T system from the periphery of an adjacent fiber. *M* indicates the M line. × 46,000.

FIGURE 14 Longitudinal section which cuts tangentially to the fiber (essentially a shaving off the surface) so that the contractile material goes out of the plane of the section in the middle of the image and is replaced by a tangential section of the sarcolemma. It is as if the observer were looking at the sarcolemma from the outside of the fiber. At the Z line level (arrows mark the Z lines of the fibrils), the round orifices of the T system cisternae (asterisks) appear in the sarcolemma at the sites where they penetrate into the fiber. Other circles of low density appearing as holes in the sarcolemma mark places where the section passes through "caveolae" in the fiber surface (5). Glutaraldehyde fixation. × 17,000.

FIGURE 15 Here the sarcolemma penetrates deeply within the fiber between the terminal sacs of the SR and forms the walls of the T system. Between the membranes limiting the two and associated especially with the terminal sacs, one can notice, in this glutaraldehyde-fixed preparation, small line densities, almost bridges, connecting the facing membranes across the narrow space. The SR membrane at the level of the triad is scalloped. This structure probably corresponds to the not-so-clearly defined density that one can observe between the elements of the triad in osmium tetroxide-fixed preparations of white fiber. × 60,000.


M
M
13
Z
15
14

observed by negative staining technique without osmium tetroxide fixation are larger (100 to 200 A) than the same dimension measured on osmium tetroxide-fixed preparations. This observation suggests further that some material is lost during such fixation of muscle.

Sarcoplasmic Reticulum

The SR of the black Mollie muscle after glutaraldehyde differs only slightly from the image following osmium tetroxide fixation. As in the case of the contractile material, the differences are mostly due to the more faithful preservation by glutaraldehyde of symmetry in the disposition of the sacs and cisternae along the sarcomere. Apparently the sarcoplasmic reticulum is less responsive to OsO_4 in terms of morphological change than are derivatives of the plasma membrane such as the T system (see below).

T System

Where the glutaraldehyde-fixed image assumes a special significance, however, is in the demonstrated preservation of continuity in membranous structures which usually appear interrupted after osmium tetroxide. This is evident in a comparative examination of the T system. Whereas in osmium tetroxide-fixed preparations this system appears as a string of vesicles or short tubules, after glutaraldehyde it has the form of a flat cisterna, which runs for some distance in the fiber without interruptions. At the periphery of the fiber, this cisterna is in continuity with the sarcolemma and its cavity is open to the outside. The openings are, in the black Mollie, very numerous and thus can be seen easily in different section orientations relative to the fiber axis (see Figs. 8, 11, and 13 to 15).

A structural feature peculiar to the black Mollie is of special interest because it allows us to conclude that the glutaraldehyde image of the T system is the one which more faithfully represents the native form of that system. This is the presence of numerous finger-like projections which extend from one fiber deeply into the T system cisternae of neighboring fibers. Since it is difficult to believe that such interdigitations between cells could be induced by any type of fixation, we may reasonably conclude that their presence describes a native association of great intimacy. As was mentioned earlier, similar conclusions relative to the unreliability of OsO_4 derive from the observations of Rosenbluth (53) which demonstrate that during its penetration this fixative may act specifically on invaginations of the plasma membrane in other cells, inducing interruptions in their continuity. Also in parallel with what happens in the case of the invaginations of the neuronal plasmalemma described by Rosenbluth, calcium ion added to the fixative prevents in part, in the experience of Porter, this disturbance to the T system. It seems also that the buffer used with the OsO_4 is influential in the preservation of the T system. For example, in the preparations shown by Fawcett and Revel (13) and Fahrenbach (12) following the use of *s*-collidine–buffered osmium tetroxide, the T system appears most frequently in the form of a continuous tubule.

2. Interpretation of the New Image of the T System

It is evident from the above considerations that glutaraldehyde provides a more faithful preservation of the T system than does OsO_4. Also from the available observations the different responses of the SR and T system to osmium tetroxide serve to emphasize the differences in the character and origins of the two systems. That the T system is unique was already indicated by the greater thickness and prominence of its membranes and by the absence of any direct connection and communication of content between it and the SR (3–5, 13, 46, 48, 50). The SR is easily recognized as homologous to the ER of other cell types. No connections with the sarcolemma can ever be traced, and its homology and continuity with the nuclear envelope are very evident, particularly in the well preserved glutaraldehyde-fixed preparations.

The T system is, on the other hand, a sarcolemmal derivative. In place of the numerous suggestions of this in the literature, we can now provide a direct demonstration, at least in the case of the black Mollie and three other muscle fibers that have been examined. It can be visualized as a system consisting of a series of finger-like invaginations of the sarcolemma, which penetrate into the fiber and run in a direction transverse to its long axis. The openings of the T system to the outside are in the form of a round orifice of slightly larger diameter than the immediately adjacent cisterna within the fiber. The openings occur, in the black Mollie, at every point where a Z band and an interfibrillar sarcoplasmic ray intersect with the sarcolemma. Thus they may be estimated

to number 20 to 30 at each Z line level, around the circumference of a 12 μ fiber, such as are found in the body of the black Mollie.

The T system may therefore be considered, as suggested by Peachey and Porter (41), the morphological element between the contractile material and the sarcolemma required to explain the results of experiments by Huxley (22), Huxley and Straub (23), and Huxley and Taylor (25). Those authors were able to excite a localized contraction in the fiber by inducing a localized depolarization on the sarcolemma. The contraction occurred only when the depolarization was applied at the level of the triad. This compelling evidence for a role of the triad, or some part of it, in the excitation-contraction coupling, is now supplemented by further evidence derived from electrophysiological data for the existence of an electrical connection of the T system with the sarcolemma and its possible involvement in the spreading of excitation into the fiber. For example, in recent studies on ion permeabilities of muscle membranes in frog fibers, Hodgkin and Horowitz (21) tentatively conclude that there is a small compartment ($\frac{1}{500}$ of the fiber volume) within the fiber which exchanges much more rapidly than the sarcoplasm when the external solution is changed, but lags behind the change of external solution by several seconds. This compartment was identified with the T system, whose volume relative to the fiber's would correspond to the one calculated. Adrian and Freygang (2) have also proposed a three-compartment system to explain certain of their results on the impedance of muscle fibers; one of the compartments should be, according to their speculations, separated by a membrane both from the sarcoplasm and from the outside spaces which would be the other two compartments. Adrian (1) has now modified this model proposing that the first compartment might be composed of a number of 300 A tubules which open freely to the outside. With the reservation that we have not looked at the muscles used for the above mentioned physiologic studies, we suggest that the T system, existing as an invagination of the sarcolemma around a space in direct continuity with the outside, provides the electrical continuity into the fiber necessary to explain their results. To what extent this morphology is universally present remains at present in doubt, but the discovery of it as previously noted in three other kinds of muscle fibers strengthens the supposition that it is common if not ubiquitous. In most instances the connections with the sarcolemma may not be so numerous as in the black Mollie, and in some cases the T system may be connected with the sarcolemma either intermittently or in only a few places along the periphery of the fiber. Whether this would bear any relation to the differences in the physiology or activity of the fibers observed is difficult to say at the moment. It is important in this connection to be reminded that in Huxley's and coworkers' experiments only a few places around the periphery of the fiber at each triadic level were able to be excited locally. This may reflect a morphological situation in which very few connections of the T system with the sarcolemma are present.

Girardier *et al.* (15) have arrived at similar conclusions from a study of crayfish muscles in which they also followed derivatives of the sarcolemma into the fiber. The pecularity of this muscle resides in the fact that the outward current is carried by Cl^- and that only part of the tubules have the necessary permeability properties.

The demonstration of the T system as a sarcolemmal invagination in skeletal muscle fibers of vertebrates prompts us to compare it with homologous structures in other types of muscle fibers. In heart muscle, for example, invaginations of the sarcolemma have been demonstrated in several cases to penetrate into the fiber (11, 36, 55, 56). Since these start at the periphery of the fiber at the Z line level and run at this level across the fiber for at least a few microns, their homology with the T system is evident.

More difficult is the comparison with the fibers of insects and other arthropods, where the sarcolemmal invagination penetrates into the fiber apparently at any level and runs randomly between the fibrils (12, 49, 57, 58). The homology to the T system is to be traced in the common nature of the two systems: in both cases these sarcolemmal invaginations bring the extracellular milieu and the sarcolemma (or a membrane continuous with it) into close proximity to the contractile material. We may, in a search for further correlations, consider the T system of vertebrate fibers as homologous to the sarcolemmal sheets which envelop the single, flat ribbon-like fibrils of the body musculature of the Amphioxus (see Peachey, reference 39). Thus, some device to bring the sarcolemma or its derivatives into close proximity with the contractile material seems to be universally present. Tubular structures probably of sarcolemmal origin

have been described by Pucci and Afzelius (47) and also by Röhlich (52) in the muscle fibers of different species of leeches. It would seem further that the numerous tubules which pervade the crayfish stretch receptor muscles are homologous to the T system, since they are sarcolemmal derivatives. The use of the term sarcoplasmic reticulum by Peterson and Pepe (42) in describing such structures is to be deplored, since it adds confusion in an area where some degree of order and understanding is beginning to emerge.

The only instance so far described in which structures homologous with the T system have not been found to occur in a plurifibrillar fiber having diameters of several microns is that of the frog slow fibers described by Peachey and Huxley (40). This kind of fiber differs significantly, however, from the rest of the vertebrate skeletal muscles in that the contraction and relaxation times are of the order of seconds and the sarcolemma is incapable of developing an action potential.

The Triad

Once able to recognize the T system as a component common to most muscle fibers, we can also recognize its principal characteristics. One of these is that, in all muscles examined, the T system or part of it is arranged in close relationship to the elements of the SR. In vertebrate skeletal muscle the areas of proximity are at the level of the triad, where the T system is bordered on both sides by the terminal sacs of the SR. In cardiac muscle and in certain insect muscles the homologue of the triad is to be found in a dyad, a structure composed of one element of the SR closely apposed to part of the T system tubule (46, 57, 58). Further to be noted is the fact that the two apposing membranes of the T system and SR are somewhat denser at the level of the triad (or dyad) than elsewhere in the fiber (46). In osmium tetroxide-fixed preparations some dense material seems to occupy the space between these membranes, but glutaraldehyde fixation shows better than OsO_4 a peculiar structure of the membranes limiting the SR and T system in the triad. In this structure both membranes appear scalloped, and an ordered structure occupies the space between them. This morphology, described by Revel (50) in the bat cricothyroid muscle, corresponds probably to the dense, ill defined material noticeable after osmium tetroxide fixation. The presence of an organized structure in the space separating the elements of the SR and T system in the triad probably means that there is a close functional relationship between the two systems at such a level. In this regard it is interesting to notice that both systems are probably present in the relaxing factor, *i.e.*, the microsomal fraction of the muscle homogenate (Nagai, *et al.*, 34; Muscatello *et al.*, 33; Ebashi and Lipmann, 10) which has an ATP-dependent Ca binding activity (Ebashi and Lipmann, 10; Hasselbach and Makinose, 18). It is this latter property that is thought to be responsible for relaxation of contracted fibrils (6–9; 62–64). The observations of Revel (44) are important in suggesting that the triad may be a primary site of Ca binding, whereas those of Hasselbach (17) suggest that Ca accumulates in some non-specified part of the reticulum. These data obviously lend possible significance to the peculiar structured material which binds the SR and T system together in the triad.

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