CORRELATED MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES ON ISOLATED SINGLE MUSCLE FIBERS

I. Fine Structure of the Crayfish Muscle Fiber

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ABSTRACT

Single fibers isolated from walking leg muscles of crayfish have 8- to 10-μ sarcomeres which are divided into A, I, and Z bands. The H zone is poorly defined and no M band is distinguishable. Changes in the width of the I band, accompanied by change in the overlap between thick and thin myofilaments, occur when the length of the sarcomere is changed by stretching or by shortening the fiber. The thick myofilaments (*ca.* 200 A in diameter) are confined to the A band. The thin myofilaments (*ca.* 50 A in diameter) are difficult to resolve except in swollen fibers, when they clearly lie between the thick filaments and run to the Z disc. The sarcolemma invaginates at 50 to 200 sites in each sarcomere. The sarcolemmal invaginations (SI) form tubes about 0.2 μ in diameter which run radially into the fiber and have longitudinal side branches. Tubules about 150 A in diameter arise from the SI and from the sarcolemma. The invaginations and tubules are all derived from and are continuous with the plasma membrane, forming the transverse tubular system (TTS), which is analogous with the T system of vertebrate muscle. In the A band region each myofibril is enveloped by a fenestrated membranous covering of sarcoplasmic reticulum (SR). Sacculations of the SR extend over the A-I junctions of the myofibrils, where they make specialized contacts (diads) with the TTS. At the diads the opposing membranes of the TTS and SR are spaced 150 A apart, with a 35-A plate centrally located in the gap. It appears likely that the anion-permselective membrane of the TTS which was described previously is located at the diads, and that this property of the diadic structures therefore may function in excitation-contraction coupling.

INTRODUCTION

The combined study of the physiology and morphology of single muscle fibers has proved itself capable of relating data obtained from electrophysiological and osmometric studies with morphology, and some of the results, primarily in their physiological context, have been reported earlier (13, 35). It played an essential role in demonstrating (13) the existence of a transverse

tubular system (TTS) in crayfish muscle fibers. The morphological observations supported the physiological findings that the TTS is the site of a specialized anion-permselective membrane and that the system, which is thus distinguishable from the sarcoplasmic reticulum (SR), is open to the exterior. Since the TTS carries the extracellular environment deep into the muscle fiber, it was further suggested (13) that the anion permselectivity of the membrane of the TTS offers a particularly efficient mechanism for excitation-contraction coupling.

The morphological evidence mentioned in the previous paper (13) regarding the connections of the TTS with the external medium, as invaginations of the cell membrane, will be presented here. Also to be presented are detailed morphological data which relate the TTS to other structural elements of the muscle fiber. Thus, they demonstrate the location and structure of diads, which are a highly ordered conjuncture of the TTS and SR.

The "normal" architecture of the muscle fibers will be the main subject of this paper, and subsequent reports will deal with structural changes that are induced by varying the physiological conditions. However, the changes which occur with different experimental treatments often helped to clarify structural relationships in the normal muscle fiber. Therefore, some effects of experimental procedures are also included in the present work.

MATERIALS AND METHODS

Crayfish of the genus *Procambarus* were used, but probably of several species which were not further identified. Single muscle fibers were prepared by dissection and were maintained approximately at their resting length by preserving a bridge of chitin across the gap made in the exoskeleton (13). They were exposed to a crayfish saline (43), but with varying K concentrations depending on the experimental design. The majority of the fibers were fixed by replacing the saline with ice cold 1 per cent OsO_4 in Veronal-acetate buffer at pH 7.4 (30). Other preparations were fixed with glutaraldehyde (39) or permanganate (38) solutions.

After fixation in the cold for about 1 hour the fibers were carried through 30, 50, 70, 95, and 100 per cent alcohols in 5-minute steps at 4°C, the 100 per cent step being repeated 3 times. The fibers were then transferred to propylene oxide for 5 minutes and thence to ⅓ Epon and ⅔ propylene oxide for 1 hour. The ratio of the two compounds was reversed for overnight soaking of the preparations. The next day they were soaked in 100 per cent Epon at room temperature for the day, transferred into polyethylene capsules with fresh Epon, and polymerized for 24 hours at 60°C. The mixture of Epon components was varied in an effort to find one particularly suited to the single fiber preparations. The best ratio we have found is 5 parts of Epon 812 (Shell Chemical Co.), 5 parts of dodecenyl succinic anhydride, and 2 parts of nadic methyl anhydride. This is polymerized with DMP-30 (Rohm & Hass) which has been kept dry, since water, which mixes readily with this compound, is an accelerator of the Epons. The amount of DMP-30, usually 1.5 per cent, was adjusted to give a firm but not brittle block after 1 day in the oven.

Sections were cut with an LKB microtome. They were mounted on Formvar-coated grids and stained for 1 hour in 1 per cent sodium borate saturated with uranyl acetate. They were washed briefly and transferred to Karnovsky's mixture A lead stain (23) for 5 minutes. The sections were examined with an RCA (EMU 3F) or a Siemens (Elmiskop I) microscope.

The sarcomere spacings were measured in a number of fibers before, during, and after fixation and dehydration. The maximum shrinkage observed was 20 to 25 per cent. Since almost all cell compartments showed some differences relative to one another after various fixatives, and since the subcellular structures could be modified by various experimental procedures carried out before fixation of the fibers, no absolute definition of "normal" morphology can be given. The degree of change in gross structure appeared to be smallest in material fixed in OsO_4, and these preparations are therefore considered to be probably closest to the normal morphology. However, preparations which were fixed in glutaraldehyde or sodium permanganate were useful in elucidating some relationships of small structures which were difficult to resolve in OsO_4-fixed material.

RESULTS

FIXATION: The three fixatives used in the present work (osmium tetroxide, permanganate, and glutaraldehyde) produced quite different results on single fibers which had been prepared under identical conditions. The data relating fixation changes with changes induced experimentally before fixation will be reported later. Here we shall only indicate the reasons for regarding the OsO_4-fixed material as the least distorted.

Observation of the muscle fibers with the light microscope before and during fixation disclosed that only minimal changes in diameter or appear-

ance occurred when the fibers were fixed with OsO_4. The fixation appeared to be rapid. Less than 1 minute after the fixative was applied, the fiber was rigid and could be cut without contracting. Except for the rapid tinting produced by the reduction of OsO_4, the optical appearance of the fibers did not change and contractures were absent. However, a 10 per cent diminution in sarcomere spacing occurred. These findings are in agreement with observations on frog muscle fibers (29).

When glutaraldehyde was used as the fixative the muscle fibers invariably swelled, approximately 50 per cent above their initial volume, as calculated from diameter changes. The swelling occurred despite the fact that the fixing solution was hyperosmotic. When the glutaraldehyde was buffered with phosphate it also became difficult to see the striations. The fibers swelled to the same degree when cacodylate buffer was used, but changes in the appearance of the striations were less marked. The alterations in diameter and appearance of the muscle fibers took place slowly, lasting for about 15 minutes after the glutaraldehyde was applied, an indication that rapid fixation of the original structure of the fibers was not achieved. In contrast to fibers fixed in osmium tetroxide, the glutaraldehyde-fixed fibers remained soft even after 15 minutes of fixation. There were large differences in the volumes of various intracellular compartments in comparison with the volumes in muscle fibers which had been fixed in osmium tetroxide. Some of the effects obtained with glutaraldehyde fixation could be obtained in OsO_4-fixed fibers if they were exposed to highly modified saline solutions before fixation (data to be published).

Fibers which were fixed in permanganate also showed differences in the form of certain compartments when compared with OsO_4-fixed material. For example, after OsO_4 fixation the radial tubules (RT; reference 13) appeared to be convoluted adjacent to the sarcolemma, but were straight within the peripheral mitochondrial zone (*T*, *T'*, Fig. 5) and could be followed for several micra. In permanganate-fixed fibers, however, the membrane of the RT could be more frequently observed continuing without convolutions or breaks into the plasma membrane. The latter morphology was also observed in OsO_4-fixed cells in which the TTS had been caused to swell before fixation (13). Thus, the differences between control fibers fixed in OsO_4 and in permanganate may have been due to a mechanical factor, the straightening of the tubules as a result of a change in the relative volumes of the various compartments. Studies now in process on the alterations which are induced in different compartments by ionic fluxes may help to clarify another problem, namely, to what extent ionic fluxes which may accompany fixation contribute to the final morphology.

General Description of the Muscle Fiber

The fiber preparations came from the flexor and extensor muscles in the meropodite. The muscles have two kinds of fibers which are distinguished by their different sarcomere lengths (13) and are of quite different fine structure (unpublished data). The present study used only those fibers in which the sarcomere pattern repeated every 8 to 10 μ when the fibers were at their resting length in the living state. The repeat interval of the sarcomere could be altered approximately in proportion to the change in fiber length by stretching the fibers or by causing them to contract. The fibers were 3 to 6 mm long and 100 to 400 μ in diameter.

A cortical zone of sarcoplasm beneath the plasma membrane, which is up to 5 μ thick, contains most of the nuclei and mitochondria (Figs. 1 to 4). The myofibrils are 1 to 2 μ in diameter and each is surrounded by a complex of membranes (Figs. 2, 13, and 14). There are two independent systems of these membranous organelles, the longitudinal sarcoplasmic reticulum (SR) and the transverse tubular system (TTS). The latter originates from invaginations of the plasma membrane. Each myofibril shows the characteristic structures of the sarcomere: A, I, and Z bands (Figs. 1, 3, and 7). However, the sarcomeres of adjacent myofibrils are not strictly in register, faults appearing at intervals in the longitudinal sections (Fig. 1). Because of the lack of register between different myofibrils, cross-sectional micrographs show regional differences in structure which are characteristic of the different bands of the sarcomere (Figs. 2, 14, and 25). A reconstruction of a portion of a fiber which is based on the electron microscope data is shown in Fig. 28.

The Sarcolemma

In all major respects the sarcolemma of crayfish muscle fibers resembles that of frog skeletal muscle

fibers (26, 33, 36, 37). It has an outer coat, about 0.1 to 0.3 μ thick, which overlies an inner "unit" plasma membrane. The two structures are generally in contact. The coat is lamellated in control preparations which were fixed in osmium tetroxide (Fig. 5), and more clearly so in fibers which were fixed in permanganate (Figs. 6 and 16) or in OsO_4-fixed fibers which had been first exposed to KCl-rich media (Fig. 26).

The unit structure of the plasma membrane was seen clearly in permanganate-fixed material, and consisted of three equal bands which had a total width of about 60 A (Figs. 6, 16, and 24). Nearly identical measurements were obtained in OsO_4-fixed preparations, but the components of the unit membrane structure were not well resolved.

The Segments of the Sarcomere

The number of subdivisions that could be observed in the crayfish sarcomere depended on the treatment prior to fixation, on the fixative, and on whether the light or the electron microscope was used to examine the sections. Not all these variables will be explored in this report.

Z DISC: The Z disc is the shortest and the densest element of the sarcomere. It is 0.15 to 0.2 μ thick in sections through its shortest axis (Figs. 7 and 9). The disc wanders across the fiber and

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Note on the Figures

The first four figures are light micrographs of 0.5-μ sections stained with toluidine blue and photographed in the phase microscope. All the remaining figures are electron micrographs, except the last, which is a drawing. In all the figure legends the fixative is mentioned as well as the solutions to which the isolated fibers were exposed before fixation. The following terms are used to describe the different solutions.

Control = Van Harreveld solution.

20 K, 50 K, 100 K = control plus indicated amount of KCl instead of 5 mM KCl; 0 K indicates no K in the Ringer's solution.

−100 NaCl = control minus 100 mM NaCl.

iso Ca = control with Ca^{++} substituted for all Na^{+}.

iso K = control with 50 mM KCl substituted for 50 mM NaCl.

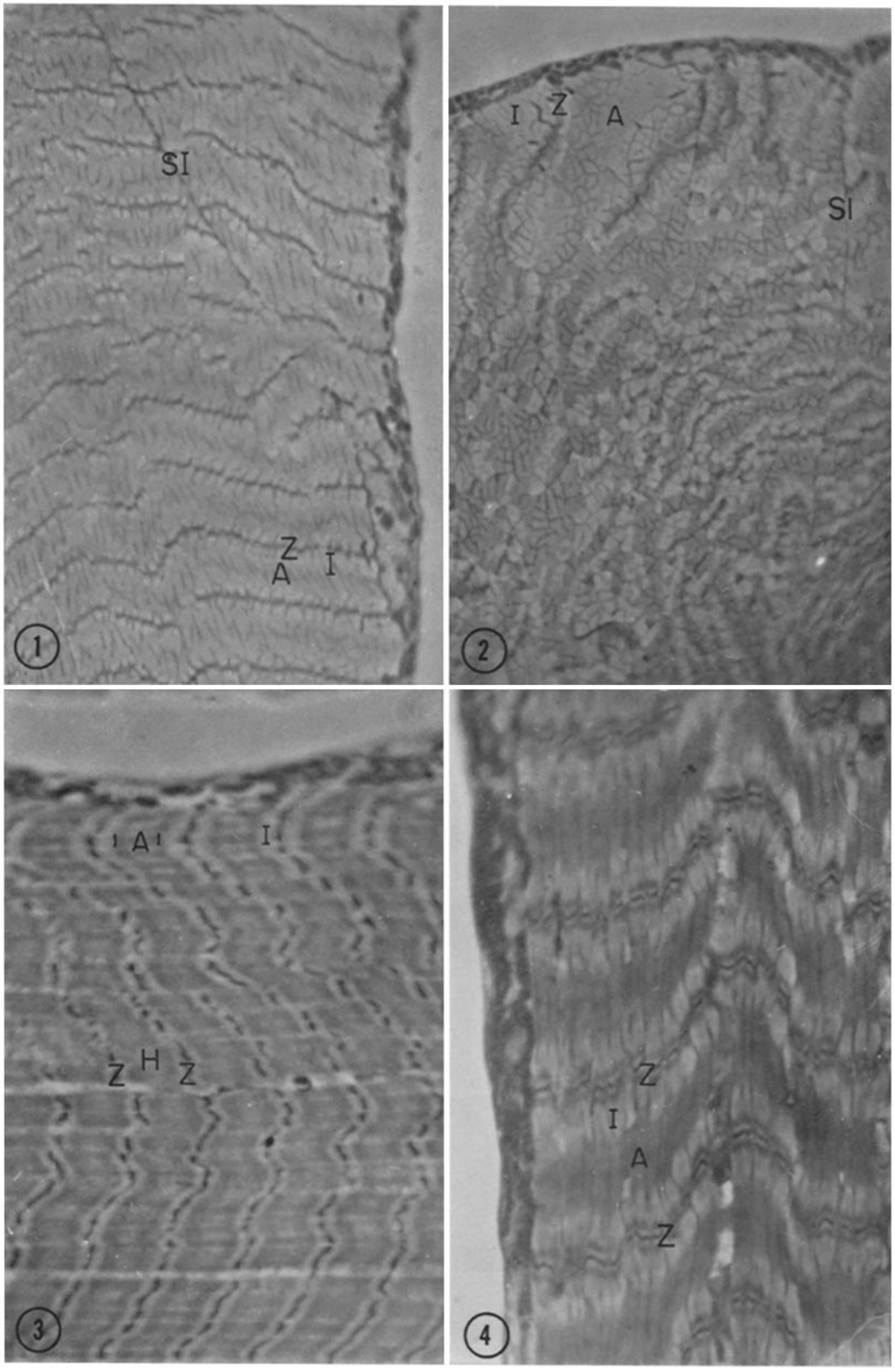
Abbreviations

A, A band
CM, collection of membranes
D, diad
F, fibrous layers of sarcolemmal coat
FS, fenestrations of sarcoplasmic reticulum
H, H zone
I, I band
M, mitochondrion
PM, plasma membrane
RT, radial tubule
S, sarcolemma
SI, sarcolemmal invagination
SR, sarcoplasmic reticulum
T, tubule
U, unidentified cell process
Z, Z line

FIGURES 1 AND 2 A cortical zone rich in nuclei and mitochondria is found under the sarcolemma. In both figures the line crossing the sarcomere pattern is a sarcolemmal invagination (*SI*). This is oblique in the longitudinal section (Fig. 1) and radial in the cross-section (Fig. 2). The Z, A, and I bands, or zones, can be recognized, but no H or M zone is seen. In both sections myofibrils are demarcated by lines of greater density at their borders Control, OsO_4. × 1650.

FIGURE 3 In addition to the Z, A, and I bands seen in Figs. 1 and 2, an H zone is seen in glutaraldehyde-fixed fibers. The demarcations between myofibrils are reversed in contrast as compared with Figs. 1, 2, and 4, and are less dense than the fibrils. The fiber was also used for the electron micrograph in Fig. 9. 0 K, glutaraldehyde. × 1650.

FIGURE 4 The stretched fiber has an additional band within the I band, and the Z lines are more irregular than in the fiber at resting length. The A band has a rather ragged edge, and the demarcation lines between myofibrils are dense and irregular. 0 K, OsO_4. × 1650.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does not fall in a single plane even in the span of one myofibril (Figs. 1 and 3). It may run almost along the longitudinal axis of the fiber or it may end abruptly opposite the A band of an adjacent myofibril (Fig. 1). The greatest irregularity in the plane of the Z disc is shown by stretched fibers (Fig. 4), but there is little change in the thickness of the Z disc with changes in sarcomere length.

The Z disc of crayfish muscle fibers lacks the "zigzag" appearance of the disc in vertebrate fibers (24). It is obviously made up of thin (50 A) filaments, closely packed in parallel arrays which lie in the plane of the fiber axis. In places the Z disc seems to consist of a simple interdigitation of the thin myofilaments of the I bands from the adjacent sarcomeres (Figs. 9 and 17).

I BAND: In fibers fixed at resting length the I band occupies about 3 μ of the sarcomere, divided into two equal sections on the two sides of the A band (Fig. 7). The width of the I band varies directly with the changes in fiber length. When the fiber shortens to 60 per cent of its resting length the I band disappears. Unlike the thin myofilaments of frog muscle fibers, those of crayfish fibers are difficult to resolve in OsO_4-fixed preparations. The major part of the I band is almost homogeneous in appearance (Fig. 7), except near the Z disc, where there is some increase in the density of the thin myofilaments. In this region they have diameters of about 50 A (Fig. 17).

In fibers which are stretched by about 50 per cent over their resting length the I band develops a subbanding in the region close to the Z disc. The subband is easily resolved with the light microscope (Fig. 4) as well as in low power electron micrographs (Fig. 10). It appears to arise from clumped thin myofilaments which are dispersed near their origin at the Z disc, and which lose contrast in their course toward the A band. However, occasional thin filaments are visible in this latter, apparently homogeneous part of the I band. They seem to be embedded in a dense matrix which is confined to the myofibrils and does not extend into the spaces between the myofibrils. The spaces, however, are partly occupied by membranes.

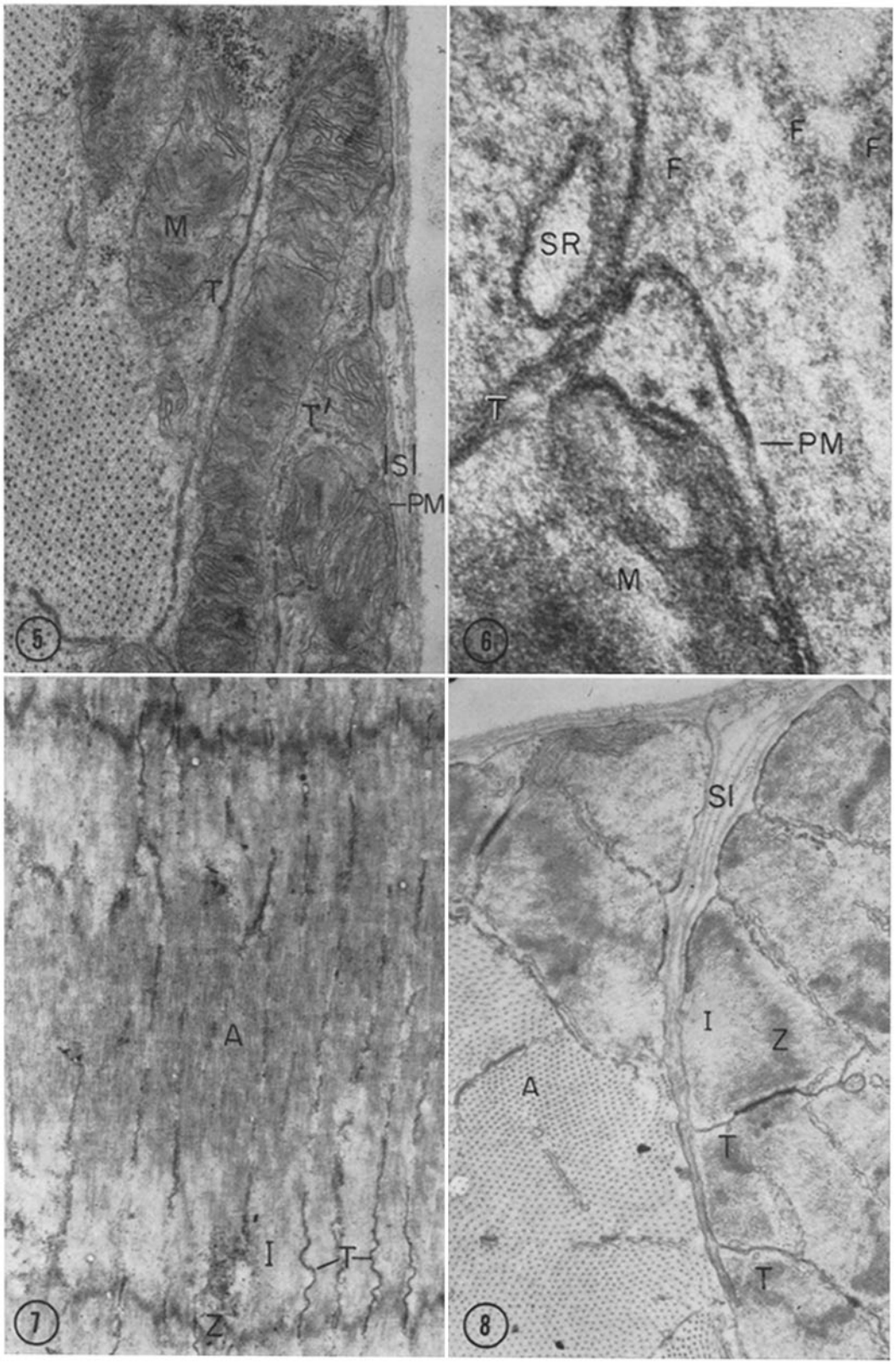
Thin myofilaments can be demonstrated throughout the I band in OsO_4-fixed muscle fibers which had been swollen by exposure to a solution made hyposmotic by removing part of the NaCl from the control saline or by exposure to an isosmotic medium in which KCl had been substituted for NaCl. The appearance of the preparations was similar to that of control fibers which

FIGURE 5 The sarcolemma (*S*) is thick and consists of two components, an outer coat and an inner plasma membrane. The outer coat is lamellated, as is more obvious in other experimental conditions. Beneath the sarcolemma is a layer of mitochondria (*M*). A component of the TTS (*T*) is seen leaving the vicinity of the sarcolemma and running obliquely inward for almost 4 μ. A row of vesicles at *T'* may represent a tubule of the TTS which is highly convoluted, twisting in and out of the plane of section. 0 K, OsO_4. × 24,000.

FIGURE 6 At high magnification the sarcolemma is seen to be bordered on the cytoplasmic surface by a unit membrane (*PM*) about 60 A thick, with a central light part about 20 A wide. Owing in part to the fixative, the outer part of the sarcolemma is layered into three coarse bands (*F*). A tubule (*T*) running into the cytoplasm can be seen which is formed by the invagination of the plasma membrane. Also visible are part of a mitochondrion (*M*) and some SR. 0 K, permanganate. × 257,000.

FIGURE 7 This low power micrograph demonstrates the tubules (*T*) which run from the Z disc inward toward the A-I band junction. They are higher in contrast than the SR which surrounds the A band. At this magnification little can be seen in the I band, although the thick filaments are apparent throughout the A band. 20 K, OsO_4. × 10,000.

FIGURE 8 In this cross-section a sarcolemmal invagination (*SI*) travels close to the Z disc for about 6 μ before leaving the plane of the section. Layers of the sarcolemmal coat can be followed into the invagination. Tubules (*T*) leave the invagination and run toward the A-I junctions. In places where the membranes of the invagination and tubules are close to the Z disc, they become thicker and denser. iso K, OsO_4. × 17,000.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had been swollen by fixation with glutaraldehyde (Fig. 9). In the I bands of such fibers fine filaments 50 A in diameter run from the Z disc into the A band. These filaments are relatively dense near the Z disc, but become somewhat finer and less dense at a distance of about 1 μ from the disc. The parts of the thin myofilaments which have the lower contrast appear to be more labile than the denser parts. They can be completely extracted by treating the fibers with 50 per cent glycerol, or, alternatively, they can be more readily visualized by causing the fiber to swell. When the thin filaments are made visible there is a parallel diminution in over-all contrast between the I band and the interfibrillar spaces.

A BAND: The central part of the sarcomere contains thick myofilaments about 150 to 200 A in diameter (Fig. 17 and reference 31) and constitutes the A band. This region is further differentiated by an abundant wrapping of SR. At the edge of the A band the thick myofilaments are irregularly staggered in preparations fixed at their resting length (Fig. 7). They appear more uneven in stretched fibers (Fig. 10). When the I band almost disappears in fibers which are contracted to about 60 per cent of their resting length, the thick myofilaments touch the Z disc. When fibers are shortened further, the tips of the thick filaments become disoriented and bent aside.

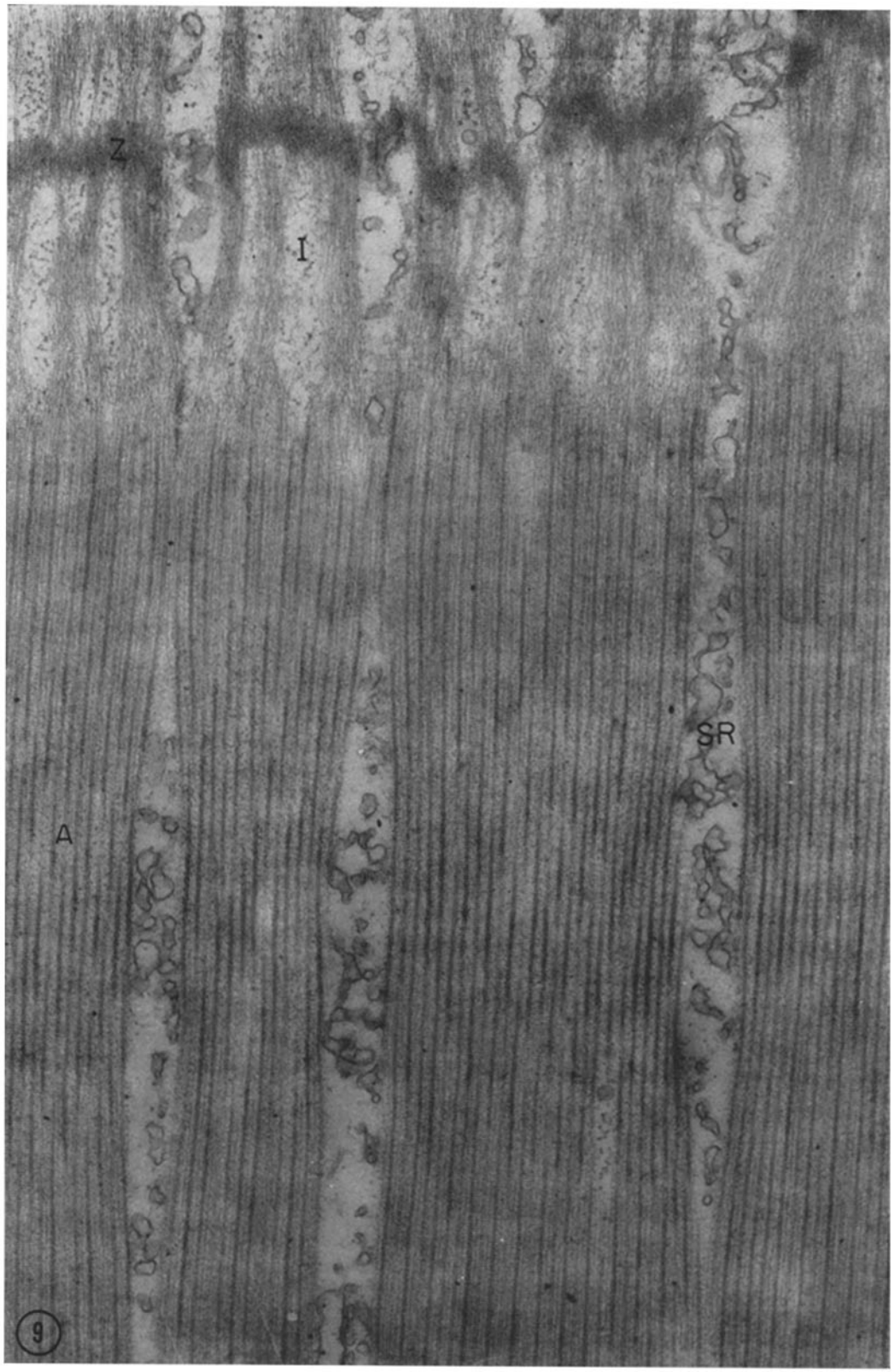
Thin filaments may be seen among the thick myofilaments, but they are not well resolved in OsO_4-fixed preparations of unstretched control fibers. They cannot be seen at all in preparations which have been stretched by 50 per cent above their resting length. However, in fibers which have been caused to swell the thin myofilaments become very evident and 6 to 8 filaments form a ring around each thick myofilament (Fig. 11).

Electron micrographs of OsO_4-fixed fibers in longitudinal sections do not disclose variations in the density of the A band which might denote an H zone and M band. This absence of subbanding within the A band is independent of the sarcomere length (Figs. 7, 10 and 17). Subbands within the A band of crayfish muscle fibers have been reported, based mainly on observations with light microscopy (42). In the present work, what might correspond to an H zone could be shown in 0.5 μ thick sections of fibers which were fixed in glutaraldehyde, embedded in epoxy, stained with toluidine blue, and photographed in phase contrast (Fig. 3). When the same fibers were examined with the electron microscope the H zone was most difficult to delineate (Fig. 9). If the H zone is limited to the part of the A band which lacks thin myofilaments (1 to 1.5 μ, Fig. 9), it does not correspond in width to the H zone visualized in the light microscope (3 μ, Fig. 3). It is conceivable that changes in the extrafibrillar spaces may contribute to the optical effects which demarcate an H zone in light microscopy.

The Sarcoplasmic Reticulum

This system of membranes forms a flat cistern which envelops each myofibril in the A band, where the thick myofilaments are found. It is sparse or absent in the I band (Figs. 13 and 14). Owing to the poor register of adjacent myofibrils, the restriction in coverage of the SR is not readily seen in thick grazing sections in the longitudinal plane, the out-of-register effect causing the SR of one myofibril to appear to cover the I band or Z disc of an adjacent fibril. However, the association of the SR with the regions of the thick filaments is seen particularly clearly in cross-sections of preparations which had been fixed while stretched (Fig. 14). The SR envelope is essentially a cylinder with a wall formed of two concentric membranes. The envelope is fenestrated at multiple sites, by pores which are formed by the fusion of the two layers of the envelope (Figs. 12 and 15). Thus, the pores in the SR of crayfish muscle fibers do not expose the interior of the SR to the general sarcoplasm, but are areas of communication between the interfibrillar compartment and the myofibrils. The pores occupy from

FIGURE 9 The thin myofilaments of the I band are visible at relatively low magnifications in glutaraldehyde-fixed fibers. These myofilaments extend a limited distance between the thick myofilaments of the A band. However, their ends do not clearly define an H zone. At the other end the thin filaments continue into the Z line. Fragments of the SR are found in the interfibrillar spaces, which are rather large and may account for the light spaces around the myofibrils seen in the same fiber in Fig. 3. 0 K, glutaraldehyde. × 33,000.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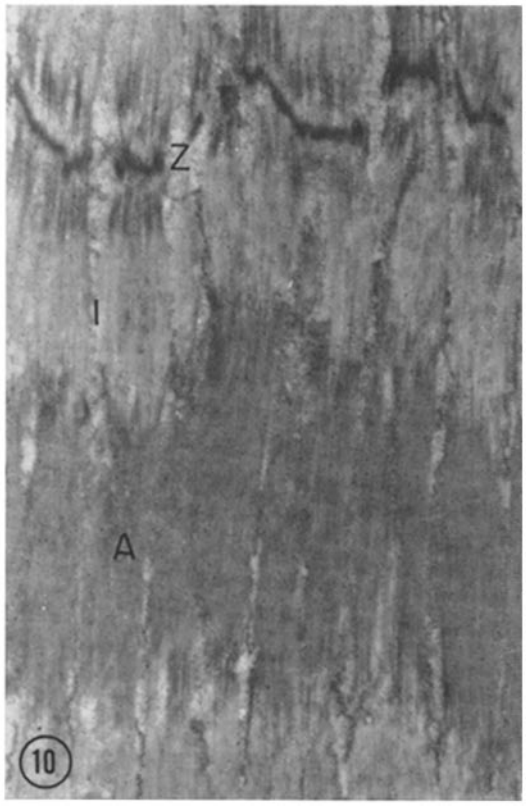
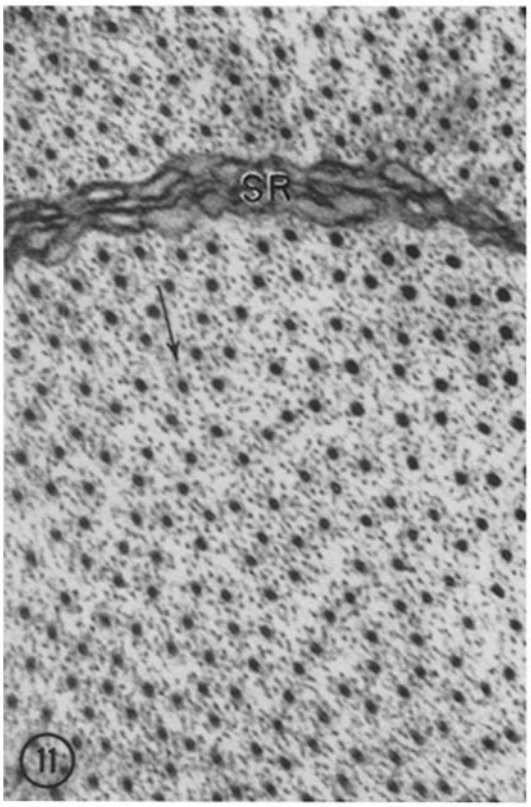




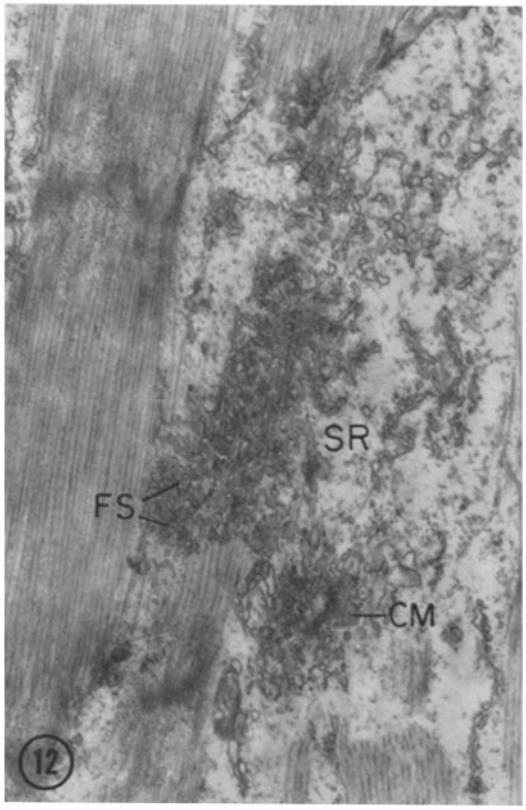


FIGURE 10 The extra band within the I band is the most obvious feature of the stretched fiber (see also Fig. 4). It is apparently due to the increase in contrast of the thin myofilaments at this region and to their organization into clumps. The remainder of the I band is rather dense and homogeneous. The uneven edges of the A band show the lack of register between the thick myofilaments. 0 K, OsO_4, × 6350.

FIGURE 11 The thin myofilaments (arrow) are readily visible among the thick myofilaments of the A band in swollen fibers. The filaments do not appear to fit into an over-all lattice, but rather to be grouped, with one thick filament surrounded by six to eight thin myofilaments. — 100 NaCl, OsO_4. × 65,000.

FIGURE 12 A grazing section of the surface of a myofibril shows the form of the SR around the A band. It is apparently fenestrated (*FS*) at numerous points. In the lower right of the field is a collection of membranes (*CM*) which may have TTS components in it, but the complexity of the group does not permit analysis. iso Ca to 50 K, OsO_4. × 14,000.

one-eighth to one-fourth of the surface area of the SR. When the SR is swollen under some experimental conditions the structure takes on more of the appearance of anastomosing tubules. Saclike projections of the SR which lack pores extend to the I band and make frequent contact with the TTS. As will be described below (see also Fig. 28), the contacts form diads. The SR envelopes of adjacent myofibrils are continuous with each other at their margins of contact (Figs. 13 and 25).

A complication for the interpretation of longitudinal sections with respect to the localization of the SR is the presence of the tubules of the TTS. As described below, these tubules run in the interfibrillar spaces adjacent to the I band, and often penetrate the Z disc (Fig. 7). When the sarcomere shortens, these tubules become contorted (Fig. 15) and may resemble the SR.

The Transverse Tubular System

On the basis of more extensive morphological studies it appears advisable to modify the terminology used in our previous paper (13). In that work, primary emphasis was placed on the differentiation of special anion-permselective characteristics of some regions of the TTS, and the term TTS was applied to these specialized regions. The connections of these regions with the exterior were termed radial tubules (RT). The TTS as now defined contains two morphologically distinctive elements: *sarcolemmal invaginations* and *tubules*. Many of the tubules do originate directly from the sarcolemma, and the term RT may be retained for these connections. However, by far the largest number of tubules originate from the sarcolemmal invaginations (Fig. 28). There are about 10,000 tubules per sarcomere, and of these about 90 per cent originate from the sarcolemmal invaginations and only about 10 per cent from the sarcolemmal surface.

SARCOLEMMAL INVAGINATIONS: At intervals over the surface of the muscle fiber the sarcolemma forms invaginations (SI) 0.1 to 0.4 μ in diameter (Fig. 8). The initial part of the invagination is funnel-shaped. The invaginations may be flattened in one plane. The sarcolemmal invaginations run radially or obliquely inward (Figs. 1 and 2), and it is estimated that per sarcomere length of fiber there are between 50 and 200 invaginations of the surface. The continuation of a portion of the sarcolemmal surface coat together with the plasma membrane into the invaginations distinguishes the latter from the tubules of the TTS, where no such coat is apparent. Because of the coat, the invaginations are readily identifiable within the muscle fiber, even when their connection with the fiber surface is not in the plane of the section (Fig. 17). Since this report is based on the study of isolated single fibers, the SI cannot be confused with spaces between fibers.

The radially oriented sarcolemmal invaginations may form side branches within the fiber (Fig. 28), which run parallel to the long axis of the fiber for considerable distances (Fig. 17). Branches have been traced over a length of up to 100 μ before they were lost from the plane of the section, although most of the longitudinal branches run in and out of the section plane every few sarcomeres. Whenever the invaginations or the tubules are in close proximity to the Z disc, but not necessarily in contact with it, the membrane increases in density (Figs. 8, 17, and 19). The invaginations appear to be irregularly distributed with respect to the sarcomere pattern.

TUBULES: Tubules, 200 to 400 A in outside diameter (the diameter depending upon the experimental procedure), leave the sarcolemmal invaginations as closely as every 0.25 μ (Figs. 8, 18, 19, 20, and 23) and run toward the A-I junctional zone. As already noted, they lack the sarcolemmal coat found throughout the invaginations. At the edge of the A band the tubules curve around the myofibrils and as parts of the diads make intimate contact with the SR at numerous discrete sites (Figs. 13, 18, 20, 22, and 23).

The membrane of the tubule walls is continuous with that of the SI and of the sarcolemma. The continuity of the different parts which is shown in Figs. 6, 8, 16, 18, 19, 20, 23, 24, 26, and 27 thus confirms the physiological evidence (13) that the lumen of the TTS is an extension of the extracellular space. Physical evidence of the fact that the extracellular environment penetrates deep into the fiber by way of the TTS is provided by the diffusion of ferritin from the bathing medium into the TTS (Fig. 27). It does not enter the SR. The continuous membrane of the TTS has identical "unit" dimensions (*ca.* 60 A) throughout, whereas membranes of other intracellular organelles are thinner (*ca.* 50 A).

The tubules which originate at the plasma membrane have the same structure as do those which start from the invaginations. They run radially inward (hence their designation as RT;

see 13), penetrating the peripheral zone of nuclei and mitochondria and entering the cortical zone of the myofibrils (Figs. 5, 16, 24, and 26). Like the tubules which start from the sarcolemmal invaginations, they also make intimate contact with the SR at the A-I junction. The contacts between SR and tubules appear to be the same whether the latter originate at the cell surface or from an invagination.

The Diads

The diads are sites of specialized contact between the TTS and SR which occur invariably at the edges of the A band, close to the A-I junctions (Fig. 25). The membranes of the two systems maintain a regular spacing of 150 A, with a dense intermediate plate evenly dividing the gap (Fig. 22). The plate, which has not been described in diad or triad structures of other cells, is about 35 A thick, and the spaces between it and the membranes of SR and TTS are accordingly about 60 A wide. The SR makes diadic contact with the sarcolemma (Fig. 26) as well as with the sarcolemmal invaginations (Figs. 17 and 21), but most frequently with the tubules (Figs. 13, 16, 18, 20, 22, 23, and 26). "Dyads" or contacts between plasma membrane invaginations and isolated vesicles of SR have been reported in insect muscle (41).

The diads occur at multiple sites around a single myofibril at the ends of the A band (Fig. 13). Frequently two or three diads may be seen clustered about one fibril in a single section, and it is likely that several more occur out of the plane of the section. The longest diads observed have been about 0.5 μ, but it is also likely that still longer diads would leave the plane of the section. The membranes of the TTS and SR may be in close proximity at other sites without forming the complex structure of the diad.

DISCUSSION

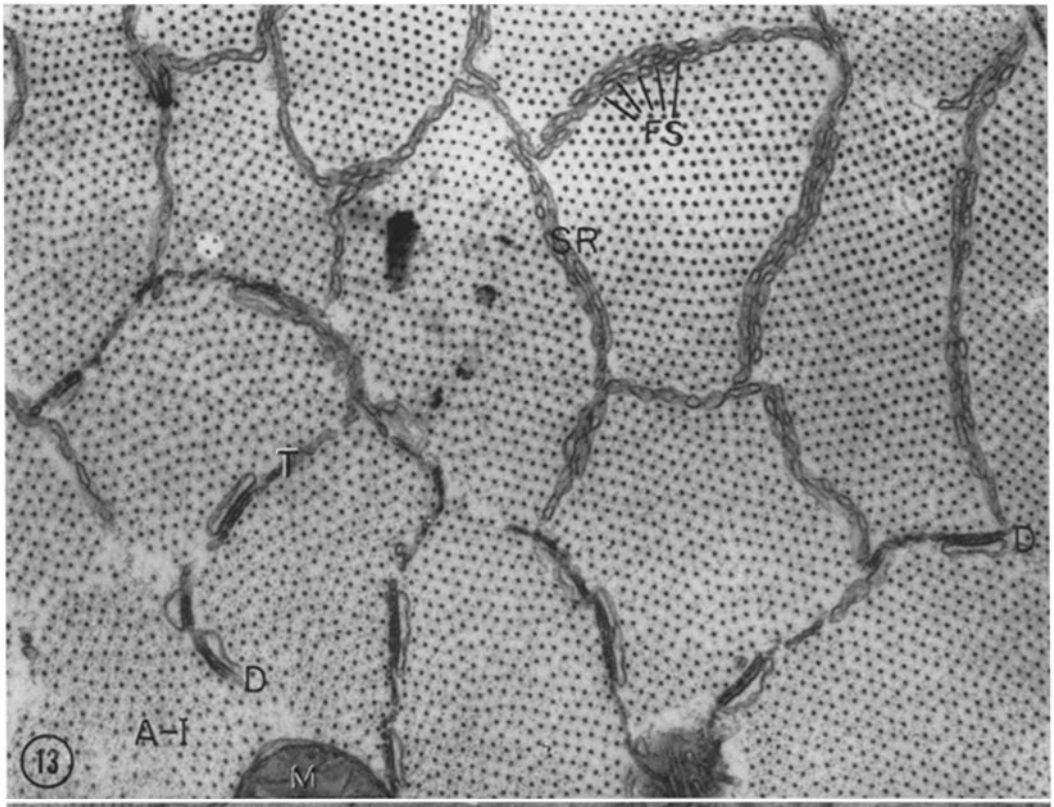
The A Band

In longitudinal sections of control fibers which had been fixed in osmium tetroxide it was not possible to demonstrate unequivocally that thin myofilaments are always present between the thick filaments. Nor does the A band show a clear subbanding into an H zone and an M band under the same conditions (Figs. 1, 7, and 17). If the fibers were swollen prior to fixation, thin myofilaments and a rather poorly defined H zone could be discerned. However, no M band was observed (Figs. 3 and 9). In cross-sections of unswollen fibers thin myofilaments could be seen in parts of the A band, and were absent in other parts (Fig. 25). Hence an H zone must be present, but its width cannot be measured from cross-sections. Since interdigitating thick and thin myofilaments could be demonstrated, one of the conditions of the sliding filament model (18, 21) is met. When the muscle shortens, the I bands on both sides of the A band tend to diminish and disappear, also in conformity with the model.

Since the A band does not show a clear division by an H zone in longitudinal section, a direct proportionality between the length of the thin filaments and the width of the H zone over a range of sarcomere lengths could not be demon-

FIGURE 13 The myofilaments are grouped into myofibrils by the membranes of the SR and transverse tubular system (TTS). The upper half of the field is occupied by fibrils surrounded by SR, the lower half by fibrils surrounded by both SR and TTS. This concurrence of the two systems takes place within the A band near the junction with the I band. At intervals the tubules (*T*) of the TTS make a characteristic contact (diads, *D*) with the SR. Several fibrils can be seen to be bordered by two or more diads. The SR is a double-membrane envelope around each myofibril, and fusions between the two layers occur at the fenestrations; therefore the interior of the SR compartment is closed off from the sarcoplasm. At the lower edge of the field are parts of two mitochondria. iso K, OsO_4. × 30,000.

FIGURE 14 When a fiber is stretched, the I band becomes longer than that of a fiber at resting length. In cross-section it can be seen that stretching also tends to confine the SR to the A band, while the I band is almost devoid of membranes. Within the I band an occasional profile of a tubule (*T*) is found. 0 K, OsO_4. × 23,000.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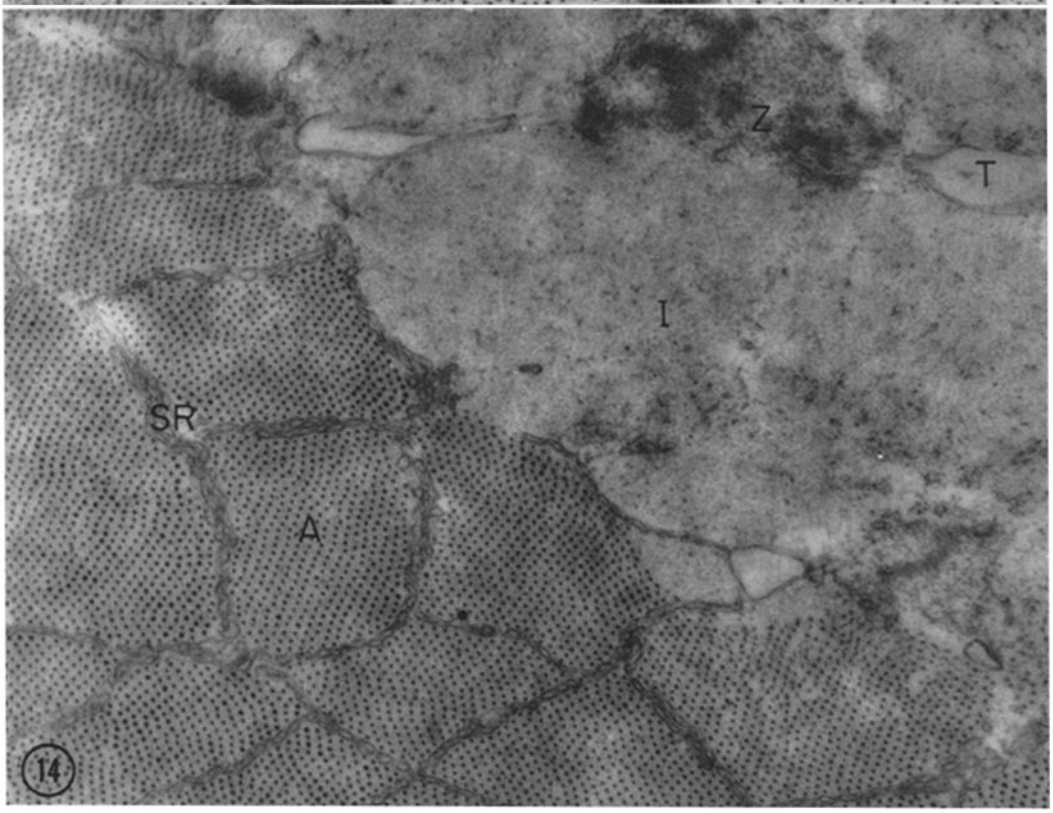
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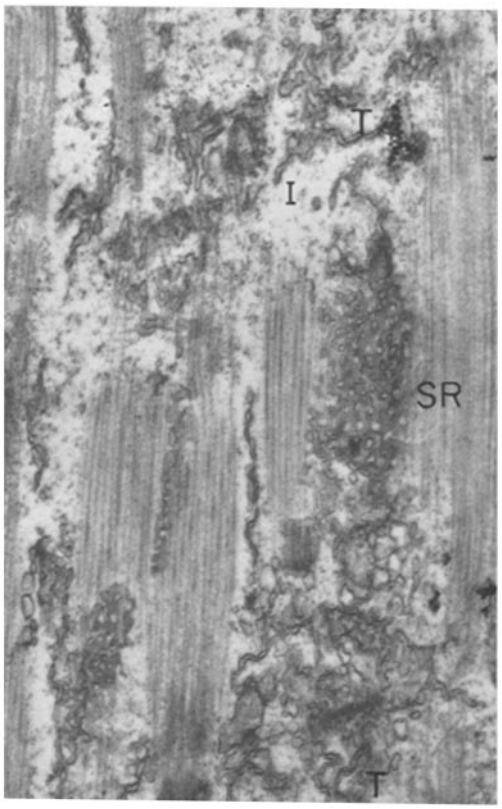


FIGURE 15 The surface view of the SR in this figure demonstrates its fenestrated nature. The tubules (*T*) which are found at the borders of the SR near the I bands form a very complex pattern in fibers contracted to about 60 per cent of resting length. iso Ca to 50 K, OsO_4. × 19,000.

strated. Swan (42) has reported that a correlation could be made between the sarcomere length and the width of the H zone. He found it necessary, however, to postulate at least four different fiber types, each with a different A band width. Though the difficulty in defining the H zone, particularly from longitudinal sections, may be due to a number of factors, it appears to be related most directly to the difficulty encountered in resolving the thin myofilaments.

When the muscle fibers were permitted to shorten to less than 60 per cent of their resting length, only the A band was visible, but its thick myofilaments now appeared to be crumpled against the Z disc. The thick filaments of contracted glycerinated insect myofibrils are coiled at the Z disc (10). In the "supercontracted" giant muscle fibers of the barnacle (27) the thick filaments also appear to be crumpled against the Z disc.

The I Band

The texture of the I band is different in different regions. In control fibers fixed in osmium tetroxide the I band close to the Z disc appears more fibrillar and the fibrils are clumped together. The myofilaments in the remainder of the I band appear to be masked by a matrix. The matrix is best visualized in a fiber soaked in low K Ringer's solution and fixed under stretch, after which the I band appears dense and in high contrast against the relatively transparent spaces between the myofibrils (Fig. 10). In general, the thin myofilaments of crayfish muscle fibers appear to be less dense and may be smaller in diameter than are those of frog muscle fibers (24). Various experimental procedures or glutaraldehyde fixation accentuate the appearance of fine filaments in the I band and between the thick myofilaments of the A band.

The thin myofilaments assume much higher contrast in swollen fibers, while over all the I band loses contrast. The thin myofilaments are believed to contain actin (3, 14, 15, 21), and in invertebrate muscles tropomyosin is believed to be bound to the actin (16). However, this binding appears to be rather weak and reversible and subject to changes in ionic conditions, and is decreased in media of lowered ionic strength (25).

The Sarcoplasmic Reticulum

A detailed study of the properties of the SR will be presented in a later paper. Here it is only relevant to note that the interior of the SR is not exposed to the sarcoplasm. In the muscles of some fish, however, the pores of the SR apparently perforate only one surface of the envelope (7), so that the interior of the SR appears to be exposed to the sarcoplasm. The SR of the crayfish muscle fibers accumulates Ca rather selectively (unpublished data).

The Transverse Tubular System

The electron microscopic evidence presented here and summarized in Fig. 28 agrees with the physiological finding (13) that the membrane of the TTS is in continuity with the plasma membrane. The continuity, which results in the introduction of the extracellular milieu deep into the

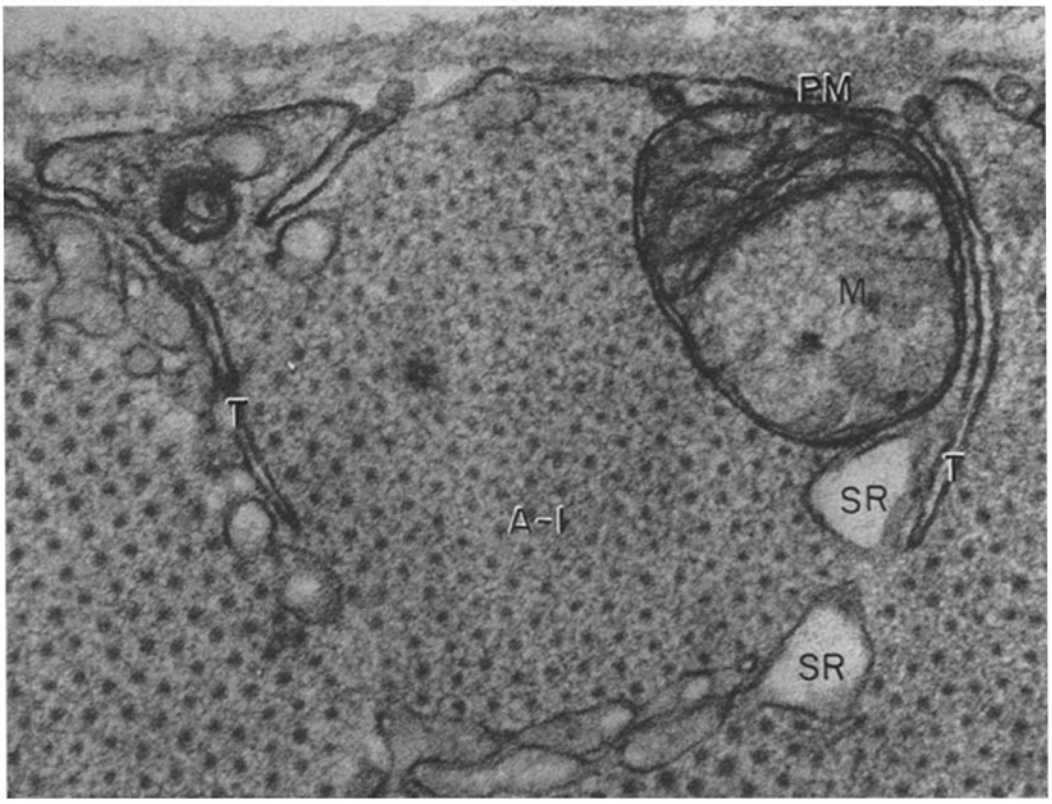


FIGURE 16 Two tubules (*T*), their membranes a continuation of the plasma membrane (*PM*), run into the fiber at the A-I junction. The tubule at the right comes in close contact with the SR, which is swollen in the permanganate fixation. This contact may be a diad, but in this fixative the diadic detail usually resolved by OsO_4 fixation is not so clearly seen. A mitochondrion (*M*) can be seen. 0 K, permanganate. × 110,000.

interior of the muscle fiber, had not been observed in the earlier studies on the vertebrate T system (2, 6). However, recent studies on vertebrate muscle fibers (4, 8, 20, 22) do report continuity between the plasma membrane and T system tubules. These findings suggest that the T system is strictly homologous with the TTS of crayfish muscle fibers and is also an "extracellular" space.

In crayfish muscle fibers the TTS includes a dense system of sarcolemmal invaginations from which most of the fine tubules originate. In this respect therefore the TTS differs somewhat from the T system of vertebrate skeletal muscle (2). However, invaginations are also prominent in heart muscle, and tubules are given off from these invaginations (12, 28, 40), as was demonstrated by the fact that ferritin diffuses into the tubules from the external medium (12). The myocardial tubules may play a role in excitation-contraction coupling (11) such as that which has been suggested for the TTS in crayfish muscle fibers (13). Numerous tubules originate directly from the plasma membrane in crayfish muscle fibers (13; Figs. 5, 6, 16, 24, and 26), and presumably it is these which are more directly comparable with the T system tubules described by workers on vertebrate skeletal muscle. Invaginations, which however were interpreted as being confluent with the SR, were observed in the muscle fibers associated with crayfish stretch receptors (32).

The TTS not only is transversely oriented, but also spreads longitudinally for considerable distances, and thereby ties together many sarcomeres of a given myofibril. Shorter longitudinal extensions of the T system tubules have been recently observed in frog muscle fibers (20).

Some consequences, with respect to the electrical and electrophysiological properties of the muscle fiber, of such an extensive system of intracellular canals which open to the exterior have been discussed in an earlier paper (13). Since the TTS is an even more elaborate system than was envisaged in the earlier work, the effects of the electrical shunting by the channels of the TTS on the action currents of the plasma membrane should be very marked, indeed. Measurements of the AC impedance in frog as well as crayfish muscle fibers (5) support our previous finding (13) of the existence in crayfish muscle fibers of an electrical shunting network which is in parallel with the resistance of the plasma membrane. Falk and Fatt (5) also suggested that the shunting is provided by a network of transversely oriented tubules. The shunting effect was observed in our earlier paper (13), under steady state (DC) conditions, whereas with the AC measurements the shunting was evidenced only at higher frequencies, above about 50 CPS in the frog fibers (5, p. 98).

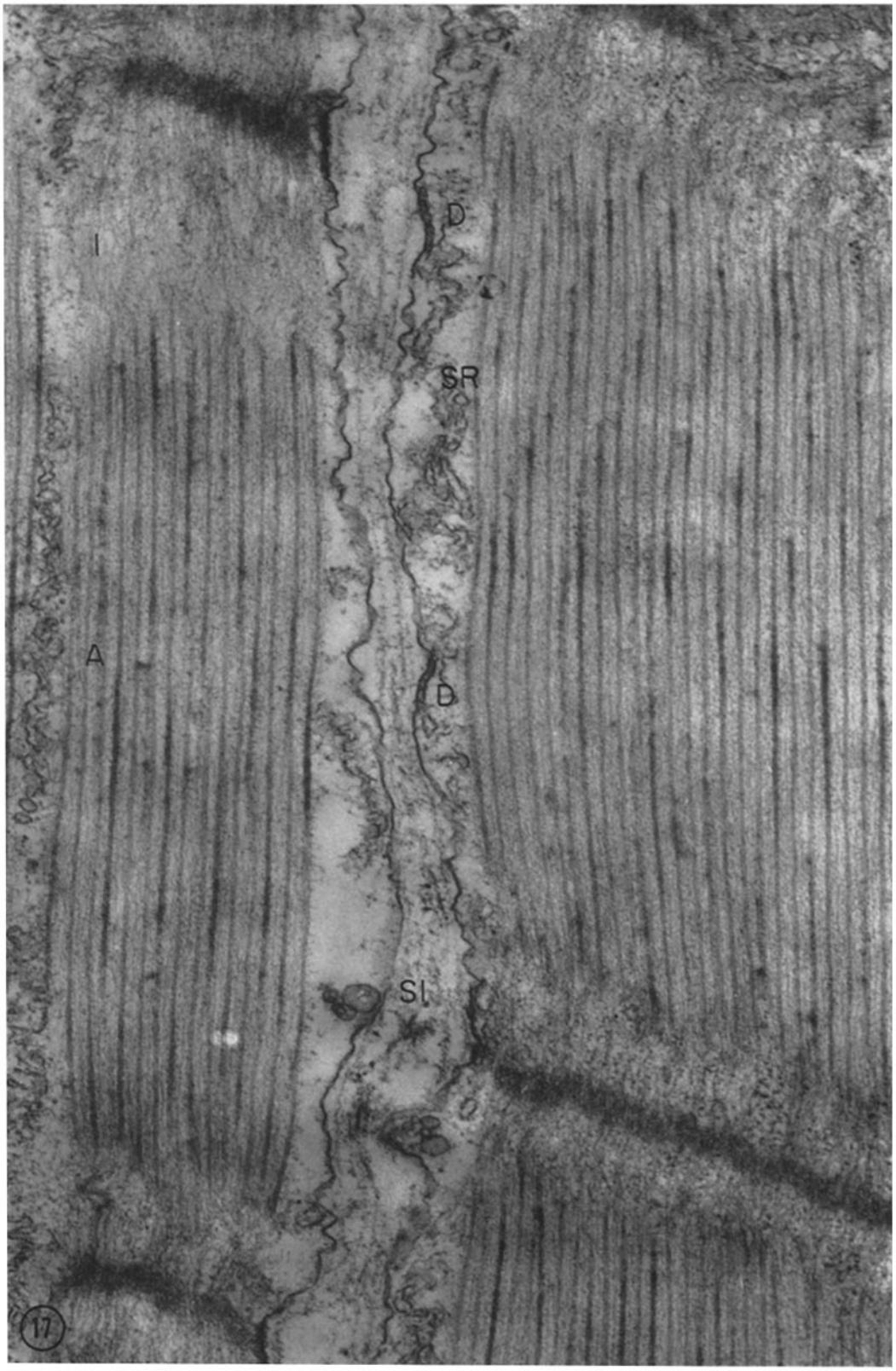
In our earlier report (13) on crayfish muscle fibers, evidence was presented concerning the existence and location of anion permselectivity in the membrane of the TTS. Only the extremities of the TTS appeared to have anion-permselective membrane, whereas the membrane lining the radial tubules which originate from the plasma membrane appeared relatively impermeable. The morphological data presented now do not permit a comparable characterization of the membrane of the sarcolemmal invaginations. However, the specialized complexes (diads) which are formed at conjunctions of the tubules and the SR are also present at numerous sites along the sarcolemmal invaginations as well as at the plasma membrane. If, as seems likely, the diadic complexes are the sites of the specialized anion permselectivity, there may also be local specialization in membrane properties along the plasma membrane and the sarcolemmal invaginations. To account for some electrophysiological data, Adrian and Freygang (1) suggested that the T system of frog muscle fibers has membrane which is selectively permeable to K, but not to Na or Cl. However, they also postulated that the channels of the T system are closed at the surface of the fiber by membrane which is permeable to the cations but not to Cl.

Interrelations of the TTS and the SR

The TTS components of the regular, structured contacts which we have termed diads are the regions where vesicles begin to form (13) when Cl is made to leave the muscle fiber (Fig. 25; publication in preparation). Morphological continuity between the sarcolemmal invaginations and the SR has been described in the specialized muscle fibers of the crayfish stretch receptor organ (32). However, in the present study on skeletal muscle fibers no direct morphological continuities have been observed between the TTS and the SR. The evidence is rather to the contrary. Our previous findings (13) demonstrated that the TTS swells greatly under conditions which leave the volume of the SR compartment comparatively unchanged. In fact, whereas the TTS swells when Cl leaves the cell, the SR appears to shrink somewhat (Fig. 25). Conversely (Fig. 13), exposure of the cell to isosmotic KCl causes the SR compartment to swell while the TTS shrinks. The finding (Fig. 27) that ferritin diffuses into the TTS, but not the SR, provides additional evidence for the independence of the two systems. In vertebrate muscle the independence of the T system from the SR was shown by Andersson-Cedergren with electron micrographs of serial sections (2). The more recent evidence on the selective penetration of ferritin into the tubules but not the SR in cardiac muscle of the rat (12) and skeletal muscle of frog (20) confirms those findings. The latter type of data also demonstrates that the T system, like the TTS, introduces the external environment deep into the muscle fiber.

Thus, both the physiological and the morphological observations strongly suggest that the TTS

FIGURE 17 A sarcolemmal invagination (*SI*) which runs between two myofibrils is identified by the coating material in its lumen. At the Z disc of each fibril the membrane of the invagination becomes denser and is perhaps attached to the Z disc. Two diads (*D*) are shown. In places the thin myofilaments can be seen between the thick myofilaments. In the I band the thin filaments are poorly resolved. Among the myofibrils in the I band are numerous unidentified granules. Control, OsO_4. × 42,000.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and the SR do not form a continuous system. The extracellular environment is led to the diads by way of the TTS. There it comes into intimate contact, but not continuity, with the SR, which is itself confined to the sarcoplasm in the region of the A band. If there be a functional interrelation between the TTS and the SR, it is mediated and regulated by interposed membranes at the diadic contacts.

The specialized anion permselectivity of the membrane in the crayfish tubules (13) offers a particularly effective mechanism by which spread of excitation may be achieved when the plasma membrane is depolarized. This mechanism involves a redistribution of the Cl across the anion-permselective membrane during depolarization with a flow of current through the TTS. Recent studies (9; unpublished data) on contractures of crayfish muscle fibers demonstrate that redistribution of Cl plays a direct role in excitation-contraction coupling. In view of the often expressed surmise that the diads or triads and the T system are involved in excitation-contraction coupling (17, 19, 34), and since the T system and the TTS are apparently homologous, it seems reasonable to suspect that the mechanism of excitation-contraction coupling suggested earlier (13) may also be extended to other muscles. Evidence to support this conclusion is now available for cardiac muscle (11).

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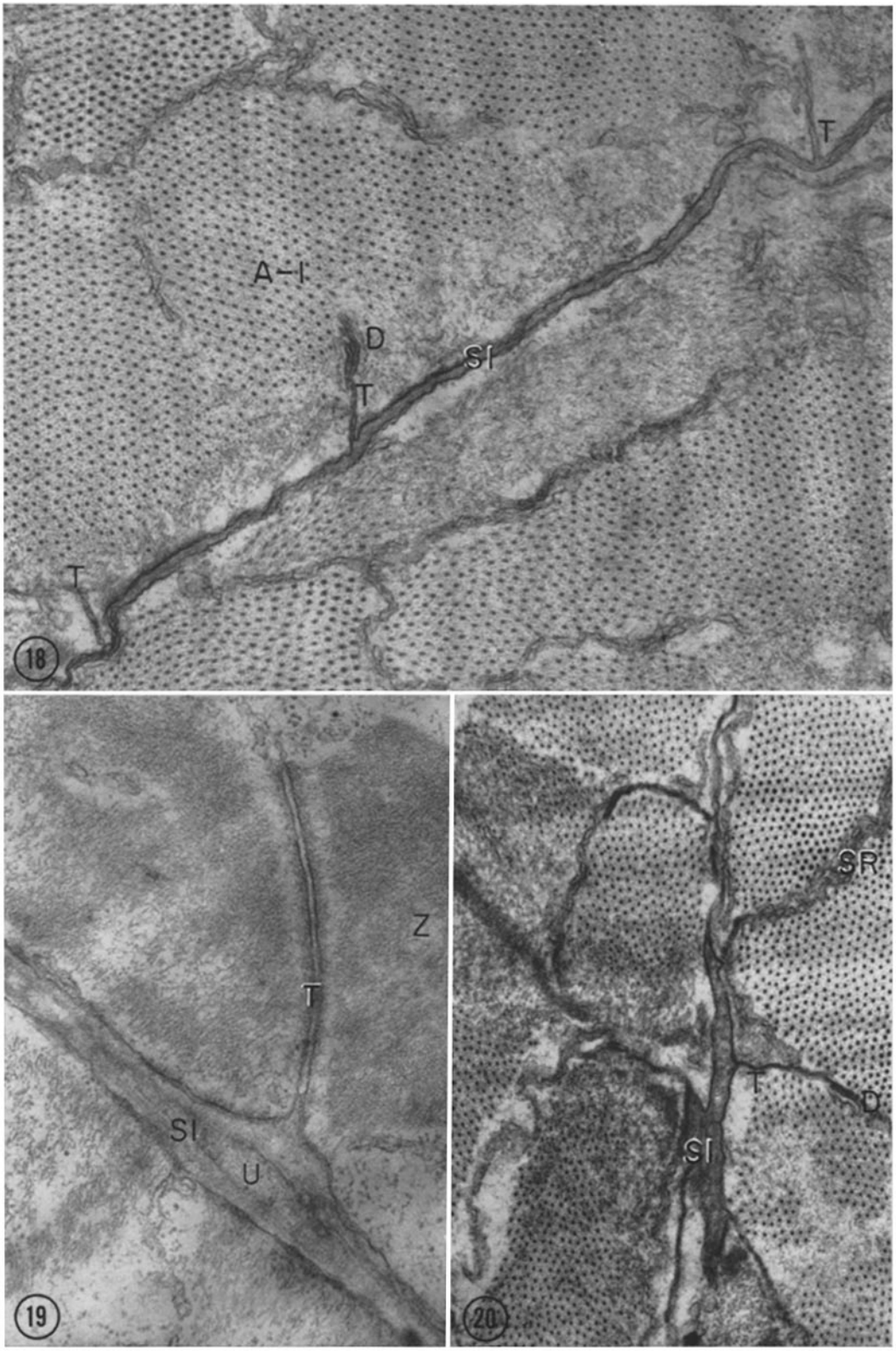
For References, see page 254.

FIGURES 18 to 20 This group of micrographs demonstrates different forms of the branchings of the TTS. From the sarcolemmal invaginations (*SI*) smaller branches or tubules (*T*) run in various directions. Whenever the membrane of a tubule or invagination comes into contact with the Z disc it becomes coarser and denser. The tubules are usually higher in contrast than the SR, partly because of their geometry and partly because the plasma membrane derivative, which lines them, is thicker (*ca.* 60 A) than the SR membrane (*ca.* 50 A). In several instances the tubules contact the SR in a diadic junction (*D*). In Fig. 19 a cell process (*U*) of unknown origin is found lying within the invagination. All in OsO_4. Fig. 18, iso K, short exposure, 31,500; Fig. 19, procaine, × 37,000; Fig. 20, iso K, short exposure, × 24,500.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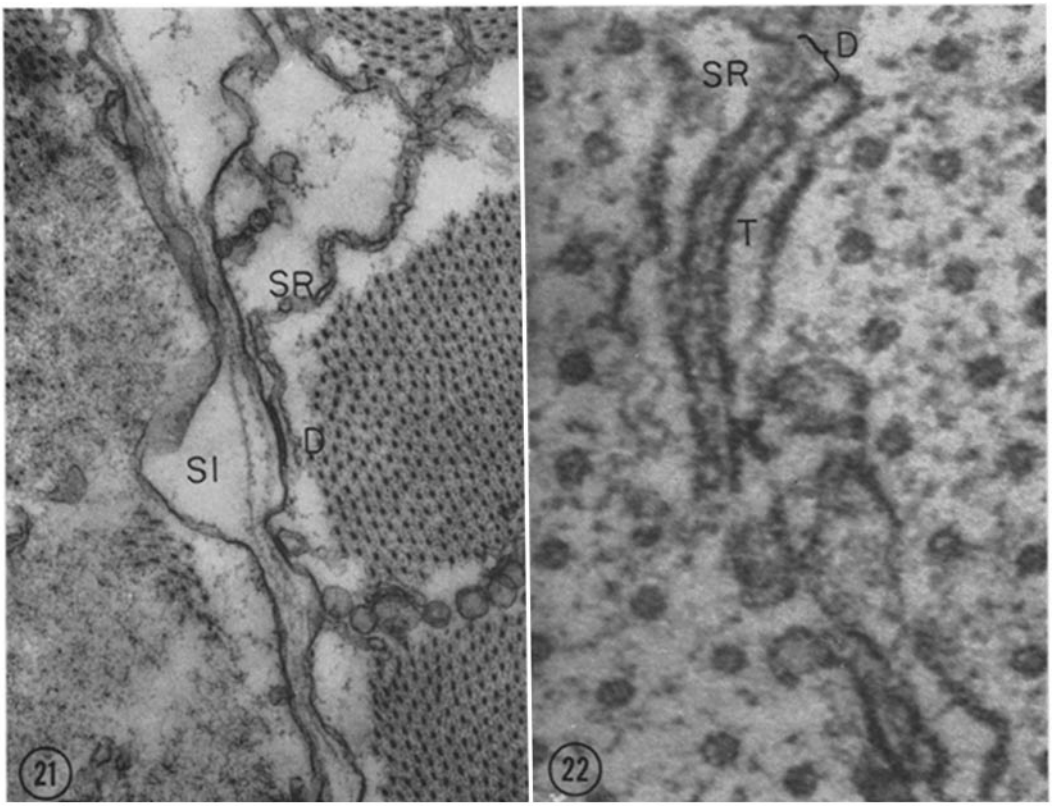

FIGURE 21 The experimental condition has swollen the sarcolemmal invagination (*SI*), but it can still be identified by the presence of fibrous material in its lumen. At one point the SR makes a diadic contact (*D*) with the invagination which is identical with the other forms of the diadic contact between the transverse tubular system and the SR. The distortion of the fiber permits the connection between the diad part of the SR and the regular form of the SR to be easily traced. 100 K to 0 K, OsO_4. × 32,000.

FIGURE 22 At higher magnification the diad (*D*) is seen to have a uniform 150-A gap evenly divided by a plate 35 A thick. In the OsO_4 fixative the unit membrane structure of the diadic membranes demonstrated by permanganate is unresolved. Control, OsO_4. × 230,000.

FIGURE 23 A cross-section of a longitudinally running sarcolemmal invagination (*SI*) gives off five tubular branches (*T*) as it passes near the A-I junction. Several of the tubules contact the SR at diadic junctions. In numerous places the unit membrane of the TTS is resolved, and has the same dimensions as the plasma membrane (60 A). The membranes of the SR are more difficult to resolve and are thinner. 0 K, permanganate. × 132,000.

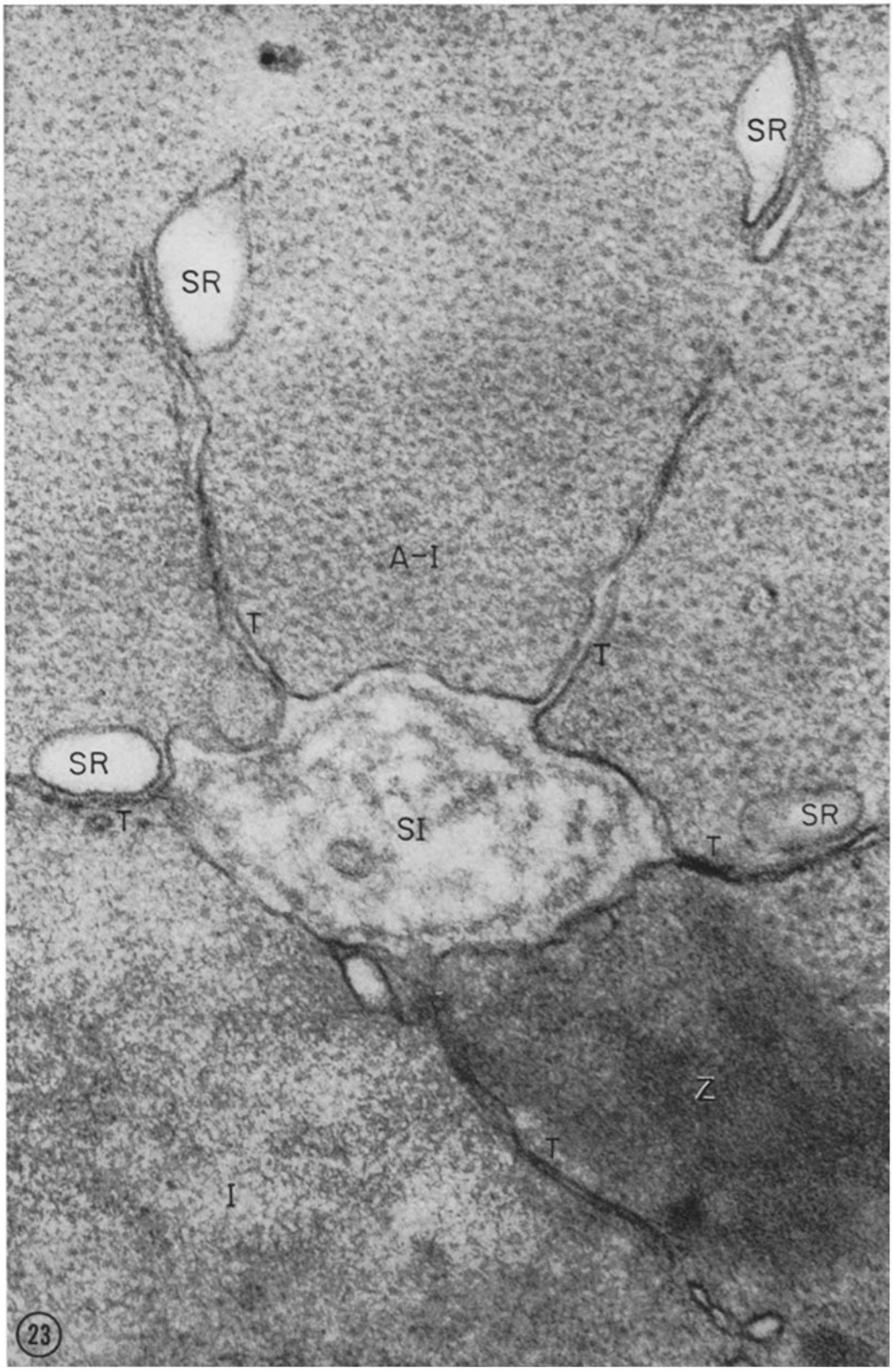
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FIGURE 24 A tubule (*T*) runs from the cell surface into the sarcoplasm. The membrane of the tubule is a continuation of the plasma membrane (*PM*), and the lumen can be traced to the extracellular space. An unidentified cell process (*U*) lies adjacent to the throat of the tubule. Within the sarcoplasm the tubule eventually comes into contact with the SR at what is probably a diadic junction, but owing to the fixative does not show exactly the same detail as do the diads of fibers fixed in OsO_4. The mitochondrion (*M*) more closely resembles those fixed with OsO_4 than those fixed with glutaraldehyde. 0 K, permanganate. × 141,000.

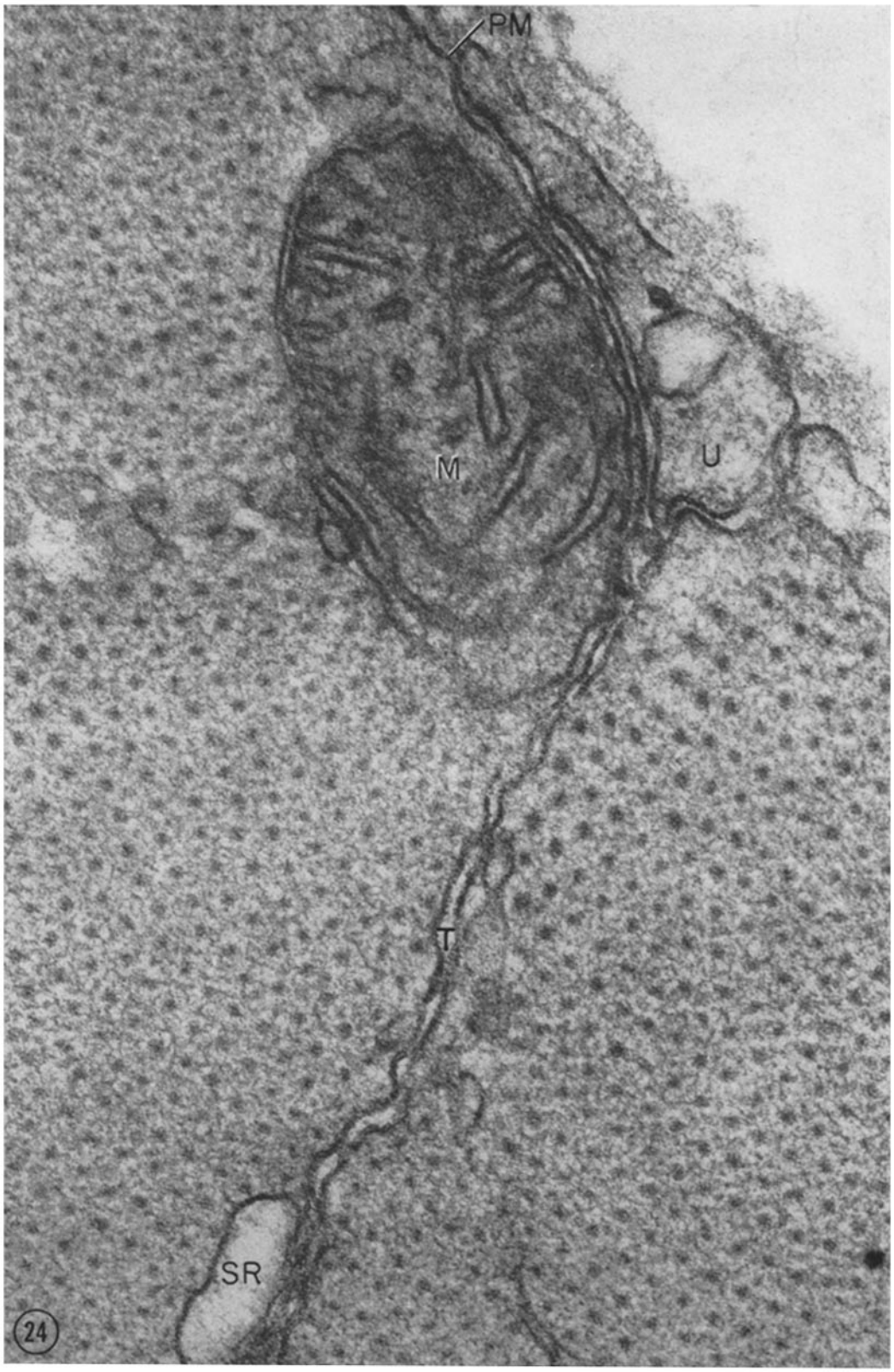
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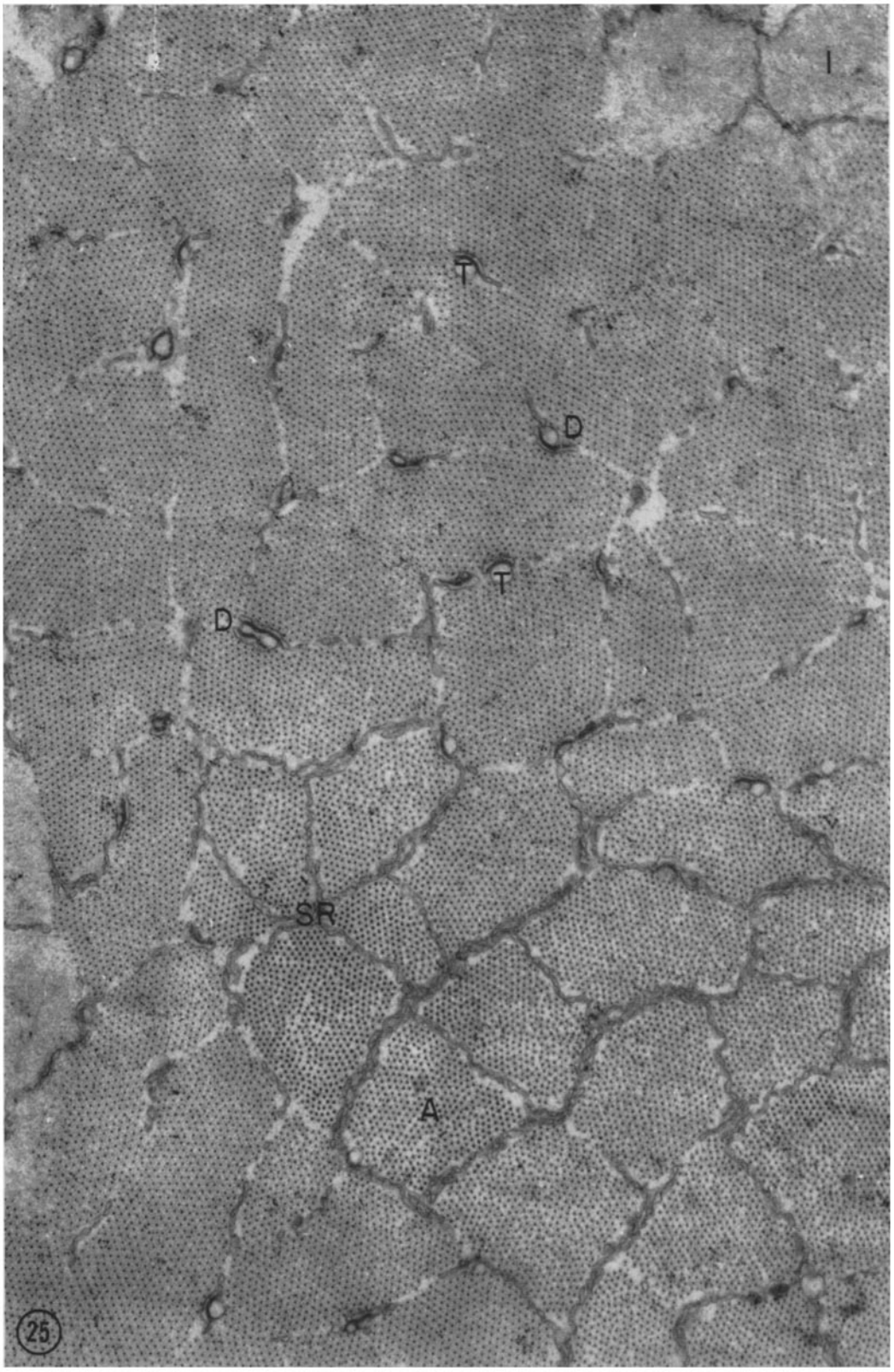
FIGURE 25 The locations of the diadic (*D*) contacts between the SR and the tubules (*T*) are seen clearly in this experimental condition which swells the tubules slightly. Sometimes a tubule lies between two elements of the SR and forms a diadic contact with both in the same plane (the diad on the left). The bulk of the diads are within the A band near the A-I junction. They are completely absent deeper within the A band where the SR is most concentrated. The location of the diads is apparently coincident, in this figure, with the overlap of the thick and thin myofilaments. The I band appears in places around the margins of the figure. 50 K to 0 K with 0.1 per cent procaine, OsO_4. × 12,500.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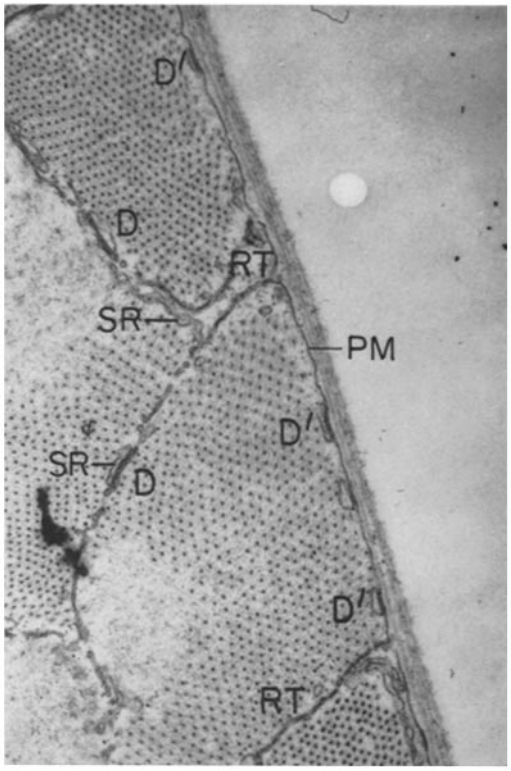
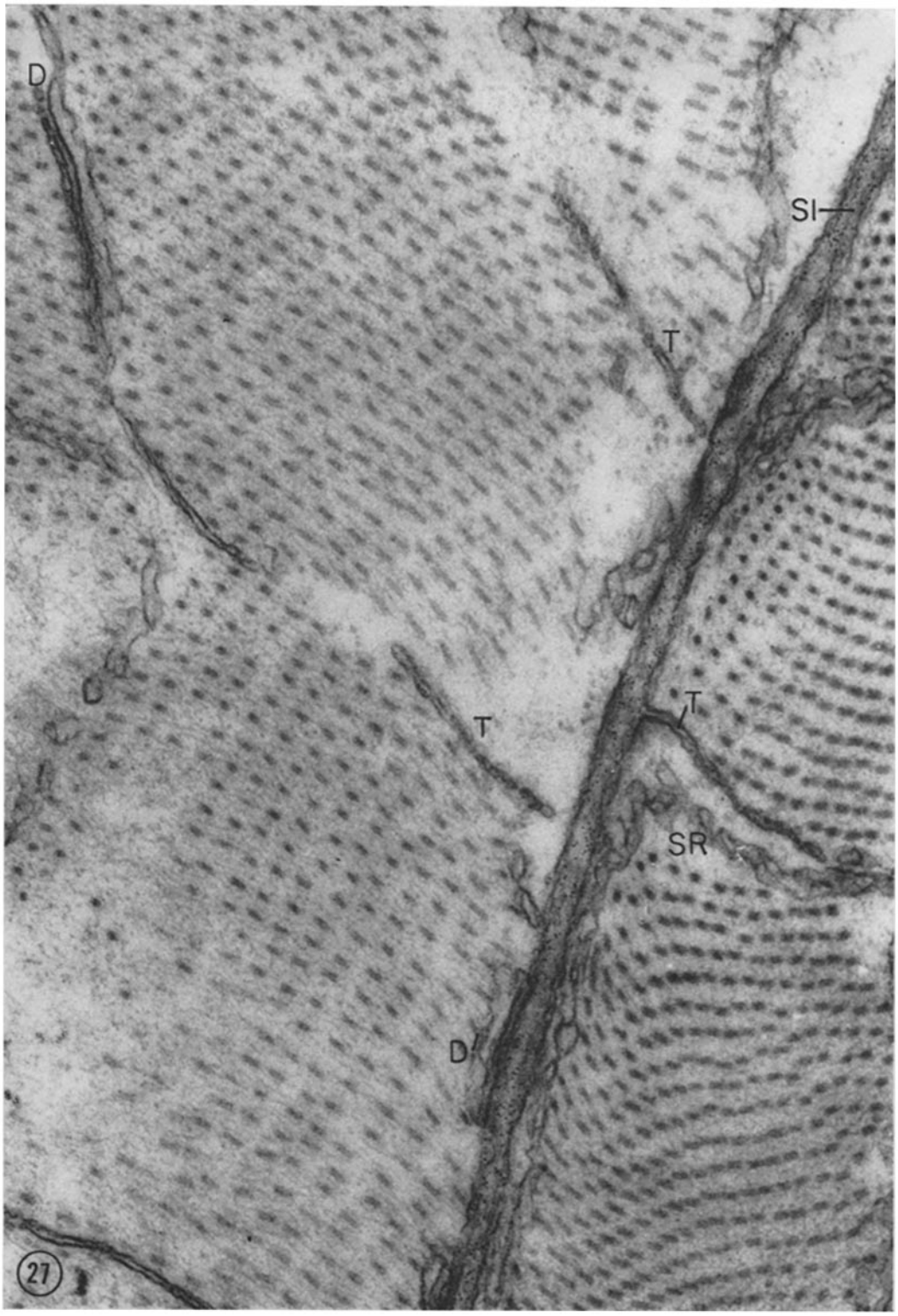


FIGURE 26 Several radial tubules (*RT*) leave the sarcolemma and curve inward where they contact the SR at the diads (*D*). Diadic junctions (*D'*) between sacculations of the SR and the plasma membrane are located beneath the sarcolemma. Because of the orientation of the SR along the fiber axis, the SR component of the diads appears in cross-section as isolated vesicles. The discontinuities in the radial tubules are due to the convolutions in and out of the section plane. iso K, OsO_4. × 20,000.

FIGURE 27 Ferritin diffuses from the bathing medium throughout the elements of the transverse tubular system (*SI*, *T*), but not into the SR or the general cytoplasm. The tubule which forms a diad (*D*) with the SR contains several ferritin particles. *D'* is a diad formed between the sarcolemmal invagination (*SI*), which is loaded with ferritin particles, and the SR. Control plus ferritin, OsO_4. × 60,000.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27

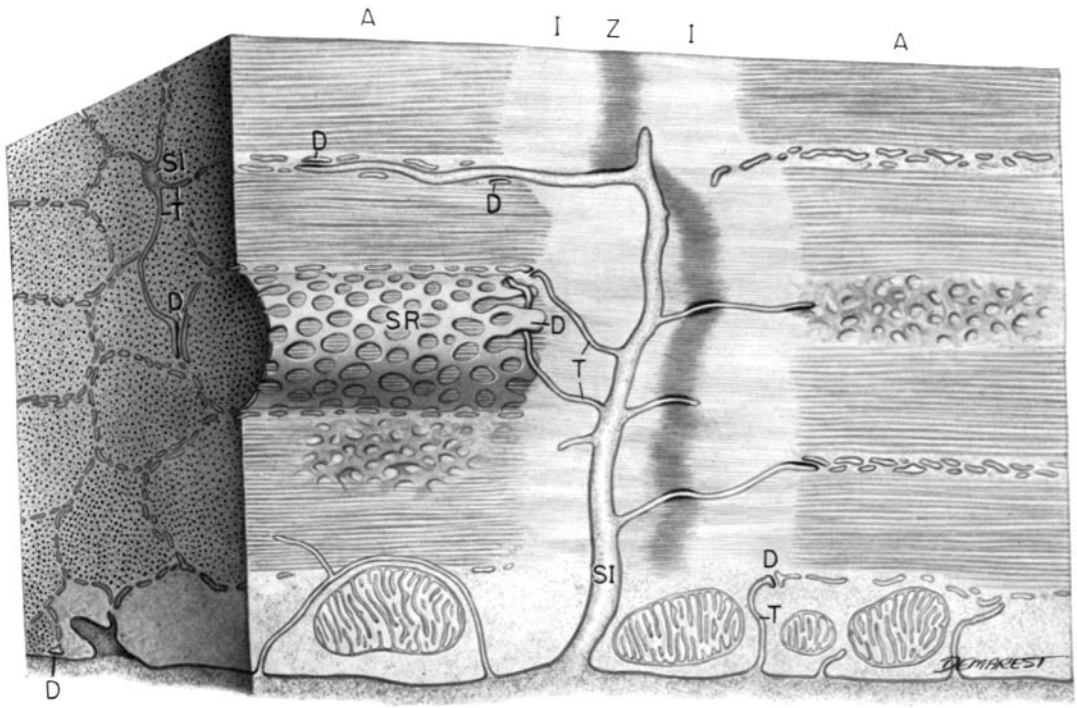

FIGURE 28 Composite representation of the fine structure of a crayfish muscle fiber. A fenestrated envelope of sarcoplasmic reticulum (*SR*) is shown surrounding one myofibril at the juncture of the longitudinal and cross-sectional views. Two types of invagination of the plasma membrane are distinguished by the presence of the sarcolemmal coat in the sarcolemmal invaginations and their large diameter (*SI*), and the absence of the sarcolemmal coat in the finer radial tubules (*T*). The sarcolemmal invaginations and their branches also give rise to many tubules. Where the invaginations or tubules penetrate the Z disc their membrane becomes denser. Diads (*D*) may be formed between the SR and the tubules, the sarcolemmal invaginations, or the plasma membrane.