CORRELATED MORPHOLOGICAL AND PHYSIOLOGICAL STUDIES ON ISOLATED SINGLE MUSCLE FIBERS

II. The Properties of the Crayfish Transverse Tubular System: Localization of the Sites of Reversible Swelling

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ABSTRACT

Living muscle fibers of crayfish become dark during efflux of Cl^-. This change in appearance is correlated with occurrence of vacuolation in the fixed fibers. The vacuoles begin at and are mainly confined to the terminals of the transverse tubular system (TTS) which are in diadic contact with the sarcoplasmic reticulum (SR). In electron micrographs swellings more than 1 μ in diameter may be seen connected to the sarcolemma or sarcolemmal invaginations by relatively unswollen tubules about 300–500 A wide. Darkening of the living fibers can be reversed by causing an influx of Cl^-. Vacuoles are then absent in the fixed preparations. These findings accord with the conclusion that the membrane of the TTS is anion permselective. Localization of the selectivity to the membrane of the terminals of the TTS strengthens the hypothesis that a channeling of current flow is responsible for initiation of excitation-contraction coupling. During the swelling, and upon its reversal, the area of the membrane of the terminals must change reversibly by about two to four orders of magnitude. The absence of changes in the dimensions of the unit membrane indicates that the expansion of the membrane and its subsequent shrinkage involve reversible incorporation of cytoplasmic material into the membrane phase.

INTRODUCTION

Crayfish muscle fibers become opaque and their striations become difficult to resolve when Cl^- is caused to flow out of the cells under the drive of either a concentration gradient or an electric field (8). In electron micrographs it was found that the opaque cells, contrasted with control fibers, have what appear to be numerous large vacuoles that could have caused the darkening of the living fibers by an increase in light scattering. The previous physiological and morphological studies (3, 8) showed, furthermore, that the vacuoles are formed by swelling of the transverse tubular system (TTS), particularly at the terminals of the tubules.

Vacuoles are produced by an inward current applied through an intracellular microelectrode filled with KCl. The vacuoles are localized to the region of the microelectrode, i.e. where the density of the current is highest and where the largest efflux of Cl^- occurs. When the microelectrode

contains the salt of an impermeant anion, no vacuoles develop. Thus, the vacuoles result only from an efflux of anion across the membrane of the TTS (8).

The present work, which also combines electron microscopic and physiological data, shows that the swellings induced by efflux of Cl^- begin at and are mainly confined to the diadic elements (3) of the TTS. These swellings disappear under experimental conditions that institute an influx of Cl^- and cause disappearance of the darkening in living muscle fibers. Thus, the new findings provide further evidence for the identification of the terminal portions of the TTS as the sites of anion-permselective membrane. Thereby they further support the "channeled current" hypothesis of excitation-contraction (e-c) coupling (8, 11, and data in preparation). The volume of the terminals can undergo very large changes on swelling or on reversal of the swelling. The area of the membrane of the TTS must change by several orders of magnitude. However, no changes could be detected in the thickness of the unit membrane, either in the distended state or upon reversal of the swellings of the TTS.

MATERIAL AND METHODS

All the animals were of the genus *Orconectes*, as were the specimens used in the previous studies (3, 8) although they were incorrectly called *Procambarus* in those studies. Single fibers were dissected from the flexor muscles of the meropodite of the walking legs. The fibers were equilibrated in a control saline prior to the application of experimental solutions. This saline was buffered with Tris at pH 7.6 and contained 5 KCl, 200 NaCl, 13.5 $CaCl_2$, all values given in millimoles per liter. A chloride-free control solution made with propionate salts had the same cationic composition as the control chloride saline. Isosmotic KCl-enriched or K propionate-enriched solutions were made by substituting K^+ for Na^+ in the respective Cl and propionate control salines as specified in the text and legends. Changes in the muscle fiber, living and during fixations, were observed and photographed in the light microscope.

Fixation for microscopy was carried out either by dripping cold Palade's osmium tetroxide fixative (10) directly onto the cell after the final bathing fluid had been removed, or by first prefixing the fiber by slowly adding glutaraldehyde to the saline until this fixative constituted 0.2% by volume of the solution.[1] After 10 min of prefixation, the fibers were stiff and slightly yellow in color. After the prefixation, the fibers were placed in Palade's fixative for about 1 hr. After removal from the latter fixative, fibers were dehydrated in a series of refrigerated ethanols (30, 50, 70, 95, and 100%), then they were transferred at room temperature to propylene oxide and to mixtures of this compound with Epon. The final Epon mixture contained five parts Epon 812, five parts dodecyl succinic anhydride, two parts Nadic Methyl Anhydride (Allied Chemical Corp., Plastics Div., Morristown, N.J.), 4% dibutyl phthalate, and 1.5% DMP-30 (Rohm & Haas Co., Philadelphia, Pa.). This embedding mixture was hardened at 60°C for 20 hr.

[1] This prefixation procedure was developed by Dr. Lucien Girardier, at the University of Geneva, Switzerland. (Personal communication.)

Effects of Fixation

The light microscopic observations of fibers in vivo and during fixation correlated well with the electron microscopic changes in the TTS. However, it should not be assumed that no significant fixation artifacts were introduced.

It is desirable that fixation be rapid and not accompanied by contraction, relaxation, or changes in volume or structure. No method tried to date fulfills all these criteria. Fixation in Palade's fixative is very rapid for single fibers and usually is not accompanied by excitatory effects or structural changes visible in the light microscope. In sections, however, a progressive shortening of the sarcomeres towards the core of the muscle fiber is observed and, suggests that some degree of contraction had occurred. Connections of the tubules of the TTS with the sarcolemma or with sarcolemmal invaginations are difficult to trace, since the tubules are usually coiled near their origins. Fixation techniques which increase the diameter of the tubules or minimize contractile activity greatly increase the frequency with which tubules are found to be in open continuity with the extracellular space. When control fibers are presoaked in solutions containing procaine (1 mg/ml) or in high concentrations of potassium which render them less excitable, no selective shortening of the central sarcomeres occurs, and the tubules, although small in diameter (120–200 A, Fig. 3), are frequently in continuity with the sarcolemma or its invaginations (see Figs. 18–20 in reference 3).

Fixation in 2% or higher concentrations of glutaraldehyde causes the fibers to contract and swell (3). Glutaraldehyde in concentrations lower than about 0.05% does not fix the fibers, whereas in a concentration of about 0.2% it fixes fibers in less that 30 min with no sign of contraction. The membrane potential in control saline plus 0.2% glutaraldehyde falls slowly from −80 to about −55 mv during a period of 30 min. Increasing the K^+ concentration during this period results in a further, partially reversible depolarization and a small contraction. After 30 min in the

glutaraldehyde solution the membrane potential stabilizes at about −55 mv, but it can be reversibly reduced by increasing the K^+ concentration in the medium. However, tension no longer accompanies the K^+ depolarization.

Preliminary light diffraction studies indicate that the sarcomeres lengthen slightly during this fixation. In most respects, fibers fixed in 0.2% glutaraldehyde and postfixed in osmium tetroxide resemble procaine-treated or potassium-depolarized fibers fixed by direct immersion in osmium tetroxide. In some respects, therefore, fixation in low concentrations of glutaraldehyde appears to be the method of choice, but fixation must be considered to be very slow since the fibers remain excitable for some time.

The tubules of the TTS can range in diameter from about 120 A to as much as 500 A (in high concentrations of glutaraldehyde) under conditions of no experimental variation except the fixation. The swelling and vacuolation of the TTS, which correlate with the optical changes induced by a Cl^- efflux, are several orders larger and are beyond any effects of the fixatives. During fixation in the low concentration of glutaraldehyde or in osmium tetroxide, none of the optical changes that are correlated with swelling of the TTS during chloride efflux are observed (Fig. 1 *g*).

RESULTS

In Vivo Observations

Properties of the TTS are demonstrated in vivo by the experimental conditions under which the fiber changes its optical properties. The changes in a single fiber sequentially exposed to six different salines are recorded in Fig. 1. Light and electron micrographs of fixed fibers treated in a parallel manner prior to fixation are shown in Figs. 2–9. The light micrographs of Fig. 1 illustrate that optical changes are induced in the muscle fiber by a redistribution of chloride ions. Initially (Fig. 1 *a*), the fiber was equilibrated in the control saline. It was then exposed for 23 min (Fig. 1 *b*) to an isosmotic saline in which 100 mmoles/liter KCl replaced an equivalent amount of NaCl. The swelling which results from the entry of water and KCl (12) leads to increased transparency of the fiber (15). Upon returning to the control saline and thus undergoing an efflux of KCl, the fiber became opaque and began to shrink (Fig. 1 *c*). It became still darker (Fig. 1 *d*) when the Cl^- of the saline was replaced by propionate, a change that enhances the efflux of Cl^- (5, 8, 12). Elevating the K^+ in the propionate saline to 100 mmoles/liter isosmotically did not abolish the darkening (Fig. 1 *e*) or cause swelling of the fiber (12). However, a rapid increase in transparency and swelling (Fig. 1 *f*) occurred upon substitution of an isosmotic chloride saline containing 100 mmoles/liter KCl. The same fiber is shown in (Fig. 1 *g*) after fixation for 6 min in 0.2% glutaraldehyde dissolved in the bathing saline. Although there was a slight yellowing and hence a darkening of the photographed, fixed fiber (Fig. 1 *g*), this coloration is readily distinguished from the in vivo darkening of the fiber (Fig. 1 *c–e*).

Observations on Fixed Material

Two different fibers are shown in cross-section in the light micrographs in Fig. 2. The fiber in Fig. 2 *a* was dark, and before fixation it was similar in appearance to the fiber shown in Fig. 1 *d*. The fiber in Fig. 2 *b* had been dark but subsequently became transparent upon immersion in an isosmotic KCl (100 mmoles/liter K) saline prior to fixation (see Fig. 1 *f*). The fiber in Fig. 2 *a* is peppered with small clear vacuoles, whereas the fiber in Fig. 2 *b* is free of these vacuoles. In Fig. 2 *b* the large dark areas are the regions of the A bands, and the lighter areas are the I bands which are traversed by Z bands. Even at low magnification, a pattern of distribution of the vacuoles found in dark fibers (Fig. 2 *a*) is apparent. The vacuoles are least numerous in areas that are occupied exclusively by the A bands. The TTS of the crayfish fiber is most abundant near the A–I band junctions and is almost absent from the A band, whereas the converse is true of the sarcoplasmic reticulum (SR) (3).

The periphery of the fiber is relatively free of vacuoles (Fig. 2 *a*). It is also relatively free of myofibrils and is occupied mainly by mitochondria (3, 8; also see Fig. 5). Where the A bands and SR do approach the fiber surface, the SR makes diadic contacts with the sarcolemmal membrane (3). These membrane sites presumably take on the anion-permselective properties of the TTS. No gross morphological change such as the swelling of the TTS could occur during the efflux of Cl^- at these sites. The radial tubules (*RT*, Fig. 5) which penetrate through the periphery of the fiber to make diadic contacts with the SR do undergo swelling. However, their number per unit of fiber surface is small compared with the number of tubules that proliferate from the sarcolemmal invaginations (3).

As has already been described (3), the major

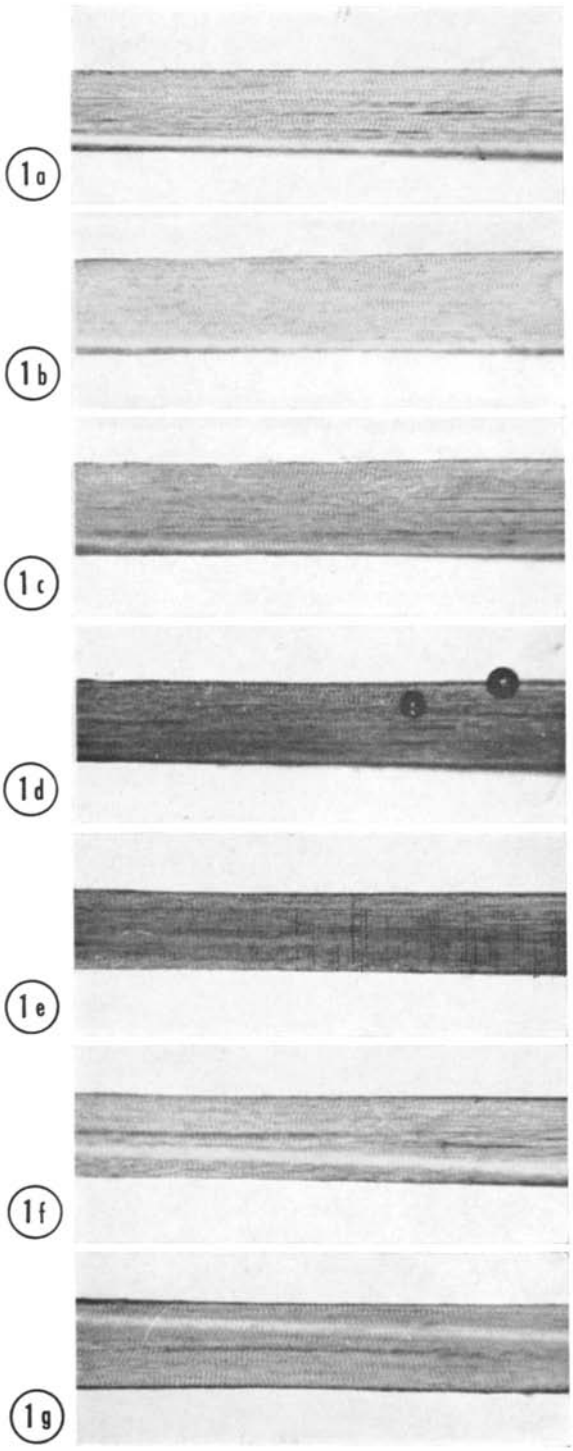

FIGURE 1 Changes in optical appearance of a single fiber under various experimental conditions. *a–f*, living fiber; *g*, after fixation. *a*, in control saline the striations are readily seen. *b*, after 23 min exposure to isosmotic saline containing 100 mmoles/liter KCl. The fiber swells and becomes somewhat more transparent. *c*, 2 min after return to control saline. Increased opacity accompanies shrinkage. *d*, after 6 min in a control propionate saline the darkening is very marked. *e*, after exposure for 10 min to isosmotic propionate saline enriched with 100 meq/liter K^+ The fiber did not swell (*b*), and it remained darkened. *f*, 10 min after return to isosmotic chloride saline containing 100 mmoles/liter KCl (as in *b*). The increased transparency is accompanied by some swelling. *g*, after 6 min fixation in 0.2% glutaraldehyde added to the KCl-enriched medium of *f*. ×65

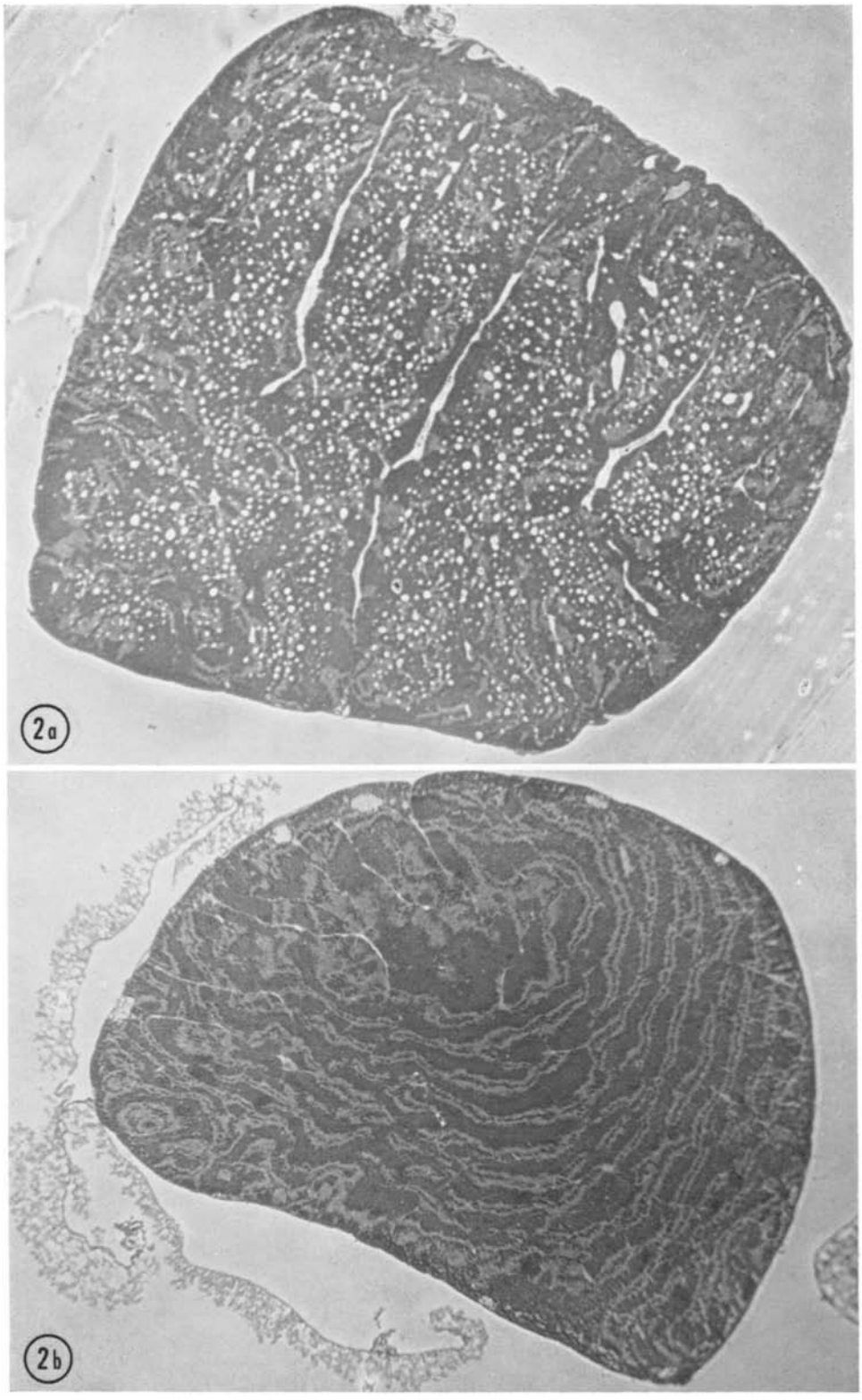

FIGURE 2 Low power light micrographs of cross-sections of two muscle fibers. *a*, in the opaque state; *b*, after reversal of the opacity. *a*, The fiber had been exposed to 30 mmoles/liter KCl (isosmotic) and was fixed 5 min after return to the control saline when it was opaque (as in Fig. 1 *c*). Note the numerous vacuoles. See further description in text. *b*, This fiber had been exposed first to 50 mmoles/liter KCl (isosmotic), then returned to the standard saline in which it had become markedly opaque, but the opacity was reversed by reexposure to 50 mmoles/liter KCl (as in Fig. 1 *f*). Both fibers were irregular in shape in the living condition, and these shapes were maintained despite the prior swelling in *a* or during the swollen state in *b*. *a*, × 600; *b*, × 400.

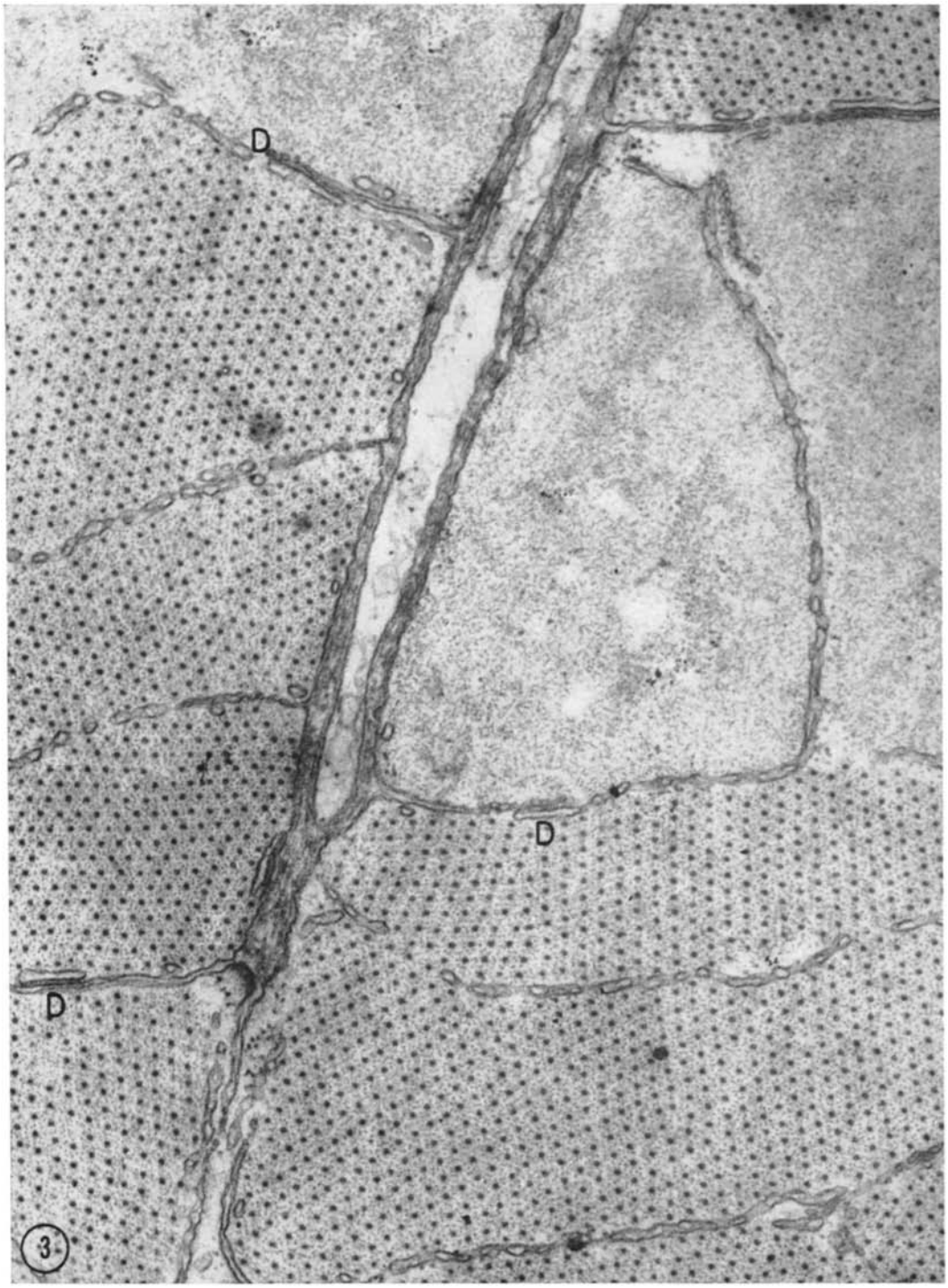

FIGURE 3 Electron micrograph of muscle fiber prefixed with 0.2% glutaraldehyde in control saline for 10 min. The cross-section includes myofibrils near the A-I junctions in which both thick and thin myofilaments are present, as well as some regions of transition to I bands in which there are only a few thick myofilaments. In the middle of the figure is a sarcolemmal invagination that is identified by the inclusion of the characteristic fibrous inner coat. A number of tubules arise from the invagination and run along the borders of the myofibrils where they make diadic contacts (*D*) with the sarcoplasmic reticulum. × 40,000.

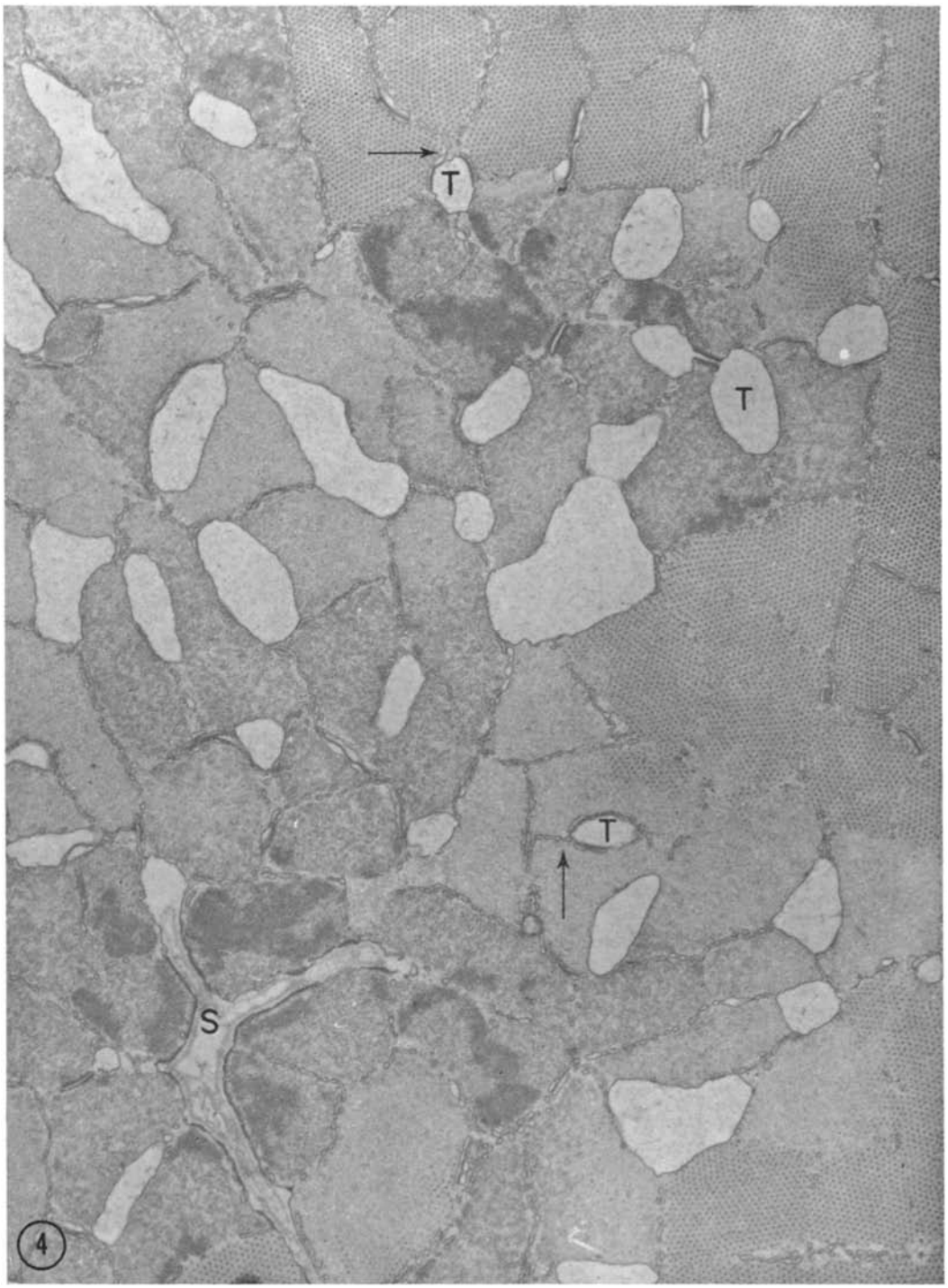

FIGURE 4 Electron micrograph of a fiber darkened upon return from 30 mmoles/liter KCl to control saline. The numerous vacuoles are in the I bands and the A-I junctions. The sarcolemmal invagination (*S*) is slightly swollen, but the tubules (*T*) are considerably swollen. The arrows indicate two places where the sarcoplasmic reticulum makes diadic contact with swollen tubules. The sarcoplasmic reticulum is not swollen. The labeled tubule on the upper right is connected to another swollen section by a tubule that passes through the Z band and has a characteristic membrane density. Minimally swollen tubular components of diads are seen in the upper right. × 15,000.

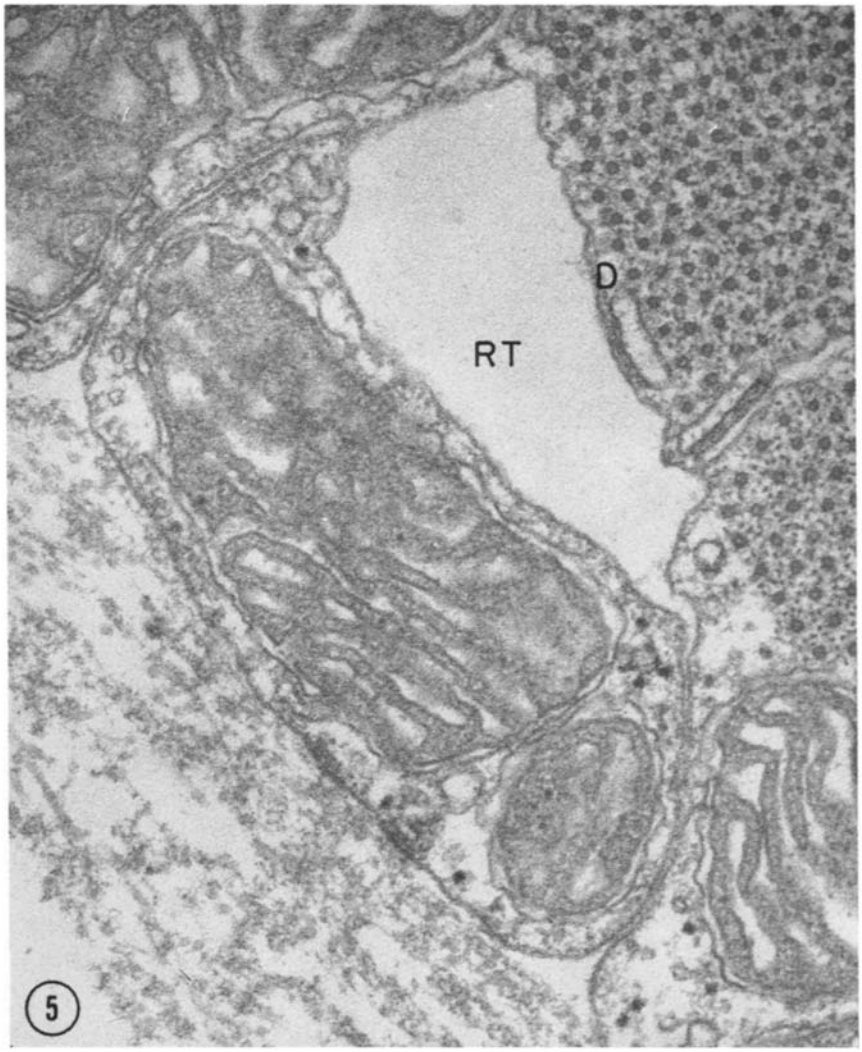

FIGURE 5 Radial tubule (*RT*) arising from the fiber surface. The fiber was exposed to KCl (100 mmoles/liter) for 15 min, then returned to the control saline for 20 min before prefixation in 0.2% glutaraldehyde. After fixation in osmium tetroxide the fiber was washed for 10 min and stained in half-saturated uranyl acetate. The unit membrane can be traced from the fiber surface into the swollen portion. The latter makes diadic contact (*D*) with the sarcoplasmic reticulum. × 54,000.

part of the TTS of crayfish fibers consists of sarcolemmal invaginations and their associated tubules (Fig. 3). The tubules run to the SR and form diadic contacts with it approximately at the junctions of the A-I bands. The invaginations are distinguished from the tubules by the presence of a fibrous inner coat which is continuous with the basement membrane-like material overlying the fiber surface.

Darkened fibers can be identified in low magnification electron micrographs by the presence of numerous swollen tubules, as seen in Figs. 4–6. Within many of the swellings are strands of the fibrous coat which identify the sarcolemmal invaginations (*S*, Fig. 4). In several places in Figs. 4–6, enlarged tubular structures are continuous with the sarcolemmal invaginations and make diadic contact with the SR. In areas where the

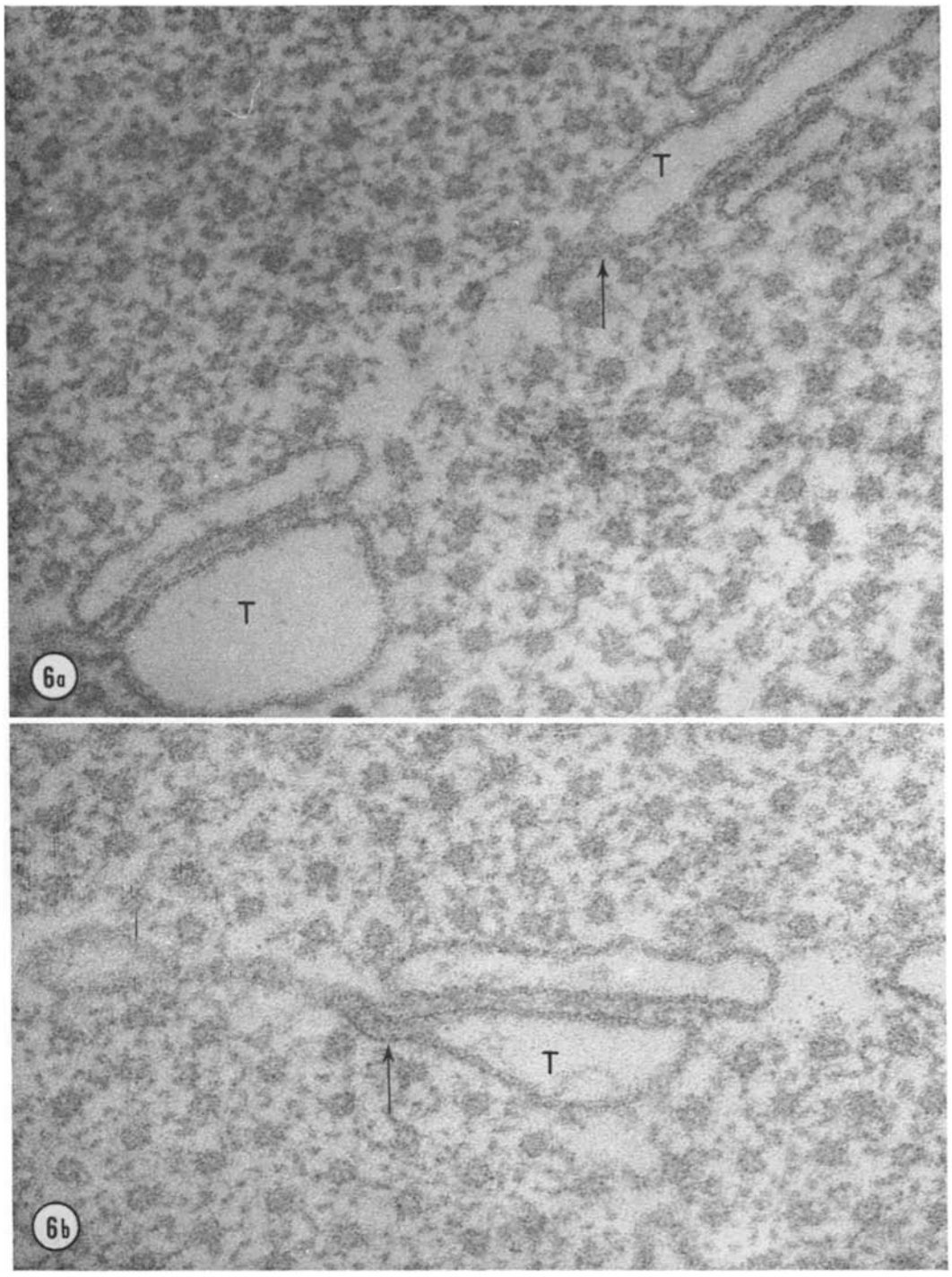

FIGURE 6 *a* and *b* Higher magnification electron micrographs showing unit membrane structure and diadic contacts. The slightly swollen diadic components of the TTS (*T*) are connected with unswollen portions of the tubules at the arrows. Same fiber as in Fig. 5. × 250,000.

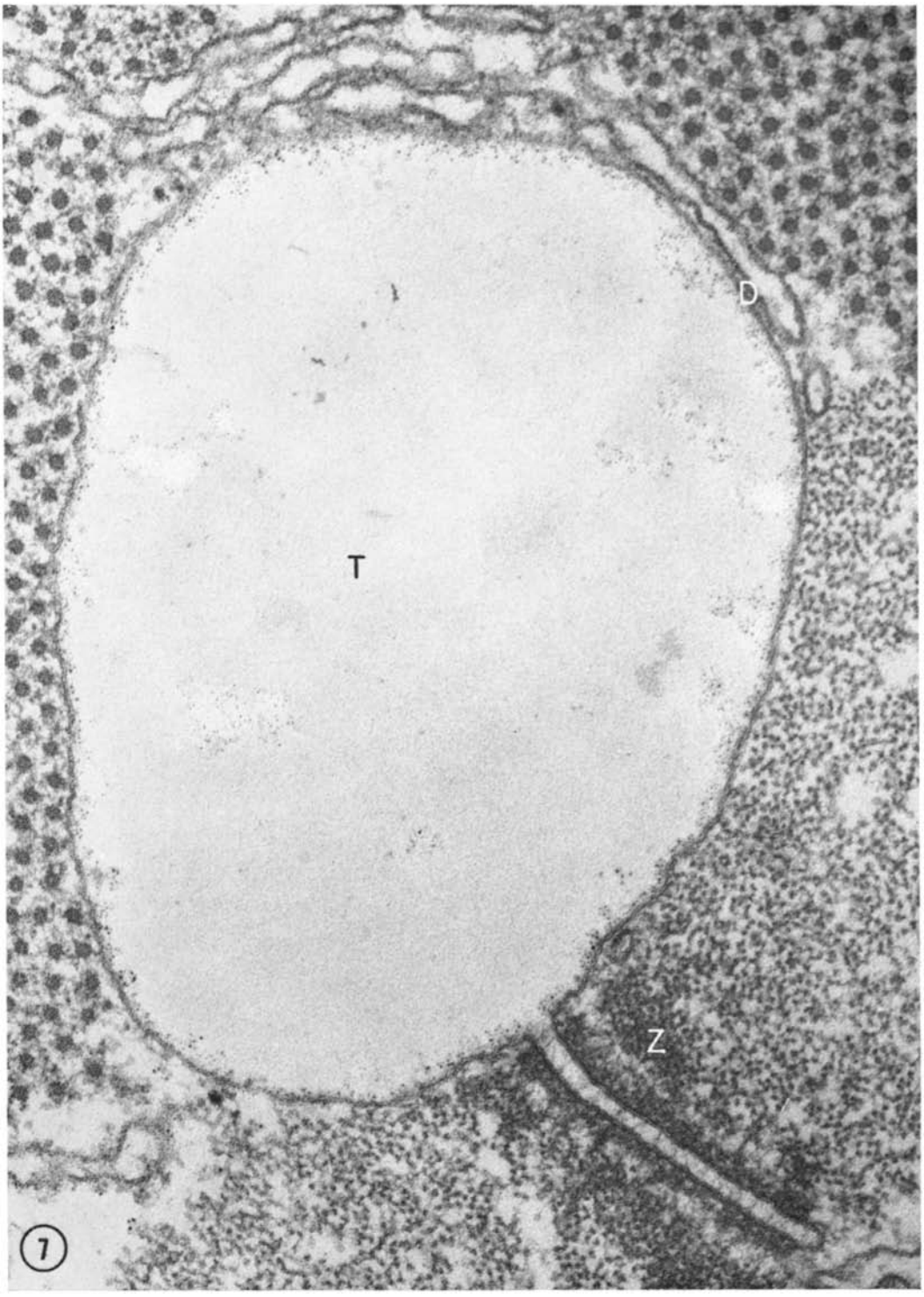

FIGURE 7 Greatly swollen diadic terminal (*T*) connected with an unswollen portion of the tubule which penetrates through the Z band (*Z*). The unit membrane of the swollen diadic vesicle remains intact despite the extension of the surface area by several orders of magnitude. The unswollen sarcoplasmic reticulum makes a diadic contact (*D*) with the swollen tubule. Same fiber as in Fig. 5. × 95,000.

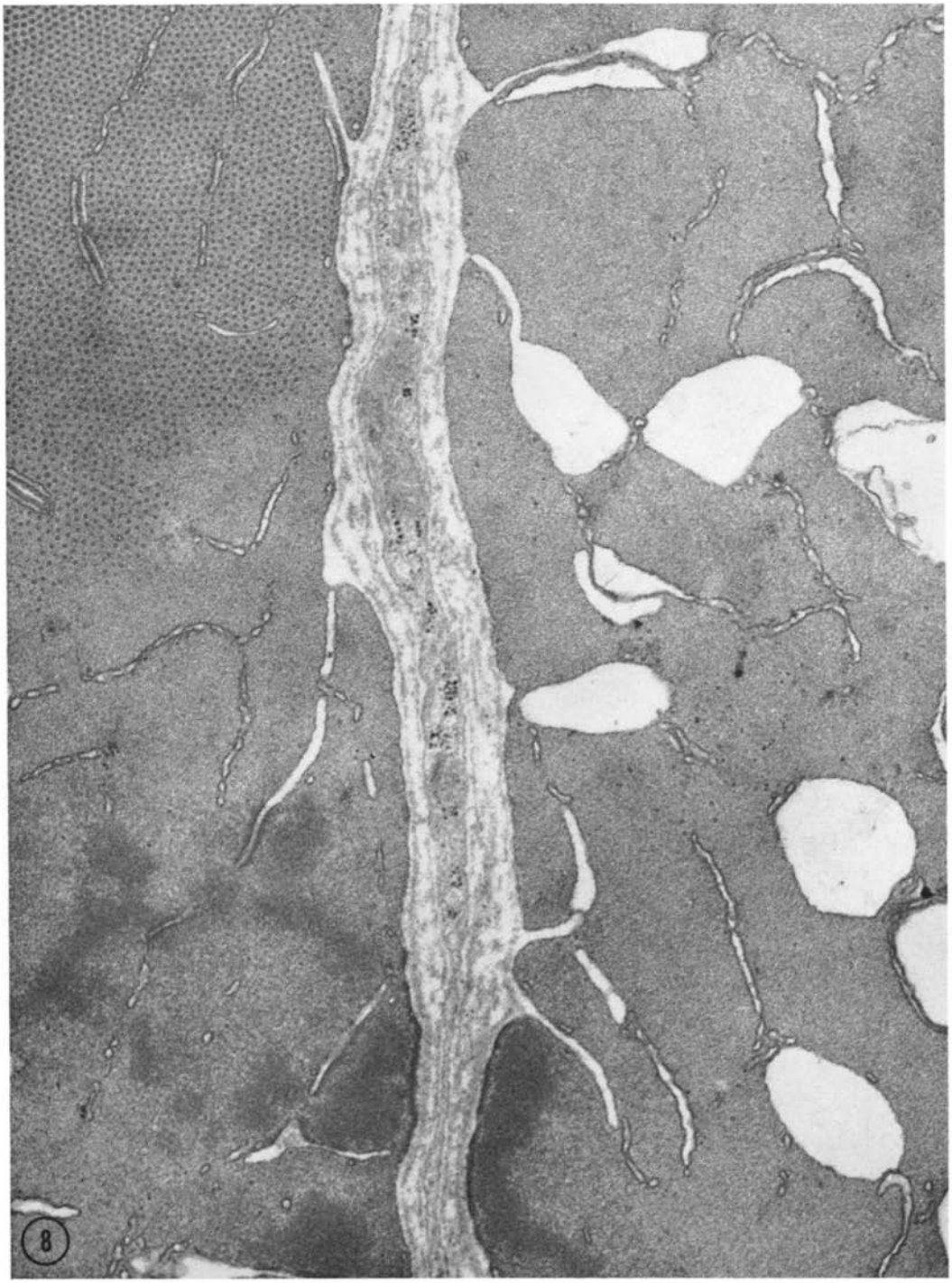

FIGURE 8 Low-power electron micrograph of a darkened fiber that was fixed in an isosmotic propionate saline containing 170 mmoles/liter K. Numerous tubules arise from the sarcolemmal invagination. The greatest swelling occurs in the terminations of the tubules which are the sites of diadic contacts with the SR. Numerous large vacuoles are also seen which represent swollen diadic components cut in a plane that does not include the connecting tubules. Note that the SR remains unswollen. Note also the increased density of the membrane where the sarcolemmal invagination passes through the Z band region. × 40,000.

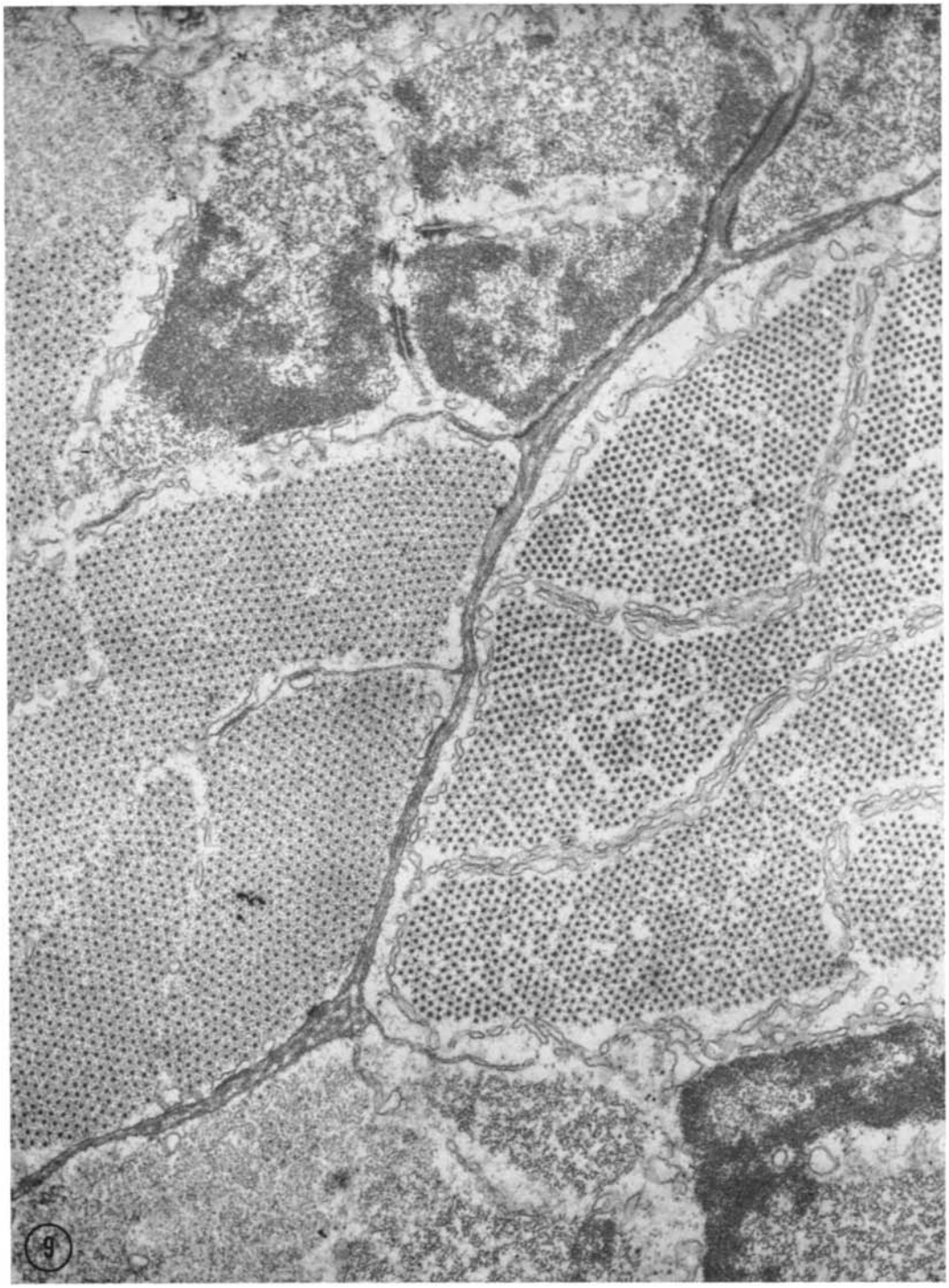

FIGURE 9 Cross-section of a fiber that had been initially treated like the fiber of Fig. 8 but was then reversed from the darkened state by reexposure to 100 mmoles/liter KCl. No vacuoles are to be seen. The uptake of water during exposure to the KCl-enriched isosmotic saline caused a widening of the spaces between myofibrils and myofilaments and some swelling of the SR, but the tubules and the diadic components of the TTS are not swollen. × 27,000.

tubular component of the diads is not connected to the invaginations, owing to the plane of the section, only one component of the diad is swollen (Fig. 4, arrows). The other component of the diad is frequently identifiable by its continuity with the SR (see also Fig. 7). Some of the vacuoles are connected to dense-walled tubules which have been identified (3) as TTS tubules that penetrate the Z band (Figs. 4 and 7). In addition to the swollen segments of the invaginations and tubules, there are a number of membrane-bounded vacuoles which can not be positively identified; but none of them are located in areas inconsistent with the assumption that these vacuoles are elements of the TTS.

Under conditions in which the in vivo darkening of the fibers is minimal at fixation, or in areas of dark fibers where the swelling of the TTS is small, the swelling is highly localized to the portion of the tubules in diadic contact with the SR. The electron micrographs shown in Figs. 5–7 were selected to demonstrate the connection of unswollen nondiadic tubule segments to swollen diadic segments. The vacuole in Fig. 7 is large in comparison to the vacuoles in Fig. 6 *a* and *b* and is connected to a tubule passing through the Z band. In all four examples (Figs. 5–7), however, the unit membrane bounding the swollen tubular segments is not notably different in dimension than the membrane bounding the unswollen tubules.

The fibers in Figs. 8 and 9 were exposed to a sequence of solutions which induced a darkening, as in Fig. 1 *c* and *d*, but prior to fixation the fiber in Fig. 8 was exposed to 170 mmoles/liter K propionate whereas the fiber in Fig. 9 was exposed to 100 mmoles/liter KCl. The fiber in Fig. 8 remained opaque in spite of its exposure to high potassium (see Fig. 1 *e*), whereas the fiber in Fig. 9 (see Fig. 1 *f*) became transparent rapidly, although exposed to a lower concentration of potassium but in a chloride saline. It is obvious that the TTS is swollen in Fig. 8, in contrast to that in Fig. 9 in which the tubules and sarcolemmal invaginations are of about the same diameter as the control TTS seen in Fig. 3.

DISCUSSION

The present work demonstrates that it is the tubular component of the TTS which swells when the muscle fiber is losing Cl^- (Figs. 4–7) and shrinks when the fiber is gaining Cl^- (Fig. 9). The SR does not participate in the swelling, although it can be made to swell by other methods (Unpublished data.). The swelling of the TTS is initiated and becomes largest at the terminal portions of the tubules (Figs. 5–7). The adjacent segments of the tubules and perhaps also the invagination from which the tubules originate dilate secondarily and to a lesser degree (Fig. 4). Not all the diadic portions of the tubules are swollen to the same degree (Fig. 4). At present the properties that are responsible for this variation are not understood.

The darkening of the living muscle fibers (Fig. 1 *c–e*), the vacuolation (Fig. 2 *a*), and the swelling of the TTS (Figs. 4–8) are all induced by an efflux of KCl, independent of the membrane potential (8). The decisive factor in causing the fiber to darken and the TTS to swell is an efflux of Cl^-, since the changes are induced by an intracellularly applied current which carries K^+ *inward* and Cl^- *outward* across the cell membrane. The changes are not induced when the current is flowing in the opposite direction or when the intracellular electrode contains an impermeant anion and the inward current is then carried only by K^+ (8).

The swelling of the TTS during the outward passage of Cl^- is not dependent upon the absolute level of intracellular Cl^- and is accounted for by the obligatory redistribution of water (8). The present data support this explanation in that reexposure of a darkened fiber to KCl results in an influx of Cl^-. The fibers become lighter in appearance (Fig. 1 *f*) and lose their vacuoles (Fig. 2 *b*), and the swelling of the TTS is no longer evident (Fig. 9). The presence of an inward driving force for Cl^- is obligatory for these changes. The changes do not occur when the fiber is exposed to a high concentration of K^+ but in the presence of an impermeant anion (Figs. 1 *e* and 8).

The experimental conditions under which all these changes are induced have provided physiological evidence that the terminal portions of the TTS are the sites of specialized anion-permselective membrane. On the other hand, the membrane on the surface of the fiber is largely cation permselective (12). The conclusion that the cell membrane consitutes an electrochemically heterogeneous system led to the suggestion of the channeled current hypothesis for excitation-contraction coupling (8). The present evidence localizing the anion-permselective membrane at the diadic por-

tions of the TTS and our further studies on e–c coupling in crayfish muscle fibers (11; Data in preparation.) provide strong support for this hypothesis.

The efflux of Cl^- causes similar morphological changes in muscle fibers of crab (14; Data in preparation.) and insect (4). Thus, it is likely that their membranes are heterogeneous and that the channeled current mechanism is applicable to e–c coupling in other arthropods (2, 13, 16). While the properties of the transverse tubules of frog muscle are not well defined (6, 7), these tubules also appear to have different permselectivity than the surface membrane (1). Thus, it is likely that the channeled current mechanism may also apply to vertebrate muscle (8).

According to this hypothesis, anionic currents are directed across the diadic portions of the tubules where these diadic components make contact with the SR at the A-I junctions. Thus, ionic changes are effected at critical sites of each sarcomere segment of each myofibril. The channeling of the current through the TTS by virtue of the special ionic permselectively of the diadic component offers a model for e–c coupling that differs from the model of Huxley and Taylor (9) in which the tubules mediate passive electrotonic spread of potential into the depths of the fiber.

It is apparent in the electron micrographs that the volume of the terminal portions of the tubules can change by several orders of magnitude when they swell (Figs. 3–8) and when the swelling is reversed (Fig. 9). The surface area of the tubules must therefore change proportionately, and thus causes expansion and contraction of the membrane. It is generally assumed that an increase in the surface area of cells reflects an unfolding of the cell membrane by a smoothing out of irregularities in the surface and perhaps also by conformational changes of macromolecules. In the swollen TTS, however, the surface area of the membrane of the terminals must have been expanded by as much as two to four orders. There are no conspicuous irregularities in the outlines of the unswollen tubules that might provide the slack required during the distension (Fig. 3), nor is there any observable thinning of the unit membrane (Figs. 5–7).[2] Once swollen, the tubules can maintain their volume indefinitely (Fig. 1 *c–e*), unless a restorative Cl^- influx (Fig. 1 *f*) is initiated. Thus, the distended tubular membrane appears to be a plastic element with little or no elastic properties. When the swelling is reversed (Fig. 9), the membrane exhibits its original smooth outline and shows no sign of the very considerable distension that it had undergone during the swelling. Thus, the tubular membrane behaves as a highly labile and reversibly extensible interface.

These findings seem to imply that the interfacial film which constitutes the cell membrane is capable of rapidly and reversibly incorporating intracellular matter to maintain the continuity of the cell surface and the integrity of the cell. At the present time, this behavior can only be noted as a formidable challenge for theories of membrane structure and formation, and it provides new methods for studying the problem.

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swollen, and upon reversal from swelling), it seems unlikely that the dimensional constancy is due to fixation artifacts. However, we cannot at present evaluate more precisely the possible role of such artifacts.