STUDIES OF THE TRIAD

II. Penetration of Tracers into the Junctional Gap

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ABSTRACT

Ferritin and Imferon molecules were introduced as tracers inside "skinned" muscle fibers to test which part of the triadic junction gap is freely exchangeable with the sarcoplasm. At least 50% of the T-system surface is freely accessible from the sarcoplasm. Of the remainder, 30% of the total T-system surface is covered by the junctional feet, and 20% in the center of the junction may or may not be accessible. The possibility is discussed that the triadic junction may not function as an electrical coupling.

INTRODUCTION

In frog twitch fibers the transverse T-system is composed of flattened tubules of oval cross-section. The short axis of the oval is parallel to the fiber axis and the flat faces of the tubule are closely apposed to two cisternae of the sarcoplasmic reticulum (SR), the lateral sacs of the triad (Page, 1965; Peachey, 1965; Eisenberg and Eisenberg, 1968).

SR and T-system membrane are separated by an approximately 120 A junctional gap. Each lateral sac of the triad is attached to the T-system by two rows of discrete junctional spots or "feet." Each foot is composed of a projection of the SR membrane towards the T-system in the form of a hemispherical scallop and of a small lump of amorphous material which crosses the remainder of the junctional gap (Franzini-Armstrong, 1970 *a*). The two rows of junctional feet running along each T-system flattened face are parallel to one another and are separated by a distance of approximately 200 A. The T-system surface is thus divided into three areas: (*a*) The portion of surface which lies in the center of the junction, between the two rows of feet. This is approximately 20% of the total T-system area. (*b*) The area covered by the rows of feet, which is about 30% of the total surface (Franzini-Armstrong, 1970 *a*). (*c*) The remainder of the T-system surface, which faces in part towards the lateral sacs of the triad and in part towards the fibrils.

In conventionally treated sections, no visible substance fills the junctional gap aside from the amorphous material forming the feet, so that it may be presumed that areas a and c of the T-system are freely exposed to the sarcoplasm (Franzini-Armstrong, 1970 *a*). To test this hypothesis, electron-opaque tracers were introduced inside the fiber to see if large molecules can indeed penetrate these portions of the junctional gap. The results show that area c of the T-system is freely accessible and that area a may also be accessible. It is doubtful that ferritin molecules can penetrate within the feet (area b). Thus no "adhesive material" or "cementing substance" of the type postulated by Walker and Schrodt (1965) and Kelly (1969), respectively, fills the junctional gap.

MATERIALS AND METHODS

Experimental Procedure

Sartorius muscles from *Rana pipiens* were immersed in a relaxing solution (Endo, 1967) and single fibers were then skinned, that is, deprived of the sarcolemma over a short portion of their length, essentially following the Natori (1954) procedure. Usually, a superficial layer of fibrils was also ripped off in the procedure.

The relaxing solution was then blotted away from the surface of the fiber, and the skinned portion was covered with a few drops of one of the tracer-containing solutions (see below) for 15 min. The marker solution was then removed and the fibers were fixed in 2% glutaraldehyde, rinsed in buffer, and postfixed in 1% OsO_4 . 0.1 M cacodylate buffer, pH 7.0–7.4, was used in all solutions. Each step was of 30 min duration. The fibers were quickly (in 25–30 min) dehydrated in alcohol and propylene oxide and embedded in Araldite. The sections were cut on a Cambridge (A. F. Huxley pattern) microtome, stained in lead citrate (Venable and Coggeshall, 1965), and examined in an AEI 6B microscope.

Commercially available solutions of cadmium-free horse spleen ferritin (Nutritional Biochemicals Corporation, Cleveland, Ohio) and Imferon (Lakeside Laboratories, Milwaukee, Wisc.) were prepared for intracellular application by sedimenting three times at 15,000 *g* and 50,000 *g*, respectively. The tracers were resuspended in the relaxing solution. Even following this procedure, ferritin-containing solutions tended to produce contractures of the skinned regions of the fiber. The composition of the original ferritin solution is not known, but it is possible that it contains sufficient calcium to produce this effect even when diluted.

Choice of Marker

Of the currently used ions and molecules which are directly or indirectly visible in the electron microscope (tracers), ferritin and Imferon were the final choice, because even single molecules can be unequivocally located in the section. Horseradish peroxidase was not used because the diffuse density resulting from its presence might not be distinguishable from the density due to the amorphous material forming the feet. Lanthanum tends to precipitate in the relaxing solution, and colloidal iron gels with both the relaxing solution and the sarcoplasm.

The over-all diameter of the ferritin molecule (approximately 90–100 A, Richter, 1957) is not much smaller than the space available in the junctional gap. For this reason, Imferon was also used because it is smaller (mol wt 180,000; Ricketts et al., 1965) and it was thought that it might more easily penetrate into the junction. However, the exact diameter of the molecule is not known, since only the dense iron core, 15–20 A in diameter, is visible in the electron microscope, and the dextran shell cannot be detected (Richter, 1959; Muir, 1960).

RESULTS

The Triads in Skinned Fibers

It is interesting to notice that T-system tubules are present in the triads of the skinned region of the fiber, even when near the periphery (Figs. 1 and 2). This indicates that the tubules break very close to the point where the sarcolemma and the peripheral layer of fibrils are stripped off during the skinning procedure. Also, few tracer molecules are enclosed in the T-system near the skinned region. Presumably these penetrate into the T-system either through temporary interruptions or from nearby intact openings at the sarcolemma.

Except at the periphery of the fiber, where the T-system is interrupted, the structure of the triadic junction in the skinned region of the fiber is essentially intact (Costantin et al., 1965), i.e. the distance separating SR and T-system elements is, in most triads, the same as in the intact fiber. The shape of SR and T-system, on the other hand, is slightly distorted in most triads. This is attributable to a shift out of register of the Z lines of adjacent fibrils, due to unsynchronized contractions, particularly those induced by the ferritin solutions (Figs. 3, 5, and 6).

In order to identify single tracer molecules it was found necessary to stain the sections weakly, with lead only. Unfortunately, this results in scarce visibility of the amorphous material forming the feet, and identification of intact triads must rely on the assumption that, where SR and T-system membranes run parallel and at an approximate distance of slightly over 100 A, the feet are still present to hold the junction together. Where SR and T-system separation is wider and variable, it is likely that some of the feet have been damaged.

The dense content of the lateral sacs of the triad in these preparations is often in the form of small granules, not dissimilar in size from the tracer molecules. However, these SR granules are usually less dense than ferritin (Figs. 3–9) and they are also present in the skinned regions of fibers not treated with a tracer-containing solution. Thus in most cases the penetration of the SR by ferritin can be excluded.

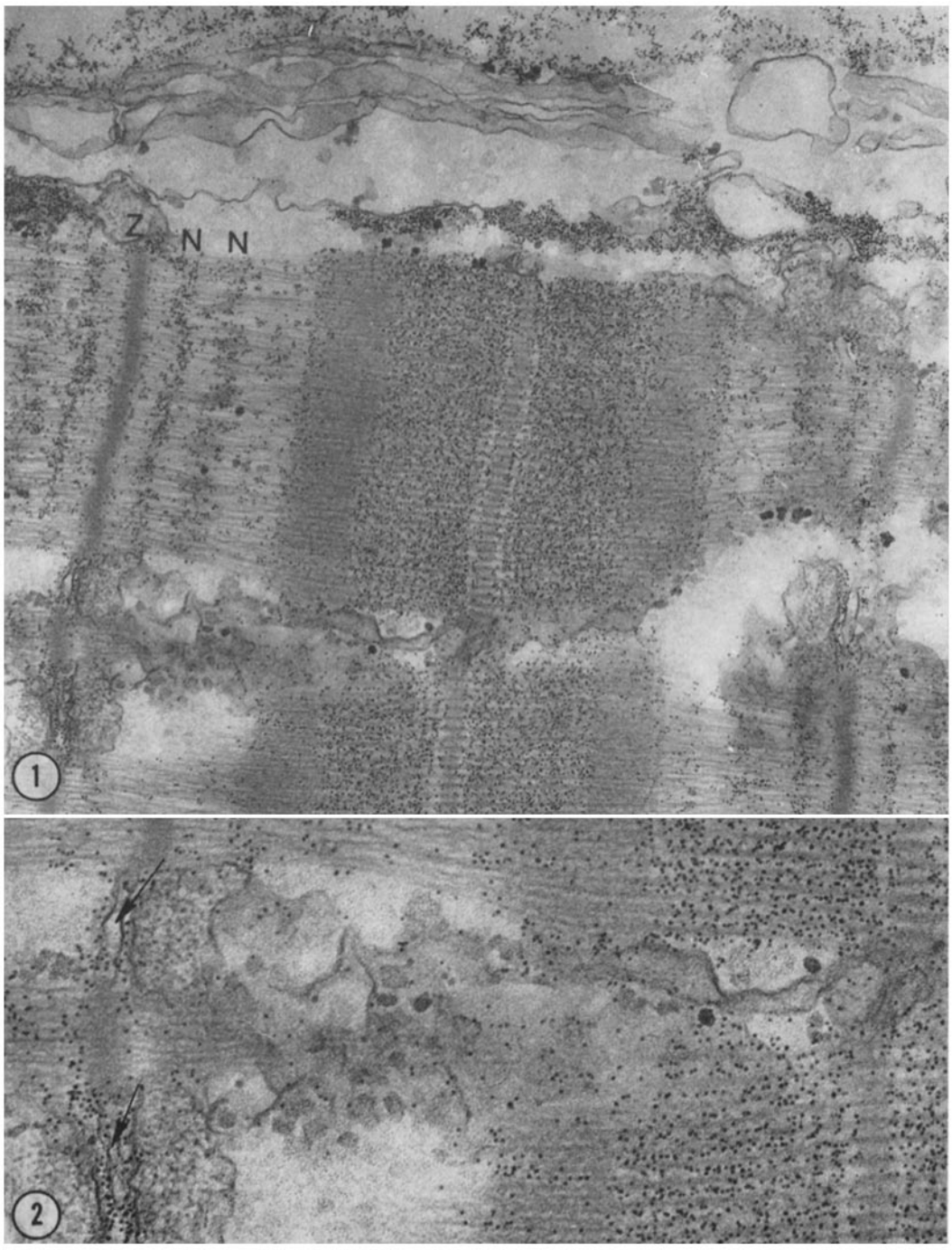


FIGURE 1 Periphery of a skinned fiber infiltrated with ferritin. At top are remnants of the SR and the outside. Ferritin granules are within the fibrils and near the SR. *N* and *Z* indicate the corresponding lines in the sarcomere. × 45,000.

FIGURE 2 Detail of a portion of Fig. 1, showing preferred location of ferritin within fibril and at the triadic junction. Arrow, T-system. × 75,000.

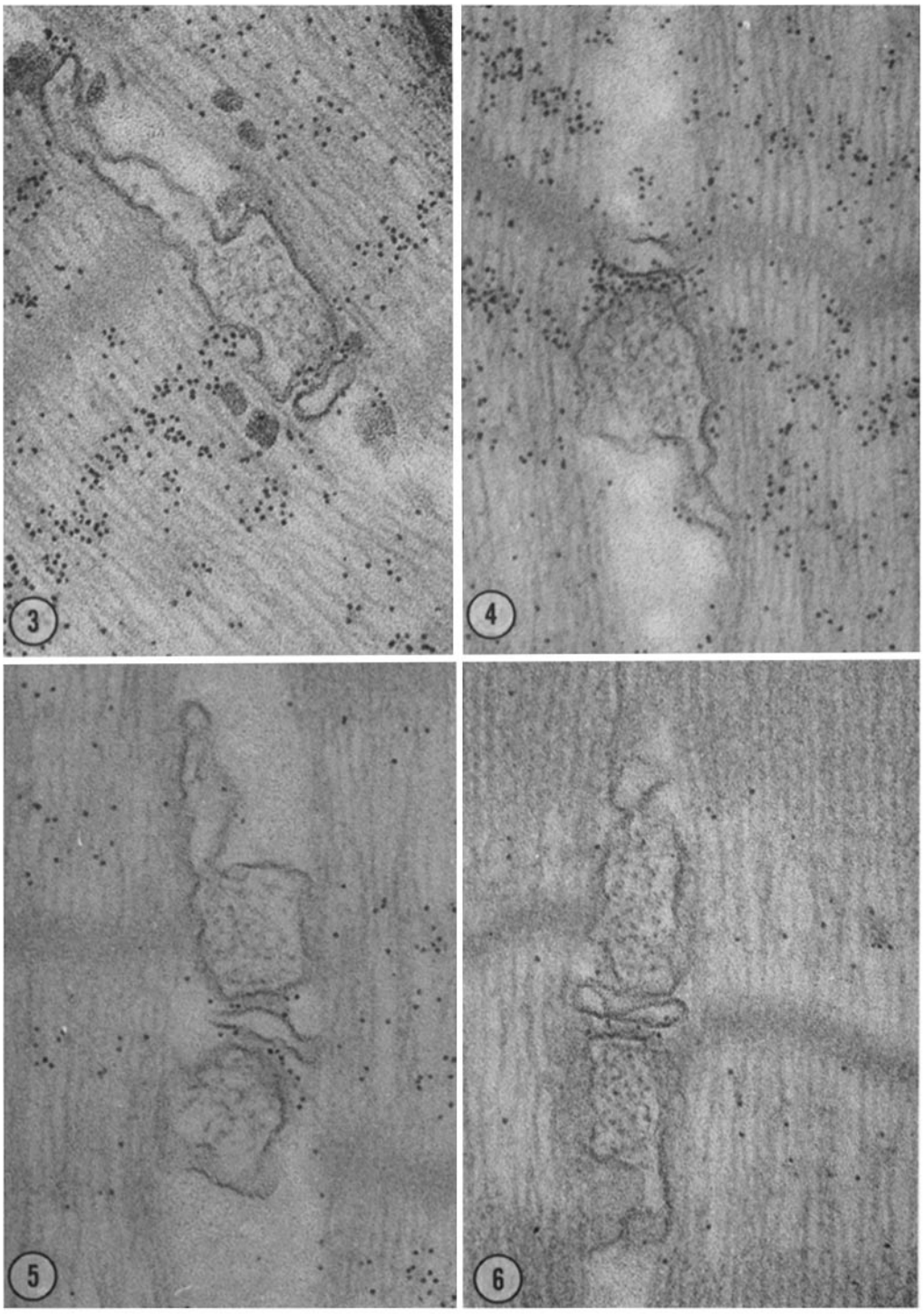


FIGURE 3 Triad in ferritin-rich region near surface. Numerous granules penetrate the junctional gap. × 100,000.

FIGURE 4 Numerous granules are in this triad, which is probably damaged. × 100,000.

FIGURES 5 and 6 Well preserved triads, with ferritin in zone c. One granule in Fig. 6 may be in zone a. × 100,000.

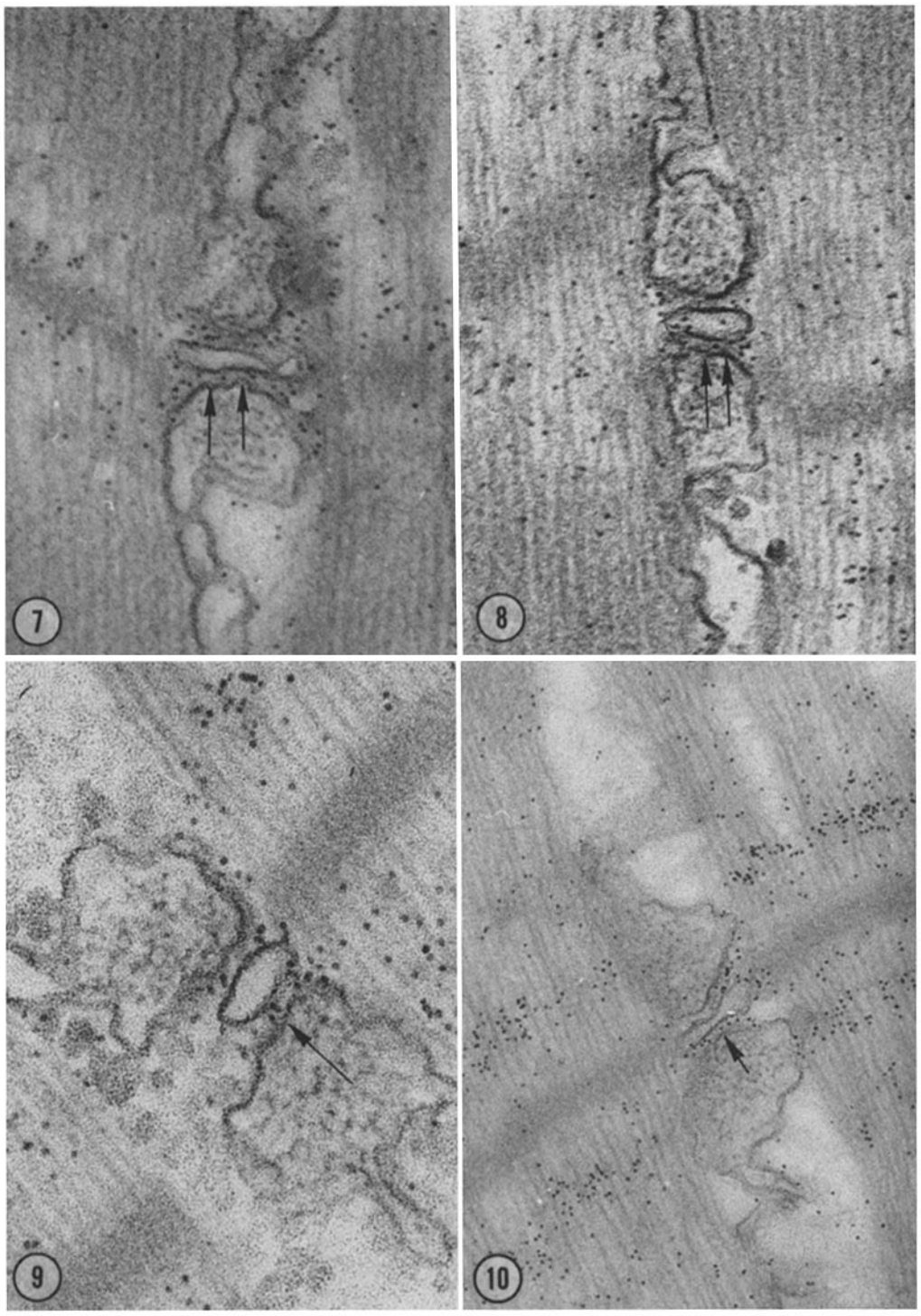


FIGURE 7 Two arrows indicate SR scallops which correspond to the position of the two feet. In the center is a ferritin granule. × 100,000.

FIGURE 8 Arrows indicate two feet. Between them is a ferritin granule. × 100,000.

FIGURES 9 and 10 A ferritin granule (arrow) is in the approximate center of the junction. Feet are not clearly visible, but the space between ferritin granules is sufficient to accommodate them. × 150,000 and × 75,000, respectively.

Distribution of Ferritin

The amount of ferritin found inside the skinned portion of the fiber varies considerably from one preparation to another, and the micrographs presented here have been selected from the most successful preparations.

As noted elsewhere (Franzini-Armstrong, 1970 *b*), ferritin penetrates inside the fibrils, where it is particularly located along the N lines (Fig. 1). Even where ferritin granules are abundant (Figs. 1 and 2), none of them are found free in the sarcoplasm, although they are in close proximity to the membranes. In particular, clusters of granules are attached to the triadic junction area (Fig. 2). A preference of the ferritin molecules for the junctional area is also noticeable in the interior of the fiber, where far fewer granules are present. This is particularly fortunate for the purposes of the present investigation.

On the basis of their possible significance relative to the solution of the problem proposed here, triads in ferritin-infiltrated fibers may be divided into three groups. A total of 318 triads have been thus classified. (*a*) Triads which occur in areas where ferritin is present in large quantities (Figs. 1 and 2). These are usually at the periphery of the fiber, corresponding to the area where the sarcolemma has been ripped off. Ferritin is clustered at the junction in large numbers, and it has also penetrated the junctional area (Figs. 2 and 3). It is likely that triads located very close to the area of skinning are only apparently intact; thus evidence from these peripheral triads that ferritin can enter the junctional gap is to be taken with caution. About 10% of the triads examined fit into this category.

(*b*) Other triads within the fiber are probably damaged, as indicated by the fact that SR-T-system separation is variable. Clusters of granules are often visible in the junctional gap, but again they cannot be taken as evidence for penetration of ferritin into an intact junction (Fig. 4). These triads comprise about 17% of those examined.

(*c*) In the case of triads which have been little distorted by the procedure and which are cut crosswise or very nearly so, ferritin granules are present within the junctional gap, but a space is left in the center of the junction which is sufficient to accommodate the two rows of feet (Figs. 5 and 6). Thus ferritin has penetrated up to the periphery of the two rows of feet, but probably not within the feet, nor between them. About 17% of the triads are of this type.

(*d*) Well preserved triads, with ferritin present within the junctional gap, are cut either tangentially or at an odd angle, so that it is not possible to tell where the granules are in relation to the two rows of feet (Fig. 10). Usually the granules are single and separated by spaces sufficient to accommodate one foot, and are thought to be in either area a or area c. About 24.5% of the triads belong to this category.

(*e*) In well preserved triads, cut crosswise, with a ferritin granule occupying theapproximate center of the junction (Figs. 7–9), these granules have presumably penetrated between the two rows of feet in space a. Only about 3% of the triads are in this group.

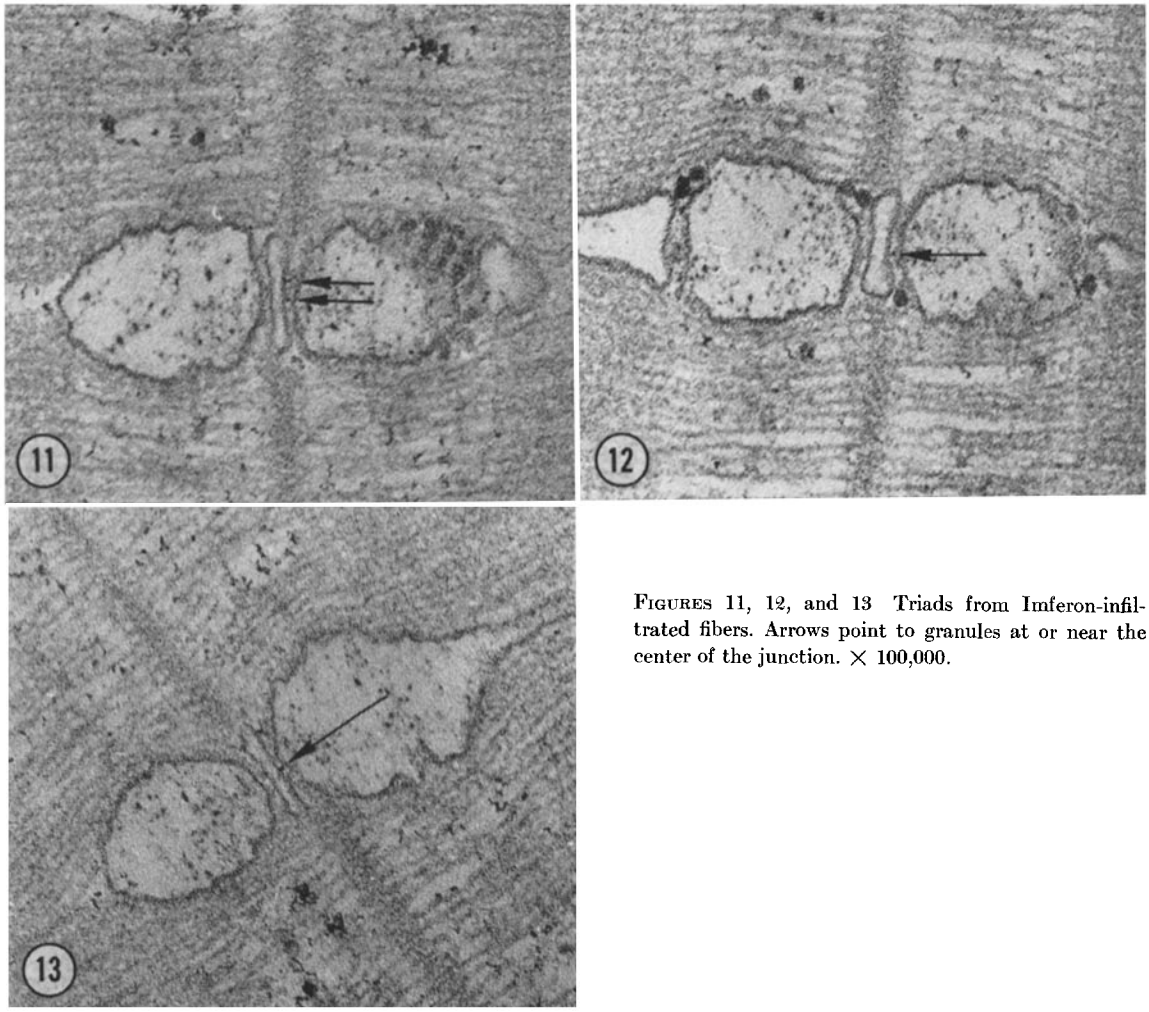
Distribution of Imferon

Imferon readily penetrates into the fiber, and it offers the advantage over ferritin suspension of not producing localized contractures of the skinned fibers. The granules are visible in the I band of the fibrils (Figs. 11–13), where they are seen mostly with a 400 A period, which is attributed to tropomyosin (Ebashi et al., 1969). Unfortunately, Imferon does not have a tendency to concentrate at the triad. Indeed it seems to be concentrated preferentially elsewhere, and Imferon granules are found only rarely within the junctional gap. A few examples are illustrated in Figs. 11–13. In all three cases the granules are located close to the center of the junction and thus may have penetrated between the two rows of junctional feet. As noted in the case of ferritin, the scarce visibility of the feet and the rarity of the observed cases make these results less reliable.

DISCUSSION

The penetration of ferritin molecules within the junctional gap up to the very periphery of the two rows of junctional feet indicates that at least 50% of the T-system surface area is freely accessible from the sarcoplasm. It is not altogether clear from this study if ferritin and Imferon granules can easily penetrate into the space that lies between the two rows of feet. Thus, a central strip comprising 20% of the T-system flat face may or may not be covered by some cementing substance.

In a previous paper (Franzini-Armstrong, 1970 *a*), two possible mechanisms of electrical coupling



FIGURES 11, 12, and 13 Triads from Imferon-infiltrated fibers. Arrows point to granules at or near the center of the junction. × 100,000.

between SR and T-system tubules were discussed. It was stated that the structure of the junction makes it highly unsuited for a capacitative coupling, due to the relatively large separation between the two conductors (SR and T-system lumina) and the small area of junction. One could argue that a medium of high dielectric constant filling the junctional gap would increase the capacitance of the junction. This study shows that such hypothetical material may only be located either in the feet (covering 30% or less of the T-system surface area) or at most in the feet and in the central portion of the junction (thus covering at most 50% of the T-system surface). The occasionally observed penetration of ferritin and Imferon into the junctional gap between the two rows of feet makes the second possibility less likely. In either case, the over-all capacitance would not be greatly increased.

The other possibility for electrical coupling is that the triad is a low resistance junction and that ionic current can flow directly from SR to T-system. This study clearly demonstrates that the only possible pathway for such flow would be via the amorphous material of the feet, since current flowing across the rest of the T-system membrane would escape into the sarcoplasm, following a path which is undoubtedly lower in impedance than the alternate path through the SR membrane. This raises the interesting possibility that aqueous channels occupy the center of each foot and that these channels are open at the moment

of excitation to allow flow of current between SR and T-system lumina.

On the other hand, on the basis of morphology alone, a hypothesis alternative to electrical coupling cannot be excluded. Such is, for example, the "trigger" hypothesis, first stated by Bianchi (1961) (see also Bianchi and Bolton [1967]). In this hypothesis, it is proposed that the change in the permeability of SR to calcium ions, which occurs at the moment of excitation, may be induced by the action of a substance which is released from the T-system. The small separation between SR and T-system membranes at the triad would make the junction suitable for this type of transmission. Delay due to diffusion would be well within the limits of excitation-contraction coupling times even for the fastest fibers, since diffusion of acetylcholine across the neuromuscular junction gap, which is ten times wider than that at the triad, takes less than 1 msec (Katz and Miledi, 1965).

Recent experiments on skinned fibers have indeed shown that a sudden release of calcium from the reticulum may be induced by changing the ionic composition of the medium bathing the SR (Endo et al., 1968; see also Ebashi et al., 1969; Podolsky and Ford, 1970).

I wish to thank Mrs. Lillian Peracchia for her generous assistance.

Supported by National Science Foundation Grant No. GB 6714.

Received for publication 15 July 1970, and in revised form 4 November 1970.

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