THE SUBCELLULAR LOCALIZATION OF CALCIUM ION IN MAMMALIAN MYOCARDIUM

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ABSTRACT

This study was designed to investigate the proposition that subcellular calcium is sequestered in specific sites in mammalian myocardium. 29 functioning dog papillary muscles were fixed through the intact vascular supply by means of osmium tetroxide containing a 2% concentration of potassium pyroantimonate ($K_2H_2Sb_2O_7 \cdot 4H_2O$). Tissue examined in the electron microscope showed a consistent and reproducible localization of the electron-opaque pyroantimonate salts of sodium and calcium to distinct sites in the tissue. Sodium pyroantimonate was found exclusively in the extracellular space and clustered at the sarcolemmal membrane. Calcium pyroantimonate, on the other hand, identified primarily by its susceptibility to removal by chelation with EGTA and EDTA, was consistently found densely concentrated in the lateral sacs of the sarcoplasmic reticulum and over the sarcomeric I bands. M zones were virtually free of precipitate. The implications of these findings with respect to various parameters of muscle function are discussed.

The special role of the sarcoplasmic reticulum as the subcellular component of muscle which serves as the link between excitation and contraction has been suggested by many investigators (1, 2). The postulation has been made that this role is achieved by the sarcoplasmic reticulum's ability to sequester calcium ion and to release it to the area of the myofibrils in response to the depolarizing impulse. Portions of the sarcoplasmic reticulum, moreover, actively pump calcium from the surrounding media in vitro; relaxation is thought to be largely effected by the removal of this ion from the actomyosin complex and the restoration of it to an inactive site in the cell (3, 4).

In 1965, Costantin et al. (5), working with skinned skeletal muscle fibers, precipitated calcium as the electron-opaque salt of calcium oxalate and localized this ion to the area of the lateral sac. They postulated that the lateral sac was that portion of the sarcoplasmic reticulum in skeletal muscle which served as an intracellular sink for calcium and was the morphological site to which calcium was displaced during the process of relaxation.

In the present study, the observation by Komnick and Komnick (6) that certain ions form electron-opaque pyroantimonate salts in the presence of potassium pyroantimonate was adapted to effect the precipitation of calcium in functioning dog papillary muscle. It was determined that there are distinct subcellular sites in cardiac muscle, such as had been demonstrated in skeletal muscle, to which calcium pyroantimonate could consistently be localized. The sarcolemmal membrane was intact in these cells, and the tissue was functional at the time of fixation.

METHOD

By means of a method previously described in detail (7), the septal artery of a dog heart was cannulated within 2–3 min after the sacrifice of the animal, and perfusion of the septum and papillary muscles of the right ventricle was begun with an isotonic, oxygenated solution containing NaCl 130 mM, $CaCl_2$ 5 mM, $MgCl_2 \cdot 6H_2O$ 1 mM, NaH_2PO_4 0.435 mM, $NaHCO_3$ 14 mM, and glucose 5 mM. Evans blue dye was added to the perfusate in order to outline the septal vasculature. The afferent artery to a papillary muscle, chosen for its lack of Purkinje fibers, could then be identified and cannulated without interruption of the perfusion. The muscle was then isolated, mounted in a chamber, and attached to an isometric transducer, stimulating electrodes pacing the muscle when required at six beats per min. Perfusion was maintained at a rate of 1 ml/g/min for the duration of the experiment while a continuous record of function was obtained.

At the conclusion of the experiment, a 1% solution (pH 7.4–7.6) of osmium tetroxide containing a 2% concentration of potassium pyroantimonate ($K_2H_2Sb_2O_7 \cdot 4H_2O$) (Fisher Scientific Co., Pittsburgh) in 0.01 N acetic acid replaced the perfusate. The muscle was instantaneously fixed through its own vasculature, usually within a single contraction cycle. The muscle was then taken from the chamber, cut into pieces approximately 0.2 × 0.2 × 1 mm in size, and dehydrated in graded concentrations of acetone. The tissue was embedded in Araldite, sectioned on a Sorvall Porter-Blum ultramicrotome MT II, and stained with uranyl acetate and lead citrate. It was then examined in the Siemens Elmiskop II microscope.

RESULTS

Control Muscles

Three muscles were perfused with standard perfusate. All of them maintained good (8–10 g) systolic tension. After perfusion for a period of time varying from 15 min to 1 hr, standard solution was replaced with osmium tetroxide containing potassium pyroantimonate, and the muscle was fixed instantaneously through its own vasculature.

All tissue examined showed four distinct sites of precipitate deposition, two of which formed the primary subject matter of the present study. One of the four sites was extracellular, and the remaining three were intracellular.

1. LATERAL SAC PRECIPITATE: The lateral sacs of the sarcoplasmic reticulum in observed triads were completely filled with an electron-opaque precipitate. This finding was such a consistent one that the precipitate served as a marker for the lateral sac. Rarely, a sac could be seen which was not densely full; in such sacs, the electron-opaque precipitate could clearly be seen adherent to the inner surface of the lateral sac membrane (Figs. 1 *a*, *b*).

2. MYOFIBRILLAR PRECIPITATE: In relaxed sarcomeres, I bands showed a distinct line of precipitate reproducibly located at the approximate midpoint between the Z band and the beginning of the A band (Fig. 2). (The distance from the Z band to the line of precipitate was 50–56% of the total Z-A band distance in sarcomeres which measured over 1.8μ.) In contracted sarcomeres, where no I band was apparent, precipitate was heaviest at the Z band, but was scattered along the A-band filaments up to the area of the M zone (Fig. 3). The M band, on the other hand, showed a conspicuous absence of precipitate relative to the rest of the sarcomere (Fig. 4).

3. PRECIPITATE AT THE SARCOLEMMAL MEMBRANE: The extracellular space was marked by granules of precipitate which were individually much larger than those filling the lateral sacs. These granules were found primarily clustered at the sarcolemmal membrane (Fig. 5), but occasionally were also noted to fill the space bounded by the membrane systems of the intercalated disc (Fig. 6). Even more rarely, they extended down into the lumen of a transverse tubule (Fig. 5), but this was the exception. In general, the T system was empty.

4. MITOCHONDRIAL PRECIPITATE: Small, uniformly round granules of precipitate were seen to pepper the mitochondria. These granules were consistently of a smaller size than those seen in the extracellular space, and more closely approached the dimension of those seen in the lateral sac (Fig. 1 *a*).

Identification of the Precipitate

The following series of experiments were performed to identify the nature of the precipitate found in the sites of primary interest, the lateral sac and the I band:

1. PERFUSION WITH SOLUTION CONTAINING NO CALCIUM: Five muscles were perfused with solution containing no calcium for periods of time varying from 20 min to 90 min. In all muscles, systolic tension began to fall within seconds after perfusion with calcium-free solution was initiated. A significant decrease in active tension was apparent by 3 min of perfusion in these muscles.

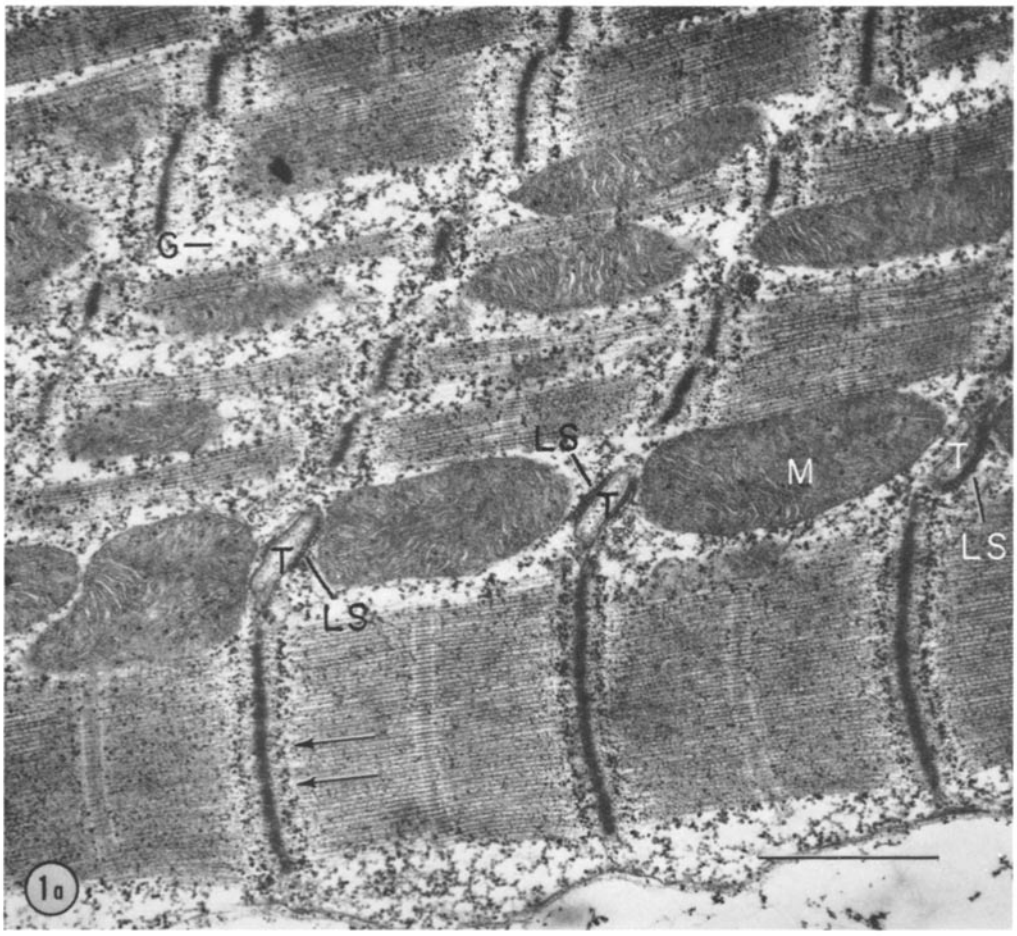


FIGURE 1 *a* This section illustrates the three primary intracellular sites of electron-opaque pyroantimonate salt formation in control myocardium fixed with OsO_4 solution containing potassium pyroantimonate ($K_2H_2Sb_2O_74H_2O$). Granules of precipitate apparently fill the lateral sacs (*LS*) of triads and diads. Note that the large central T vesicles (*T*) have no precipitate accumulation. The lighter, more uniformly round granules of glycogen (*G*) are easily distinguishable from the dark, irregularly shaped particles of pyroantimonate salt. This section also illustrates the dense line of precipitate (arrows) present in sarcomeric I bands. Note that small granules of precipitate are also present in mitochondria (*M*), which show no evidence of vacuolization or autolysis and have well preserved cristae. Scale, 1.0 μ. × 26,000.

Electron microscope examination of this tissue revealed that precipitate, although still present in some areas of the tissue to some degree, was significantly diminished in comparison to that in control tissue (Fig. 7). Of primary interest was the greatly diminished amount or absence of precipitate in the lateral sac and over the I band. This was in marked contrast to the dense clusters of precipitate granules found in these sites in control tissue.

2. PERFUSION WITH SOLUTION CONTAINING NO SODIUM: In this experiment, a papillary muscle was perfused with standard solution labeled with radioactive Na^{24}. The general technique of this protocol has been previously described in detail (7). As perfusion proceeded, the gradually increasing radioactivity of the muscle was monitored by means of a Geiger-Müller probe until it reached asymptotic values, which occurred by the end of 10 min of perfusion. At this point, it was es-

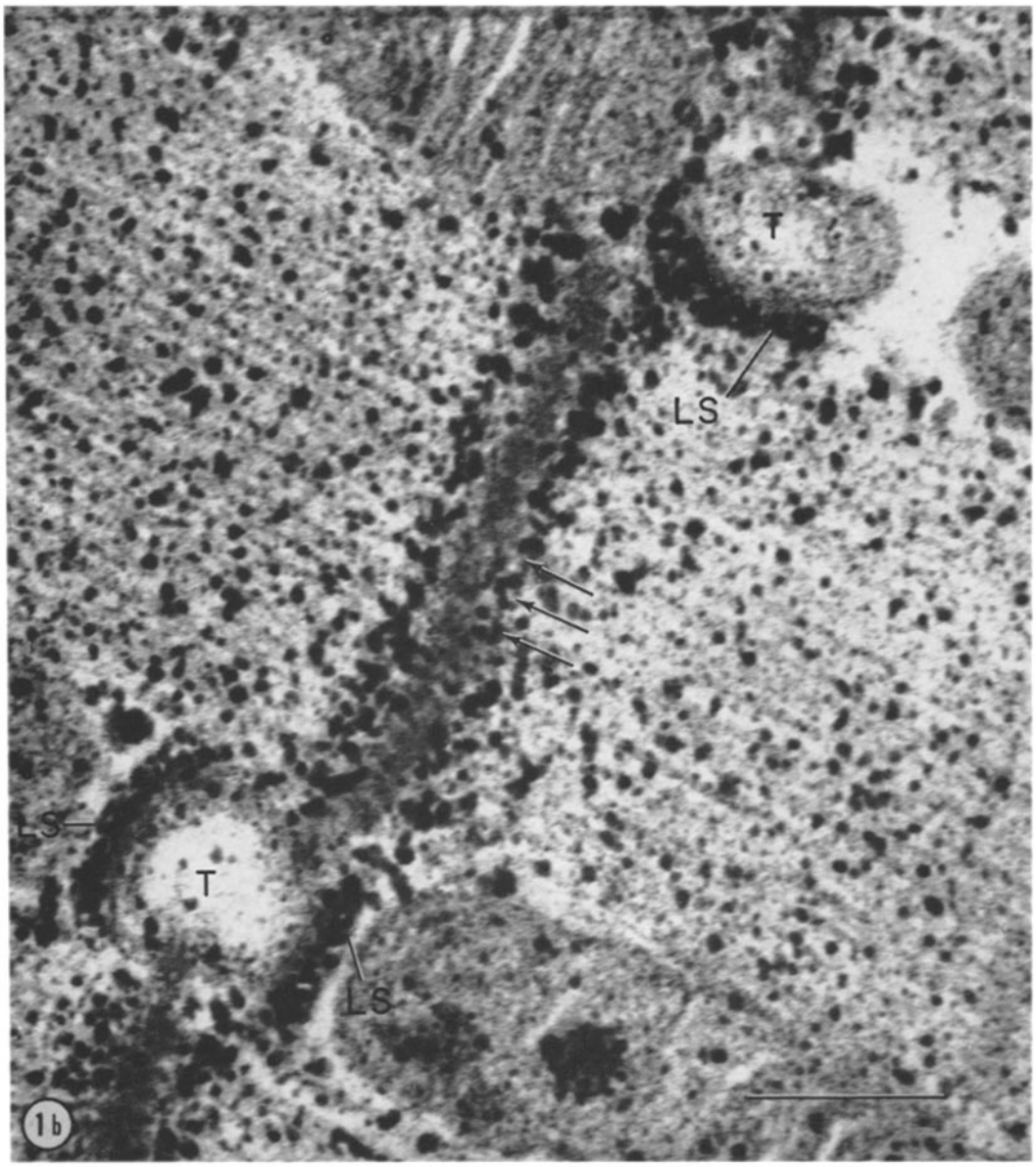


FIGURE 1 *b* This high magnification view of control myocardium illustrates the dense granules of precipitate filling the lateral sacs (*LS*) of both a triad and a diad. Again, note the empty T vesicle (*T*) and the accumulation of granules in the I band (arrows). Scale, 0.25 μ. × 84,000.

timated that all of the exchangeable muscle sodium was replaced with labeled perfusate sodium. Isotonic solution, identical with the standard perfusate except that NaCl had been replaced by 130 mM choline chloride, was then used to wash out the muscle. (It should be noted that this solution still contained 14 mM of sodium as the bicarbonate salt. This perfusate contained 10% of the original Na^{24} activity by volume, so the specific activity of the remaining Na (14 mM) was essentially equal to that of the original labeling solution.) Each washout droplet was collected, and its radioactivity was determined. The fall in radioactivity of muscle effluent was plotted against time. The

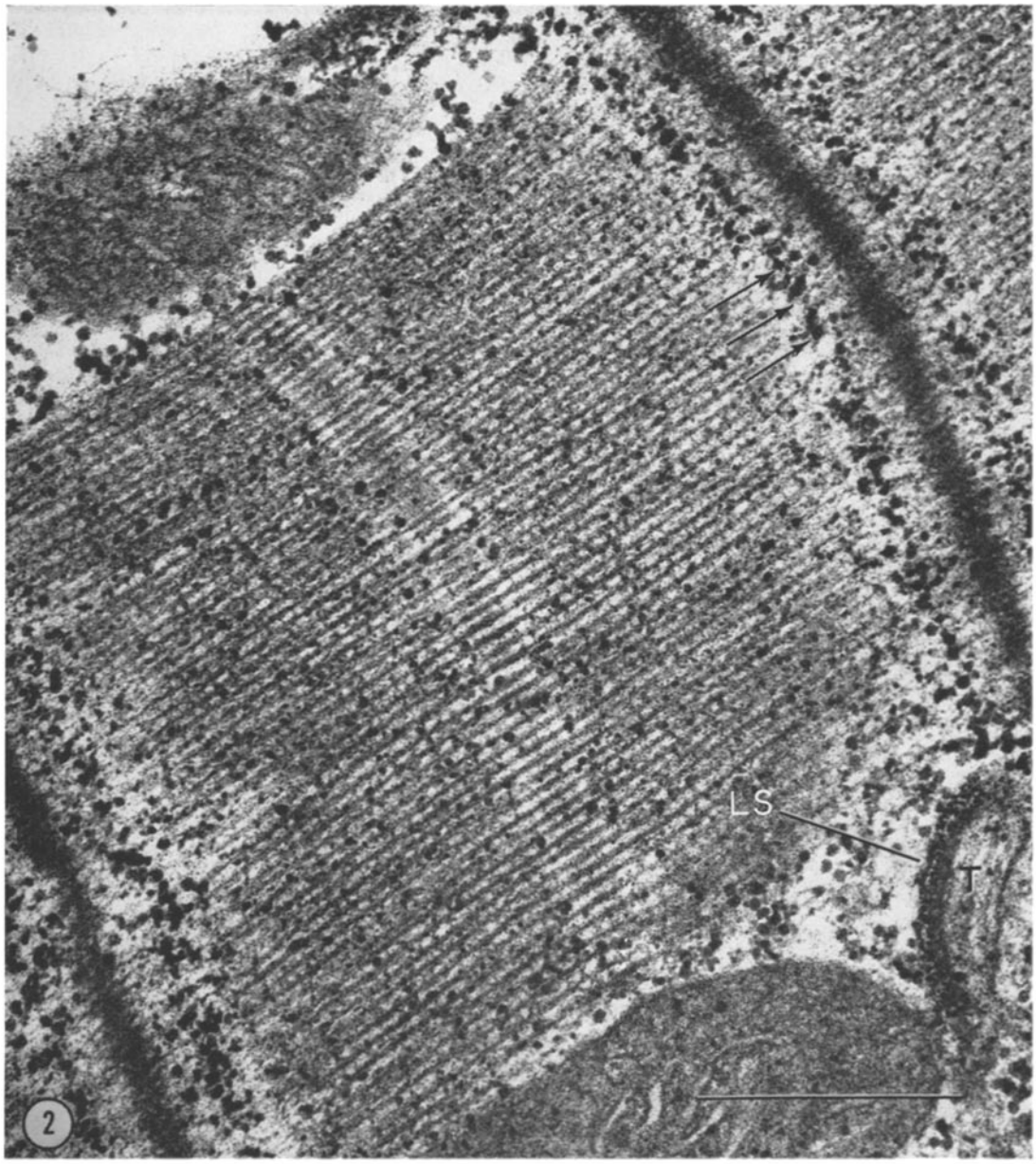


FIGURE 2 This section of control myocardium illustrates the dense line of precipitate (arrows) present in sarcomeric I bands. There is relative absence of precipitate in the M band itself. A granule-filled lateral sac (*LS*) is seen applied to an empty T vesicle (*T*) in a diad below the sarcomere. Scale, 0.25 μ. $\times$ 108,000.

effluent radioactivity became stable. Tissue Na^{24} activity stabilized at 10% of its asymptotic value, indicating that 90% of tissue sodium had been washed out. The tissue was then fixed in the usual manner.

This tissue, when examined in the electron microscope, showed a heavy deposition of precipitate both in the lateral sacs and in the sarcomeres in all fields examined. The distribution of this precipitate was identical with that of the precipitate

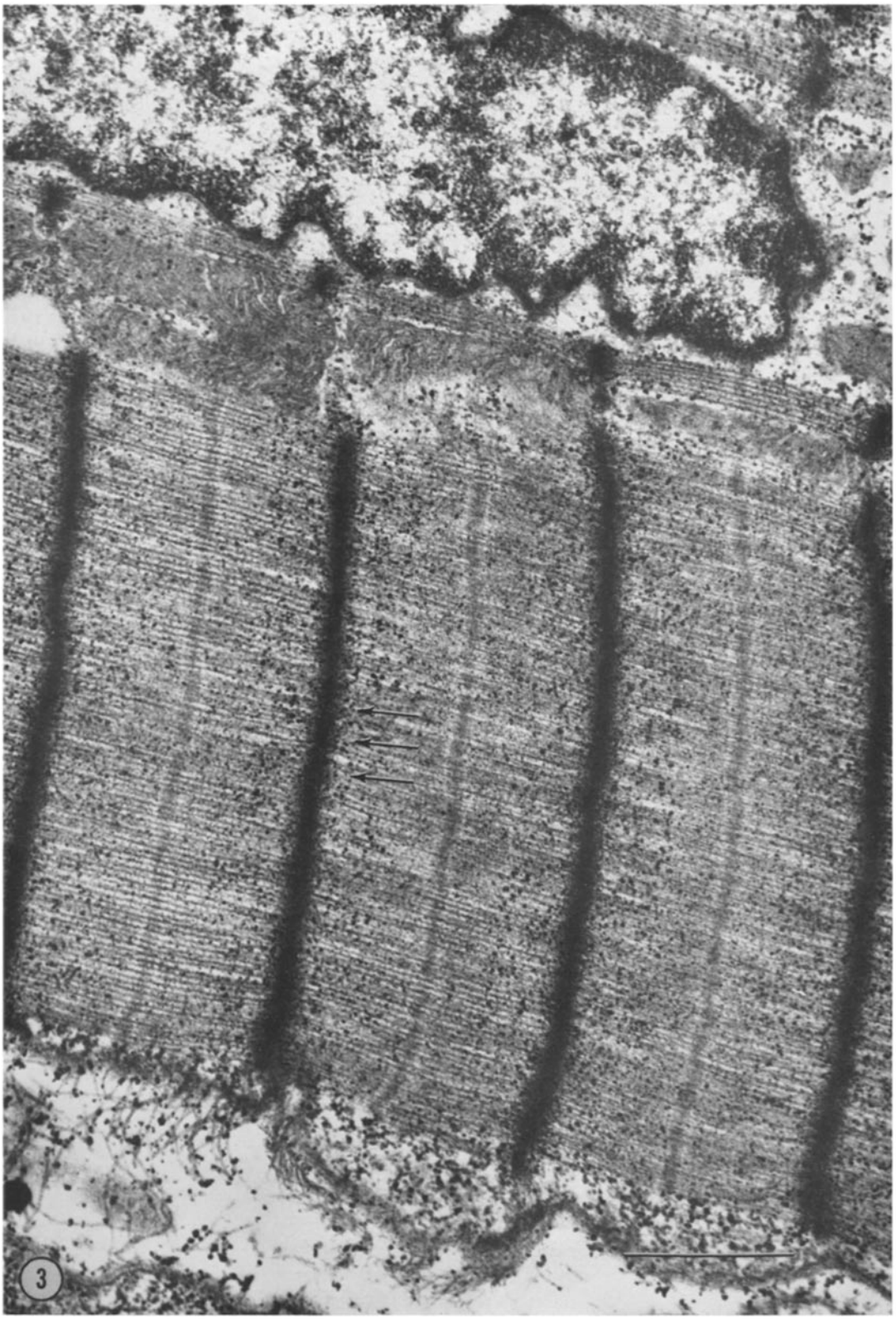


FIGURE 3 This section illustrates that when I bands are not present in contracted sarcomeres of control myocardium, precipitate granules are concentrated at the Z-band area (arrows), their number diminishing towards the center of the sarcomere. Scale, 1.0 μ. $\times$ 60,000.

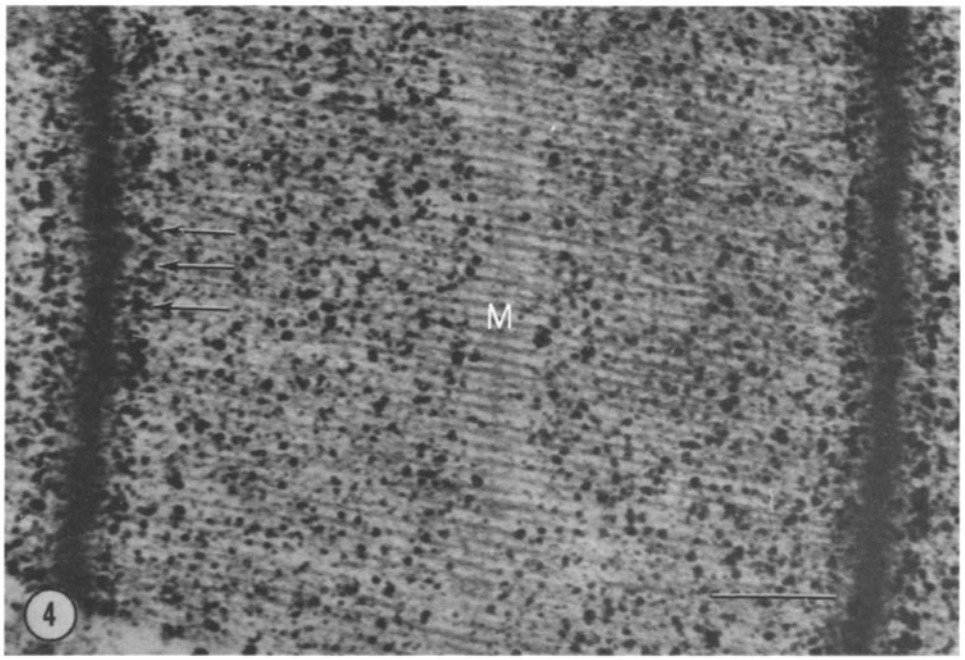


FIGURE 4 This higher power view of a sarcomere in control myocardium shows the M band (*M*) clearly devoid of precipitate granules. The usual concentration of pyroantimonate salt at the Z band, however, is preserved (arrows) and granules are present along the remainder of the myofilaments. Scale, 0.25 μ. × 80,000.

seen in control tissue (Figs. 8 *a*, *b*). Since it was known that all but 10% of muscle sodium had been washed out, it could be assumed that lateral sac and sarcomeric precipitate was not sodium pyroantimonate, but rather magnesium or calcium pyroantimonate.

It was now clear that the precipitate in the lateral sac and over the I band could be significantly diminished by perfusion with calcium-free solution. On the other hand, the amount of precipitate was unchanged or actually increased in a muscle in which all but 10% of total sodium had been removed. It should be recalled that perfusion with low sodium concentrations, moreover, has been shown to increase calcium uptake in functioning muscle (8).

These data supported the conclusion that the precipitate observed in these areas was not sodium pyroantimonate and might well be calcium pyroantimonate. However, since magnesium as well as calcium is known to be present in muscle in concentrations sufficient to produce precipitation with pyroantimonate anion under these experimental conditions, a series of experiments was done which demonstrated whether or not the precipitate could be removed by chelation with EDTA or EGTA.

3. PERFUSION WITH EDTA AND EGTA: In these experiments, the tissue was perfused with normal perfusate for a control period which varied from 10 to 60 min. At the end of this time, normal perfusate was replaced by a solution identical in all respects to normal perfusate except that the 5-mM calcium chloride had been replaced by either 5-mM sodium EGTA or 5-mM sodium EDTA.

Both EDTA and EGTA invariably produced a reduction of muscle contractile force to zero within a few minutes of the initiation of perfusion. If, however, perfusion was continued for more than 30 min, a gradual increase in tension became apparent and the muscle went into contracture. Electron microscope study of such tissue revealed widespread destruction of membrane systems in muscles subjected to such prolonged perfusion. For this reason, muscles were perfused with chelating agents for only as long as needed to reduce muscle tension to zero.

Three muscles were perfused with solution containing 5 mM of EGTA. In the first muscle so treated, active tension had decreased to zero within

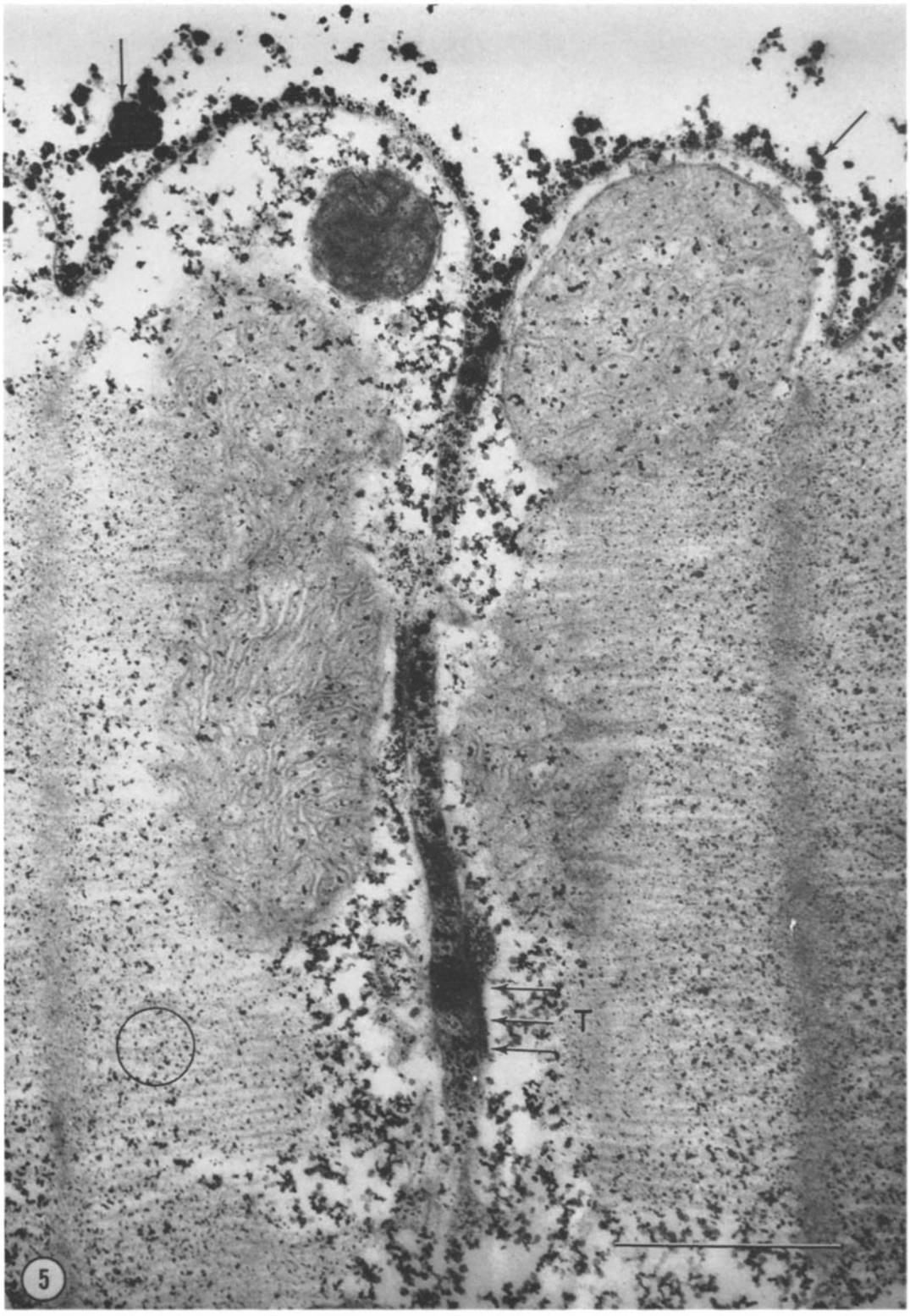


FIGURE 5 This micrograph of control myocardium shows the large, round granules of sodium pyroantimonate (arrows) clustered at the sarcolemmal membrane and filling the extracellular space, and enclosed by the cell membrane as this membrane invaginates to form a transverse tubule (*T*). Note the relatively large size of the sodium pyroantimonate particles compared to the smaller granules of calcium pyroantimonate (circle). Scale, 1.0 μ. × 30,000.

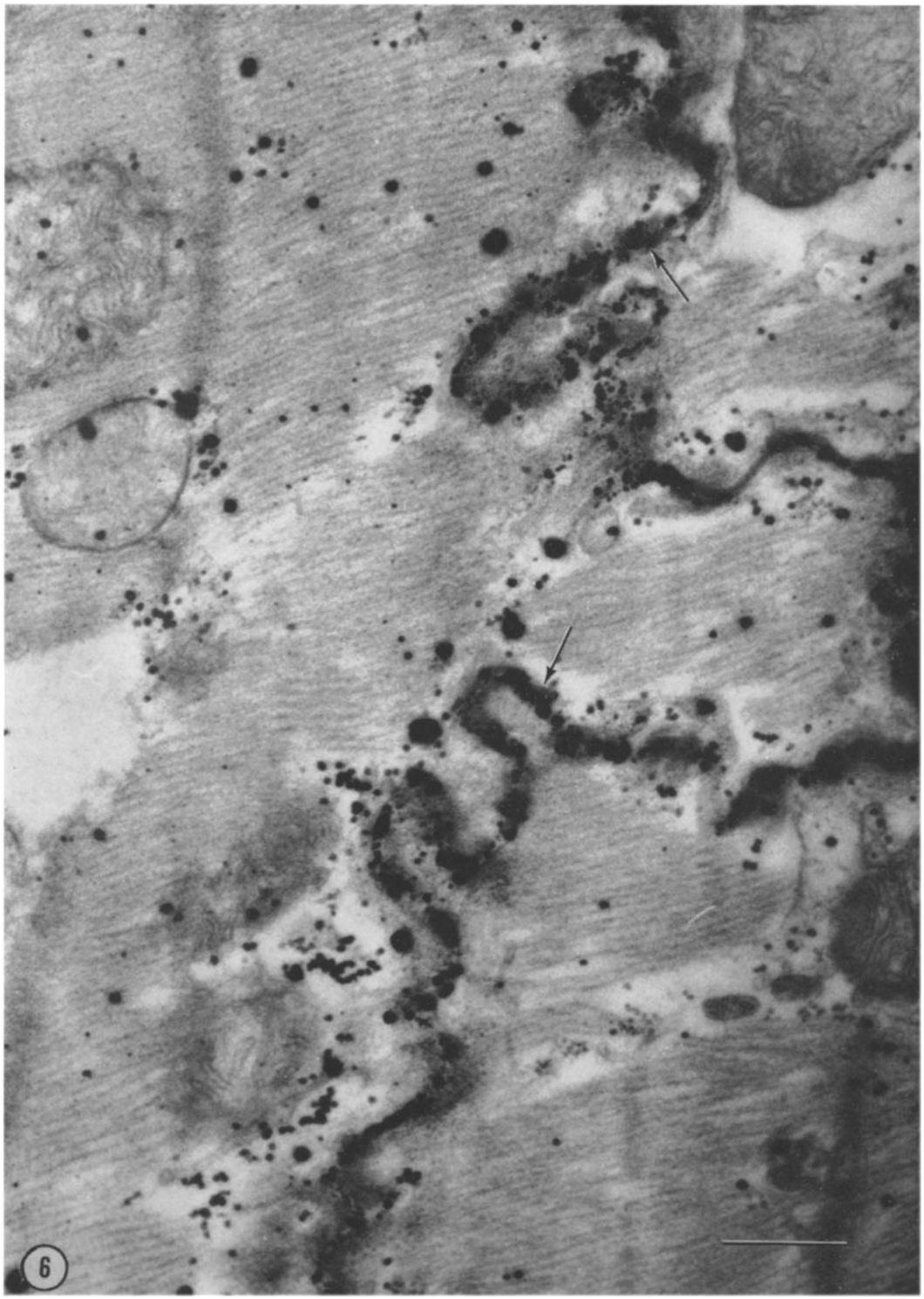


FIGURE 6 This micrograph of control myocardium shows that a portion of the extracellular space enclosed by the intercalated disc is filled with large, black granules of sodium pyroantimonate (arrows). Scale, 0.5 μ. $\times$ 30,000.

FIGURE 7 Striking diminution of precipitate at I band (arrows) and lateral sac (*LS*) is seen in this view of myocardium perfused with calcium-free solution. (Compare to Fig.1 *a*.) Scale, 1.0 μ. × 24,000.

FIGURE 8 *a* The usual dense concentration of I-band precipitate is seen in this section of myocardium perfused with low concentration of sodium. (Compare to Fig. 1 *a* and Fig. 2.) Scale, 1.0 μ. × 25,000.

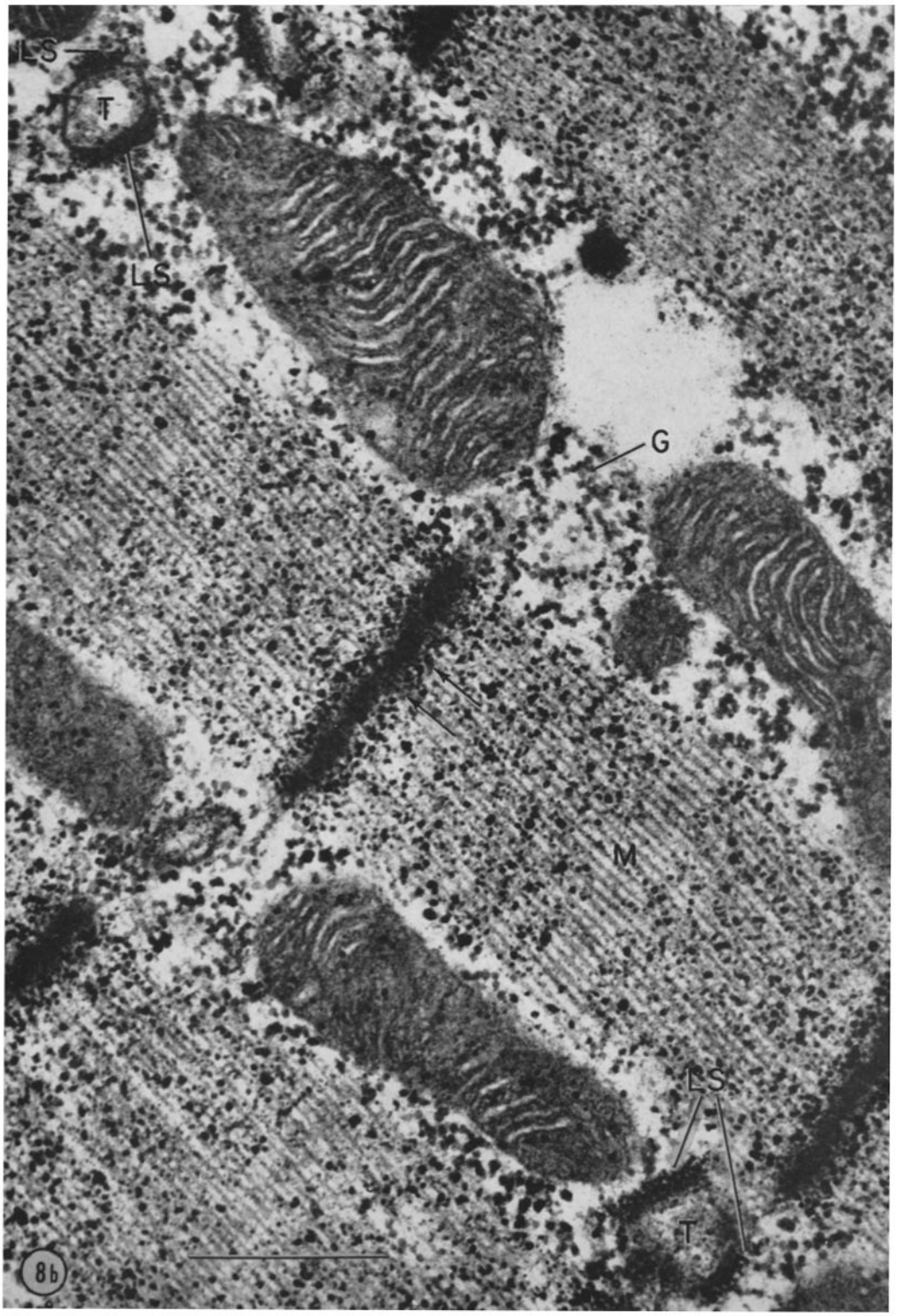


FIGURE 8 *b* The dense concentration of precipitate in the lateral sacs (*LS*) flanking empty T vesicles (*T*) is apparent in this myocardium perfused with low concentration of sodium. Distribution and amount of intrasarcomeric precipitate, with its characteristic high concentration at the Z line (arrows) and absence of particles at (*M*), are identical with those of control myocardium. (Compare with Figs. 1 *a*, 1*b*, and Fig. 2.) *G*, glycogen. Scale, 0.5 μ. × 60,000.

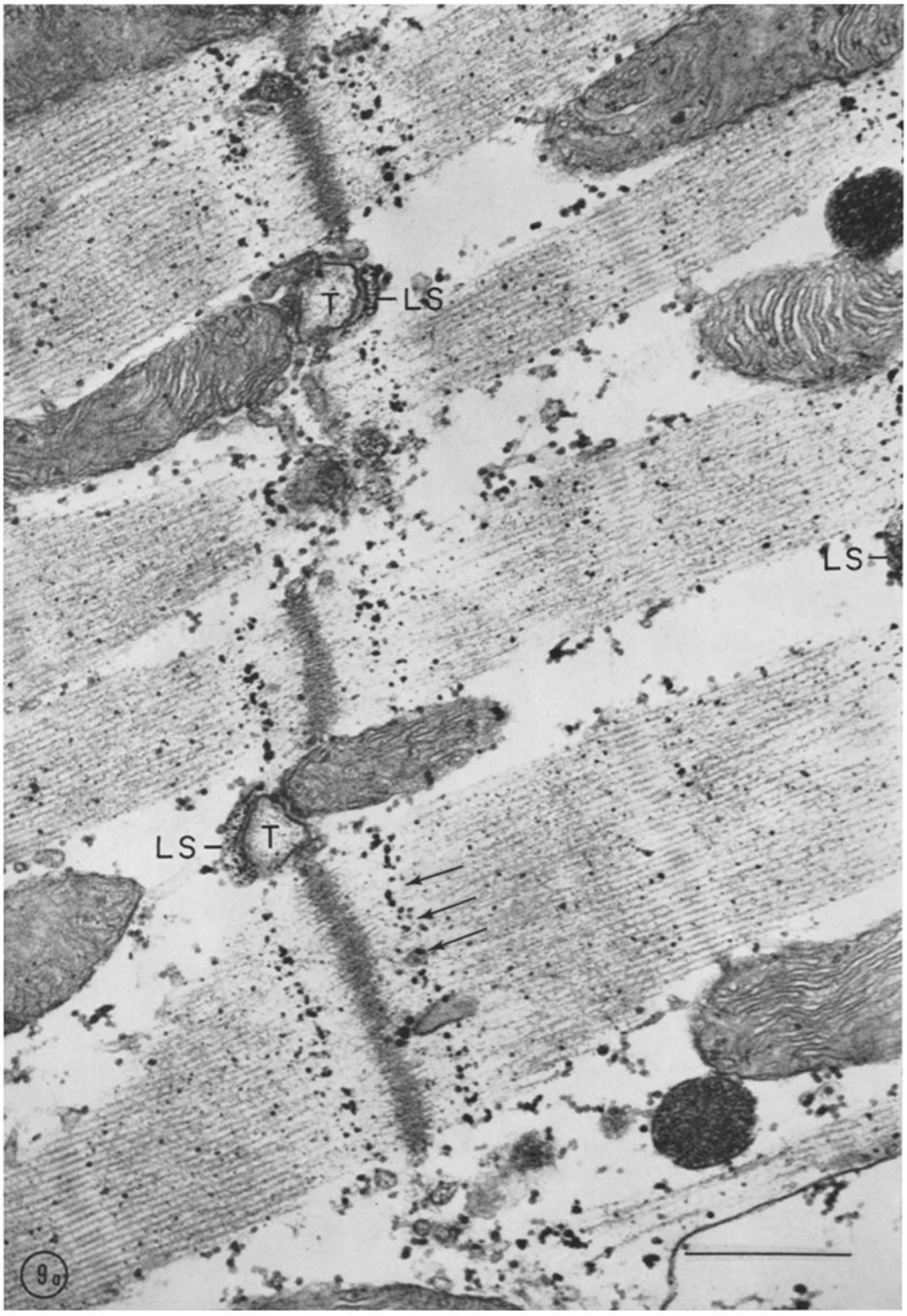


FIGURE 9 *a* Lateral sac precipitate (*LS*) was significantly diminished in myocardium perfused with 5mM concentration EGTA prior to fixation. Note as well the striking diminution in concentration of I-band precipitate (arrows). Scale, 0.5 μ. × 45,000.

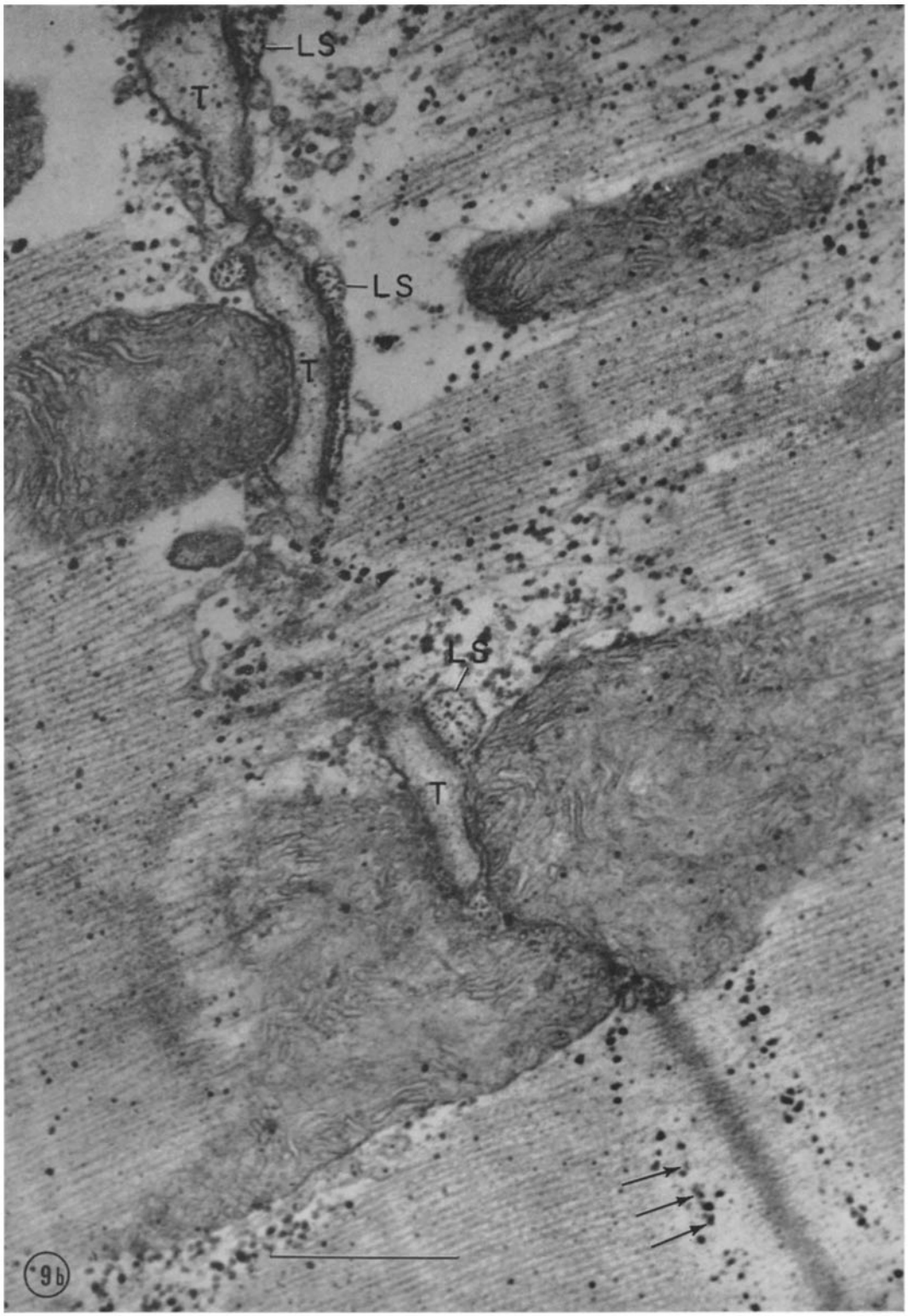


FIGURE 9 *b* This myocardium, perfused before fixation with OsO_4 solution containing potassium pyroantimonate with 5 mM EGTA, again illustrates the depletion of granules in the lateral sacs (*LS*) seen along the course of the long transverse tubule (*T*). (Note also depletion of I band precipitate [arrows].) Scale, 0.5 μ. × 36,500.

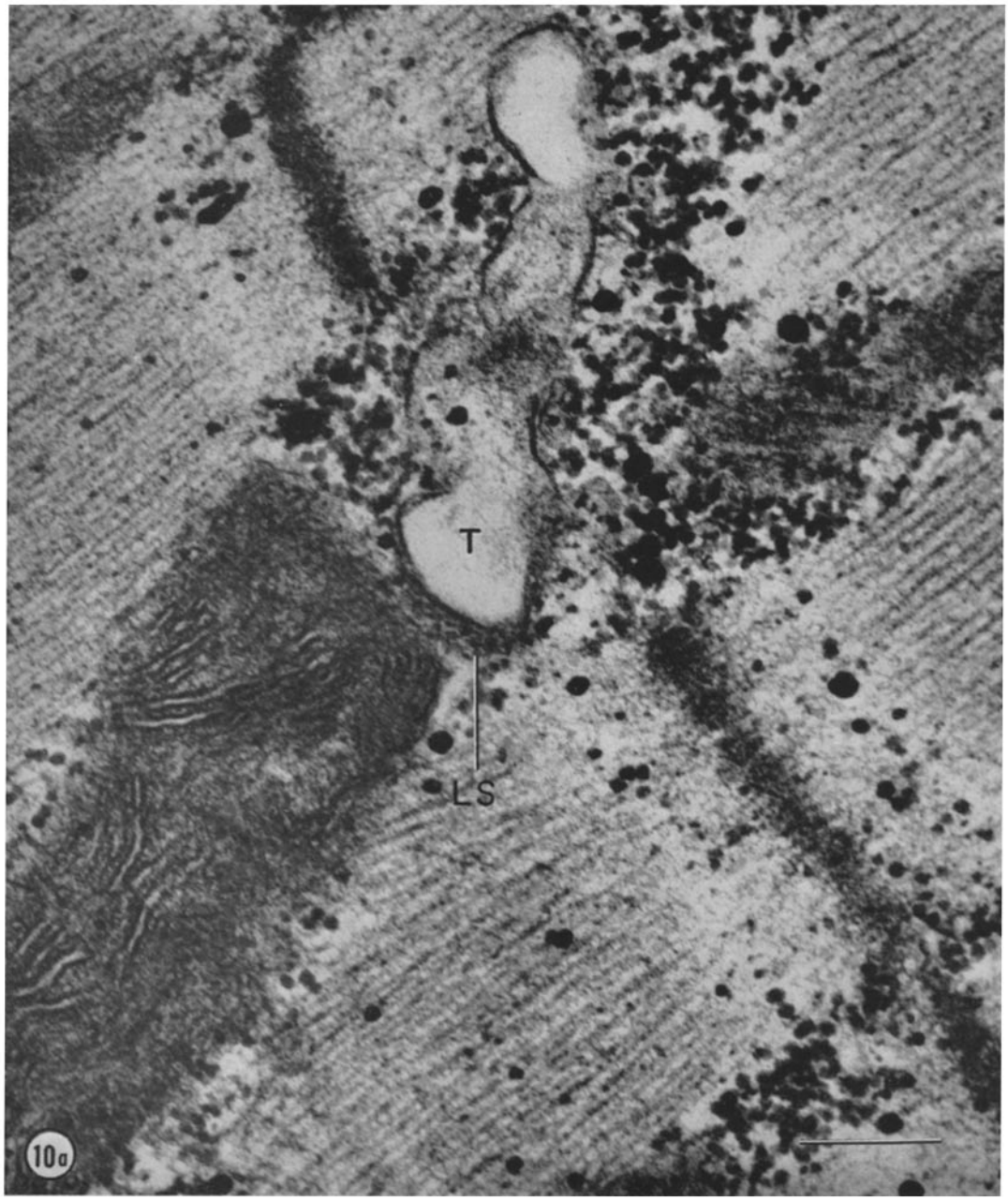


FIGURE 10 *a* This section shows a long portion of transverse tubule (*T*) to which is applied a lateral sac (*LS*). The depletion of lateral sac precipitate (*LS*) in this myocardium, which was perfused with 5 mM EDTA prior to fixation, as compared to the precipitate in control and low-sodium perfused myocardia, is apparent. Scale, 0.25 μ. × 60,000.

35 sec of muscle perfusion, and immediately at that point potassium pyroantimonate in osmium tetroxide was injected. In the other two muscles, within a 2–3-min period after EGTA perfusion had begun, tension reached zero. Electron microscope examination of the tissue in all three cases showed a striking diminution of intracellular precipitate, although the extracellular space

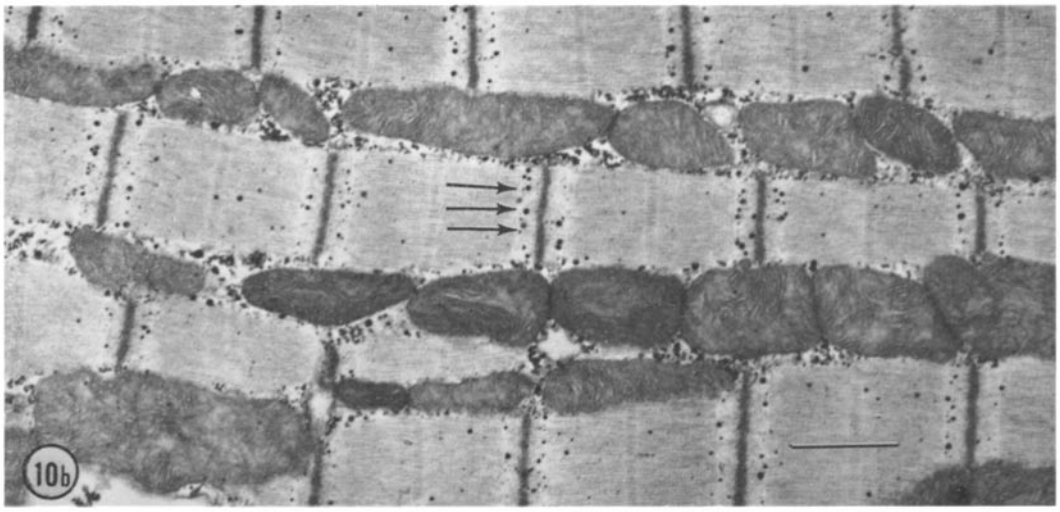


FIGURE 10 *b* This section illustrates the striking depletion of precipitate granules in the sarcomere, both at the I band (arrows) and along the myofilaments of the A band in myocardium perfused with 5 mM EDTA prior to fixation. Scale, 1.0 μ. × 10,200.

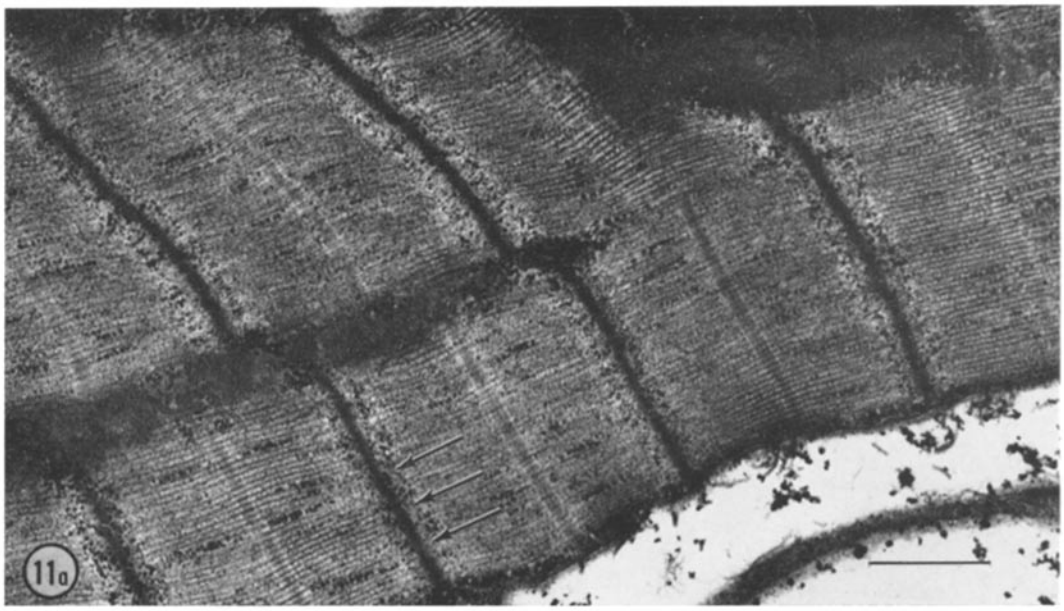
continued to exhibit amounts of precipitate commensurate with control levels. Lateral sacs were strikingly empty of precipitate (Figs. 9 *a*, *b*). The granules of precipitate that remained in sarcomeres were randomly scattered along the myofilaments. Even when distinct I bands were present, there was, in most fields, a striking diminution of the usual dense and linear concentration of I-band precipitate observed in control tissue, though some vestige usually remained.

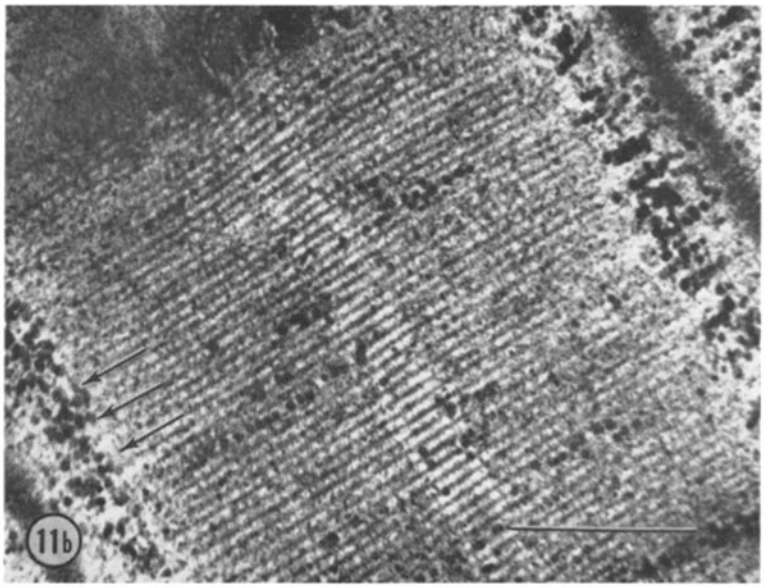
Perfusion of one muscle with EDTA for 4 min resulted in a total loss of tension generated during the control periods. This tissue showed exactly the same phenomena as had been observed in EGTA-perfused tissue. Again, lateral sacs were empty of precipitate (Fig. 10 *a*). I bands showed virtual absence of precipitate granules (Fig. 10 *b*).

It remained to be established that the intracellular calcium found in our tissue was not shifted from the extracellular space, at the time of perfusion with osmium tetroxide, through membranes whose permeability to calcium had been altered by this fixative. There is evidence to suggest that osmium tetroxide may affect membrane permeability under special circumstances (9). These circumstances include a long (10-min) perfusion with osmium tetroxide followed by perfusion with solution containing calcium for 30 min.

We perfused two muscles, therefore, with sucrose-substituted isotonic perfusate with sodium concentration 25% of normal for a 40-min period. This was done to load fully the intracellular compartment with calcium. We then substituted an isotonic solution identical in every respect to our usual perfusate except that it was completely calcium free. Langer's studies on calcium flux in mammalian myocardium indicated that, after 3.5 min of perfusion with calcium-free solution, the calcium concentration of the interstitial space has been reduced to less than 5% of control levels (10). Therefore, after 3–4 min of perfusion with solution containing no calcium, when the systolic tensions of the muscles had reached 20–25% of control levels, the tissue was fixed. Muscles were never subsequently reexposed to calcium-containing solution, but were immediately dehydrated, infiltrated, and fixed.

Tissue from these experiments demonstrated precipitate in exactly the same intracellular loci as in control tissue and in an amount comparable to that demonstrated in control tissue (Figs. 11 *a–d*). It was apparent from this work that the precipitate seen was not due to a shift of calcium from the extracellular space, but was, indeed, the consequence of truly intracellular cation.





FIGURES 11 *a–d* These micrographs illustrate tissue that was perfused for 3–4 min with isotonic solution containing no calcium before it was perfused with OsO_4. The extracellular compartment of these muscles was virtually calcium free at the instant of fixation.

FIGURES 11 *a* and *b* These micrographs show sarcomeric I band precipitate (arrows) comparable in amounts to that in control muscle. Compare to Fig. 1 *a* and Fig. 2. Scale, Fig. 11 *a*, 0.75 μ; Fig. 11 *b*, 0.5 μ; Fig. 11 *a*, × 23,000; Fig. 11 *b*, × 53,000.

FIGURE 11 *c* In this micrograph, precipitate-filled lateral sacs (*LS*) flank empty T tubules (*T*). The black arrows indicate the granules of precipitate in the two lateral sacs formed by the branching of a sarcotubule. As is always the case, the sarcotubule itself contains no precipitate (see text). Scale, 0.5 μ. × 32,000.

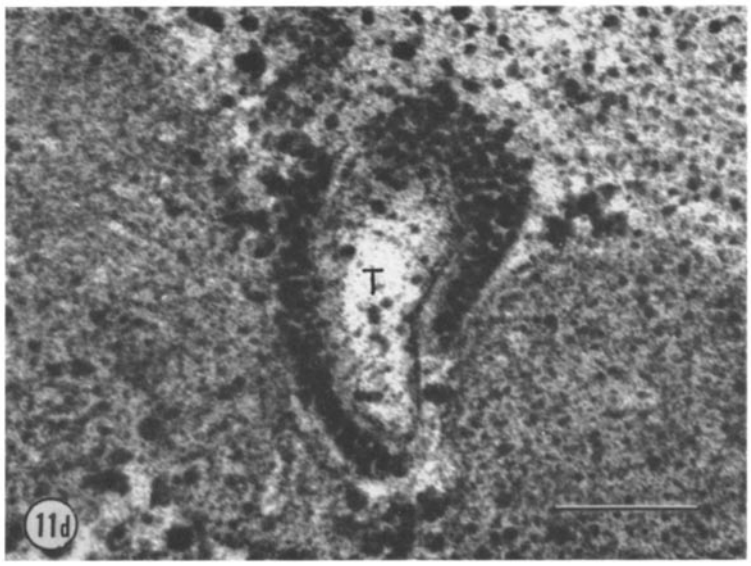


FIGURE 11 *d* This high-power view of a triad shows precipitate granules packed into the lateral sacs flanking the T vesicle (*T*). Compare to Fig. 1 *b*. Scale, 0.25 μ. × 73,000.

DISCUSSION

Method

We have attempted to evaluate carefully the usefulness of this method as an accurate tool for the localization of subcellular sites of ions in functioning mammalian myocardial tissue. Analogous previous studies based on the formation of an electron-opaque precipitate of calcium oxalate (5) have been done on single fibers of skeletal muscle from which the sarcolemma had been removed. The tissue preparation used in this study has the advantage of being intact and functioning whole dog papillary muscle, well oxygenated and perfused at a constant pressure through its own vasculature. Although the large, negatively charged pyroantimonate anion was not able to cross the intact cell membrane when the muscle was perfused with only a solution of potassium pyroantimonate made isotonic by adding sucrose, or with a solution of potassium pyroantimonate in combination with glutaraldehyde as a primary fixative, osmium tetroxide apparently changed the membrane permeability enough to allow entry of the pyroantimonate anion into the intracellular compartment.

Even though this tissue was fixed within a single contraction cycle, it is apparent that rapid diffusion or shifts of intracellular ions at the moment of fixation could have been effected. This would, of course, vitiate the usefulness of the resultant localization of the precipitate formed as an indication of the actual position of the ion in vivo. Two facts made such an artefact unlikely in the present study. First, lateral sac precipitate is membrane enclosed. No precipitate particles were ever seen either near or even just outside the confining wall of this structure. Significant shifts at the time of precipitate formation, therefore, were unlikely to have occurred.

Secondly, as we have noted, the I-band precipitate is extremely reproducible in its location, no matter what sarcomere I-band width was in evidence in the tissue examined. If this localization of precipitate was actually the result of ionic shift at the time of fixation, one might anticipate considerable variation in the locus of these electron-opaque particles. The extreme consistency of the results obtained makes it unlikely that significant diffusion of either calcium or calcium pyroantimonate had occurred as an artefact of this method.

Although the transverse tubular system is known to be in direct communication with the extracellular space (11), only an occasional tubule at the cell surface contained precipitate. The reason for this is not clear. One fact about the morphology of the T tubule in cardiac muscle is pertinent, however. Unlike the small transverse tubule in skeletal muscle, the transverse tubule

in the cardiac system has a relatively wide lumen. The precipitate, if not membrane enclosed (as in the intracellular particles), may be mechanically washed out during postfixation dehydration and infiltration.

Identification of Precipitate

In their original work on the electron-opaque salts formed by a combination of cations with pyroantimonate anion, Komnick and Komnick (6) made the point, largely overlooked by subsequent investigators who have adopted their method, that the solubility characteristics of sodium pyroantimonate, calcium pyroantimonate, and magnesium pyroantimonate, among other salts, are such that any or all of these salts, under the proper circumstances, may form electron-opaque particles of precipitate. Not only sodium, but also calcium and magnesium are present in sufficient concentrations in muscle to form electron-opaque and highly insoluble precipitates when combined with pyroantimonate anion (12). In the case of calcium, we found that a grossly visible precipitate was formed when concentrations of this cation from 0.3 mM to 2 mM were added to equal volumes of a 2% solution of potassium pyroantimonate. Hence it becomes essential to establish the identity not only of the precipitate in the extracellular space clustered along the sarcolemma, but also of the intracellular lateral sac and I-band precipitate.

As has been stated, perfusion of muscles with isotonic solution containing no calcium greatly diminished, but did not totally eliminate in all areas, the precipitate located in the lateral sac and over the I band. Perfusion with EDTA and EGTA, however, virtually eliminated the precipitate densely concentrated in these two locations. While magnesium can be chelated by EDTA, EGTA is specific for calcium. Hence it seemed likely that the small (150-A) granules of precipitate scattered along the I band and filling the lateral sacs were calcium pyroantimonate. It is of interest to note that the larger granules of precipitate localized to the extracellular space and clustered along the sarcolemma were not altered in locus or concentration by chelation when compared to the precipitate in control tissue; this precipitate was thought to be sodium pyroantimonate and to reflect the known high extracellular concentration of sodium in muscle.

The problem of precipitate identification was attacked by another means in the study utilizing radioactive sodium to load the muscle. It is known that low sodium concentration enhances calcium uptake in functioning dog papillary muscle (8). By monitoring the tissue during subsequent perfusion with isotonic, sodium-free perfusate, it could be ascertained that all but 10% of total muscle sodium had been washed out of this tissue. Muscle function in this experiment demonstrated the classic low sodium effect: loss of spontaneity, increase in systolic tension, and after-relaxation. This tissue, when examined, showed as heavy a concentration of precipitate in the I-band region and filling the lateral sac as the control tissue. Indeed, it seemed that the densest precipitate concentration in the series of experiments was seen in the lateral sacs of the triads of this muscle.

It is apparent, then, that a precipitate, probably calcium pyroantimonate (since its deposition is fostered by low-sodium perfusion, depleted by prolonged perfusion with calcium-free solution, and virtually eliminated by EGTA- and EDTA-chelating solutions), is sharply, consistently, and densely localized to two primary intracellular areas: the lateral sac and the I band. Moreover, the source of this precipitate is intracellular calcium: its amount and localization are not changed if the extracellular space is calcium free prior to fixation.

Precipitate in the Lateral Sac

The postulate that the lateral sac and sarcoplasmic reticulum network may be the link between excitation and contraction by virtue of its ability to pump and store calcium has been put forward by many investigators. Hasselbach and Makinose (3) and Weber et al. (13–15) have demonstrated that vesicles of isolated sarcoplasmic reticulum from skeletal muscle have an ability to pump calcium against a concentration gradient. Costantin et al. (5) have demonstrated that calcium is precipitated in the lateral sac of skeletal muscle sarcoplasmic reticulum as its oxalate salt. Our experiments seem to indicate that there is a large and preferential concentration of calcium in the lateral sac of functioning cardiac muscle. Interestingly, moreover, the longitudinal tubules of the sarcotubular system itself have never been demonstrated, in any of our experiments, to contain any suggestion or fragment of precipitate. Either the pyroantimonate anion cannot penetrate

the SR membrane or calcium is not stored in the longitudinal network itself for any prolonged period or in amounts sufficient to form the electron-opaque precipitate. The latter possibility would fit with the concept of the sarcotubular network as a pump, removing calcium from the area of the myofilaments and shunting it to the inactive site of the storage area, where it can be released by virtue of cell depolarization. That the lateral sac calcium is the calcium probably involved in triggering the contractile event is suggested by its close approximation not only to the T tubule but also to the intercalated disc and the sarcolemmal membrane. Moreover, the fact that lateral sac precipitate was densest in the tissue perfused with solution containing no sodium suggests that the lateral sac may be at least one of the anatomic sites of sodium-calcium competition in muscle.

In more primitive muscle, such as the frog ventricle, Niedergerke (16) has pointed out that there is no elaborate morphological system which could serve to effect the active transfer of calcium in the muscle. Electron microscope studies of Fawcett and Selby (17) and Grimley and Edwards (18) have shown a very primitive and rudimentary SR in cardiac tissue of lower vertebrates. Niedergerke has proposed that in these tissues probably the sarcolemmal membrane itself functions as a surface for transport of calcium from the extracellular space and that there is, therefore, no need for an intracellular sink or storage area for calcium. In higher vertebrates, such as dog, however, the lateral sac, shown in our experiments to contain calcium, may serve as an intracellular source of this ion. It is from this site, presumably, that calcium can be made available for actin and myosin interaction.

Calcium at the I Band

The sharp, consistent, and dense localization of calcium to the area of the I band was a totally unexpected finding in our experiments. There are no data to suggest that the I-band's actin filaments have a specialized morphology at any one isolated site which would enhance or effect calcium binding. When the tissue is closely examined, however, one never sees any elements of the sarcoplasmic reticulum or any other membranous structure enclosing the granules of precipitate; the granules of precipitate seem to be at the level of the myofilaments. The sharp localization of precipitate to an area approximately at the midline between Z and A bands may indicate that there is some entity which is either incorporated into or immediately applied to the surface of the actin filaments and which specifically binds calcium.

Recent work by Fuchs and Briggs (19) has offered data which help to explain a possible reason for the sharp localization and dense precipitation of calcium in the I band. An investigation of the calcium-binding properties of both I- and A-band myofilaments revealed that purified myosin A does not bind calcium. I-band filaments, on the other hand, have two classes of binding sites. It is the first of these classes which is postulated to be involved in supplying the calcium utilized in the actin-myosin interaction.

Further purification of I-band filaments established the fact that tropomyosin-free actin does not bind calcium at all, and that any calcium-binding activity of actin is secondary to its association with tropomyosin. Ebashi and Kodama (20) have further demonstrated that tropomyosin actually is associated in the sarcomere with another heretofore unrecognized protein which they have called troponin. It is actually troponin (which has an affinity constant of $10^6\ \Delta LM^{-1}$ for Ca) that is responsible for the Ca-binding ability of tropomyosin; purified tropomyosin without troponin does not bind calcium. Fuchs and Briggs have postulated that it is this protein which binds calcium and which is thus involved in initiating interaction between actin and myosin. Whether this calcium is derived from the lateral sac is not commented upon.

The dense line of calcium pyroantimonate precipitate in the I band may be due, then, to I-band troponin, and may actually serve to localize this protein within the sarcomere. Ebashi, however, has recently pointed out that a more likely locus of troponin might be along the actin filament at 400-A intervals at the potential site of cross-bridge formation (S. Ebashi, personal communication).

Alternatively, the line of I-band precipitate might label an as yet unrecognized intermediate binding site for calcium in this area of the sarcomere, and may not represent either the calcium bound to troponin or the calcium of the lateral sac.

Precipitate in the A Band

The finding of granules of precipitate along the A-band filaments is not so unpredictable. It has

been long established that calcium is probably involved in the formation of cross-bridges between the myofilaments. The absence of M-band precipitate raises the possibility of an absence of binding sites in this portion of the sarcomere or the existence of a barrier to calcium diffusion at the M band. The latter proposition is given more weight by the fact that precipitate granules can be seen piled up at the M band on either side, apparently unable to diffuse across the cross-bridges between the myosin filaments to reach the other side of the sarcomere. That actin filaments, on the other hand, apparently traverse the M-band region seems to militate against the existence of an impenetrable spatial barrier at the M zone.

The apparent inability of calcium to diffuse through the sarcomeric midzone furnishes an interesting mechanism to explain the experimental result of Huxley and Taylor (21), who, when applying calcium to the area of the Z band, produced contraction in adjacent halves of sarcomeres, but not a complete, two-sided, as it were, contraction in either sarcomere.

Finally, the discrete line of precipitate in the I bands of relaxed sarcomeres should be contrasted with the more generalized distribution of precipitate along the area of actin and myosin overlap in contracted sarcomeres (compare Figs. 1 *a* and 2 with Fig. 3). This difference in distribution correlates with the observation of Winegrad (22) in skeletal muscle that calcium moves from the area of the I band into the area of myofilament overlap, presumably because of calcium's involvement in cross-bridge formation during the contractile event. In relaxed sarcomeres, there is relatively little precipitate in the area of actin and myosin interdigitation.

The authors wish to acknowledge with thanks the kindness of Dr. David Spiro, who made the facilities of his laboratory available to us in the preparation of a portion of this work.

We also wish to acknowledge the expert technical assistance of Miss Gloria McCord.

This work was supported by grants HE 11074-01-02 from the United States Public Health Service and grants 66-626 from the American Heart Association.

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Received for publication 21 June 1968, and in revised form December 12 1968.

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