CYTOCHEMICAL STUDIES OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE SARCOPLASMIC RETICULUM

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INTRODUCTION

In an earlier study (Padykula and Gauthier, 1963) two distinct ATPases[1] (mitochondrial and myofibrillar ATPase) were localized within skeletal muscle fibers of the rat diaphragm, using the light microscope. A third ATPase active at pH 7.2 and dependent on sulfhydryl groups appeared to have its origin in the sarcoplasmic reticulum. In transverse sections of the muscle fibers, the final reaction product of this enzymic activity formed a more or less continuous network surrounding myofibrils. Localization in the diaphragm was not conclusive, however, because mitochondria and the sarcoplasmic reticulum have a similar distribution in this muscle. It was hoped that more precise localization could be achieved by studying a fish muscle in which mitochondria are separated spatially from the sarcoplasmic reticulum. This morphological situation occurs in the striated muscle fibers of the gas bladder of the toadfish (Fawcett and Revel, 1961). Enzymic activity originating in these organelles can, therefore, be distinguished with the light microscope, a situation which permits analysis of cytochemical properties in frozen sections. Mitochondria are confined to thin circumferential rims and small central cores of cytoplasm. The remainder of each fiber consists of radially arranged myofibrillar sheets separated by intervening sheets of the sacroplasmic reticulum. A similar configuration has been described in other fish muscles (Bergman, 1964; Franzini-Armstrong and Porter, 1964). In the present study, ATPase activity was demonstrated at sites known to be occupied by the sarcoplasmic reticulum in the toadfish muscle. The favorable morphological

[1] The following abbreviations will be used in this report: ATPase, adenosine triphosphatase; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine-5-phosphate.

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relationships in this muscle have permitted a description of some of the properties of sarcotubular ATPase *in situ*, using frozen sections instead of the more complex systems used for ultrastructural cytochemistry. An attempt was made to use this information in an ultrastructural characterization. The preparative procedures necessary for electron microscopy yielded more complex results, but certain consistent findings are reported.

MATERIAL AND METHODS

ATPase activity of the gas bladder muscle of the toadfish, *Opsanus tau*, was examined by using both light microscopic and electron microscopic procedures. The animals were killed using an overdose of MS 222 (tricaine methanesulphonate, Sandoz). Narrow strips of muscle were dissected free, tied to prevent contraction, and then either frozen in dry ice and ethanol at −70°C, or fixed in 10 per cent neutral buffered formalin or 1 per cent osmium tetroxide buffered to pH 7.4 with veronal-acetate. ATPase activity was demonstrated according to the Gomori principle, using lead at pH 7.2 and calcium at pH 9.4 (Wachstein and Meisel, 1957; Padykula and Herman, 1955; Padykula and Gauthier, 1963). Response to activators and inhibitors was tested, using cysteine as a source of sulfhydryl groups or *p*-hydroxymercuribenzoate as a mercurial compound. The light microscopic observations are based primarily on unfixed cryostat sections incubated for 30 minutes. ATPase activity of the sarcoplasmic reticulum does, however, survive formalin fixation, although demonstration of this activity requires that the time of incubation be increased to several hours.

Ultrastructural localization of enzymic activity was achieved by using similar procedures except that the muscle was first fixed in 5 per cent glutaraldehyde, buffered with cacodylate, for 60 minutes, according to methods described by Sabatini, Bensch, and Barrnett (1963). Frozen sections (40 to 80 μ) of this glutaraldehyde-fixed material were incubated in the lead medium in the presence of cysteine for 30 minutes (Padykula and Gauthier, 1963), and then postfixed in 2 per cent osmium tetroxide and embedded in Epon. Structural features of the muscle were examined by using material fixed in 1 per cent buffered osmium tetroxide or in glutaraldehyde followed by 2 per cent osmium tetroxide. Thick Epon sections (about 2.0 μ) were stained with toluidine blue and examined with the light microscope. Ultrathin sections were examined with a Siemens Elmiskop I or an R.C.A. EMU-3F microscope.

RESULTS

In the present study, emphasis is placed primarily on the demonstration of ATPase activity using the light microscope. These findings are supplemented by certain preliminary ultrastructural observations.

Morphological Features

Transverse sections examined with the light microscope consist, for the most part, of alternating light and dark radial stripes (Fig. 1). These represent the radial arrangement of longitudinal myofibrillar sheets and the intervening sarcoplasmic reticulum (or sarcotubular system). Mitochondria are densely stained with toluidine blue and are usually confined to thin circumferential rims and to small central cores of cytoplasm. Equivalent sections examined with the electron microscope confirm this interpretation of the radial pattern and of the restriction of mitochondria to peripheral and central regions of the fibers. Broad myofibrillar sheets are separated by narrower spaces occupied by membranes of the sarcoplasmic reticulum, and mitochondria do not occur among these structures (Figs. 3 and 5). The longitudinal banding pattern of the myofibrils is similar to that seen in most mammalian skeletal muscles (Figs. 4 and 5), and the triads of the sarcoplasmic reticulum are located near the A-I junctions (Fig. 5). The alternating pattern of myofibrils and the sarcoplasmic reticulum is especially apparent in longitidunal sections that pass across the radially arranged sheets so that membranes of the sarcoplasmic reticulum are seen in profile between the myofibrils (Fig. 5).

ATPase Activity

ATPase activity demonstrated at pH 7.2 in the presence of cysteine is associated with radially arranged stripes of the sarcoplasmic reticulum (Figs. 2 and 6) and also with peripheral and central mitochondria (Fig. 6). That the radial pattern of enzymic activity represents a site between myofibrils and not within them was confirmed by examination with a polarizing microscope. This was further demonstrated by comparing the longitudinal distribution of this enzyme with that of myofibrillar ATPase. Myofibrillar ATPase, demonstrated at pH 9.4, occupies the broad A band regions of the fibers (Fig. 8), as described for the rat diaphragm (Padykula and Gauthier, 1963). On the other hand, the enzymic activity demonstrated at pH 7.2, is marked by thin paired transverse lines of reaction product (Fig. 7). When these cytochemical preparations are examined with

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polarized light, it is evident that the lines occur approximately at the A-I junctions, which are the sites of the triads of the sarcoplasmic reticulum. This is the same activity that has a radial distribution in transverse sections, and is thus most likely sarcotubular in origin.

The sarcotubular ATPase is sulfhydryl dependent. If the mercurial compound, *p*-hydroxymercuribenzoate, is added to the medium, mitochondrial activity survives, but the radial pattern of sarcotubular activity is absent (Fig. 9). When cysteine is present instead, both the sarcotubular and mitochondrial activities are apparent in unfixed sections (Fig. 6). In addition, ATPase activity of both mitochondria and the sarcoplasmic reticulum is diminished if magnesium ion, which is a usual constituent of the medium, is omitted (Fig. 10). If magnesium is replaced by calcium, activity is again present, but only in the sarcoplasmic reticulum, which suggests that calcium is adequate as a source of cation for the sarcotubular enzymic activity. That the sarcotubular activity is that of a triphosphatase is demonstrated by using ADP instead of ATP. When this diphosphate is used as a substrate, no activity is observed within the fibers (Fig. 11). Similarly, activity is not observed when AMP or glycerophosphate is used or when substrate is omitted.

Ultrastructural Localization

Although the conditions of incubation for ultrastructural localization (pH 7.2, in the presence of magnesium ion and cysteine) were designed particularly to evoke sarcotubular activity as demonstrated with the light microscope, all three ATPases could be demonstrated within different regions of a given ultrathin section. These results undoubtedly reflect the gradient of fixation obtained in glutaraldehyde-preserved material. Sarcotubular ATPase activity usually occurred in better preserved regions of the section, and was, in addition, frequent at the edges of torn muscle fibers (Fig. 12). Where ultrastructural preservation was less adequate, myofibrillar ATPase activity prevailed (Fig. 13). Mitochondrial ATPase activity was less regular and had no apparent relationship to structural preservation. From our experience, it seems probable that certain of these enzymes splitting

FIGURE 1 Epon section, toluidine blue. Transverse sections of portions of several of the muscle fibers comprising the gas bladder of the toadfish. Mitochondria are densely stained and are confined primarily to thin circumferential rims (Mt_1) and to central cores of cytoplasm (Mt_2). The greater part of each fiber, which is less intensely stained, consists of alternating light and dark radial stripes which represent longitudinal myofibrillar sheets separated by sheets of the sarcoplasmic reticulum. × 1100.

FIGURE 2 ATPase, pH 7.2, cysteine. This transverse section was fixed in formol-calcium and then incubated for 4 hours. One muscle fiber occupies most of the photograph. Reaction product is deposited as fine radial lines corresponding to the radial array of the sarcoplasmic reticulum seen in Fig. 1. The superficial and central sarcoplasm is relatively unreactive. × 1800.

FIGURE 3 Electron micrograph showing portion of a fiber similar to that enclosed by the box in Fig. 1. This nearly transverse section shows a portion of a nucleus (*N*) and mitochondria (*Mt*) at the periphery. Radial myofibrillar sheets (*Mf*) converge toward the center of the fiber which is beyond the lower right of the photograph. They are separated by intervening membranes of the sarcoplasmic reticulum (*SR*). × 17,000.

FIGURE 4 Epon section, toluidine blue. Portions of two fibers are sectioned longitudinally. Transverse banding resembles that of other striated muscles. Mitochondria (Mt_1 and Mt_2) are located at the periphery and within central cores of cytoplasm. × 1100.

FIGURE 5 Electron micrograph of a longitudinal section perpendicular to the plane of the myofibrillar sheets. Membranes of the sarcoplasmic reticulum are seen in profile between myofibrils. Typical transverse bands are apparent, and triads of the sarcoplasmic reticulum (arrows) are located close to the A-I junctions. Note that mitochondria are absent from this region of the fiber. × 23,000.

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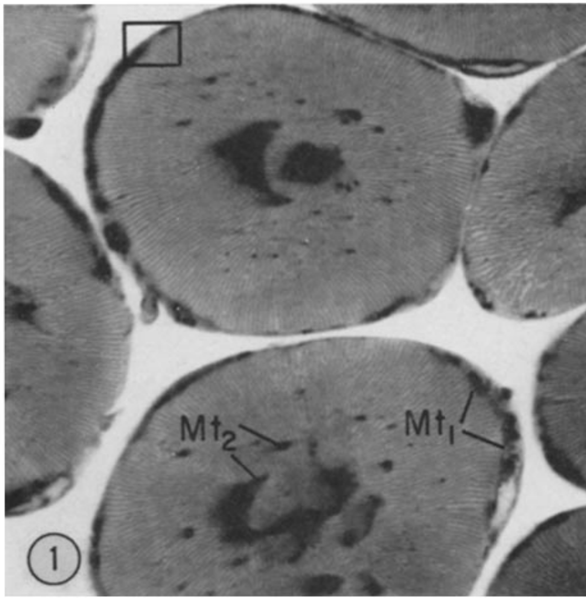

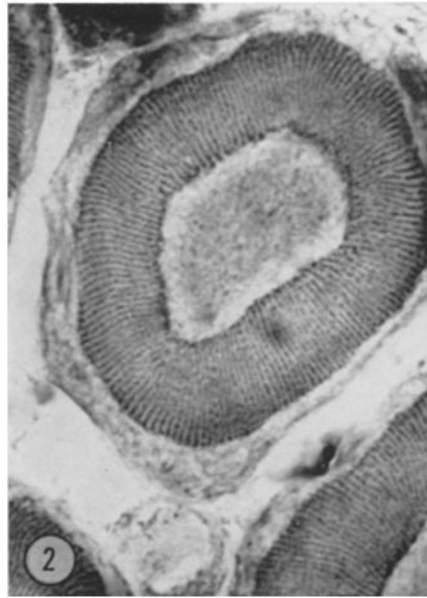

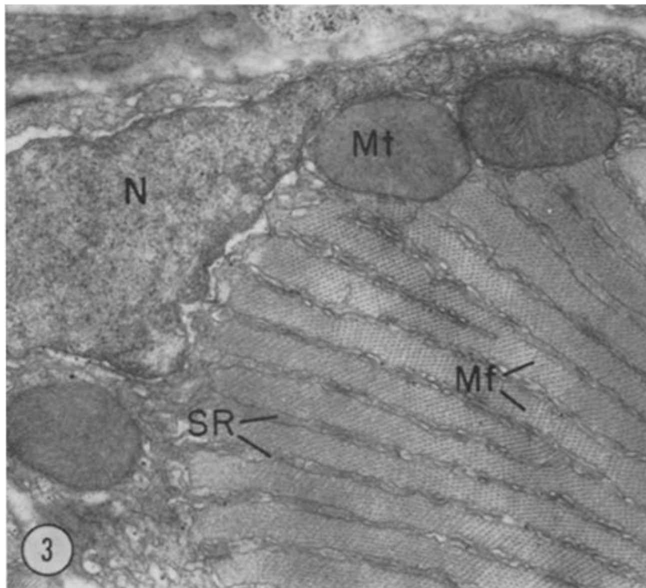

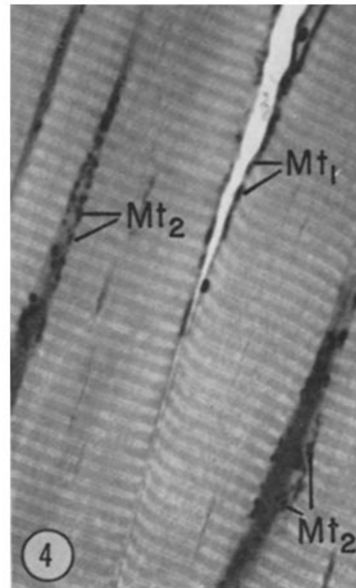

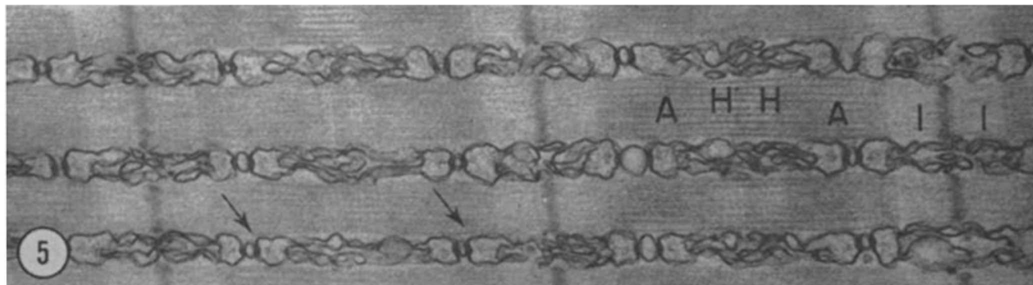

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ATP have a complex differential response to the slowly penetrating fixative, and that this results in non-uniform preservation of the three types of activity. In addition, the intense staining occurring at the cut edges of fibers (Fig. 12) suggests that there may be a barrier to the penetration of reagents under these conditions. When substrate was absent from the medium, the only deposits of lead that occurred were localized in the nuclei, particularly in association with chromatin material along the inner surface of the nuclear envelope. Thus, the nuclear staining is at least partly non-enzymic in origin. Staining elsewhere in the muscle cell is interpreted as reflecting enzymic activity. Those enzymic localizations which were obtained consistently will be described below.

In well preserved areas, sarcotubular ATPase activity was associated primarily with membranes at the level of the H bands, with terminal cisternae, and, to a lesser degree, with ill defined loci near the Z line. At the H band level, discrete foci of activity were easily identifiable on the outer surface of the membrane system; they were marked usually by mounds of lead phosphate (Figs. 14 and 15). Wherever this activity prevailed, these well defined deposits of reaction product were distributed at the regular intervals expected from the repetitive structure of the membrane system (Figs. 5 and 14). Since there was no visible sign of H band activity with the light microscope (Fig. 7), it is possible that this particular locus is one of low activity. The region of the terminal cisternae of the triads possessed sarcotubular ATPase activity also (Fig. 16). This activity most likely represents the ultrastructural definition of the two delicate lines of activity visible at the A-I junctions with the light microscope (Fig. 7). A significant feature of this ATPase localization is the general absence of activity at the intermediate component (Fig. 16).

Relatively large patches of myofibrillar ATPase activity were frequently encountered, and the reaction product was localized mainly in the A bands (Fig. 13). Only sparse and scattered deposits were present in the I bands. In such areas of high myofibrillar ATPase activity, it is probably significant that the intervening membranes appeared to be relatively inactive. This suggests a differential response of the various ATPases to the fixation procedure.

In summary, ATPases present in the sarcoplasmic reticulum, myofibrils, and mitochondria of a fish muscle have characteristics similar to those described earlier in the rat diaphragm. Favorable structural features have permitted more precise

FIGURE 6 ATPase, pH 7.2, cysteine, transverse section. Enzymic activity is associated with sites of central and peripheral mitochondria (*Mt*) as well as with radially arranged stripes of the sarcoplasmic reticulum. At the periphery of the fibers, the nuclei are seen in negative image. × 1100.

FIGURE 7 ATPase, pH 7.2, cysteine, longitudinal section. Enzymic activity is reflected as thin paired lines of reaction product which were shown, using polarized light, to be located approximately at the A-I junctions. This corresponds to the position of triads of the sarcoplasmic reticulum. Compare with Fig. 8. × 1800.

FIGURE 8 ATPase, pH 9.4, longitudinal section. Myofibrillar ATPase activity is located in the broad A band regions of the fiber. Compare with Fig. 7. × 1800.

FIGURE 9 ATPase, pH 7.2, *p*-hydroxymercuribenzoate, transverse section. The presence of a mercurial compound has abolished the enzymic activity of the radially distributed sarcoplasmic reticulum, but mitochondrial activity survives, indicating that the sarcotubular ATPase is sulfhydryl dependent. Compare with Fig. 6. × 1100.

FIGURE 10 ATPase, pH 7.2, magnesium omitted, transverse section. Enzymic activity of both mitochondria and the sarcoplasmic reticulum is diminished. × 1100.

FIGURE 11 ADP, pH 7.2, transverse section. No enzymic activity is present under these conditions. × 1100.

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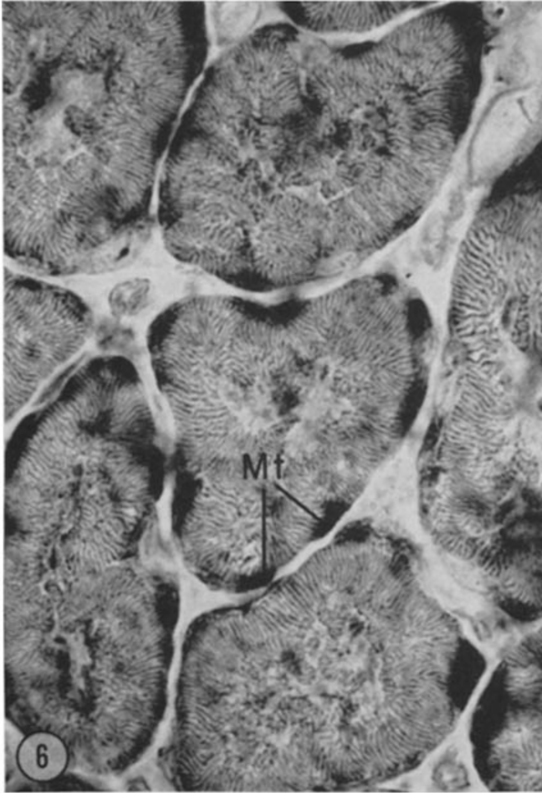

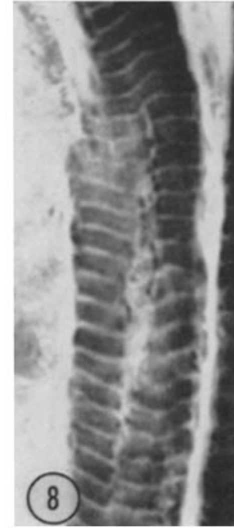

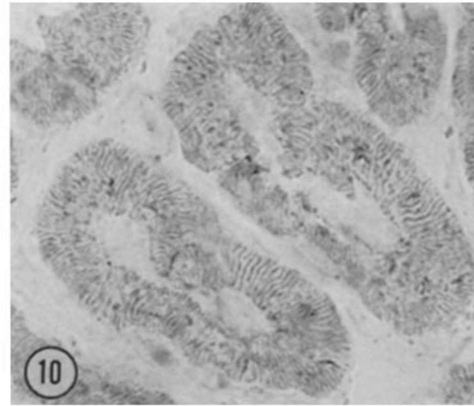

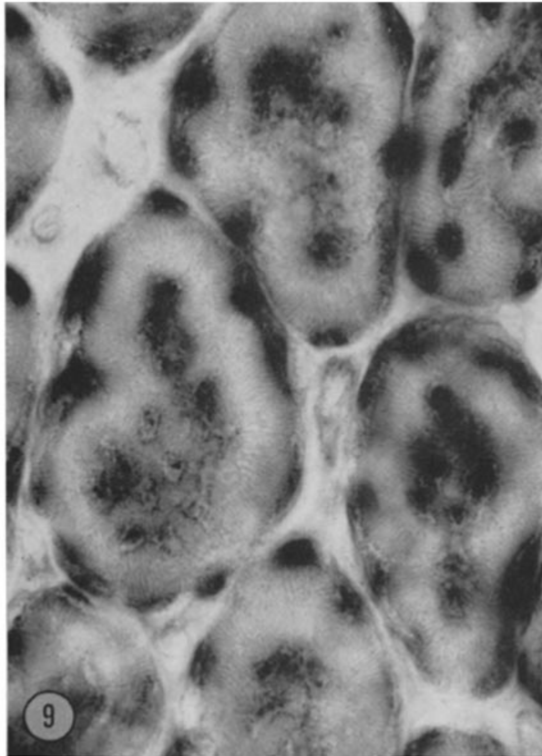

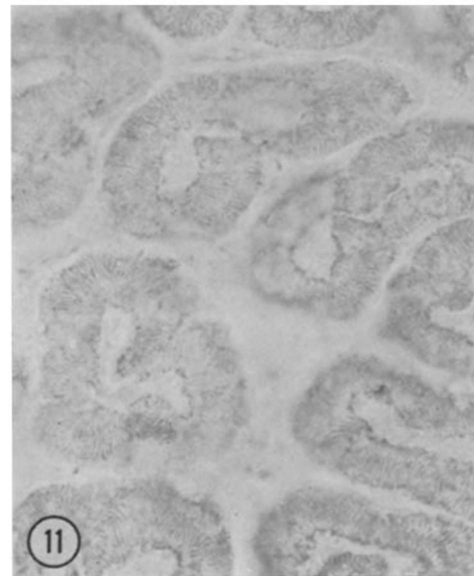

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identification of sarcotubular activity in the fish muscle. Since this sarcotubular ATPase activity is readily apparent at the level of the light microscope, its properties can be analyzed using unfixed frozen sections, preparations which approach the natural state. This enzyme is a true triphosphatase, and can be demonstrated at pH 7.2. It is, in this respect, similar to mitochondrial ATPase of both the rat and fish muscles. In contrast to mitochondrial ATPase, however, it is sulfhydryl dependent. Sarcotubular activity is apparent, at the ultrastructural level, at the H band as well as near the triads.

DISCUSSION

It is probable that ATPase in the sarcoplasmic reticulum of the toadfish muscle is, in some way, involved in the relaxation of the muscle following contraction. Some of the properties demonstrated are characteristic of an ATPase present in isolated preparations of the sarcoplasmic reticulum which also contain relaxing activity (Ebashi, 1961; Muscatello *et al.*, 1961; Ebashi and Lipmann, 1962; Muscatello *et al.*, 1962). In the present study, localization of sarcotubular ATPase was especially apparent in the region of the triads lateral to the intermediate component, and this corresponds to observations in mammalian cardiac muscle reported by Sommer and Spach (1964); Essner, Novikoff, and Quintana (1965); and Goldfischer, Essner, and Novikoff (1964). Additional ATPase activity has been demonstrated in the sarcoplasmic reticulum of the H band region of the fibers.

It is known that contraction of skeletal muscle is dependent on the release of calcium ion. Since membranes of the sarcoplasmic reticulum bind calcium by a mechanism dependent on the splitting of ATP (Hasselbach and Makinose, 1963), then an ATPase present in the sarcoplasmic reticulum might be involved in relaxation. Both physiological and morphological data suggest that transverse conduction of impulses into skeletal muscle fibers occurs at the level of the triad (Huxley and Taylor, 1958; Franzini-Armstrong and Porter, 1964; Huxley, 1964) and that calcium may become accumulated in terminal cisternae as a result of uptake through the intermediate com-

FIGURE 12 Electron micrograph, ATPase. Transverse section near cut edge of a fiber showing heavy deposition of lead phosphate primarily in the radial bands of the sarcoplasmic reticulum (*SR*) between myofibrils (*Mf*). This field includes only the A band region. Reaction product is sparse over the myofibrils. × 30,000.

FIGURE 13 Electron micrograph, ATPase. Transverse section of fiber from poorly fixed central region of block. Lead phosphate is deposited within myofibrils. Because the section is somewhat oblique, it is apparent that localization is primarily within the A band regions of these myofibrils. × 6000.

FIGURE 14 Electron micrograph, ATPase. Longitudinal section showing discrete lead phosphate deposits at regular intervals along membranes of the sarcoplasmic reticulum, primarily in the H band regions (*H*). × 21,000.

FIGURE 15 Electron micrograph, ATPase. Transverse section through the H band level of a fiber. Discrete accumulations of lead phosphate form mounds at the outer surface of the sarcotubular system at this level of the fiber. The longitudinal distribution of this H band enzyme is shown in Fig. 14. × 36,000.

FIGURE 16 Electron micrograph, ATPase. Longitudinal section showing enzymic activity associated with triads of the sarcoplasmic reticulum. At the right, the plane of section is perpendicular to sheets of myofibrils, so that components of the sarcoplasmic reticulum are seen in profile between them. Lead phosphate occurs near terminal cisternae (*TC*). At the left, the plane of section coincides with that of the myofibrils. Here terminal cisternae extend transversely across the surface of the myofibrillar sheet. Note that, in both areas, the intermediate component between cisternae is relatively free of lead phosphate (arrows). × 34,000.

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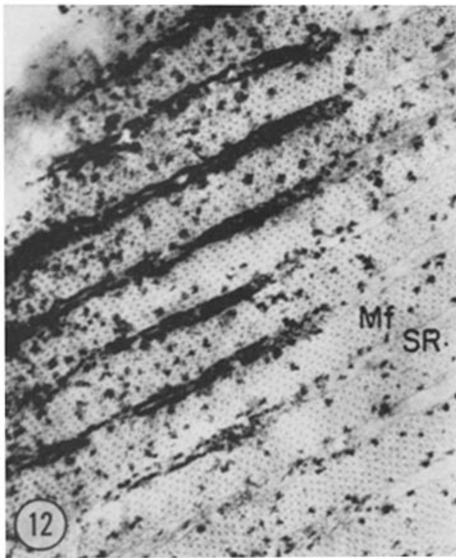

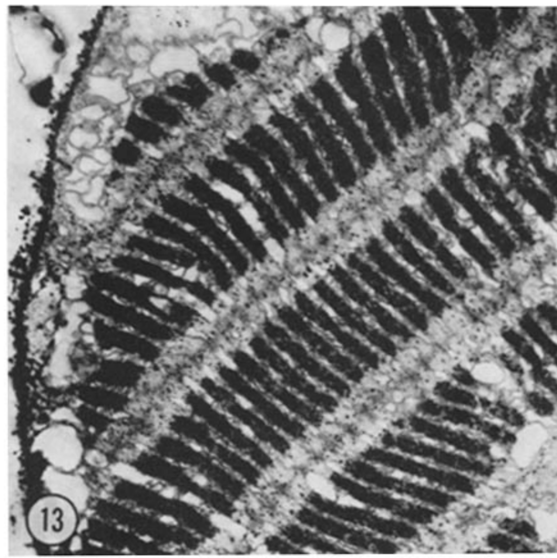

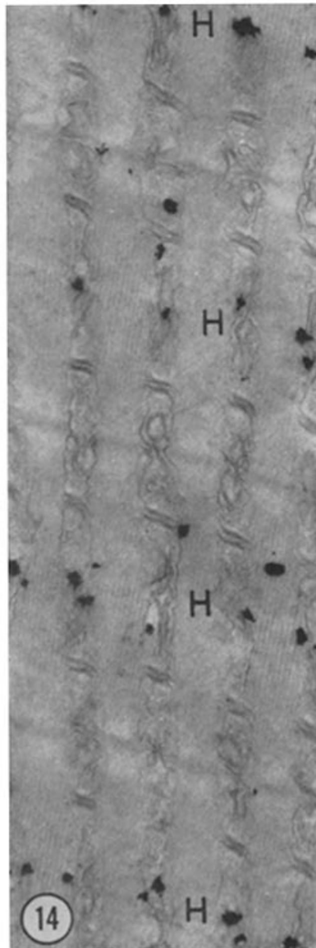

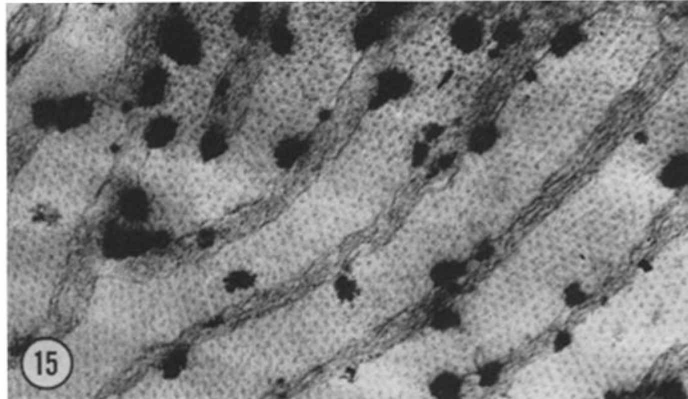

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ponent or T system (Constantin, Franzini-Armstrong, and Podolsky, 1965). The presence of an ATPase at the triads, especially at the terminal cisternae, might reflect active binding of calcium at this site, and might, therefore, account for the availability of calcium at the time of the initial stimulus for contraction. The same ATPase could account also for the subsequent rebinding of calcium, and thus relaxation of the muscle. Alternatively, a second sarcotubular ATPase, possibly that which is present at the H band, might be more specifically involved in the relaxation phase of the contraction cycle. The present study has shown that ATPase activity can be demonstrated at both of these sites.

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