NUCLEOSIDE PHOSPHATASE ACTIVITIES IN RAT CARDIAC MUSCLE

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ABSTRACT

Localizations of aldehyde-resistant nucleoside phosphatase activities in frozen sections of rat cardiac muscle have been studied by electron microscopy. Activities are higher after fixation with formaldehyde than with glutaraldehyde. After incubation with adenosine triphosphate or inosine diphosphate at pH 7.2, reaction product is found in the "terminal cisternae" or "transverse sacs" of the sarcoplasmic reticulum, which, together with the "intermediary vesicles" (T system), constitute the "dyads" or "triads." Reaction product is also present at the membranes of micropinocytotic vacuoles which apparently form from the plasma membrane of capillary endothelial cells and from the sarcolemma. In certain regions of the intercalated discs, reaction product is found within the narrow spaces between sarcolemmas of adjacent cells and within micropinocytotic vacuoles that seem to form from the sarcolemma. With inosine diphosphate, reaction product is also found in other parts of the sarcoplasmic reticulum. After incubation with cytidine monophosphate at pH 5, reaction product is present in the transverse sacs of sarcoplasmic reticulum, in micropinocytotic vacuoles in capillary endothelium, and in lysosomes of muscle fibers and capillaries. The possible significance of the sarcoplasmic reticulum phosphatases is discussed in relation to the role the reticulum probably plays in moving calcium ions and thereby controlling contraction and relaxation of the muscle fiber.

INTRODUCTION

In attempts to identify the sites in striated muscle at which excitation is coupled to contraction, attention has focused on the "triads" and "dyads." As described by Porter and Palade (39), these generally consist of a central "intermediary vesicle" and adjacent "terminal cisternae," the "transverse sacs" of endoplasmic reticulum. In the dyad a single transverse sac encloses the intermediary vesicle; in the triad the sacs lie on either side of the vesicle. The transverse sacs are continuous with the remaining channels of sarcoplasmic reticulum that course throughout the sarcomere, but no continuities with the "intermediary vesicle" (T system) have been reported.

The unique structure of the triads and dyads, their distribution in register with specific cross-striations (Z bands or I-A junctions, depending upon the muscle), and their higher degree of development in faster acting skeletal muscles (9, 10) have suggested that they serve as pathways for conducting impulses toward the myofibrils. This view is supported by physiological studies of A. F. Huxley and co-workers (20, 21). Myofibril contractions, to a depth of 10 μ, occur when areas coinciding with the positions of the triads are depolarized by electric stimulation. In lizard muscle the triads are located at the junction of the A and I bands (40), whereas in amphibian muscle

they are found at the Z bands (39). "Thus, in both frog and lizard muscle, the region which contracts as a single unit is centered on a triad although the location of the triads is very different in these two animals" (20).

The middle component, or T system, has received increasing attention and it now appears as the likely site where the coupling of excitation and contraction is initiated. Andersson-Cedergren (1), studying serial sections, showed that images of the intermediate vesicles in skeletal muscle are, in fact, sections through a convoluted transverse tubule that extends for a considerable distance through the muscle fiber and is in contiguity with the sarcolemma. It was proposed that the T system was the component involved in transverse impulse conduction. Actual continuity between T system and sarcolemma was first shown in cardiac muscle (32, 42) and subsequently in skeletal muscle (11, 12, 44, 45). There is thus a direct continuity between extracellular space and T system. Girardier *et al.* (13) have recently shown that, in the crayfish, current can flow between the interior of the muscle fiber and the periphery through the T system and that the T system membrane, adjacent to the sarcoplasmic reticulum, is largely or exclusively anion-permselective. They consider the membrane to contain a chloride battery and to be capable of accumulating locally cations, such as calcium, which could initiate excitation-contraction coupling.

Beginning with the studies of Kielley and Meyerhof on the magnesium-stimulated ATPase[1] (23) and those of Marsh on the relaxing factor (26), an extensive literature has led to the views that the microsomal fraction of skeletal muscle contains fragmented sarcoplasmic reticulum (5, 22, 30, 31), that the reticulum *in vivo* contains a pump mechanism moving calcium ions to and from the contractile elements, and that energy for the pump is derived from ATP dephosphorylation catalyzed by enzymes of the reticulum (5, 17, 27, 28, 31, 49). Until recently it has not been possible unequivocally to demonstrate such mechanisms in cardiac muscle, but it is now evident that the sarcoplasmic reticulum plays essentially similar roles in it and in skeletal muscle (3, 8, 50).

The heterogeneity of the microsomal fractions thus far employed precludes discrimination between any localizations that might be restricted to the transverse sacs and those in other regions of sarcoplasmic reticulum. Such resolution is now being achieved by electron microscope examination of tissue sections. The transverse sacs have been shown to accumulate calcium oxalate (16) and to possess ATPase and other phosphatase activities (7, 46, 47). The situation regarding the remainder of the sarcoplasmic reticulum is less clear.

MATERIALS AND METHODS

Thin slices of ventricle, from 250- to 275-gm Sprague-Dawley rats, were fixed in one of five ways: (*a*) immersion in cold 4 per cent formaldehyde with 1 per cent calcium chloride (2) overnight; (*b*) immersion in cold formaldehyde-calcium for 2 hours following 15 minutes of intraventricular perfusion with cold fixative; (*c*) immersion in cold 5 per cent glutaraldehyde with 0.1 M cacodylate buffer, pH 7.2 (41), for 30 minutes following 15 minutes of intraventricular perfusion; (*d*) immersion in osmium tetroxide–Veronal-acetate–sucrose (4) for 60 to 90 minutes; and (*e*) immersion in osmium tetroxide–Veronal-acetate–sucrose for 60 minutes following fixation as in (*c*) and washing overnight in cacodylate buffer.

Tissues fixed by procedure *d* or *e* were used for electron microscopy but not for demonstrating sites of phosphatase activities. For the latter, frozen sections of tissue fixed by procedure *a*, *b*, or *c* were prepared.

The formaldehyde-fixed tissues (*a*, *b*, above) were rinsed briefly in cold 7.5 per cent sucrose before sectioning. The glutaraldehyde-fixed tissue (*c*, above) was washed overnight in several changes of cacodylate buffer and rinsed briefly in sucrose. Frozen sections, 40 μ thick, were prepared on a Sartorius freezing microtome. Free-floating sections were incubated for 15, 30, and 60 minutes at 37°C in the following lead-containing media, with 5 per cent sucrose added: the magnesium-ATP medium of Wachstein and Meisel (48) at pH 7.2; the manganese-IDP medium of Novikoff and Goldfischer (36) at pH 7.2; the β-glycerophosphate medium of Gomori (15) at pH 5.0, or with CMP substituted for β-glycerophosphate (Novikoff, unpublished). The effects of removing Mg^{++} or Mn^{++} or of adding Ca^{++} were not studied.

Three types of control were used: (*a*) incubation of sections in substrate-free media in the presence of Mg^{++} at pH 7.2 and without added bivalent cations at pH 5; (*b*) immersion of sections in a solution of

[1] Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate, 5′-adenylic acid; ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; CMP, cytosine 5′-monophosphate, 5′-cytidylic acid; CMPase, 5′-cytidylic acid phosphatase; IDP, inosine 5′-diphosphate; IDPase, inosine 5′-diphosphatase; PCMB, parachloromercuribenzoate; TPP, thiamine pyrophosphate.

purified intestinal alkaline phosphatase[2] at 0°C for 15 minutes, and incubation, after brief rinses in several changes of sucrose, in the β-glycerophosphate medium at pH 7.2 for 30 and 60 minutes; and (*c*) incubation of sections for 15 minutes in β-glycerophosphate medium at pH 7.2, to which a much diluted solution of purified alkaline phosphatase was added dropwise, to produce a barely perceptible cloud of lead phosphate (33).

For electron microscope examination, the incubated sections were neither rinsed in dilute acid nor treated with ammonium sulfide (14). After incubation they were rinsed in 5 per cent sucrose, postfixed for 30 minutes in osmium tetroxide–sucrose (4), dehydrated in ethanol, cut into smaller pieces with a razor blade, and embedded in Epon 812 (24). Thin sections were prepared on a Porter-Blum microtome and studied in the Siemens Elmiskop I or RCA EMU 3B microscope.

RESULTS

Unincubated Tissue

In rat cardiac muscle, as described by Porter and Palade (39), dyads are more common than triads and each consists of an "intermediary vesicle" (T system) surrounded by a "U shaped element directed toward the Z line." Dyads and triads are continuous with a "complex network of interconnected tubules and vesicles" forming strands that appear randomly oriented "except opposite the I band where they tend to be longitudinal relative to the fibril."

Incubated Controls

Controls incubated in the absence of substrate and those exposed to slowly forming phosphate ions are particularly important with structures like sarcoplasmic reticulum where metallic ions may well be present and capable of attracting enzymically liberated phosphate. Controls without substrate are completely negative. Those exposed to phosphate are also negative, except for a light precipitate on a very few triads located close to capillaries. At pH 7.2, capillary endothelium hydrolyzes β-glycerophosphate in the medium, and some of the resulting lead phosphate apparently diffuses to nearby structures. We preferred this minor complication to inactivation of the section, since the inactivating agent, *e.g.* heat (33), could change the phosphate-adsorbing capacity of cell structures. The significant finding in the controls is the absence of lead phosphate from the vast majority of dyads and triads. In addition, it appears that the minor diffusion occurring in controls may not occur when sections are incubated in the usual fashion. When glutaraldehyde-fixed sections are incubated with CMP at pH 5.0 (see below), endothelial cell micropinocytotic vacuoles show much reaction product, yet adjacent dyads or triads are without any reaction product (Figs. 13 and 14).

Sections exposed to alkaline phosphatase solution before incubation are consistently negative.

Incubation with ATP or IDP at pH 7.2

Formaldehyde-fixed sections are more active than glutaraldehyde-fixed ones, those briefly fixed (procedure *b*, in Materials and Methods) showing more activity than those fixed overnight (procedure *a*). Fine structure preservation is considerably better after glutaraldehyde than formaldehyde fixation. This is most evident in the mitochondria, but applies to a lesser extent to I bands and dyad or triad structures (Figs. 1 and 2).

With ATPase as substrate, much reaction product is present in all transverse sacs but none is present in any elements of the T system (Figs. 1 to 5). Infrequently, a small region of transverse sac is without reaction product (Fig. 5). Almost always, reaction product is within the cavities as well as upon the limiting membranes of the transverse sacs; occasionally the product is concentrated along the membrane (Fig. 5). It is often not possible, in the incubated material, to identify the sarcoplasmic reticulum elements away from the dyads and triads. When other regions are seen, they show no significant accumulation of reaction product.

The accumulations within the transverse sacs with IDP as substrate are similar to those with ATP. In contrast to ATP, with IDP as substrate reaction product is also present in elements of sarcoplasmic reticulum (Fig. 10).

With both substrates, reaction product is also found in pinocytotic vacuoles near the sarcolemma (Fig. 12) and in the endothelial cells, where it is localized mainly at the vacuole membranes (Fig. 11). Particularly with ATP, low levels of reaction product are present in certain regions

[2] The alkaline phosphatase, purchased from Sigma Chemical Co., was dissolved in water at a concentration of 1 mg/ml, and 0.4 ml was added to 2 ml of 7.5 per cent sucrose.

of the intercalated discs, in small foci within the narrow spaces separating adjacent cells (Fig. 12).

Because of their higher contrast, only lead-stained sections are included in the illustrations. It should therefore be noted that the contrast of lead phosphate, the reaction product, is essentially unaltered by such lead staining.

Incubation with CMP at pH 5.0

Much reaction product is found in the transverse sacs of dyads and triads after incubation of formaldehyde-fixed sections with CMP at pH 5.0. In contrast, after glutaraldehyde fixation there is no reaction product accumulation, up to 60 minutes of incubation. With either fixative, no reaction product is seen in the rest of the sarcoplasmic reticulum or in the T system (Figs. 8 and 9).

After either formaldehyde or glutaraldehyde fixation, reaction product is found in the lysosomes of both endothelial cells and muscle fibers (Fig. 14) and in the pinocytotic vacuoles of capillary endothelial cells (Figs. 13 and 14). There may be much product in the pinocytotic vacuoles, yet nearby dyads or triads show none.

DISCUSSION

The observations recorded above clearly demonstrate an enzymatic difference between the two components of dyads or triads in cardiac muscle: in aldehyde-fixed tissue, ATPase and other phosphatase activities are not demonstrable in the T system but are readily shown in the transverse sacs, the sarcoplasmic reticulum component. Similar findings have been reported by Sommer and Spach (46, 47).

The phosphatase activities in the rest of the sarcoplasmic reticulum are less clear. With ATP we find no reaction product other than that in the transverse sacs. On the other hand, Sommer and Spach (47) show one figure, at fairly high magnification, with reaction product in reticulum elements removed from these sacs. Because glutaraldehyde fixation was used without perfusion, there is a possibility that the area shown is from a relatively unfixed region into which the glutaraldehyde had not fully penetrated (14). The phosphatases elsewhere in the reticulum may be more sensitive to fixatives.

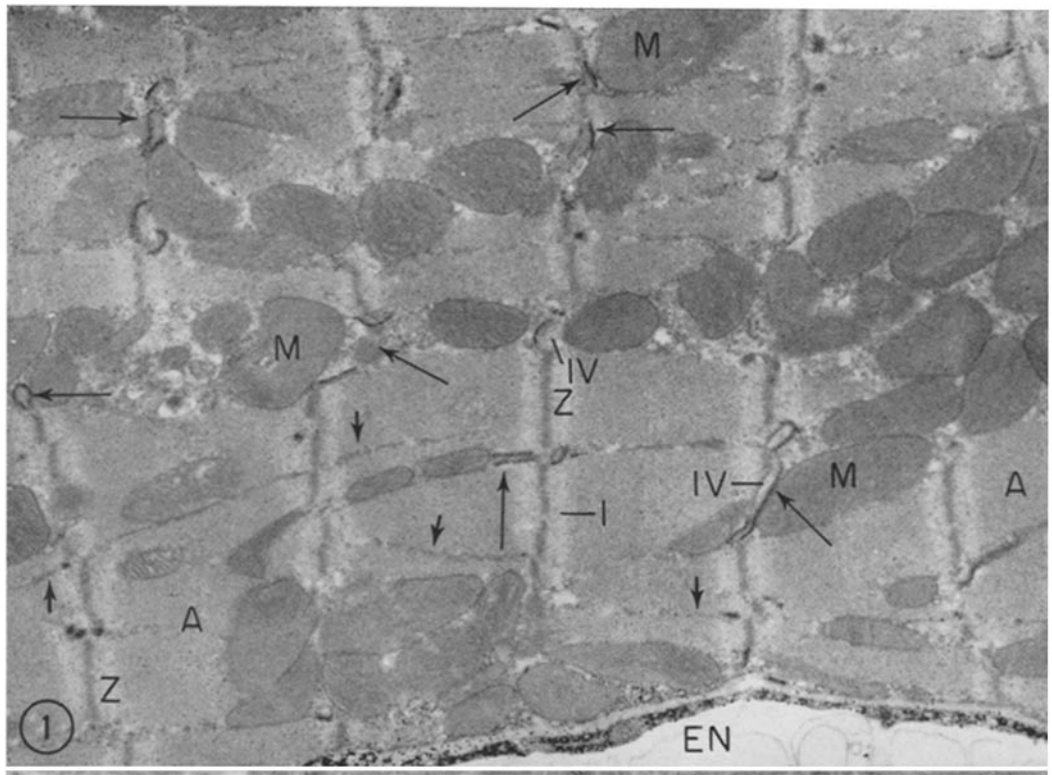
It is recognized that interpretation of negative findings in sections, particularly with fixed material, is difficult. Fixative may inhibit an enzyme activity, particularly if present at a low level; lead ions or other components of the medium may also be inhibitory. The incubation periods used in the current studies are relatively long, up to 60 minutes. Nevertheless, the possibility cannot be excluded that still longer periods might have revealed activity elsewhere.

It is, therefore, necessary to qualify the suggestion, from these staining observations, that an enzymic heterogeneity exists in the sarcoplasmic reticulum. Reported observations with unfixed tissue are limited to light microscopy and are not consistent. Padykula and Gauthier (38) find a magnesium-activated ATPase activity in the reticulum of rat diaphragm muscle. In contrast, in human skeletal muscle Engel (6) finds activity only in those areas of reticulum included in the triads.

Incubation with CMP at pH 5.0 gives results similar to those with ATP at pH 7.2. But in sections incubated with IDP at pH 7.2, reaction product is found in areas of sarcoplasmic reticu-

FIGURE 1 Glutaraldehyde-perfused frozen section incubated for 60 minutes with ATP at pH 7.2. This low-magnification, longitudinal section shows typical deposits of reaction product in the transverse channels of sarcoplasmic reticulum (long, unmarked arrows) of dyads and triads. The intermediary vesicles (*IV*) are negative. Short arrows indicate sarcoplasmic reticulum without accumulated reaction products. Reaction product is also present in a portion of capillary endothelium (*EN*) in the lower part of the micrograph. A slight degree of random precipitate is found throughout the section. Note Z, A, and I bands, and mitochondria (*M*). Unstained. × 15,000.

FIGURE 2 Formaldehyde-perfused frozen section incubated for 15 minutes with ATP at pH 7.2. This longitudinal section, like that shown in Fig. 1, shows reaction product only in the transverse sacs (unmarked arrows) of dyads and triads. As in Fig. 1, the intermediary vesicles (*IV*) and mitochondria (*M*) are negative. The random precipitate distributed throughout the section is more evident than in Fig. 1. *Z*, Z bands. Unstained. × 20,000.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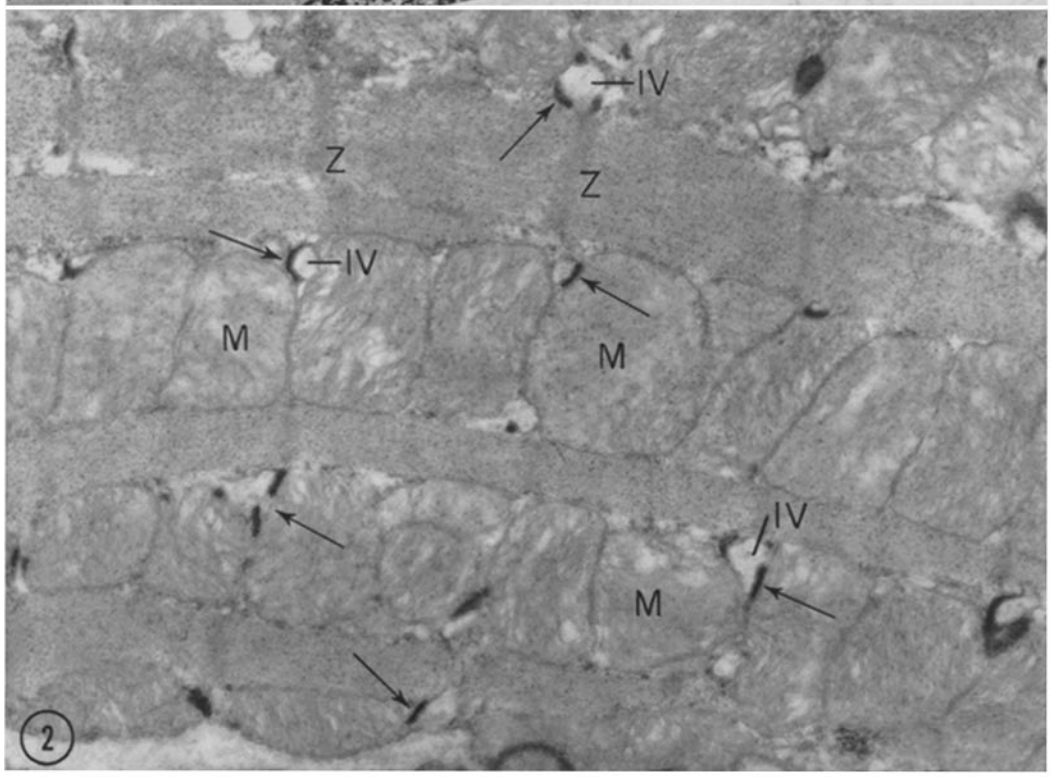
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lum away from dyads or triads as well as in the transverse sacs. No attempt was made to determine whether this is due to another enzyme, the nucleoside diphosphatase, found in many, but not all, cell types studied in our laboratory (35, 37).

Another uncertainty stems from the often inadequate preservation, in the incubated sections, of the sarcoplasmic reticulum elements away from dyads or triads.

The somewhat equivocal nature of the findings regarding the rest of the sarcoplasmic reticulum is unfortunate in light of a developing interest in localized biochemical differences along the reticulum. A. F. Huxley, in a recent review (19), discusses the "unexpected degree of heterogeneity of the reticulum." He summarizes the work of Hill (18) that suggests the concentration of ATP and creatine phosphate in restricted areas of the reticulum. We have already noted that Hasselbach (16) finds calcium oxalate concentrated in the transverse sacs and not elsewhere in the reticulum.

Uncertainty regarding the rest of the sarcoplasmic reticulum does not detract from the unequivocal demonstration of ATP dephosphorylation in the transverse sacs. These elements, lying adjacent to the T system where electrical and chemical excitatory events may be expected (13), are likely to be involved in coupling contraction with excitation. Before discussing their possible roles in such coupling, we should consider the nature of their phosphatase activities. Our experiments, with only three substrates and two pH's, are insufficient for delineating the number of phosphatases involved. Sommer and Spach (47) show reaction product in the transverse sacs after incubation with ADP at pH 7.2. They state that similar accumulations are seen with all substrates tested: ATP, ADP, IDP, AMP, TPP, and β-glycerophosphate. They find the ATP-dephosphorylating activities insensitive to PCMB and other inhibitors of SH-dependent ATPases. Both the wide substrate range and the insensitivity to SH inhibitors would appear to suggest that the ATP-dephosphorylating activities visualized in these sections are not due to an ATPase like that thought to be involved in monovalent ion transport (43).

Several relevant facts should be noted: (*a*) Fixation may well alter the sensitivities of ATP-dephosphorylating enzymes to activators and inhibitors (34). (*b*) The calcium pump mechanism has not yet been shown to be sensitive to SH inhibitors; the work of Maruyama (29) indicates that the microsomal enzyme of insect muscle is relatively insensitive to such inhibitors. (*c*) Except for ADP, the dephosphorylating capacities of isolated microsomes seem not to have been tested with nucleoside diphosphates or monophosphates (all nucleoside *tri*phosphates are hydrolyzed, references 16, 27). (*d*) There is evidence from unpublished work referred to by Martonosi and Feretos (28) that at least two ATP-dephosphorylating enzymes are present in skeletal muscle microsomes, only one of which is related to the calcium pump.

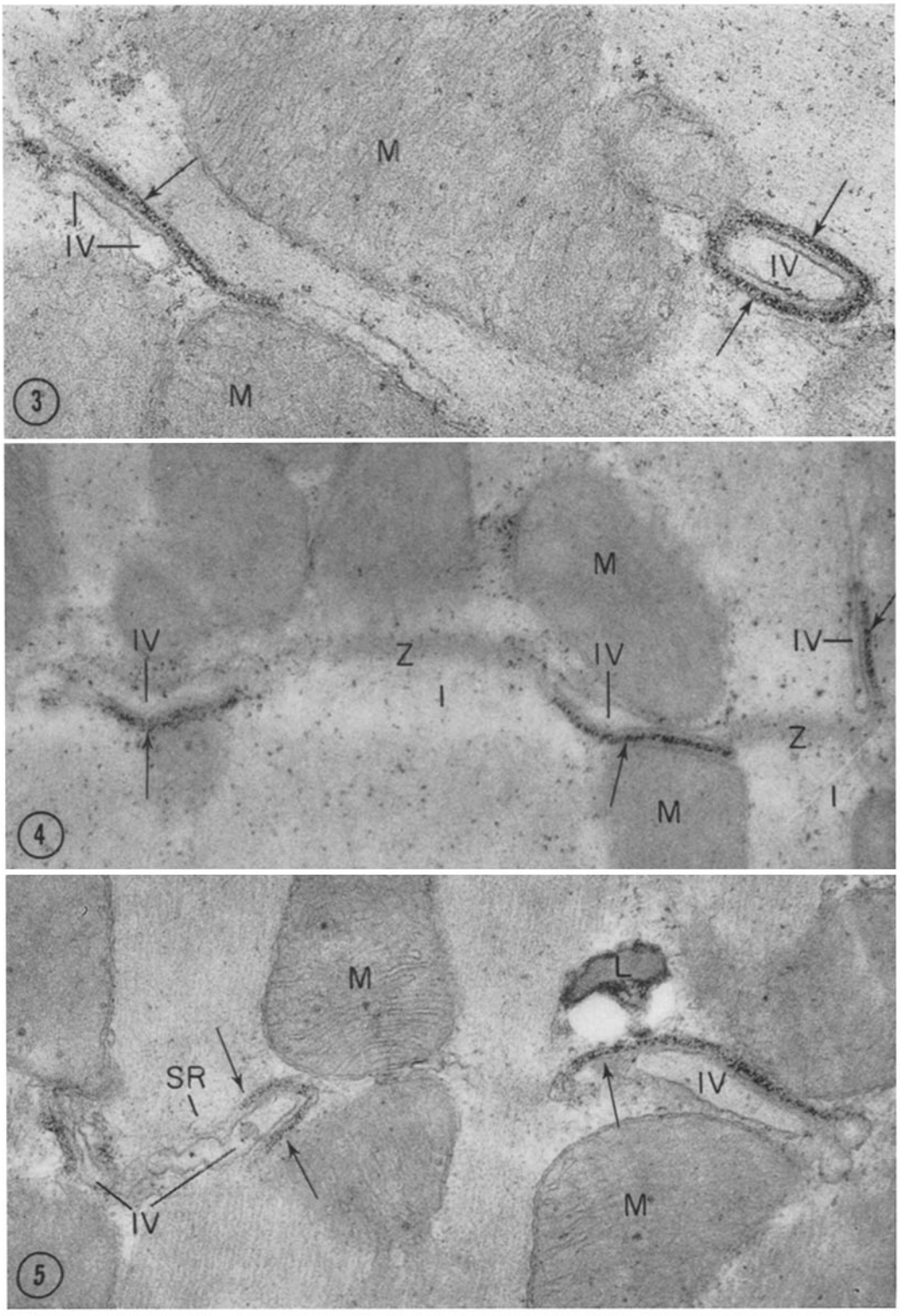
Their ability to dephosphorylate ATP, as well as their position adjacent to the T system, suggest that the transverse sacs have a role in an early event in the coupling of contraction with excita-

FIGS. 3 to 5, glutaraldehyde-perfused frozen sections incubated for 60 minutes with ATP at pH 7.2.

FIGURE 3 A dyad is seen at the left and probably a dyad (possibly a triad) at the right. Only their outer components, the transverse sacs (unmarked arrows), show reaction product. The inner components or intermediary vesicles (*IV*) are negative. *M*, mitochondria. Lead staining. × 69,000.

FIGURE 4 Reaction product (arrows) is restricted to the sarcoplasmic reticulum portion of the dyad; none is present in the intermediary vesicles (*IV*). The Z and I bands and mitochondria (*M*) show scattered random precipitate. Unstained. × 42,000.

FIGURE 5 Reaction product (arrows) is restricted to the sarcoplasmic reticulum portion of the dyads; none is present in the intermediary vesicles (*IV*). Arrows point to region where precipitate is on the membrane. A short segment without reaction product is seen at *SR*. Mitochondria (*M*) and a partially extracted lipid droplet (*L*) are also negative. Lead staining. × 51,000.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tion. It is considered that the energy driving the calcium pump is derived from ATP dephosphorylation, and that the movement of calcium ions into and out of the sarcoplasmic reticulum may cause cyclic variations in the concentration of these ions in the milieu surrounding the contractile elements such as to determine the contraction or relaxation of the muscle fiber (5, 17, 27, 28, 31, 50). As already noted, Hasselbach (16) finds calcium accumulating in the same elements in which the dephosphorylating activities are concentrated, the transverse sacs.

Phosphatases involved in ion pumping are generally considered integral parts of membrane structures. It is, however, only on rare occasions that we do not find reaction product in the cavities as well as on the delimiting membranes of the transverse sacs. However, as discussed elsewhere (14), too little is known regarding local factors that may serve as possible foci of lead phosphate precipitation to permit evaluation of this finding. In addition, it would have been desirable to employ shorter incubation times than those used in the current studies (14). Although the cytochemical evidence cannot be said to establish the localization of the phosphatase or phosphatases at or near the membrane, it is consistent with it.

Finally, a brief comment seems in order regarding CMPase activity at pH 5.0 in the micropinocytotic vacuoles of capillary endothelial cells. To our knowledge this is the first report of such acid phosphatase activity in such vacuoles. A wide variety of cells show identical localizations of activity at this pH with CMP and glycerophosphate as substrates (Novikoff, unpublished). It is thus likely that non-specific acid phosphatase hydrolyzes both substrates.

The presence of both ATPase and IDPase activities at pH 7.2 in these vacuoles is consistent with the report of Marchesi and Barrnett (25).

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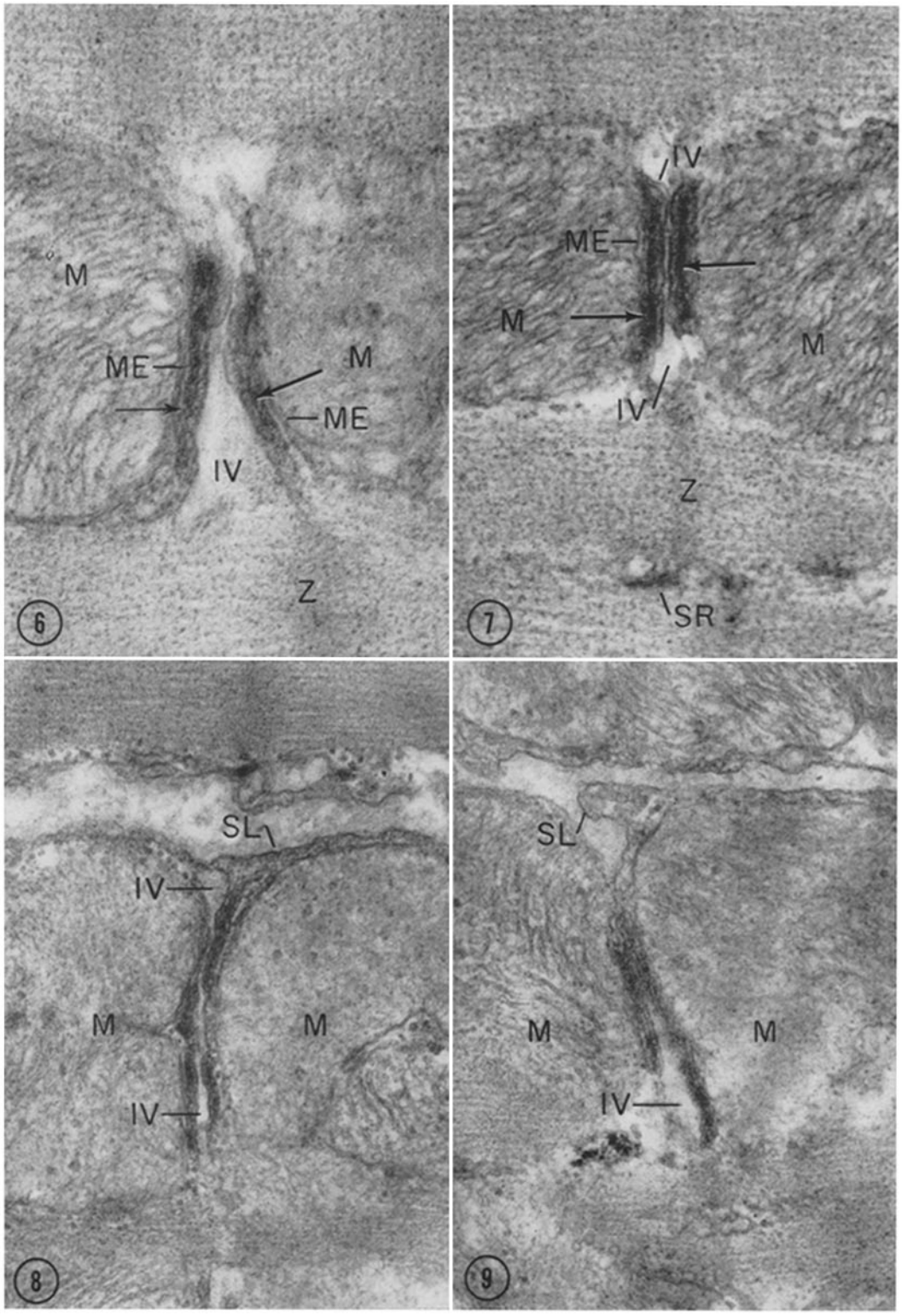
REFERENCES

1. ANDERSSON-CEDERGREN, E., Ultrastructure of motor end plate and sarcoplasmic components of mouse skeletal muscle fiber as revealed by three-dimensional reconstruction from serial sections, *J. Ultrastruct. Research*, 1959, **1**, suppl.
2. BAKER, J. R., The histochemical recognition of lipine, *Quart. J. Micr. Sc.*, 1946, **87**, 441.
3. BERNE, R. M., Intracellular localization of the skeletal-muscle relaxing factor, *Biochem. J.*, 1962, **83**, 364.
4. CAULFIELD, J. B., Effects of varying the vehicle for OsO_4 in tissue fixation, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 827.
5. EBASHI, S., and LIPMANN, F., Adenosine triphosphate-linked concentration of calcium ions in

FIGURES 6 AND 7 Formol-calcium-fixed (overnight) frozen sections incubated for 30 minutes with IDP at pH 7.2. Reaction product (arrows) is present in the transverse sacs of the sarcoplasmic reticulum and in sarcoplasmic reticulum (*SR* Fig. 7), away from the sacs but not in the intermediary vesicles (*IV*). Portions of mitochondria (*M*) and their limiting membranes (*ME*) are evident. The positions of the Z bands are indicated. Lead staining. Fig. 6, × 60,000; Fig. 7, × 46,000.

FIGURES 8 AND 9 Formol-calcium-fixed (overnight) frozen sections incubated for 30 minutes with CMP at pH 5.0. Reaction product is evident in the transverse sacs of the sarcoplasmic reticulum. The triads (or dyads?) in these figures lie close to the sarcolemma (*SL*). The continuity between membranes of intermediary vesicles (*IV*) and sarcolemma (*SL*) is suggested (see references 32, 42). Mitochondria are indicated by *M*. Lead staining. Fig. 8, × 34,000; Fig. 9, × 36,000.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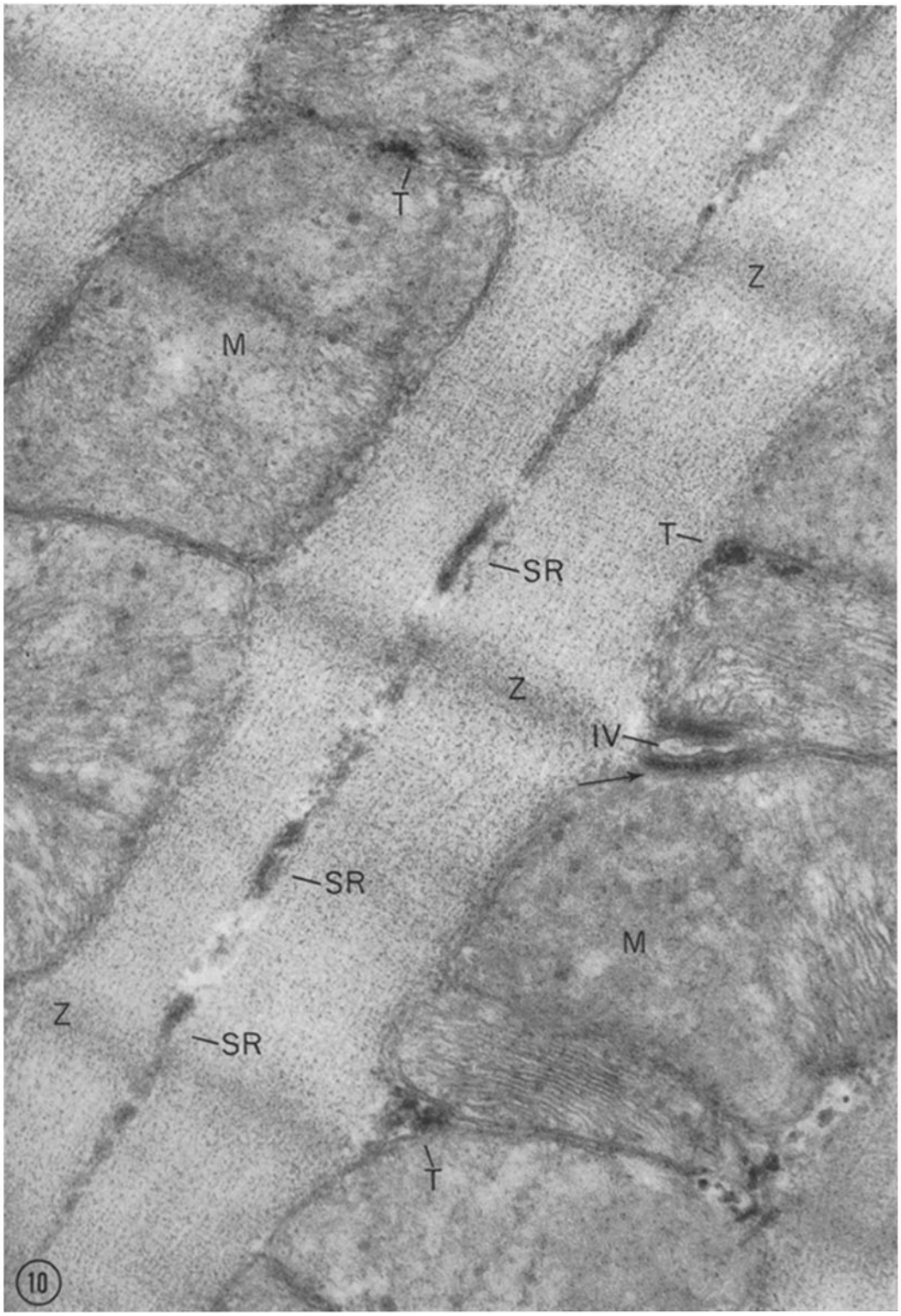
a particulate fraction of rabbit muscle, *J. Cell Biol.*, 1962, **14,** 389.

6. ENGEL, W. K., Adenosine triphosphatase of sarcoplasmic reticulum triads and sarcolemma identified histochemically, *Nature*, 1963, **200,** 588.
7. ESSNER, E., and QUINTANA, N., Nucleoside phosphatase activities in sarcoplasmic reticulum and other structures of rat myocardium, *J. Cell Biol.*, 1963, **19,** 22A.
8. FANBURG, B., FINKEL, R. M., and MARTONOSI, A., The role of calcium in the mechanism of relaxation of cardiac muscle, *J. Biol. Chem.*, 1964, **239,** 2298.
9. FAWCETT, D. W., The sarcoplasmic reticulum of skeletal and cardiac muscle, *Circulation*, 1961, **24,** 336.
10. FAWCETT, D. W., and REVEL, J. P., The sarcoplasmic reticulum of a fast-acting fish muscle, *J. Biophysic. and Biochem. Cytol.*, 1961, **10,** No. 4, suppl., 89.
11. FRANZINI-ARMSTRONG, C., Sarcolemmal invaginations and the T system in skeletal muscle fibers, *J. Cell Biol.*, 1963, **19,** 24A.
12. FRANZINI-ARMSTRONG, C., and PORTER, K. R., Sarcolemma invaginations and the *T*-system in fish skeletal muscle, *Nature*, 1964, **202,** 355.
13. GIRARDIER, L., REUBEN, J. P., BRANDT, P. W., and GRUNDFEST, H., Evidence for anion-permselective membrane in crayfish muscle fibers and its possible role in excitation-contraction coupling, *J. Gen. Physiol.*, 1963, **47,** 189.
14. GOLDFISCHER, S., ESSNER, E., and NOVIKOFF, A. B., The localization of phosphatase activities at the level of ultrastructure, *J. Histochem. and Cytochem.*, 1964, **12,** 72.
15. GOMORI, G., Microscopic Histochemistry, Principles and Practice, Chicago, University of Chicago Press, 1952.
16. HASSELBACH, W., Relaxation and the sarcotubular calcium pump, *Fed. Proc.*, 1964, **23,** 909.
17. HASSELBACH, W., and MAKINOSE, M., ATP and active transport, *Biochem. and Biophysic. Research Com.*, 1962, **7,** 132.
18. HILL, D. K., The location of adenine nucleotide in the striated muscle of the toad, *J. Cell Biol.*, 1964, **20,** 435.
19. HUXLEY, A. F., Muscle, *Ann. Rev. Physiol.*, 1964, **26,** 131.
20. HUXLEY, A. F., and STRAUB, R. W., Local activation and interfibrillar structures in striated muscle, *J. Physiol.*, 1958, **143,** 40-P.
21. HUXLEY, A. F., and TAYLOR, R. E., Local activation of striated muscle fibers, *J. Physiol.*, 1958, **144,** 426.
22. HUXLEY, H. E., Muscle cells, *in* The Cell; Biochemistry, Physiology, Morphology, (J. Brachet and A. E. Mirsky, editors), New York, Academic Press, 1960, 365.
23. KIELLEY, W. W,. and MEYERHOF, O., Studies on adenosinetriphosphatase of muscle. II. A new magnesium-activated adenosinetriphosphatase, *J. Biol. Chem.*, 1948, **176,** 591.
24. LUFT, J. H., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
25. MARCHESI, V. T., and BARRNETT, R. J., The demonstration of enzymatic activity in pinocytic vesicles of blood capillaries with the electron microscope, *J. Cell Biol.*, 1963, **17,** 547.
26. MARSH, B. B., A factor modifying muscle fibre synaeresis, *Nature*, 1951, **167,** 1065.
27. MARTONOSI, A., and FERETOS, R., Sarcoplasmic reticulum. I. The uptake of Ca^{++} by sarcoplasmic reticulum fragments, *J. Biol. Chem.*, 1964, **239,** 648.
28. MARTONOSI, A., and FERETOS, R., Sarcoplasmic reticulum. II. Correlation between adenosine triphosphatase activity and Ca^{++} uptake, *J. Biol. Chem.*, 1964, **239,** 659.
29. MARUYAMA, K., Studies on adenosinetriphosphatases of various insect muscles, *J. Fac. Sc. Univ. Tokyo, Sect. IV*, 1954, **7,** 231.
30. MUSCATELLO, U., ANDERSSON-CEDERGREN, E., AZZONE, G. F., and VON DER DECKEN, A., The sarcotubular system of frog skeletal muscle. A morphological and biochemical study, *J. Biophysic. and Biochem. Cytol.*, 1961, **10,** No. 4, suppl., 201.
31. NAGAI, T., MAKINOSE, M., and HASSELBACH, W., Der physiologische Erschlaffungsfaktor und

FIGURE 10 Formol-calcium–fixed (overnight) frozen section incubated for 30 minutes with IDP at pH 7.2. Reaction product is present in the transverse sacs of the sarcoplasmic reticulum. One triad is shown clearly at the unmarked arrow; three other sites, marked *T*, probably represent oblique sections of transverse sacs. Reaction product is also found in longitudinal elements of the sarcoplasmic reticulum (*SR*). Mitochondria (*M*) and Z bands are indicated. Lead staining. × 60,000.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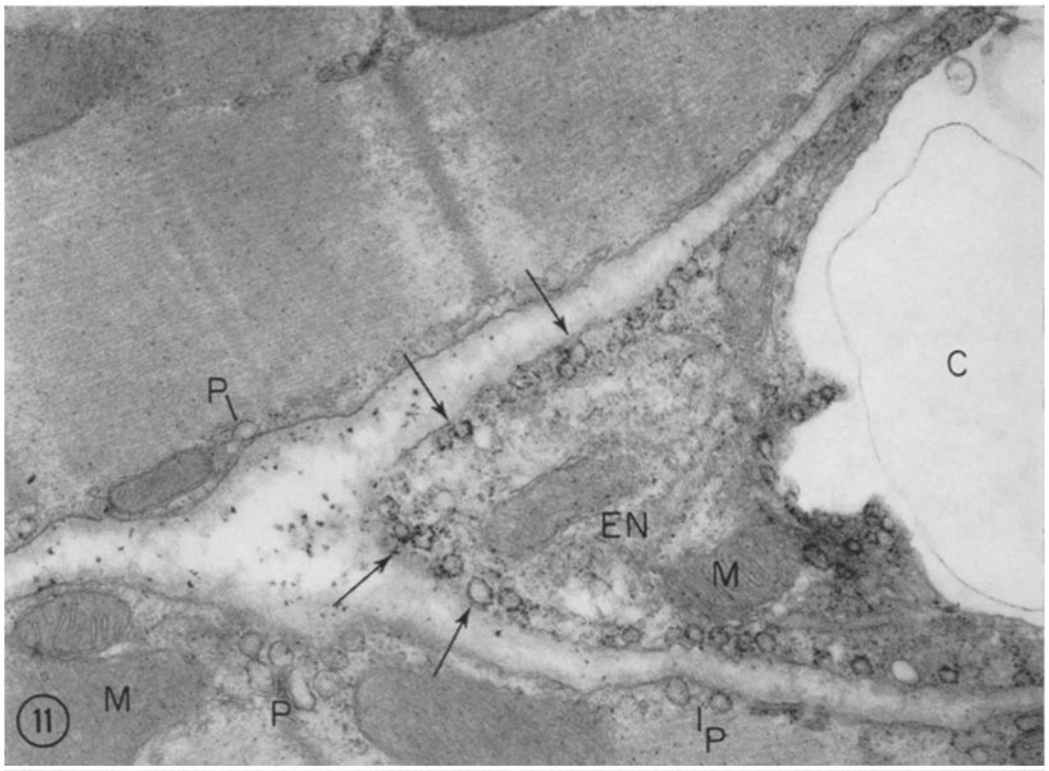
die Muskelgrana, *Biochim. et Biophysica Acta*, 1960, **43**, 223.

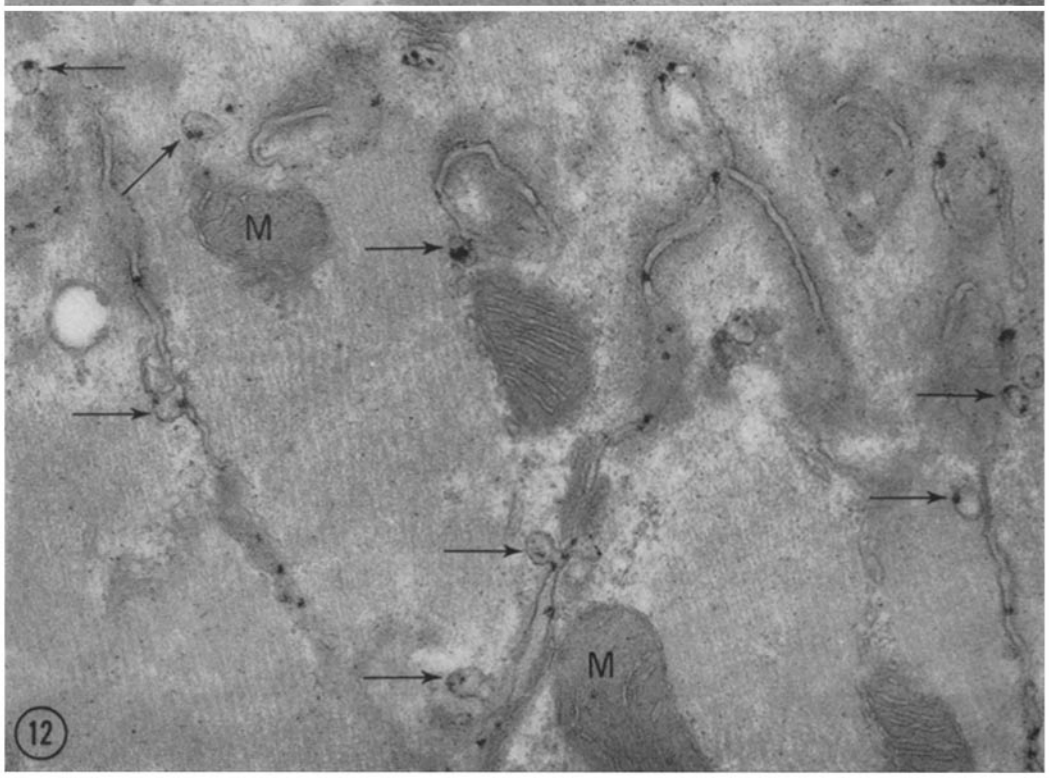
32. NELSON, D. A., and BENSON, E. S., On the structural continuities of the transverse tubular system of rabbit and human myocardial cells, *J. Cell. Biol.*, 1963, **16**, 297.
33. NOVIKOFF, A. B., The validity of histochemical phosphatase methods on the intracellular level, *Science*, 1951, **113**, 320.
34. NOVIKOFF, A. B., DRUCKER, J., SHIN, W.-Y., and GOLDFISCHER, S., Further studies of the apparent adenosinetriphosphatase activity of cell membranes in formol-calcium-fixed tissues, *J. Histochem. and Cytochem.*, 1961, **9**, 434.
35. NOVIKOFF, A. B., ESSNER, E., GOLDFISCHER, S., and HEUS, M., Nucleosidephosphatase activities of cytomembranes, *Symp. Internat. Soc. Cell Biol.*, 1962, **1**, 149.
36. NOVIKOFF, A. B., and GOLDFISCHER, S., Nucleosidediphosphatase activity in the Golgi apparatus and its usefulness for cytological studies, *Proc. Nat. Acad. Sc.*, 1961, **47**, 802.
37. NOVIKOFF, A. B., and HEUS, M., A microsomal diphosphatase, *J. Biol. Chem.*, 1963, **238**, 710.
38. PADYKULA, H. A., and GAUTHIER, G. F., Cytochemical studies of adenosine triphosphatases in skeletal muscle fibers, *J. Cell Biol.*, 1963, **18**, 87.
39. PORTER, K. R., and PALADE, G. E., Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle cells, *J. Biophysic. and Biochem. Cytol.*, 1957, **3**, 269.
40. ROBERTSON, J. D., Some features of the ultrastructure of reptilian skeletal muscle, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 369.
41. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.*, 1963, **17**, 19.
42. SIMPSON, F. D., and OERTELIS, S. J., The fine structure of sheep myocardial cells; sarcolemma invaginations of the transverse tubular system, *J. Cell Biol.*, 1962, **12**, 91.
43. SKOU, J. C., Studies on the $Na^+ + K^+$ activated ATP hydrolyzing enzyme system, *Biochem. and Biophysic. Research Com.*, 1963, **10**, 79.
44. SMITH, D. S., The structure of insect fibrillar flight muscle. A study made with special reference to the membrane systems of the fiber, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, No. 4, suppl., 123.
45. SMITH, D. S., The organization of the flight muscle in a dragonfly, *Aeshna* sp. (Odonata). *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 119.
46. SOMMER, J. R., and SPACH, M., Electronmicroscopic localization of ATPase in myofibrils and sarcoplasmic reticulum of normal and abnormal dog hearts, *Fed. Proc.*, 1963, **22**(I), 195.
47. SOMMER, J. R., and SPACH, M., Electronmicroscopic localization of adenosinetriphosphatase in myofibrils and sarcoplasmic membranes of cardiac muscle of normal and abnormal dogs, *Am. J. Pathol.*, 1964, **44**, 491.
48. WACHSTEIN, M., and MEISEL, E., Histochemistry of hepatic phosphatases at a physiological pH with special reference to the demonstration of bile canaliculi, *Am. J. Clin. Pathol.*, 1957, **27**, 13
49. WEBER, A., HERZ, R., and REISS, I., On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum, *J. Gen. Physiol.*, 1963, **46**, 679.
50. WEBER, A., HERZ, R., and REISS, I., The mechanism of the action of cardiac relaxing factor, *Fed. Proc.*, 1963, **22**, 228.

FIGS. 11 AND 12, glutaraldehyde-perfused frozen sections incubated for 60 minutes with ATP at pH 7.2.

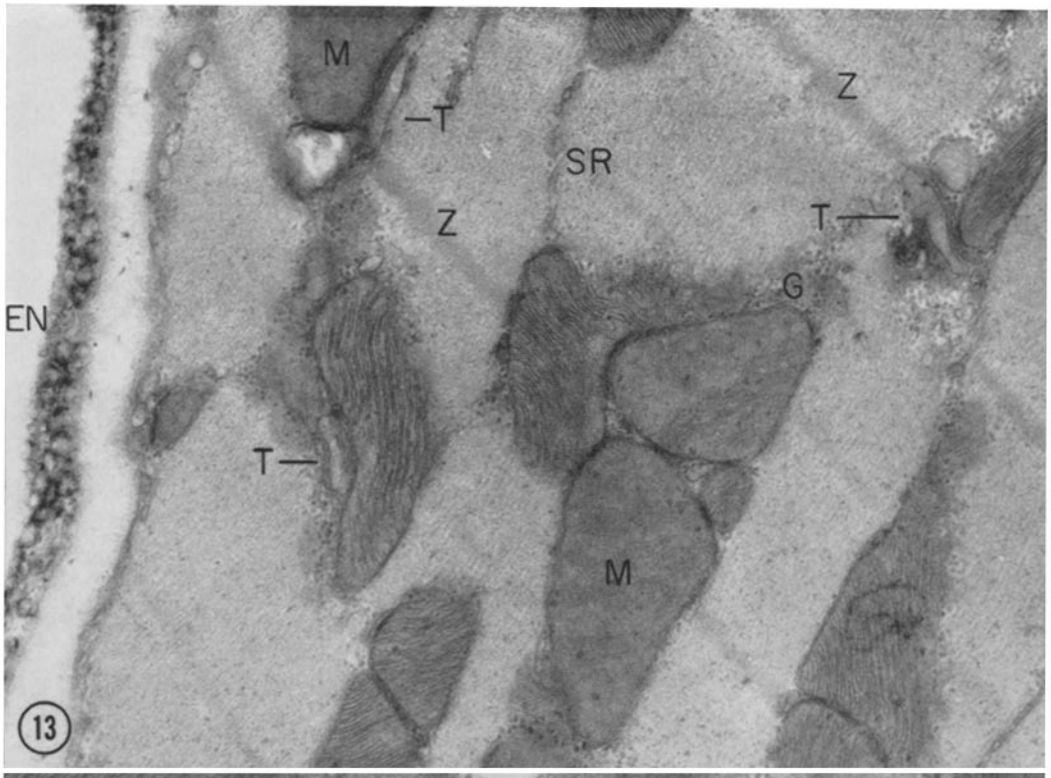
FIGURE 11 An endothelial cell (*EN*) of a capillary (*C*). Arrows indicate a few of the numerous pinocytotic vacuoles with reaction product at their membranes. Pinocytotic vacuoles (*P*), without significant accumulation of reaction product, are present beneath the sarcolemmas of the muscle fibers. Mitochondria in endothelial and in muscle cells are seen at *M*. Lead staining. × 36,000.

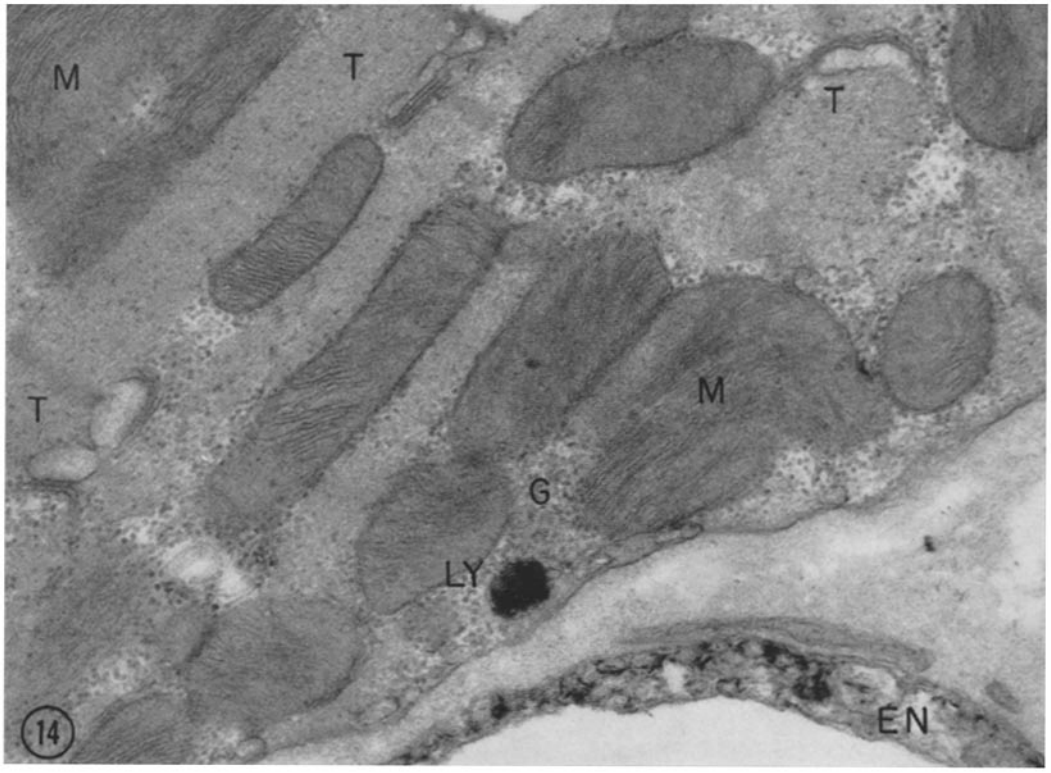
FIGURE 12 A region of the intercalated disc. Low levels of reaction product are present within the intercellular space bounded by the sarcolemmas of adjacent cells and within numerous pinocytotic vacuoles (arrows) continuous with or lying close to the sarcolemma. Mitochondria (M) are indicated. Lead staining. × 45,000.


C
P
EN
M
M
P
P
11


M
M
12

FIGURES 13 AND 14 Glutaraldehyde-perfused frozen sections incubated for 60 minutes with CMP at pH 5.0. Glutaraldehyde inhibits activity in the dyads or triads (*T*) (see Figs. 8 and 9), but, probably because of higher initial levels, activity shows at other sites: in membranes of pinocytotic vacuoles in capillary endothelial cells (*EN*) and in lysosomes (*LY*, Fig. 14). Z bands (Fig. 13), mitochondria (*M*), and glycogen (*G*) are negative. Lead staining. × 32,000.


M
T
Z
SR
Z
T
G
EN
T
M
13


M
T
T
T
M
G
LY
EN
14