CYTOCHEMISTRY OF PHOSPHATASES OF THE SARCOPLASMIC RETICULUM

II. In Situ Localization of the MG-Dependent Enzyme

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ABSTRACT

The distribution of the Mg-dependent ATPase associated with a microsomal fraction of rabbit psoas muscle was studied histochemically and its localization in relation to the vesicles of the fraction and to the structure of intact fixed muscle was determined. Although enzyme activity was retained after fixation in hydroxyadipaldehyde and in glyoxal, it was lost after fixation in glutaraldehyde or after 4 hr fixation in formaldehyde. Activity was optimally demonstrated when incubations were conducted at 17°C, in media containing 125 mM Tris-maleate buffer, pH 7.5, 5 mM ATP, 4 mM $MgCl_2$, and 1 mM $Pb(NO_3)_2$. After such incubations, activity was present throughout the sarcoplasmic reticulum, but was absent from the T system. Activation by Na or K could not be demonstrated histochemically. However, the other biochemical properties of the enzyme in the isolated vesicles and in intact muscle were similar with respect to Mg dependence, substrate specificity, inhibition by Ca, N-ethyl maleimide, *p*-hydroxymercuribenzoate, and lack of inhibition by ouabain.

In the preceding paper, the presence of a Mg-dependent, cation-stimulated ATPase[1] in a microsomal fraction of rabbit psoas muscle was reported, and some of its characteristics were described (1). The association of the enzyme with a sarcotubular fraction suggested that it was located somewhere within the sarcotubular system—either the T system, the sarcoplasmic reticulum, or possibly at the junction between the two. In this study, the biochemical findings were used to construct a histochemical method for localization of the Mg-dependent enzyme. The way in which chemical activity was affected by histochemical reagents and incubation conditions was investigated. The final product of the Mg-dependent enzyme reaction was demonstrated, first in relation to the isolated vesicles of the sarcotubular fraction, and then *in situ*.

[1] Abbreviations used: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ITP, inosine triphosphate; CTP, cytidine triphosphate; UTP, uridine triphosphate; ADP, adenosine diphosphate; IDP, inosine diphosphate; AMP, adenosine monophosphate; NEM, *N*-ethyl maleimide; PCMB, *p*-hydroxymercuribenzoate; EGTA, ethylene glycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

MATERIALS AND METHODS

PREPARATIONS USED: A microsomal fraction, consisting predominantly of smooth-surfaced vesicles when viewed in sectioned material, was isolated from rabbit psoas muscle as previously described (1). Only freshly prepared microsomes were used in histochem-

TABLE I

Effects of Temperature and Lead Concentration on ATPase Activity

Incubation temperature	Pb concentration	Cations added: None	4 mM Mg	4 mM Mg + 150 mM Na	4 mM Mg + 150 mM K	2 mM Ca
°C	mM					
37	0	8.0*	81.0	111.0	120.0	21.0
	1		11.4	12.4	13.1	16.0
	4		8.5			13.8
17	0	0.5	4.0	4.5	5.4	
	1		1.8	1.7	1.7	1.0
	4		0.9			

* Figures indicate μM of inorganic phosphate released per milligram of protein per hour.

ical experiments. The microsomes were suspended in a suitable volume of 0.25 M sucrose and added to the incubation medium to start the histochemical reaction. At the end of the incubation, after an aliquot of the reaction mixture had been taken for assay of inorganic phosphate by the method of Martin and Doty (2), the distribution of final product, precipitated lead phosphate, in relation to the vesicles was examined with negative-staining techniques (3).

For experiments with intact muscle, 1 mm–thick strips of rabbit psoas muscle, obtained from a rabbit under Nembutal anesthesia, were tied to a stick *in situ*, cut free from the muscle, and immersed in an ice cold aldehyde fixative (4). In preliminary experiments, tissues were fixed in 3, 6, and 12% hydroxyadipaldehyde for 30 min to 2 hr; in 2 and 4% glyoxal for 1 and 2 hr; in 10% formaldehyde for 1 and 4 hr; and in 3 or 6% glutaraldehyde for 30 min, 1 and 2 hr. All these aldehydes were buffered with 0.1 M Na cacodylate, pH 7.2, with the exception of glyoxal, which was used at pH 6.9, and all except glutaraldehyde contained 0.4 M sucrose. Fixation for 2 hr in 4% glyoxal was found to give the best combined preservation of enzymatic activity and morphological detail, and this procedure was used in subsequent experiments.

After fixation, the superficial fibers of the muscle strips were cut into segments about 0.5 mm long, washed in buffer containing sucrose, if this had been added to the fixative, and incubated as required on a metabolic shaker, usually for 20 min. The bits of muscle were then refixed in ice cold 6.25% glutaraldehyde in cacodylate buffer (unless the first fixation had been in glutaraldehyde or in formaldehyde), treated with 2% OsO_4 in Veronal-acetate buffer containing added sucrose for 2 hr, dehydrated through graded alcohols, and embedded in Epon. Thin sections of the embedded material, mounted on formvar- and carbon-coated grids, were stained with lead hydroxide (5) and examined in a Siemens Elmiskop I.

INCUBATION MEDIA: The media used in histochemical procedures were modified only slightly from those used in the majority of the biochemical studies. The standard medium contained 5 mM Tris-ATP, 4 mM $MgCl_2$, 125 mM Tris-maleate buffer, pH 7.5, 0.25 M sucrose, and usually 1 mM Pb $(NO_3)_2$. Departures from this standard medium are indicated in the text. When other substrates were used, they were added at concentrations of 5 mM. Inhibitors used included NEM (5×10^{-2} M), PCMB (2×10^{-5} M), and Na deoxycholate (0.1%). In inhibition studies, tissues were preincubated in sucrose containing added inhibitor for 30 min, and then transferred to incubation media which also contained the inhibitor at the same concentration.

RESULTS

The characteristics of the ATPase with which we were concerned have been discussed in the previous paper, and only those properties relevant to its histochemical identification in tissue are reiterated here. This was a Mg-dependent ATPase which had a pH optimum between 7.5 and 8. ATP splitting in the presence of Mg was significantly increased if either Na or K were added to the medium, K being somewhat more effective in this regard. No synergistic stimulation by the two cations was detected, and ouabain did not inhibit Na- or K-stimulated activity. The enzyme hydrolyzed only ATP and ITP at appreciable rates in the presence of Mg, but activation by Na or K was obtained only with ATP as substrate. CTP, UTP, IDP, AMP, and β-glycerophosphate were split at negligible rates. Inorganic phosphate was released with ADP as substrate, probably because of the myokinase activity present in the fraction. Activity could be inhibited by thiol group reagents (NEM and PCMB), by a surface-active agent (Na deoxycholate), as well as by Ca. The enzyme could be distinguished from a Ca-dependent ATPase also present in the fraction by its higher specific activity, specificity for ATP and ITP as substrates, inhibition by Ca, and its greater sensitivity to inhibition by thiol group reagents and by lead.

The sharp decrease of enzymatic activity above pH 7.5, and its sensitivity to inhibition by Ca, precluded the use of Ca-precipitation techniques

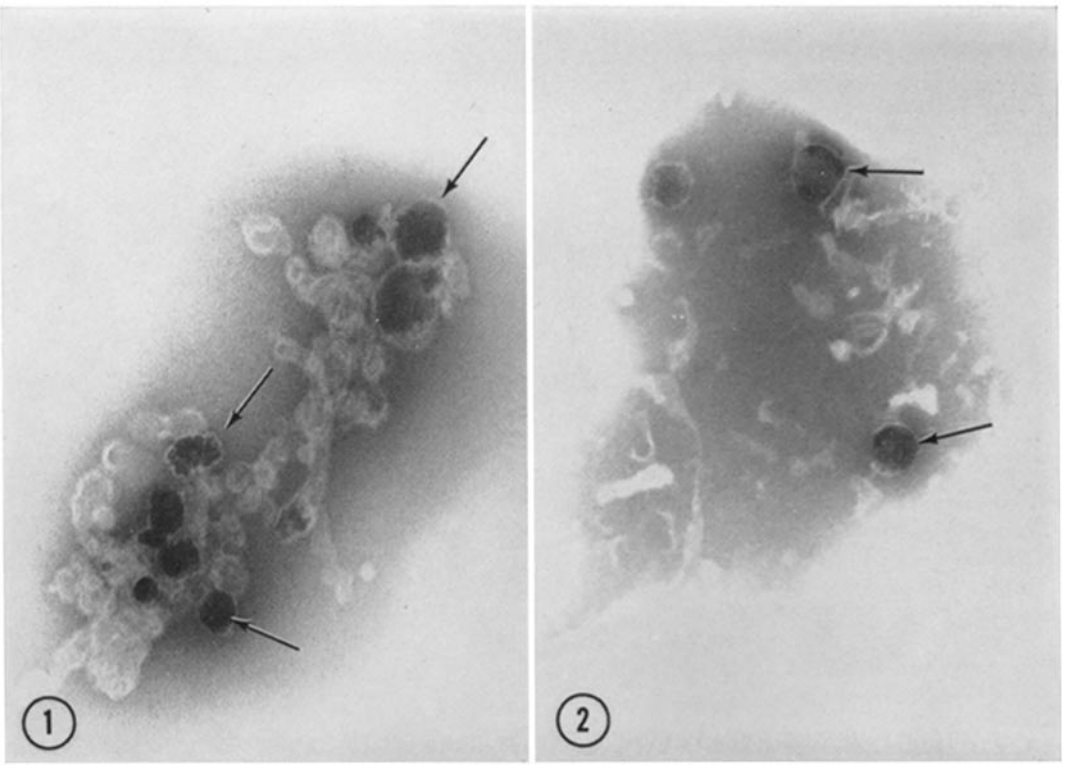


FIGURE 1 A cluster of microsomal vesicles from rabbit psoas muscle after incubation with ATP, Mg, and 1 mM Pb, 17°C. Some of the vesicles are filled with extremely dense lead phosphate (arrows) which can easily be distinguished from the moderately dense layer of PTA surrounding them. These precipitates are always found in the vesicle interiors. × 140,000.

FIGURE 2 A preparation similar to that shown in Fig. 1, although vesicular aggregation is not a striking feature in this field. Here the vesicles are only partly filled with precipitated lead phosphate (arrows) which is closely apposed to the inner side of the membrane. × 140,000.

for its histochemical demonstration. When the effects of lead were investigated, it was found that, at 37°C, 1 mM Pb inhibited ATP splitting by 90% in the presence of Mg, but some stimulation by monovalent cations was retained (Table I). With higher lead concentrations, no Na or K stimulation could be detected, and, in the presence of 4 mM Pb, stimulation of activity even by Mg was virtually lost (Table I). When incubations were conducted at 17°C, 1 mM Pb inhibited ATP splitting in the presence of Mg by only 55%, but completely inhibited stimulation by Na and K.

These results suggested that, at 37°C, activity of the Mg-dependent enzyme would not be demonstrable if incubations were conducted with conventional Wachstein-Meisel phosphatase media, which contain 3.6 mM Pb. Although at 17°C, some activity was retained in the presence of 4 mM Pb, it seemed likely that the enzyme would be more easily demonstrated if media were used which contained a somewhat lower Pb concentration.

The sarcotubular fraction was incubated in the standard medium containing 1 mM Pb, and the distribution of final product was studied with negative-staining techniques. The results of these experiments were routinely compared with the appearance of inorganic phosphate in the media. Satisfactory preparations of negatively stained vesicles were easily obtained from a final reaction mixture containing 25 to 50 μg microsomal protein per milliliter. Provided the layer of PTA surrounding the vesicles was of only moderate thickness, and provided the rate of inorganic phosphate

release exceeded 1.5 μ M Pi per milligram of protein per hour, deposits of highly electron-opaque lead phosphate were easily seen. If the rate of ATP splitting fell below this value for any reason, final product was not observed. Precipitated lead phosphate first was observable when a minimum of about 0.1 μ M Pi per milligram of protein had been released.

The histochemical results of such an experiment are shown in Figs. 1 and 2. Highly electron-opaque precipitates of lead phosphate were closely surrounded by membranes. When the precipitates were relatively small, they were found in the interior of the vesicles, lying next to the membranes (Fig. 2).

There were no differences in the localization of final product when incubations were carried out at 37°C rather than at 17°C in the presence of Mg and ATP. At 37°C, if Na or K were also present in the medium in addition to Mg, no qualitative differences in localization were noted. In either case, increased inorganic phosphate release was reflected in the size of precipitates, or, more often, in the frequency with which vesicles containing lead phosphate were encountered. No final product was seen in the absence of substrate, or if Mg was omitted from the medium containing ATP. Activity in the presence of other substrates (ITP, UTP, CTP, IDP, ADP, AMP, β-glycerophosphate) or after preincubation with inhibitors (PCMB, NEM, Ca) paralleled the biochemical results. After preincubations with Na deoxycholate, no recognizable vesicular profiles could be seen. If incubations were conducted at 17°C with Ca as the only activating cation and in the presence of 1 mM Pb, no final product was seen, apparently because the rate of ATP splitting by the Ca-dependent enzyme was below the threshold rate necessary for final product to appear.

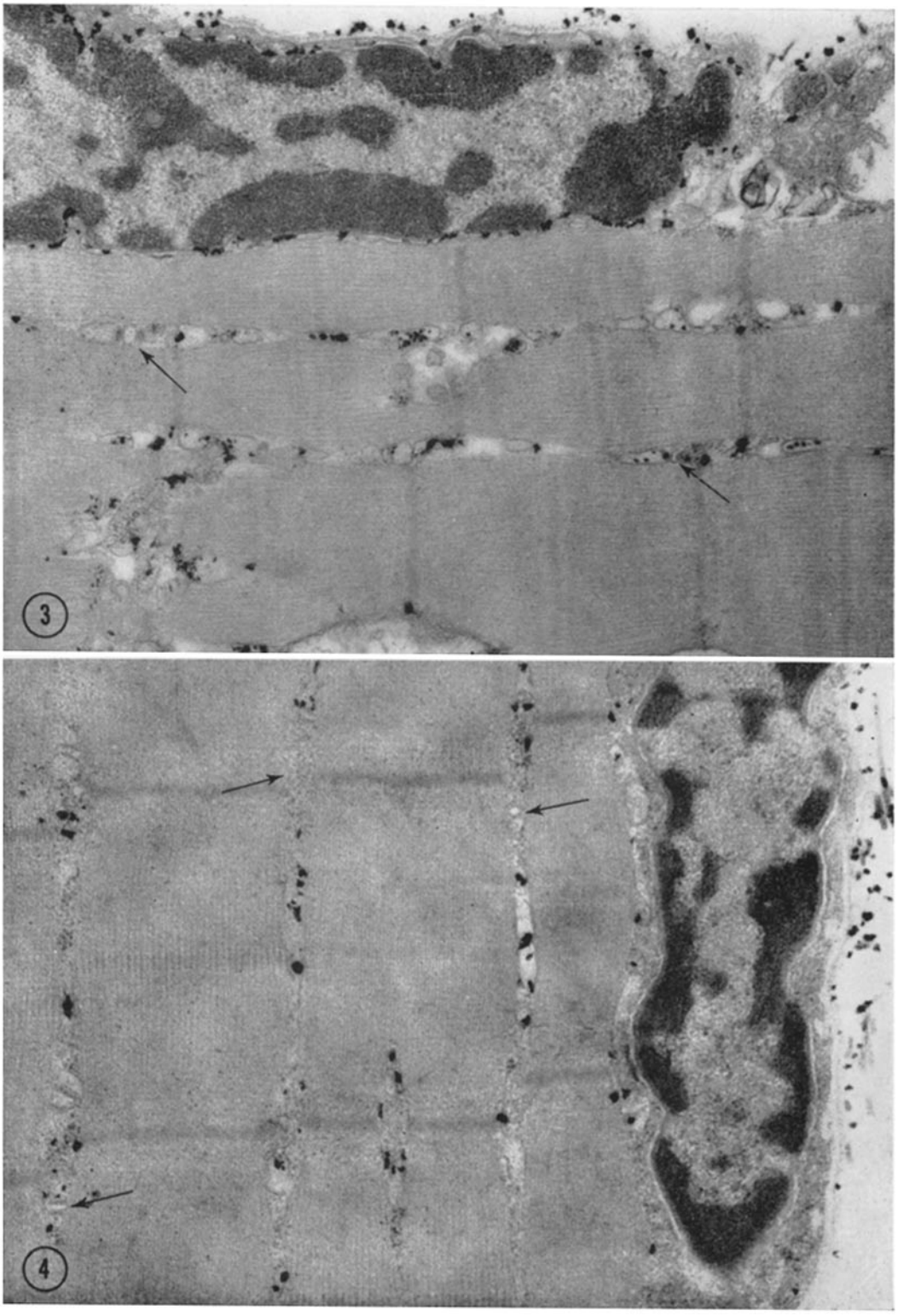
These experiments showed that when isolated vesicles were incubated at 17°C or at 37°C in the presence of 1 mM Pb, lead phosphate precipitation would occur as a result of activity of the Mg-dependent enzyme. At 17°C, activity of the Ca-dependent enzyme was suppressed, permitting differential demonstration of Mg-dependent activity. It also appeared that activation by Na and K was reflected in slight quantitative differences in activity, differences particularly hard to evaluate at a fine structural level. For this reason, experiments with intact muscle were primarily designed to localize and identify the Mg-dependent activity.

When blocks of muscle, fixed for various times in several different aldehydes, were examined after incubation for 15 to 45 min at 17°C in the medium containing 1 mM Pb, activity was found in the series fixed in hydroxyadipaldehyde or glyoxal, or in blocks fixed in formaldehyde for 1 hr. No activity was present in material fixed in glutaraldehyde, and only traces of activity were present after a 4 hr fixation in formaldehyde. Of the fixation procedures in which enzyme activity was retained, a 2 hr fixation in glyoxal gave the best preservation of morphological detail, and material treated in this way was used for morphological studies. However, regardless of the fixation employed, when activity was present it had the same localization, the same behavior with different substrates, and the same inhibition characteristics.

After incubation at 17°C in the standard medium containing 1 mM Pb, deposits of final product within the muscle fiber were found throughout the sarcoplasmic reticulum, including the terminal sacs (Figs. 3 and 4). In these sites, the lead phos-

FIGURE 3 Rabbit psoas muscle fixed in glyoxal and incubated for 20 min at 17° in a medium containing ATP, Mg, and Pb. The perinuclear region and the myofibrils immediately adjacent to it are shown. Activity, as evidenced by precipitated lead phosphate, is found at the cell surface, primarily in relation to the basement membrane. Scattered precipitates are also seen in the nuclear envelope, and are found lying within the profiles of the sarcoplasmic reticulum. These have become somewhat distorted during the course of preparation; however, no preferential distribution of activity within the sarcomere is seen. No precipitated lead phosphate is present in the T system (arrows). × 27,000.

FIGURE 4 A preparation similar to that shown in Fig. 3. Here again, deposits of lead phosphate are found at the cell surface and within the sarcoplasmic reticulum, but not in the T system (arrows). In this fiber, the nuclear envelope appears to be inactive. × 33,000.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phate precipitate was found within the tubules of the sarcoplasmic reticulum, and usually was not closely related to the inner surfaces of the membranes. There was no preferential distribution of activity along the sarcomere, and no relation was observed between the presence and distribution of final product and sarcomere length. At times, final product was also present within the nuclear envelope (Fig. 3), but no lead phosphate was ever seen within the T system, regardless of the fixation or the conditions of incubation employed.

Activity within the sarcoplasmic reticulum in the presence of Mg had the identifying characteristics noted earlier: reactivity with ATP and ITP as substrates (Figs. 5 and 9), inhibition by 2 mM Ca (Fig. 6), as well as by PCMB (Fig. 14) and NEM. Final product could be detected with ADP as substrate (Fig. 11) but not with IDP (Fig. 12), AMP (Fig. 13), CTP (Fig. 10), or β-glycerophosphate. No activity was present if substrate was omitted (Fig. 8), or if 2 mM Ca was substituted for Mg after a 20 min incubation at 17°C. Only traces of activity throughout the sarcoplasmic reticulum could be detected when Mg was omitted from the medium (Fig. 7). Localization of final product was not altered by the addition of Na or K to the medium when incubations were conducted at either 37°C or at 17°C.

Activity in other sites was found after such incubations, but it exhibited properties different from that in the sarcoplasmic reticulum. At the cell surface, activity was often closely associated with the basement membrane, and only rarely were deposits of final product found on the plasma membrane or in subsarcolemmal pinocytic vesicles. Activity at the cell surface was relatively resistant to fixation and was not abolished by fixation in formaldehyde or in glutaraldehyde. It resembled activity in the sarcoplasmic reticulum in its substrate specificity, with the exception that ADP was not split, but it was not inhibited by Ca (Fig. 6). Activity could also be observed in capillary pinocytic vesicles and was similar in its properties to that at the cell surface.

Myofibrillar activity was occasionally present in contracted portions of the fibers after incubations with ATP, Mg, and Pb, usually within poorly fixed regions in the blocks. Mitochondria occasionally contained deposits of final product associated with the cristae; this activity was more often seen in tissues incubated at 37°C.

DISCUSSION

The object of these experiments was to demonstrate, histochemically, activity of the Mg-dependent ATPase previously studied in a sarcotubular fraction of rabbit psoas muscle. Demonstration of this activity presented two main difficulties: the sensitivity of the enzyme to inhibition by lead, and the requirement that it be distinguished from activity of a Ca-dependent ATPase also present in the fraction.

In the reaction system used, a Pb concentration of 1 mM was employed. The Mg concentration

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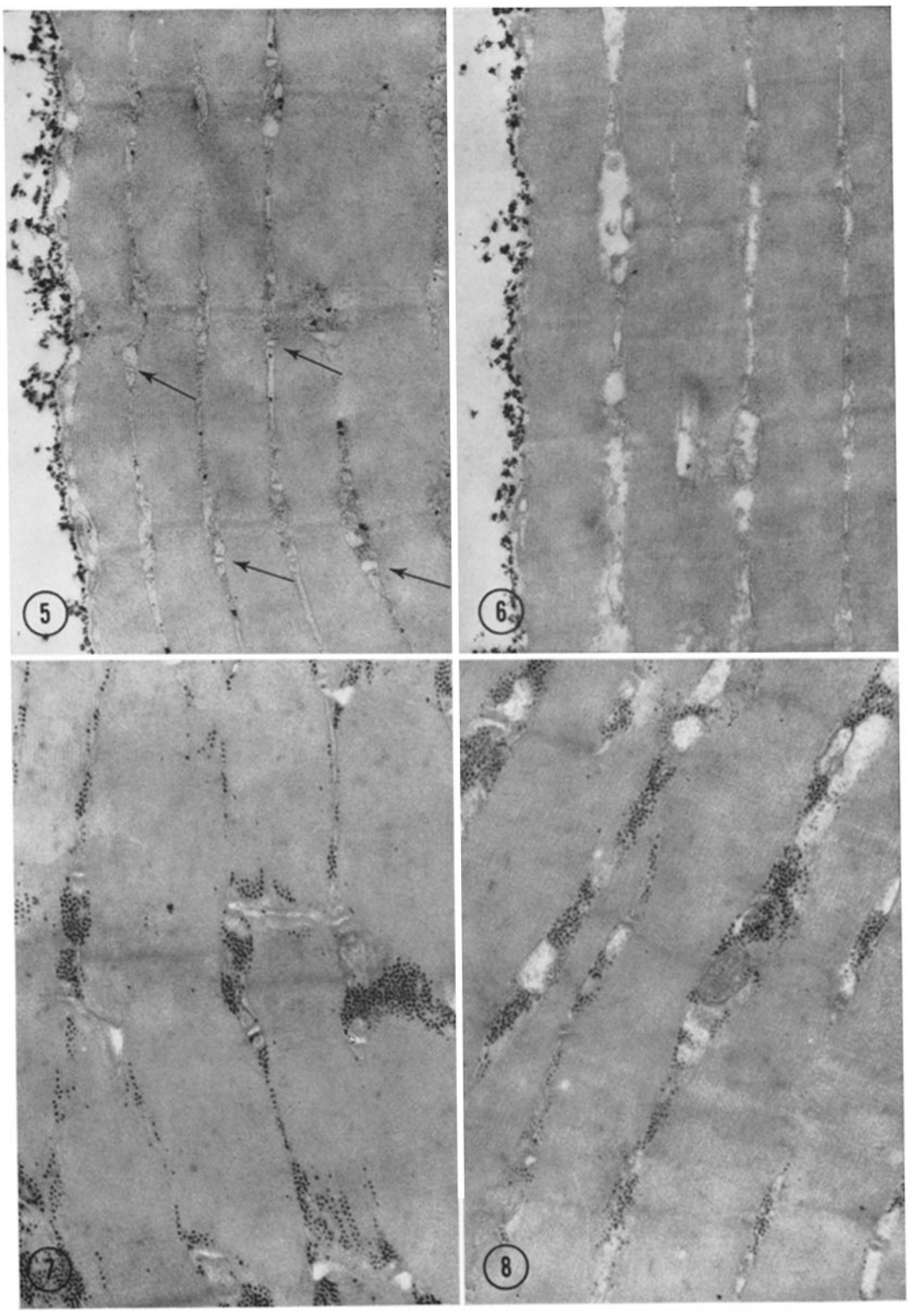
Figs. 5 to 8 and all subsequent figures are of glyoxal-fixed rabbit psoas muscle incubated for 20 min at 17°C.

FIGURE 5 Another example of the results of incubation with ATP, Mg, and Pb. Intense activity is present at the cell surface. The sarcoplasmic reticulum contains scattered precipitates of lead phosphate, usually within but sometimes closely related to the inner surface of its membranes. The T system, here clearly seen, is again totally without activity (arrows). Care must be taken to distinguish the irregularly shaped, highly electron-opaque precipitates of lead phosphate from the smaller, polygonal glycogen particles. × 23,000.

FIGURE 6 Incubation with ATP, Mg, and Pb in the presence of 2 mM Ca. Although intense activity is present at the cell surface, no activity can be detected in the sarcoplasmic reticulum. × 22,500.

FIGURE 7 Results of incubation in the medium without substrate. No final product is seen. × 33,000.

FIGURE 8 Results of incubation in a medium containing ATP, Pb, but no activating cations. No activity is seen. × 40,000.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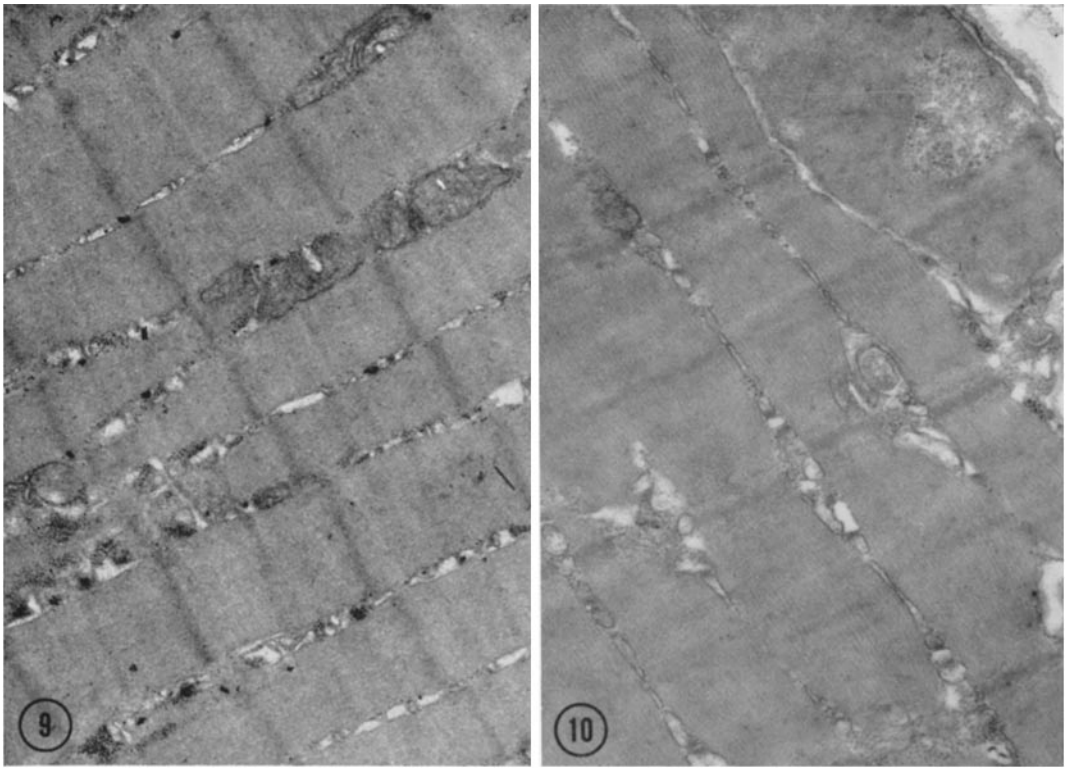

FIGURE 9 Muscle incubated with ITP as substrate in a medium containing Mg and 1 mM Pb. A moderate degree of activity is seen in the sarcoplasmic reticulum. × 16,500.

FIGURE 10. Results of substitution of CTP for ATP as substrate. No activity is seen. × 20,500.

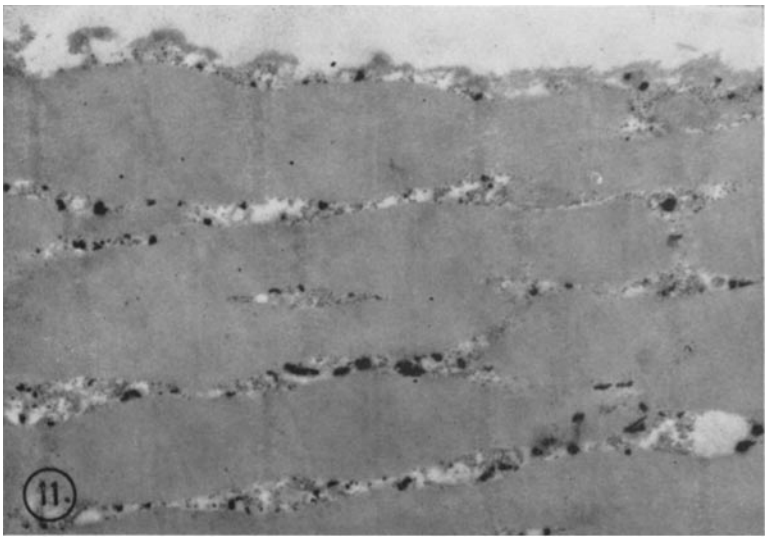

FIGURE 11 Incubation in which ADP replaced ATP as substrate. Intense activity is present throughout the sarcoplasmic reticulum, but is absent from the cell surface. × 13,000.

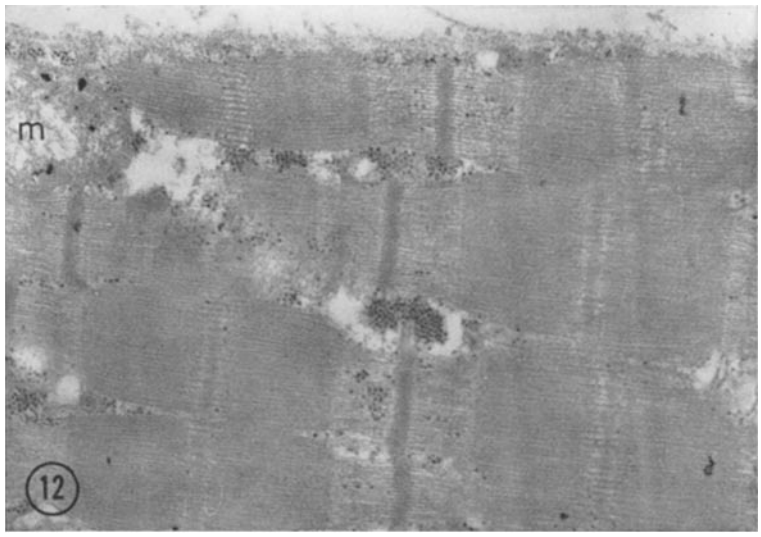

FIGURE 12 Incubation in which IDP was used as substrate. No activity is present in the sarcoplasmic reticulum or at the cell surface. However, a few flecks of precipitated lead phosphate are present in a mitochondrion (*m*). × 20,000.

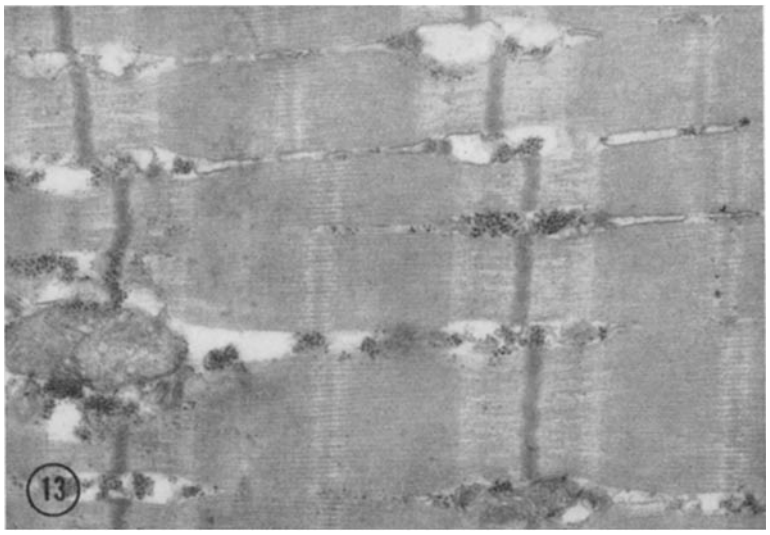

FIGURE 13 Incubation in which AMP was used as substrate. No lead phosphate is present in the sarcoplasmic reticulum. × 20,000.

and the pH were chosen to be optimal for the enzyme. A higher ATP concentration was used than that employed in the Wachstein-Meisel medium (6), lest substrate concentration become a rate-limiting factor. At 17°C, activity of the Ca-dependent ATPase was below the threshold rate necessary for precipitation of lead phosphate to result from its activity. No precipitates were seen in the presence of Ca alone, or in the presence of substrates not hydrolyzed by the Mg-dependent enzyme at appreciable rates. Although, in the presence of 1 mM Pb, Na and K stimulation was lost, the other characteristics of the Mg-dependent enzyme were identical to its properties in the isolated sarcotubular fraction.

Under these conditions of incubation, final product was found in the lumen of all components of the sarcoplasmic reticulum: terminal sacs,

FIGURE 14 Muscle preincubated in 1×10^{-5} M PCMB. No activity is seen. × 33,000.

intermediate cisternae, and longitudinal tubules. If there were no preferential distribution of lead ions in different components of the muscle fiber, it would follow that, after ATP hydrolysis, phosphate diffused into the lumen of the tubules rather than into the sarcoplasm. This may indicate that the active site responsible for ATP hydrolysis is facing, or closely adjacent to, the luminal surfaces of the sarcoplasmic reticulum.

The uniform distribution of final product throughout the sarcoplasmic reticulum indicated that the enzyme was present throughout the organelle and was not limited to one of its components. It seemed unlikely that diffusion of final product from one active region to another could have accounted for the uniform distribution of lead phosphate which was found. The appearance and behavior of activity in the nuclear envelope was similar to that observed in the sarcoplasmic reticulum, a finding not too surprising in view of the morphological continuity of the two structures (7, 8). In contrast, the activity observed at the cell surface (and in capillary pinocytic vesicles) differed from that found in the sarcoplasmic reticulum, in that it was not inhibited by Ca. This finding indicated that the sarcolemma was the site of a different ATPase, or of more than one ATPase with different inhibition characteristics. From the evidence at hand, the two possibilities cannot be distinguished.

The presence of ATPase activity in the sarcoplasmic reticulum has been reported by a number of histochemists. At the light microscope level, Padykula and Gauthier (9), using cryostat sections of rat diaphragm, found activity which had a "reticular" distribution which was intensified when cysteine was added to incubation media containing lead. More recently, Gauthier and Padykula reported that this activity was localized in the terminal sacs and the H zone region of toadfish gas bladder muscle (10). Zebe (11), using an incubation medium which contained a relatively high concentration of tartrate (0.4 M), reported that ATPase activity was present throughout the sarcoplasmic reticulum of frog muscle which had been briefly fixed in osmium tetroxide. With a Ca-precipitation technique and cryostat sections, Engel (12) reported the presence of an ATPase in the triad region of the fiber. Hori and Takahashi (13) reported that, in rat thigh muscle prepared by the section-freeze-substitution technique, activity was present throughout the sarcoplasmic reticulum after 1 min of formaldehyde fixation. Fixation in formaldehyde for 30 min or addition of Ca to the medium inhibited activity except in the terminal sacs. At a fine structural level, Sommer and Spach (14), Essner, Novikoff, and Quintana (15), and Rostgaard and Behnke (16) demonstrated ATPase activity in the terminal sacs of the reticulum in heart muscle, using formaldehyde- or glutaraldehyde-fixed tissues and a lead precipitation technique.

As noted, a wide variety of muscles, preparative techniques, and incubation media have been used in the studies listed. However, the results may be grouped into two broad categories, based on the localization obtained. In one of these, activity is found primarily in the terminal sacs, while in the other it is distributed uniformly throughout the sarcoplasmic reticulum. It would appear that activity in the terminal sacs does not require Mg (10, 16), may be relatively nonspecific in its substrate requirements (14, 15), may not be inhibited by Ca (13, 14), (but see Gauthier and

Padykula, reference 10), and is resistant to fixation in formaldehyde or in glutaraldehyde (10, 13–16). These characteristics serve to distinguish activity in the terminal sacs from the Mg-dependent ATPase studied here.

It is tempting to identify any ATPase activity demonstrated histochemically in the sarcoplasmic reticulum with the ATPase associated with the uptake of Ca, but so far definitive evidence for such an identification has not been obtained. Unfortunately, the property of this ATPase most useful for its identification in vitro, inhibition by EGTA (17), cannot be used in histochemical systems, because of the prohibitively high affinity of this agent for Pb (affinity constant 13, as opposed to 11 for Ca) (18). Hence, other properties of the enzyme must be used for its identification *in situ*. Ca accumulation and its accompanying "extra" phosphate release is enhanced in the presence of Mg, and can occur in the presence of several nucleoside triphosphates (ATP, ITP, CTP, GTP, UTP) (19). However, activity in the terminal sacs may not require added Mg for activity to be demonstrated (10, 16) and can occur in the presence of substrates other than nucleoside triphosphates (14, 15), while activity of the Mg-dependent ATPase studied by us is specific for ATP and ITP as substrates. Furthermore, the enzyme studied by us does not exhibit the almost complete inhibition by EGTA in vitro which would be expected of an enzyme active in Ca accumulation. Neither activity, therefore, can be identified as the "extra" ATPase accompanying uptake of Ca.

The working hypothesis on which this study was based was that, if there were active transport in the sarcotubular system, then at sites where this occurred there also would be cation-stimulated ATPase activity. If such activity could be localized and identified histochemically, then, indirectly, sites of active transport could also be identified. As it turned out, a somewhat atypical cation-activated ATPase was found in a sarcotubular fraction. This activity was found to be located in the sarcoplasmic reticulum, although, because of the inhibitory effects of Pb, activation by Na or K could not be demonstrated *in situ*. Assignment of a transport function to this enzyme would be premature, both because of the atypical nature of the enzyme, and because of the lack of information regarding either the ion content of the sarcoplasmic reticulum or its functions other than Ca accumulation. Until such information is obtained, its physiological role must remain uncertain.

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REFERENCES

1. ENGEL, A. G., and TICE, L. W., *J. Cell Biol.*, 1966, **31**, 473.
2. MARTIN, J. B., and DOTY, D. M., *Analyt. Chem.*, 1949, **21**, 965.
3. BRENNER, S., and HORNE, R. W., *Biochim. and Biophysica Acta*, 1959, **34**, 103.
4. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J., *J. Cell Biol.*, 1963, **17**, 19.
5. KARNOVSKY, M. J., *J. Cell Biol.*, 1961, **11**, 729.
6. WACHSTEIN, M., and MEISEL, E., *Am. J. Clin. Path.*, 1957, **27**, 13.
7. FRANZINI-ARMSTRONG, C., and PORTER, K. R., *J. Cell Biol.*, 1964, **22**, 675.
8. PORTER, K. R., *in* Proceedings of the 4th International Conference of Electron Microscopy, Berlin, 1958, Berlin, Springer-Verlag, 1960, **2**, 186.
9. PADYKULA, H. A., and GAUTHIER, G. F., *J. Cell Biol.*, 1963, **18**, 87.
10. GAUTHIER, G. F., and PADYKULA, H. A., *J. Cell Biol.*, 1965, **27**, 252.
11. ZEBE, E., *Histochemie*, 1965, **5**, 32.
12. ENGEL, W. K., *Nature*, 1963, **200**, 588.
13. HORI, S. H., and TAKAHASHI, M., *Cytologia*, 1963, **28**, 331.
14. SOMMER, J. R., and SPACH, M., *Am. J. Path.*, 1964, **44**, 491.
15. ESSNER, E., NOVIKOFF, A. B., and QUINTANA, N., *J. Cell Biol.*, 1965, **25**, 201.
16. ROSTGAARD, J., and BEHNKE, O., *J. Ultastruct. Research*, 1965, **12**, 579.
17. WEBER, A., HERZ, R., and REISS, I., *Proc. Roy. Soc. London, Series B*, 1964, **160**, 489.
18. RINGBOM, A. J., Complexation in Analytical Chemistry, New York, Interscience Publishers, Inc., 1963.
19. CARSTEN, M. E., and MOMMAERTS, W. F. H. M., *J. Gen. Physiol.*, 1964, **48**, 183.