

Ca²⁺-SPECIFIC REMOVAL OF Z LINES FROM RABBIT SKELETAL MUSCLE

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ABSTRACT

Removal of rabbit psoas strips immediately after death and incubation in a saline solution containing 1 mM Ca²⁺ and 5 mM Mg²⁺ for 9 hr at 37°C and pH 7.1 causes complete Z-line removal but has no ultrastructurally detectable effect on other parts of the myofibril. Z lines remain ultrastructurally intact if 1 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane (EGTA) is substituted for 1 mM Ca²⁺ and the other conditions remain unchanged. Z lines are broadened and amorphous but are still present after incubation for 9 hr at 37°C if 1 mM ethylenediaminetetraacetate (EDTA) is substituted for 1 mM Ca²⁺ and 5 mM Mg²⁺ in the saline solution. A protein fraction that causes Z-line removal from myofibrils in the presence of Ca²⁺ at pH 7.0 can be isolated by extraction of ground muscle with 4 mM EDTA at pH 7.0–7.6 followed by isoelectric precipitation and fractionation between 0 and 40% ammonium sulfate saturation. Z-line removal by this protein fraction requires Ca²⁺ levels higher than 0.1 mM, but Z lines are removed without causing any other ultrastructurally detectable degradation of the myofibril. This is the first report of a protein endogenous to muscle that is able to catalyze degradation of the myofibril. The very low level of unbound Ca²⁺ in muscle cells *in vivo* may regulate activity of this protein fraction, or alternatively, this protein fraction may be localized in lysosomes.

INTRODUCTION

Although the Z line is a prominent structural entity of striated muscle, its chemical composition and its functioning in contraction remain unclear. It is known that very brief trypsin treatment (Ashley et al., 1951; Goll et al., 1969; Stromer et al., 1967 a) removes the Z line without causing other ultrastructural alterations in the myofibril. Brief treatment with chymotrypsin or papain also causes structurally specific removal of the Z line (Goll and Stromer, unpublished). Garamvolgyi's observation (Garamvolgyi, 1965, 1968; Harsanyi and Garamvolgyi, 1969) that pancreatic lipase removes Z lines from both insect flight muscle

and rabbit skeletal muscle suggests that the Z line may contain lipid in addition to protein, although it is also possible that Z-line removal by lipase is due to contamination of pancreatic lipase preparations by trypsin or chymotrypsin, both of which are also pancreatic enzymes (Stromer and Goll, unpublished). Stromer and coworkers (Stromer et al., 1967 b, 1969) found that extraction of glycerinated fibrils in 2 mM Tris, 1 mM dithiothreitol for 10–12 days at 0°C removed Z and M lines without causing other ultrastructural damage to the sarcomere. It has also been reported that 45 min in 3 M urea (Rash et al., 1968),

5 hr in 11×10^{-4} M bis-dinitrophenyllysine (Burley, 1967), brief treatment of glycerinated fibrils with 0.6 M adenosine triphosphate (ATP)¹ (Hoyle et al., 1965), or extraction with acetone (Walcott and Ridgway, 1967) will all cause Z-line removal from striated muscle.

Recently, it has become clear that, in addition to the Z-line removal procedures enumerated in the preceding paragraph, Z lines are also structurally degraded during postmortem storage of muscle (Davey and Gilbert, 1967, 1969; Fukazawa and Yasui, 1967; Henderson et al., 1970; Stromer et al., 1967 a). Postmortem degradation of the Z line has been most extensively studied by Henderson et al. (1970), who found that Z lines in rabbit and porcine muscle were more susceptible to postmortem degradation than Z lines of bovine muscle and that Z lines were completely removed from rabbit or porcine muscle 24 hr after death at temperatures of 25° or 37° C. Since these observations suggest that muscle contains an endogenous component capable of Z-line removal, we have studied postmortem Z-line degradation in an attempt to discover the agent responsible for this degradation and to learn what prevents this agent from degrading Z lines in living muscle. We have found that postmortem Z-line degradation requires Ca^{2+} concentrations greater than 0.1 mM; this suggests that the very low levels of free Ca^{2+} in living muscle may prevent Z-line degradation in vivo.

MATERIALS AND METHODS

Unless indicated otherwise, all preparations were done at 0°–3°C with precooled solutions made by using double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers.

Rabbit muscle was used for all experiments reported in this paper. Rabbits were anesthetized with sodium pentobarbital (90 mg) and *d*-tubocurarine chloride (1.5 mg) just before exsanguination.

Preparation of "Ca²⁺-Activated Sarcoplasmic Factor" (CASF)

The rationale for the following preparative procedure is based primarily on the observation that in-

¹ *Abbreviations:* ATP, adenosine triphosphate; CASF, calcium-activated sarcoplasmic factor; EDTA, ethylenediaminetetraacetate; EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane; TCA, trichloroacetic acid.

tact strips of rabbit muscle suspended in a calcium solution lost their Z lines but that myofibrils prepared by blending were unaffected by subsequent addition of identical levels of Ca^{2+} . Since the sarcoplasm is the primary component removed in myofibril preparation, it seemed reasonable that a sarcoplasmic factor was involved. Sarcoplasmic proteins were extracted in the presence of ethylenediaminetetraacetate (EDTA) to prevent autolysis, were isoelectrically precipitated, and were further fractionated by ammonium sulfate. Each fraction was incubated with myofibrils to assay for Ca^{2+} -activated Z-line removal. By this method, the activity could be localized and the preparative procedure was developed.

The back and leg muscles of rabbits were removed immediately after exsanguination, chilled in ice, and trimmed free of fat and connective tissue. The trimmed muscle was ground and then suspended in 6 vol (w/v) of 4×10^{-3} M EDTA, pH 7.0–7.6, by use of a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 60 sec. This suspension was centrifuged at 6000 *g* (max) for 40 min, and the supernatant was filtered through glass wool that had previously been washed in distilled water. The filtered supernatant was adjusted to pH 6.1–6.2 by dropwise addition of 1 N acetic acid, left at 0°C for 10 min, and then centrifuged at 6000 *g* (max) for 30 min. The pH 6.1 supernatant was adjusted to pH 4.9–5.0 with 1 N acetic acid, left at 0°C for 10 min, and centrifuged at 6000 *g* (max) for 20 min. The sediment was suspended in 70 ml of 0.1 M Tris-HCl, pH 8.2, 4×10^{-3} M EDTA per 1000 g of original muscle weight used, and the pH was adjusted to 7.0. The suspension was diluted to 200 ml final volume per 1000 g of original muscle weight used, and clarified at 205,000 *g* (max) for 2 hr. The clarified supernatant was salted out between 0 and 1.8 M ammonium sulfate concentration, the precipitate was dissolved in 0.05 M Tris-HCl, pH 7.0, 2×10^{-3} M EDTA, and the solution was dialyzed against several changes of 0.01 M Tris-HCl, pH 7.0, 4×10^{-3} M EDTA. After dialysis for 24–48 hr, the solution was clarified at 105,000 *g* (max) for 60 min. The supernatant from this clarification is designated crude CASF.

Such preparations of crude CASF normally showed five major bands on disc gel electrophoresis, one of which could be identified as phosphorylase. Although the A_{280}/A_{260} ratio was typical of a protein, it is not known if the factor is also contained in lysosomal preparations. In addition to its ability to remove Z lines, CASF will, in the presence of Ca^{2+} , cause the release of trichloroacetic acid (TCA)-precipitable material from urea-denatured hemoglobin. Preliminary experiments, however, indicate no Ca^{2+} -activated alteration in F actin by CASF.

Treatment of Myofibrils with CASF

Myofibrils were prepared from rabbit back and leg muscles by suspending ground muscle in 6 vol (w/v) of 100 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA by using a Waring Blendor for 15 sec. The myofibrils were sedimented at 1000 *g* for 10 min and resuspended in 5 vol of 100 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA by use of a Waring Blendor for 5 sec. The resuspended myofibrils were sedimented at 1000 *g* for 10 min, and the resuspension-sedimentation process was repeated three more times. After the fifth wash, the myofibrils, suspended in 100 mM KCl, 50 mM Tris-HCl, pH 7.6, 5 mM EDTA, were passed twice through a 20 mesh nylon net to remove connective tissue. The strained myofibrils were sedimented at 1000 *g* for 10 min, washed three times in 100 mM KCl, and finally suspended in 100 mM KCl.

The effect of CASF on myofibril structure was assayed by comparing myofibrils from four tubes made up as described below:

Tube 1. Control: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM EDTA, 1 mM NaN₃, 5.0 mg myofibrillar protein/ml.

Tube 2. Ca²⁺-no CASF: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM CaCl₂, 1 mM NaN₃, 5.0 mg myofibrillar protein/ml.

Tube 3. CASF-no Ca²⁺: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM EDTA, 1 mM NaN₃, 5.0 mg myofibrillar protein/ml, 0.50 mg CASF protein/ml.

Tube 4. CASF-Ca²⁺: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 10 mM CaCl₂, 1 mM NaN₃, 5.0 mg myofibrillar protein/ml, 0.50 mg CASF protein/ml.

All tubes were incubated for 24 hr at 25°C, and samples were then taken for examination by phase and electron microscopy.

Isometric Tension Development

As we have previously described (Goll et al., 1970), muscle strips suspended isometrically immediately after death will, after some period of postmortem storage, gradually develop tension. Isometric tension development increases to a maximum and then gradually declines until it again reaches zero. Maximum isometric tension is normally attained within the first 3–12 hr after death, but an additional 48–312 hr or more are usually required for isometric tension to return to zero. Isometric tension measurement was used in this study to monitor postmortem changes in muscle. These measurements were made on strips of psoas muscle obtained immediately after exsanguination of rabbits that had been anesthetized as described previously. Strips 0.1–0.3 cm² in cross-section and approximately 7 cm long were removed by carefully cutting parallel to the longitudinal axes of the

fibers in the psoas muscle. One end of these strips was attached to an isometric myograph transducer (E & M Instrument Co., Inc., Houston, Tex.) by using surgical thread, and the other end was tied securely by the same means to a glass rod projecting from the inside of an 800 ml glass beaker. The muscle strips were completely immersed by filling the beaker with a solution containing 60 mM Tris, pH 7.2, 80 mM KCl, 1 mM deoxycholate, 1 mM NaN₃, and different amounts of MgCl₂, CaCl₂, 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane (EGTA), or EDTA, as specified in the individual experiments. Immersing the strips in this solution prevented dehydration of the fibers without altering their normal pattern of postmortem, isometric tension development (Busch, 1969), and also permitted study of the effects of added Ca²⁺, EGTA, etc. on the time-course of postmortem, isometric tension development. Deoxycholate was included to increase the permeability of the sarcolemma and thereby facilitate transfer of cations between the incubation medium and the myofibrils themselves. Inclusion of sodium azide prevented bacterial proliferation during 24–48 hr incubations at 25° or 37°C without altering the pattern of postmortem, isometric tension development (Busch, 1969). Extensive testing showed that neither deoxycholate nor sodium azide at the levels used in this study had any effect on Z-line ultrastructure. Temperature was controlled by placing the isometric myograph transducer and the beaker containing the immersed strips in an environmental control chamber (Lab-Line Instruments, Inc., Melrose Park, Ill.). Output of the myograph transducers was monitored by an E & M Physiograph (E & M Instrument Co., Inc.). In a single experiment, two to six muscle strips from a single rabbit were attached as quickly and as nearly simultaneously as possible to the isometric tension monitoring setup. Preliminary experiments showed that postmortem isometric tension patterns of such strips were identical.

Microscopy

Muscle strips were removed from the isometric transducers at selected stages in isometric tension development and were either homogenized for myofibril preparation or were subdivided longitudinally before fixation for electron microscopy. Myofibrils were prepared by using the method of Goll and Robson (1967) and were examined by using a Zeiss photomicroscope (Carl Zeiss, Inc., New York) equipped with phase optics. Micrographs were taken with a × 100 planapochromatic objective and a green interference filter in the light path.

For electron microscopy, the muscle strips were subdivided along their longitudinal axes into strips 1–2 mm in diameter and 20–25 mm long. The subdivided strips were fixed as described by Stromer et al.

(1969) and were then embedded in an Epon-Araldite mixture. The embedded samples were polymerized at 60°C for 94 hr, were cured at room temperature for 2-3 days, and were sectioned using a glass knife and a LKB Ultratome III (LKB Instruments, Inc., Rockville, Md.). Longitudinal sections, 600-750 Å in thickness, were mounted on uncoated, 400-mesh grids, and were doubly stained with 2% uranyl acetate in methanol followed by lead citrate (Reynolds, 1963). Electron micrographs were taken with a RCA EMU-4 operated at 50 kv.

RESULTS

Isometric Tension

The first indication that Ca^{2+} had important effects on postmortem muscle came from comparison of the isometric tension patterns of muscle strips immersed in Ca^{2+} -containing solutions with the isometric tension patterns of strips immersed in EDTA- or EGTA-containing solutions (Fig. 1). The time axis in Fig. 1 represents hours after death. Immersion of psoas strips in solutions containing either 1 mM Ca^{2+} plus 5 mM Mg^{2+} ,

1 mM EGTA plus 5 mM Mg^{2+} , or 1 mM EDTA caused immediate isometric tension development; maximum isometric tension development was reached approximately 3 hr after death in all three solutions (Fig. 1). After maximum isometric tension development, however, strips in the 1 mM Ca^{2+} -containing solution lost the ability to maintain isometric tension very rapidly and returned to zero tension development within 7 hr after death. Strips in the solutions containing either 1 mM EGTA plus 5 mM Mg^{2+} or 1 mM EDTA maintained maximum isometric tension development nearly unchanged for 24 hr after death; in other experiments, we have found that strips in these two solutions will maintain isometric tension unchanged for as long as 48 hr after death under the conditions given in Fig. 1. On the other hand, strips immersed in 60 mM Tris, pH 7.2, 80 mM KCl, 1 mM deoxycholate, 1 mM NaN_3 (the basal medium in Fig. 1), without addition of either Ca^{2+} or a Ca^{2+} -chelator, again attain maximum isometric tension 3 hr after death, but tension development in these strips decreases gradually and reaches zero approximately 24-30 hr after

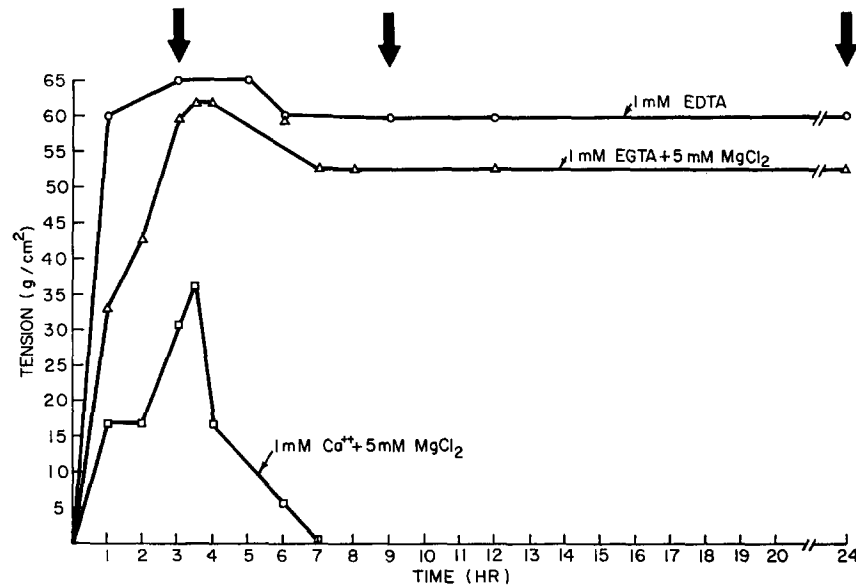


FIGURE 1 Effect of Ca^{2+} , EGTA, and EDTA on postmortem isometric tension pattern of rabbit psoas muscle incubated at 37°C. Time is plotted on the abscissa as hours after death of the rabbit, and tension is plotted on the ordinate as grams of tension per centimeters squared of muscle cross-sectional area. Muscle strips were immersed in 80 mM KCl, 60 mM Tris-acetate, pH 7.2, 1 mM NaN_3 , and 1 mM deoxycholate containing 1 mM CaCl_2 plus 5 mM MgCl_2 , 1 mM EGTA plus 5 mM MgCl_2 , or 1 mM EDTA as indicated. Isometric tension was measured by using muscle strips and E & M isometric myograph transducers as described in the Materials and Methods.

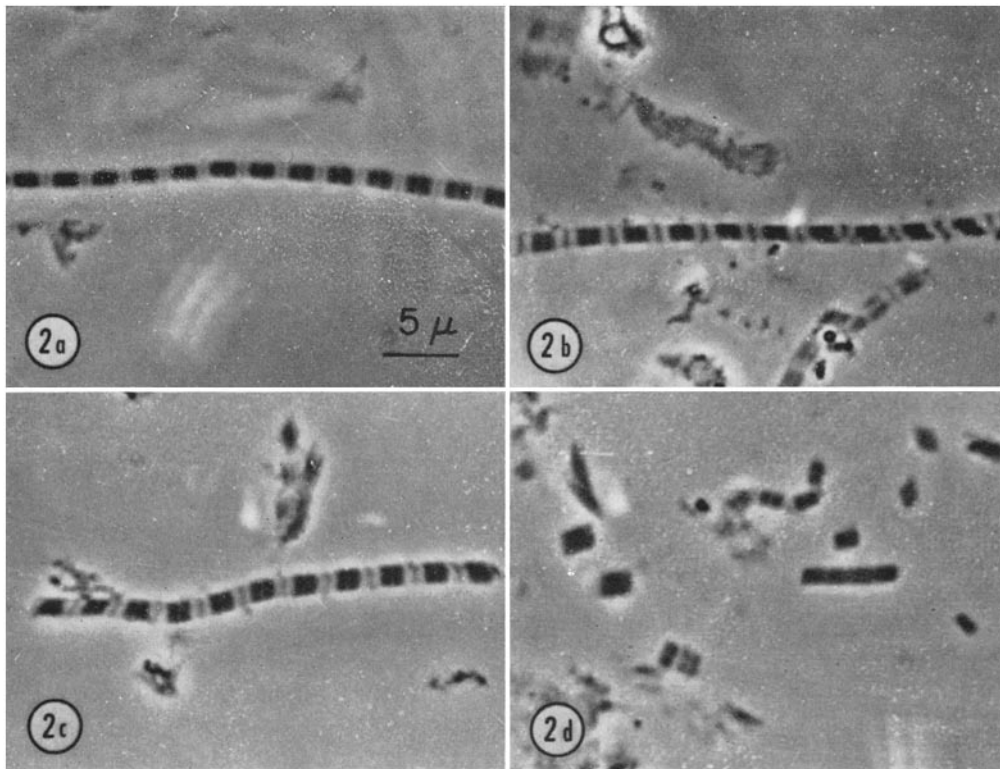


FIGURE 2 Phase micrographs of myofibrils prepared from rabbit psoas muscle strips immediately after death or after isometric suspension in different saline solutions for 24 hr at 37°C. $\times 2000$. (a) Myofibrils prepared immediately after death retain their Z lines and rest length banding. (b) Myofibrils prepared from a strip isometrically suspended in 1 mM EDTA, 1 mM deoxycholate for 24 hr are structurally similar to myofibrils prepared immediately after death. (c) Myofibrils prepared from a strip suspended in 1 mM EGTA, 5 mM Mg^{2+} , 1 mM deoxycholate for 24 hr also show no change in structure. (d) Suspension in 1 mM $CaCl_2$, 5 mM Mg^{2+} , 1 mM deoxycholate causes loss of Z lines and extensive fragmentation into 2-4 sarcomere segments during myofibril preparation.

death (not shown here). This pattern may be ascribed to the presence of contaminating Ca^{2+} in our solutions or to the release of Ca^{2+} from sarcoplasmic reticular membranes within the muscle strips or both.

It is therefore clear that Ca^{2+} has large effects on the rate at which postmortem isometric tension development is lost. Although the results in Fig. 1 suggest that strips do not develop as much tension in solutions containing 1 mM Ca^{2+} plus 5 mM Mg^{2+} as they do in solutions containing 1 mM EGTA plus 5 mM Mg^{2+} or 1 mM EDTA, it is probable that the Ca^{2+} -stimulated process that causes loss of isometric tension is operative immediately after death, and that the maximum tension developed in the presence of 1 mM Ca^{2+}

plus 5 mM Mg^{2+} represents a balance between tension development and loss of ability to maintain tension at any particular time. Since Mg^{2+} and Ca^{2+} are both obligatory requirements for contraction of myofibrils (Maruyama and Watanabe, 1962; Watanabe et al., 1964; Weber and Herz, 1963; Weber and Winicur, 1961), it is also clear that EGTA and EDTA present in the incubation solutions did not penetrate the sarcolemma, at least during the first 3 hr after death. On the other hand, since Ca^{2+} in the incubation medium altered the isometric tension patterns, it seems likely that the sarcolemma is at least partially permeable to Ca^{2+} during the first 3 hr after death. The rate of Ca^{2+} transport across the sarcolemma under the conditions in Fig. 1 must be slow

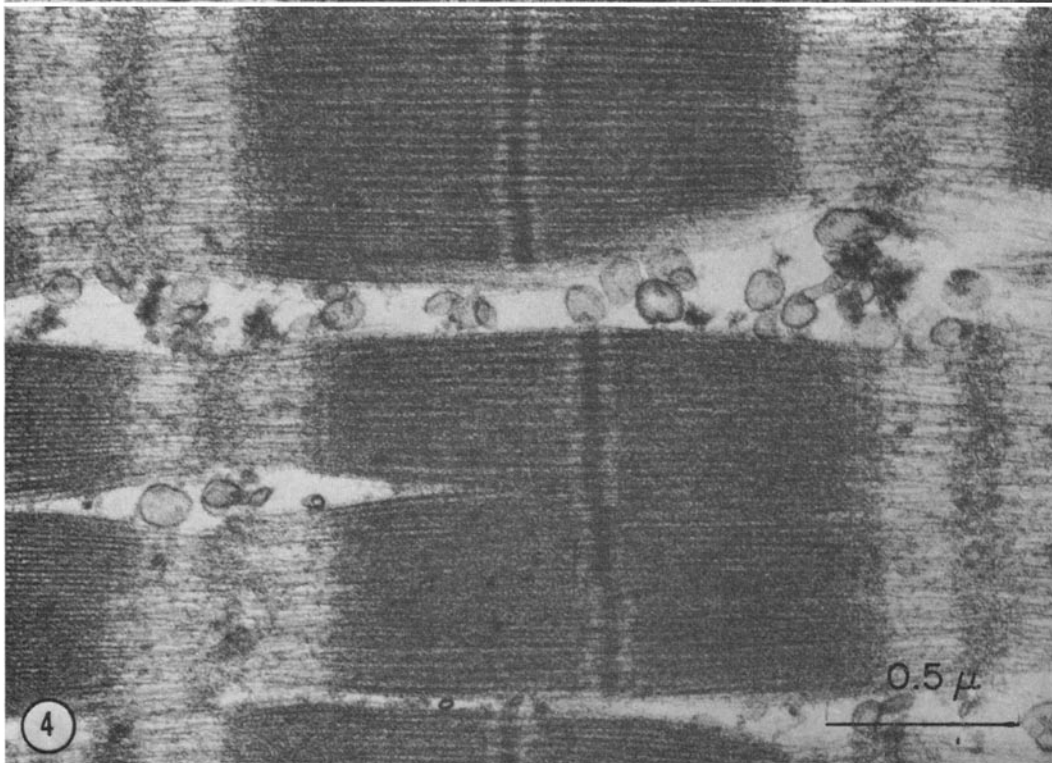
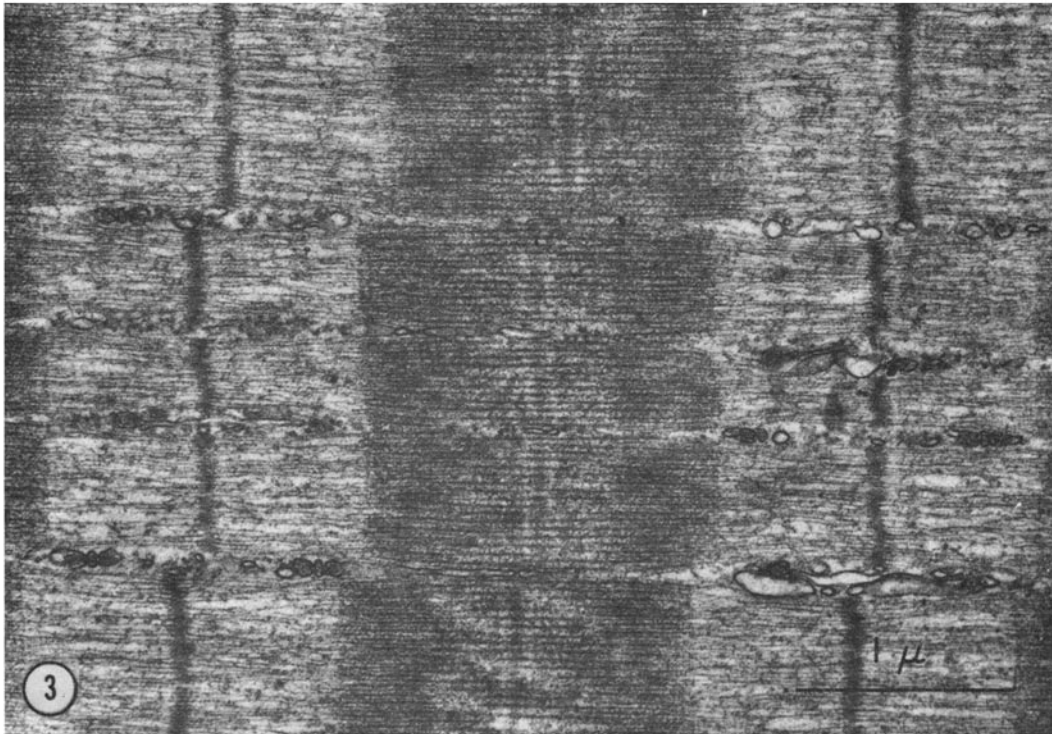


FIGURE 3 Rabbit psoas muscle sampled immediately after death. All the usual bands are present. Note particularly the dense, fibrillar Z lines. $\times 28,500$.

FIGURE 4 Rabbit psoas sampled after suspension in a saline solution containing 1 mM Ca^{2+} , 5 mM Mg^{2+} , 1 mM deoxycholate for 3 hr. This is the time required for maximum tension development. Z lines have lost their fibrillar appearance and are broad and amorphous. $\times 51,000$.

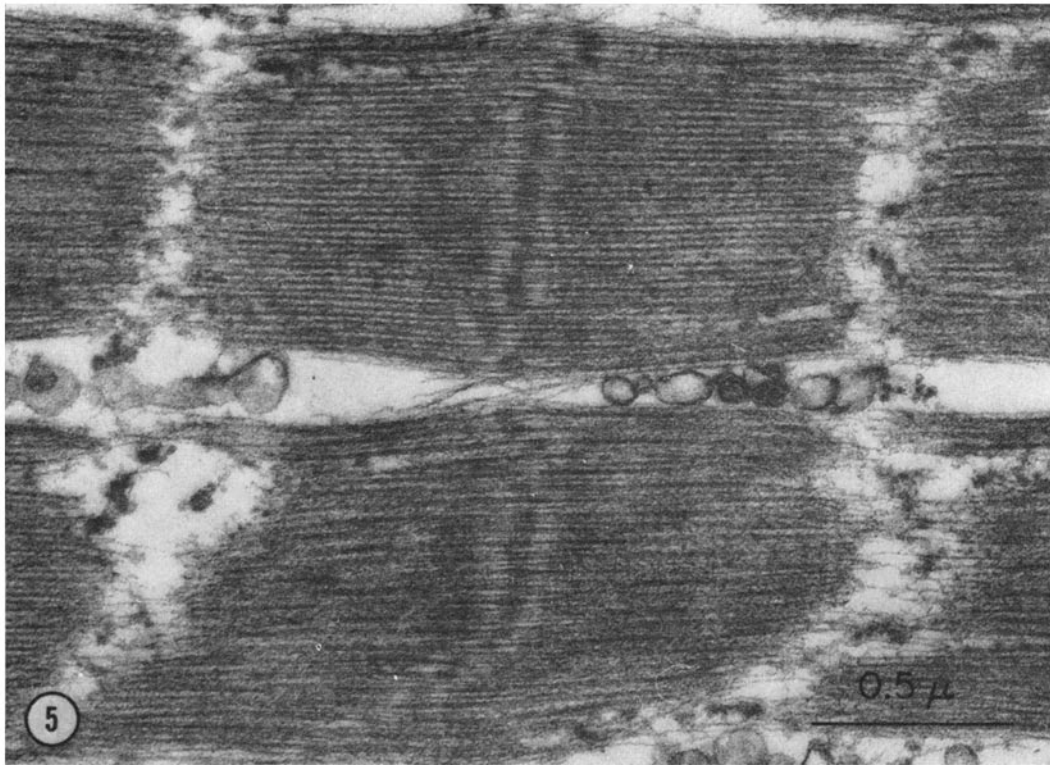


FIGURE 5 Incubation in a saline solution containing 1 mM Ca^{2+} , 1 mM deoxycholate for 9 hr results in complete Z line removal. Because these strips are held isometrically, shortening to the extent of double overlap of thin filaments in the center of the A band results in gaps between sarcomeres. $\times 52,500$.

enough to prevent escape of Ca^{2+} from the muscle cell and its subsequent chelation by EGTA or EDTA in the incubation medium before it interacts with the myofibrils to initiate tension development. Finally, it is evident that the amount of tension developed per unit of muscle cross-sectional area under the conditions in Fig. 1 is only 10–15% of the tension developed during an *in vivo* contraction.

Phase Microscopy

Since the isometric tension patterns (Fig. 1) showed that Ca^{2+} had large effects on the time course of isometric tension changes in postmortem muscle strips, strips were removed from the isometric transducers after 24 hr under the conditions described in Fig. 1, and myofibrils prepared from these strips were examined in the phase microscope to determine whether incubation in Ca^{2+} had any effect on myofibril itself. Myofibrils pre-

pared from strips incubated in solutions containing 1 mM EGTA plus 5 mM Mg^{2+} or 1 mM EDTA were structurally identical to myofibrils prepared from at-death muscle (cf. Figs. 2 *a*, *b*, and *c*). However, myofibrils prepared from strips incubated for 24 hr in a 1 mM Ca^{2+} -containing solution were highly fragmented and usually contained only two or three sarcomeres each (Fig. 2 *d*). Z lines were absent in all Ca^{2+} -treated myofibrils that were long enough and sufficiently intact to permit structural evaluation (Fig. 2 *d*). H zones were frequently evident in myofibrils prepared from strips incubated in any of the three solutions.

Electron Microscopy

Although phase microscope examination clearly showed that incubation in solutions containing 1 mM Ca^{2+} plus 5 mM Mg^{2+} caused structural alterations in the Z-line region of the myofibril, it was not possible with the resolution of the phase

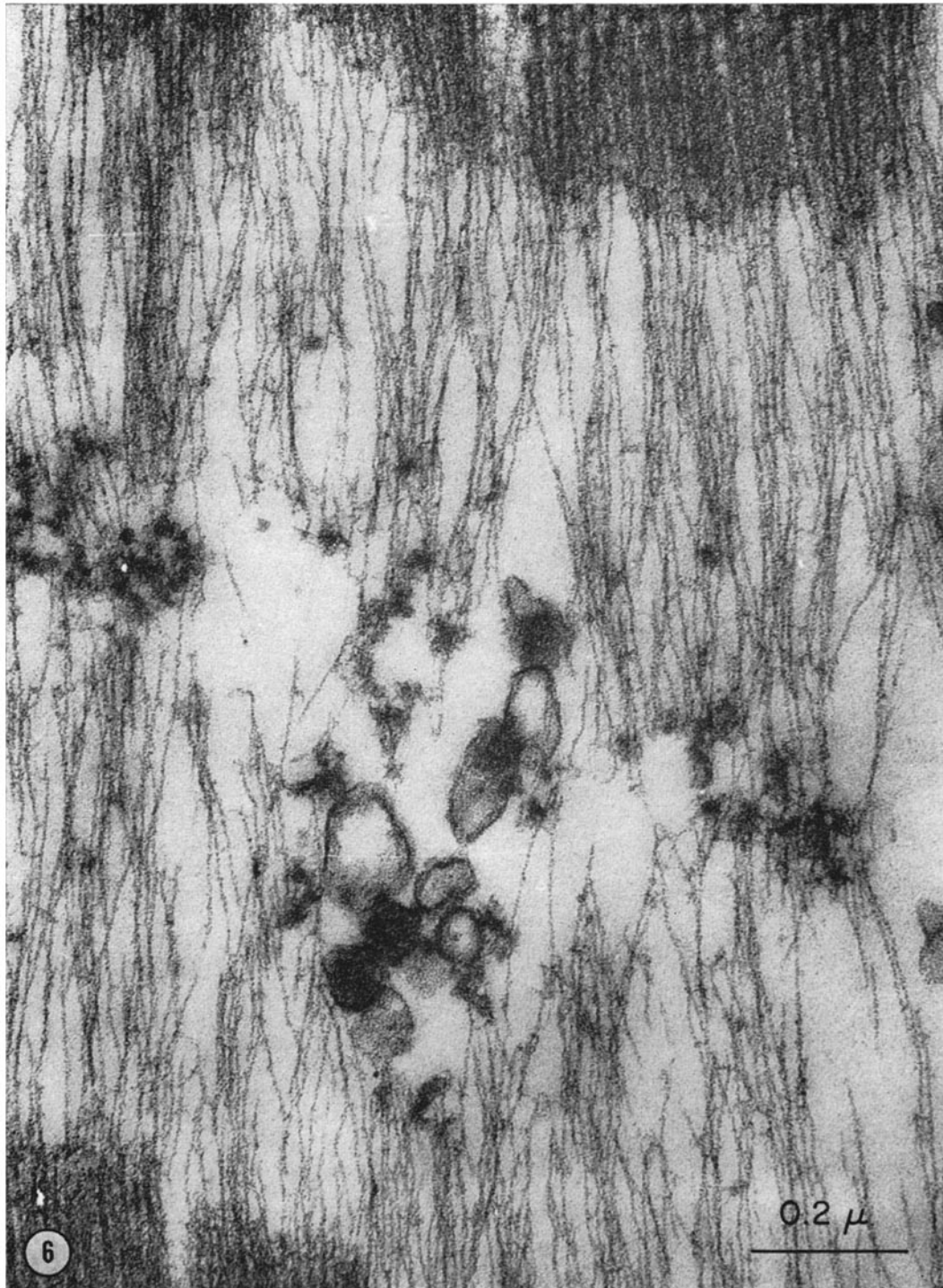


FIGURE 6 Higher magnification of the I band from a strip also incubated in the 1 mM Ca^{2+} , 5 mM Mg^{2+} , 1 mM deoxycholate-containing saline solution for 9 hr but less extensively shortened than the sample shown in Fig. 5. Suspension in Ca^{2+} seems to specifically affect Z lines rather than causing thin filament damage. $\times 114,500$.

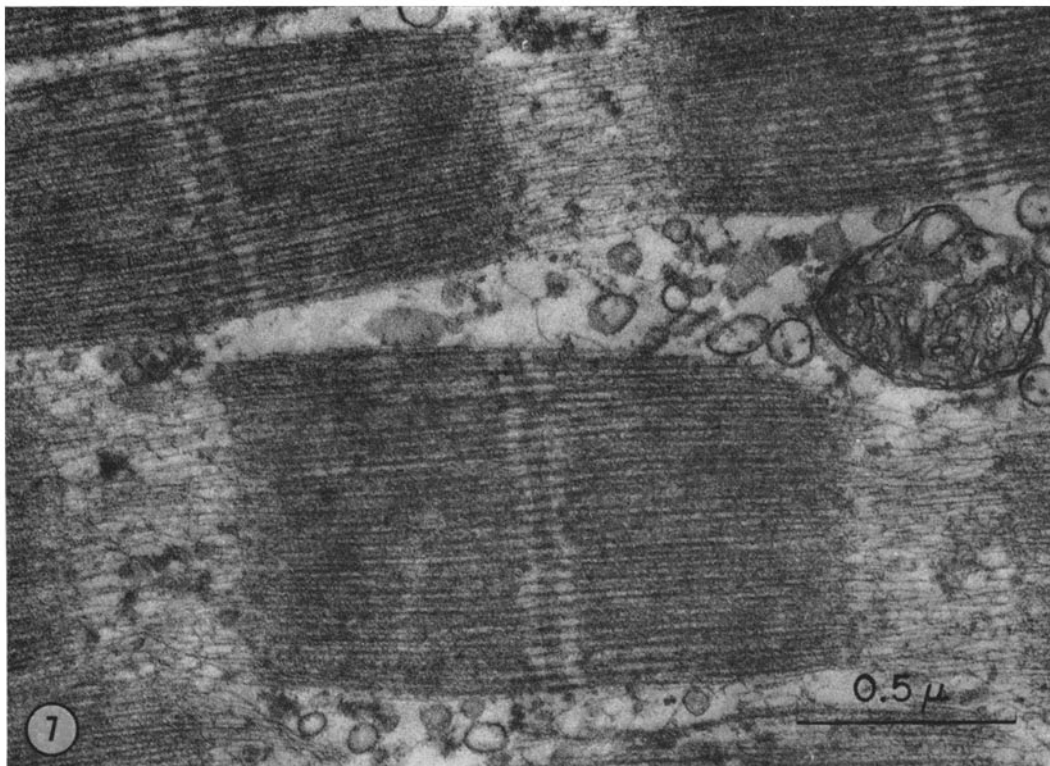


FIGURE 7 Extending the time of incubation in the 1 mM Ca^{2+} , 5 mM Mg^{2+} , 1 mM deoxycholate-containing saline solution to 24 hr causes no additional structural changes. M lines and A bands are unchanged, and I bands still contain many filaments. $\times 57,500$.

microscope to determine whether incubation in these solutions caused any other structural alterations in the myofibril, and whether the alterations in the Z-line region were due to effects of Ca^{2+} on the thin filament or to effects of Ca^{2+} on the Z line itself. Consequently, strips of muscle were removed from the isometric transducers at 3, 9, and 24 hr after death (indicated by arrows in Fig. 1) and fixed for examination by electron microscopy.

For comparative purposes, Fig. 3 shows the ultrastructure of rabbit psoas muscle fixed immediately after death. The structural features characteristic of uncontracted, striated muscle are evident. A wide H zone is present, and the wide I band is bisected by a fibrillar Z line. As shown in Fig. 4, Z lines are already clearly undergoing structural degradation after incubation for 3 hr in a 1 mM Ca^{2+} -5 mM Mg^{2+} solution (time of maximum isometric tension, Fig. 1). Z lines are broadened and amorphous, and in some instances (not shown in Fig. 4) a trough of decreased density can

be observed in the center of the Z line. The remainder of the myofibril, however, is structurally intact. After 9 hr incubation in 1 mM Ca^{2+} -5 mM Mg^{2+} -containing solutions, the Z line is completely gone, and only a few fragments of darkly stained material remain in the Z-line region (Fig. 5). Removal of the Z line has permitted the individual sarcomeres to shorten even though the ends of the fiber are fixed; this results in large gaps in the areas formerly occupied by the Z line (Fig. 5). Thin filaments overlap in the center of sarcomeres and the presence of filaments extending past edges of the A band suggest that Ca^{2+} affects the Z line itself and not thin filaments. This conclusion is substantiated by examination of a higher magnification of the 9 hr, Ca^{2+} -treated muscle (Fig. 6); this examination shows abundant thin filaments extending to the space formerly occupied by the Z line. Examination of strips incubated in 1 mM Ca^{2+} -5 mM Mg^{2+} -containing solutions for 24 hr (Fig. 7) shows that thin fila-

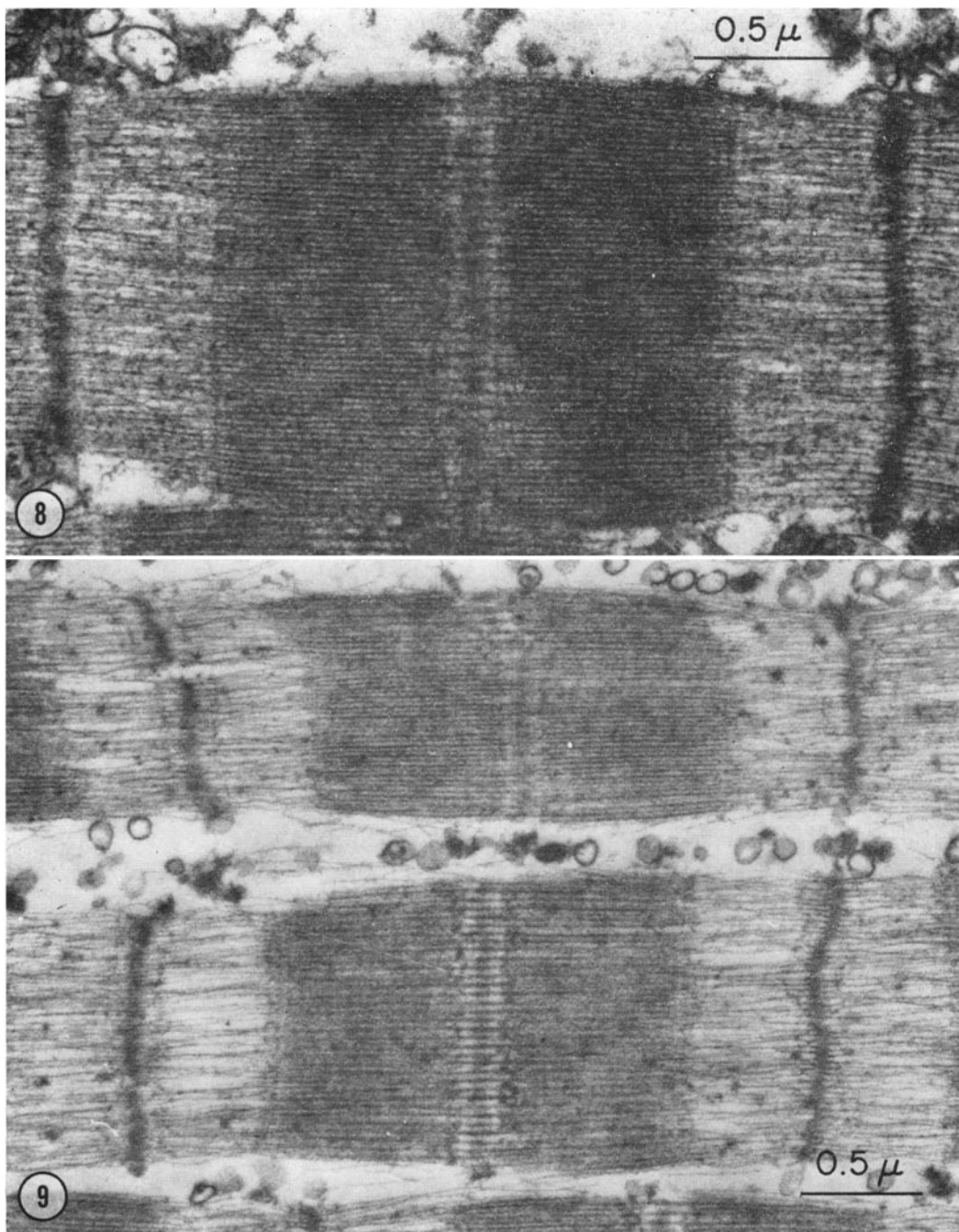


FIGURE 8 Rabbit psoas strip suspended in a saline solution containing 1 mM EGTA, 5 mM Mg^{2+} , 1 mM deoxycholate for 3 hr. When compared with Figs. 3 and 4, it is obvious that the dense, fibrillar Z-line structure persists. $\times 45,000$.

FIGURE 9 Incubation in a saline solution containing 1 mM EGTA, 5 mM Mg^{2+} , 1 mM deoxycholate for 9 hr has no effect on the Z lines of psoas strips. $\times 34,500$.

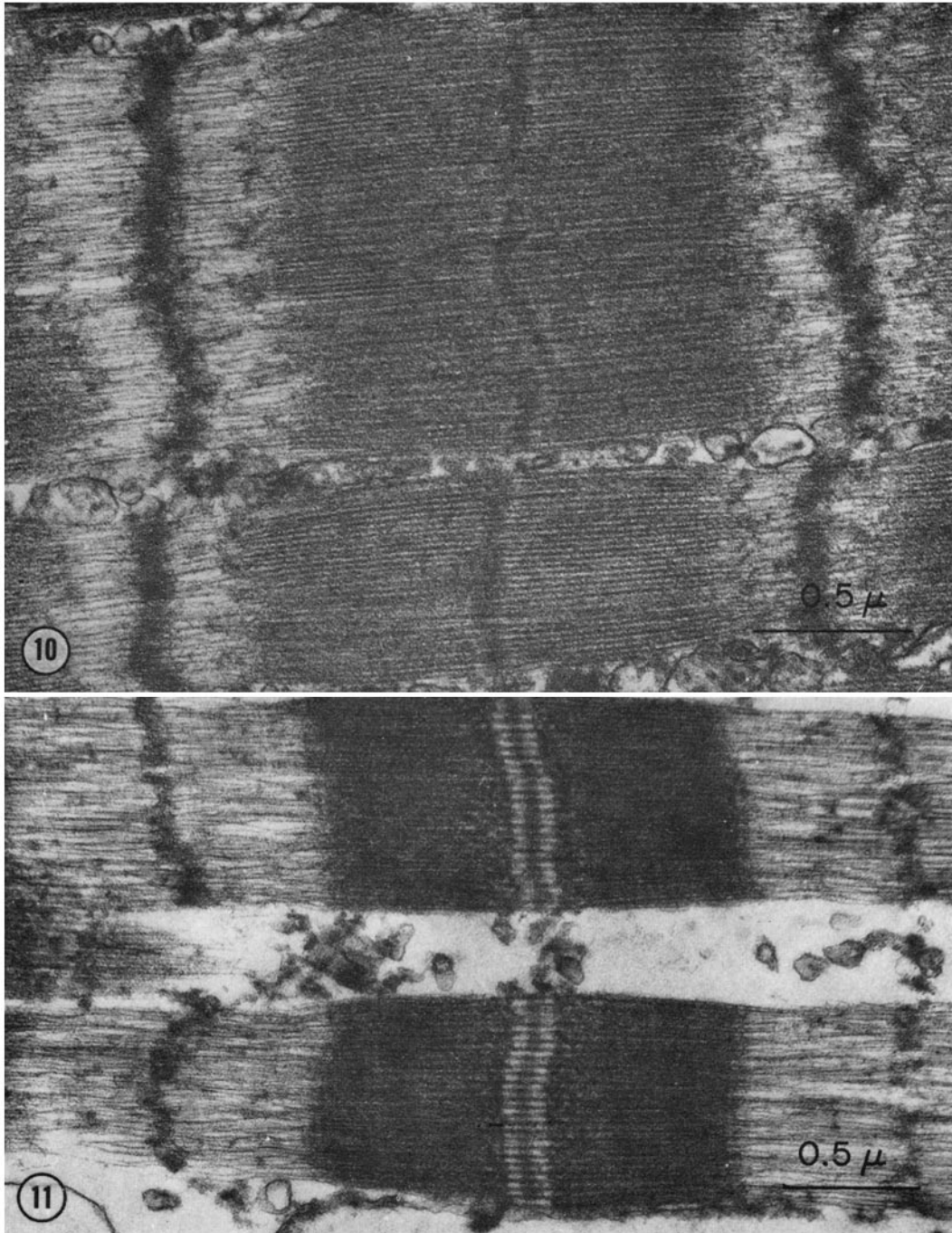


FIGURE 10 Isometric suspension of a rabbit psoas strip in 1 mM EDTA, 1 mM deoxycholate-containing saline solution for 9 hr causes Z lines to broaden slightly and become less fibrillar. $\times 47,000$.

FIGURE 11 Incubation in a saline solution containing 1 mM EDTA, 1 mM deoxycholate for 24 hr causes Z lines to become more amorphous but does not remove them (cf. Fig. 7). Loss of fibrillar Z-line structure when both Ca^{2+} and Mg^{2+} are chelated may indicate a role for Mg^{2+} in maintenance of Z lines. $\times 39,000$.

ments and M lines are still intact in this muscle even though Z lines are completely gone. Hence, incubation in a Ca^{2+} -containing solution removes Z lines specifically without affecting the rest of the myofibril.

That the Z-line degradation shown in Figs. 4–7 is specifically due to Ca^{2+} is shown by examination of strips incubated under the same conditions, but with 1 mM EGTA substituted for 1 mM Ca^{2+} (Figs. 8 and 9). After both 3 (Fig. 8) and 9 (Fig. 9) hr of incubation in 1 mM EGTA–5 mM Mg^{2+} -containing solutions at 37°C, the fibrillar appearance characteristic of Z lines immediately after death remains intact (cf. Fig. 3 with Figs. 8 and 9). Also, no other structural degradation was evident in fibers incubated in the 1 mM EGTA–5 mM Mg^{2+} -containing solutions. Incubation for 9 hr or longer in 1 mM EDTA-containing solutions causes the Z line to broaden, become amorphous, and lose its fibrillar appearance (Fig. 10). However, loss of fibrillar appearance does not presage Z-line removal in this instance since Z lines are still present after incubation for 24 hr in 1 mM EDTA-containing solutions (Fig. 11). Since Mg^{2+} is not present in the EDTA-containing solutions and since EDTA is a strong chelator of Mg^{2+} as well as of Ca^{2+} , these observations may indicate a role for Mg^{2+} in preservation of the fibrillar structure of Z lines.

Effect of CASF on Myofibrils

Phase and electron microscope examination of muscle strips incubated in Ca^{2+} -containing solutions clearly showed that 1 mM Ca^{2+} stimulated Z-line degradation in these strips. Consequently, a series of experiments were done to determine whether Ca^{2+} itself would cause Z-line removal from skeletal muscle fibrils or whether Ca^{2+}

activated a sarcoplasmic enzyme capable of removing Z lines. These experiments involved phase microscope examination of myofibrils incubated with different sarcoplasmic extracts in the presence or absence of Ca^{2+} . As shown in Fig. 12 *a*, myofibrils incubated in 100 mM KCl and 10 mM EDTA at pH 7.0 for 24 hr at 25°C were structurally intact with prominent A, I, and Z bands. Replacement of 10 mM EDTA with 10 mM Ca^{2+} , but without CASF, caused no change in myofibril structure when viewed in the phase microscope (Fig. 12 *c*). Therefore, Ca^{2+} by itself does not cause Z-line degradation in myofibrils. Incubation of myofibrils with a sarcoplasmic protein extract, prepared as described in Materials and Methods, also had no effect on Z-line integrity (Fig. 12 *b*) when incubation was done in 100 mM KCl and 10 mM EDTA. When the same sarcoplasmic protein extract was incubated with myofibrils in 100 mM KCl and 10 mM Ca^{2+} , however, Z lines were completely removed after 24 hr (Fig. 12 *d*). This result shows that the sarcoplasmic protein extract contains a Ca^{2+} -activated factor capable of removing Z lines from skeletal muscle myofibrils. On the basis of phase microscope assays, Ca^{2+} concentrations greater than 0.1 mM are necessary to obtain Z-line removal within 24 hr at enzyme to myofibrillar protein ratios of 1/10, by weight.

Although the phase microscopy experiments demonstrated that muscle cells contain a CASF capable of removing Z lines, it was impossible with the resolution of the phase microscope to determine whether CASF specifically removed Z lines leaving A and I bands and M lines intact, as was observed in the muscle strips incubated in 1 mM Ca^{2+} (Figs. 4–7). Therefore, representative portions of each of the four samples shown in

FIGURE 12 Phase micrographs of control and CASF treated myofibrils. $\times 2000$. (*a*) Control myofibrils incubated in 100 mM KCl, 10 mM EDTA, 20 mM Tris-acetate, pH 7.0, 1 mM NaN_3 , for 24 hr at 25°C show no structural changes. (*b*) Myofibrils incubated in 100 mM KCl, 10 mM EDTA, 20 mM Tris-acetate, pH 7.0, 1 mM NaN_3 , 10 mg myofibrillar protein: 1 mg CASF protein have Z lines comparable to the control. (*c*) Myofibrils incubated in 100 mM KCl, 10 mM Ca^{2+} , 20 mM Tris-acetate, pH 7.0, 1 mM NaN_3 , for 24 hr at 25°C also show no structural change. (*d*) Myofibrils incubated in 100 mM KCl, 10 mM EDTA, 20 mM Tris-acetate, pH 7.0, 1 mM NaN_3 , 10 mg myofibrillar protein: 1 mg CASF protein have no Z lines and typically have double overlap of thin filaments in the center of the A band. 1 cm = 5 μ .

FIGURE 13 Electron micrographs of sectioned myofibrils removed from the same samples as shown in Figs. 12 *b, c, d*. (*a*) Myofibrils incubated in the CASF-EDTA solution described in the legend for Fig. 12 *b* have dense fibrillar Z lines. $\times 21,000$. (*b*) Myofibrils incubated in the Ca^{2+} solution without CASF described in the legend for Fig. 12 *c* also have dense fibrillar Z lines. $\times 24,000$. (*c*) Myofibrils incubated in the Ca^{2+} plus CASF solution described in the legend for Fig. 12 *d* have no Z lines. $\times 24,000$.

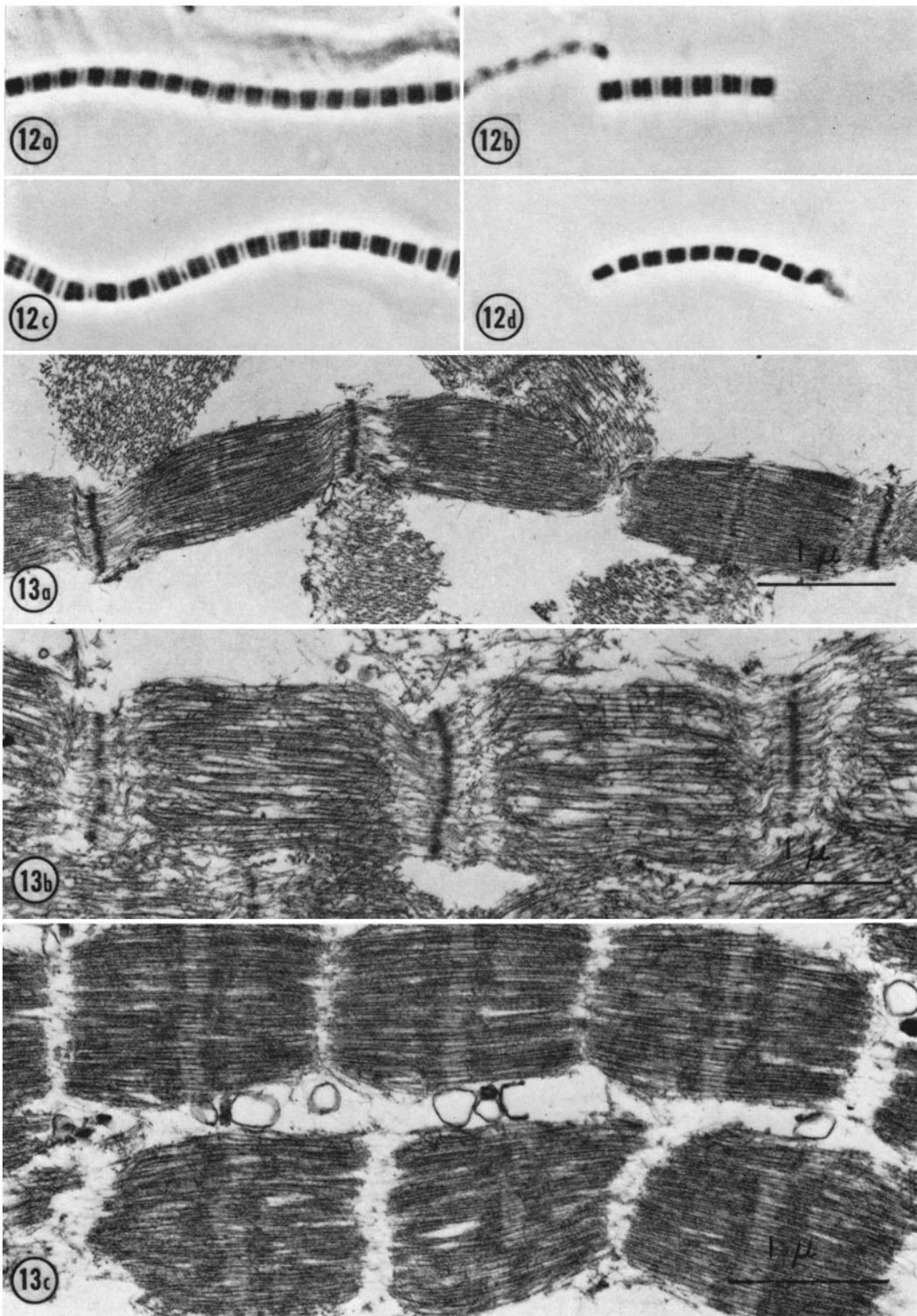


Fig. 12 were pelleted and processed for examination in the electron microscope. Myofibrils incubated for 24 hr in a 10 mM EDTA-containing solution were ultrastructurally identical to freshly prepared myofibrils (not shown here). Moreover, myofibrils incubated either in a 10 mM Ca^{2+} -containing solution or in a 10 mM EDTA plus CASF-containing solution had prominent Z lines and were structurally similar to freshly prepared myofibrils (Fig. 13 *a* and *b*). Incubation in the presence of both 10 mM Ca^{2+} and CASF, however, resulted in total removal of Z lines (Fig. 13 *c*) without evident degradation of the A band or the M line (cf. Figs. 5 and 13 *c*). Furthermore, the double overlap of thin filaments in Fig. 13 *c* shows that thin filaments remain intact even though they do not extend much beyond the lateral edges of the A band. Consequently, the effects of CASF on myofibrillar ultrastructure are very similar to the effects of incubation of intact fibrils in 1 mM Ca^{2+} -containing solutions.

DISCUSSION

The results of this study clearly show that muscle fibers contain an endogenous agent capable of Z-line removal. Although we have not yet completely purified this agent, our preliminary studies indicate that it is protein in nature, and that muscle cells contain only very small quantities of it, approximately 1–2 mg/100 g fresh muscle weight. To our knowledge, this is the first demonstration of the presence of a protein in muscle cells that will catalyze degradation of the myofibril. Although several studies (Caldwell and Grosjean, 1971; Iodice et al., 1966; Parrish and Bailey, 1966; Suzuki and Fujimaki, 1968) have shown that proteolytic enzymes can be isolated from muscle tissue, repeated and careful tests have shown that none of these preparations will catalyze degradation of the myofibril (Bodwell and Pearson, 1964; Fukazawa and Yasui, 1967; Martins and Whitaker, 1968). Since it is known that myofibrillar proteins have a predictable metabolic turnover time (McManus and Mueller, 1966), the mechanism of myofibrillar protein degradation has remained unknown. It is possible that the Ca^{2+} -activated sarcoplasmic protein factor we describe in this paper initiates myofibril degradation *in vivo* by removal of the Z line and that Z-line removal leaves the remaining myofibrillar proteins susceptible to hydrolytic action of lysosomal cathepsins found in the muscle cell (Canonica and Bird, 1970). Alternatively, the Ca^{2+} -activated sarco-

plasmic protein factor may also degrade actin and myosin *in vivo*, although we have been unable to demonstrate this ability *in vitro*. Our phase microscope observations indicate that the sarcoplasmic factor is optimally active against Z lines at pH 7.0; thus the enzyme could function *in vivo* if Ca^{2+} were available.

Regardless of the physiological role of the CASF, it is probable that this factor is responsible for the rapid Z-line degradation observed in muscle strips incubated in Ca^{2+} -containing solutions. Moreover, since sarcoplasmic reticular membranes lose their ability to accumulate Ca^{2+} during postmortem storage of muscle (Greaser et al., 1967; Nauss and Davies, 1966), it seems probable that release of bound Ca^{2+} from the sarcoplasmic reticulum may activate the sarcoplasmic factor and thereby initiate the Z-line degradation characteristically observed in postmortem muscle (Henderson et al., 1970). The mechanism for regulating activity of the Ca^{2+} -activated factor in living muscle is not yet clear. The very low levels of free Ca^{2+} inside living muscle cells and the fact that the sarcoplasmic factor requires Ca^{2+} levels above 0.1 mM for activity may act together to prevent the factor from degrading Z lines in living cells. On the other hand, our procedure for preparation of the sarcoplasmic factor does not preclude the possibility that this factor is localized in lysosomes that are ruptured by extraction in 4 mM EDTA. This latter possibility can be easily checked by attempting to isolate the CASF from muscle lysosomal preparations.

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