DEPOSIT FORMATION IN MUSCLE FIBERS FOLLOWING CONTRACTION IN THE PRESENCE OF LEAD

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The location of phosphatase sites within muscle cells is of interest since adenosinetriphosphate (ATP) hydrolysis is involved in (*a*) force generation by the myofilaments (Engelhardt and Ljubimova, 1939; Szent-Györgyi 1953; Weber, 1958; Cain et al., 1962) and (*b*) the translocation of calcium from the myofilament space into the sarcoplasmic reticulum that controls relaxation (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1963; Weber et al., 1963; Podolsky and Costantin, 1964). The present report is based on experiments designed to localize such sites for microscopic study by applying the lead precipitation technique (Wachstein and Meisel, 1957) to a limited region of a "skinned" muscle fiber (Natori, 1954). One result is that these preparations develop electron-opaque deposits associated with the sarcoplasmic reticulum, which appear to be unrelated to the transport of calcium. Another finding is a negative one which raises questions about the validity of ultrastructural localizations of phosphatase activity made with the lead precipitation technique; when the enzymatic reactions accompanying muscle contraction are activated by addition of calcium ions to the cell, the presence of lead in the contracting region does not necessarily produce myofilament-associated precipitates.

MATERIALS AND METHODS

Preparation

Experiments were made at room temperature with muscle fibers from the frog *Rana pipiens*. The semitendinosus muscle was removed from a frog and generally immersed in normal Ringer's solution; in some muscles active sodium transport was inhibited by adding 10^{-5} M strophanthidin to the Ringer's solution (Horowicz and Gerber, 1965). A fiber bundle was dissected out, blotted, placed on a glass microscope slide, and covered with paraffin oil. One fiber was dissected free, and the surface membrane was removed with a sharp needle.

ATPase Activation

The ATPases associated with both myofibril contraction and calcium transport across the internal membranes were activated by applying small droplets containing Ca^{++}, according to techniques described previously (Podolsky and Costantin, 1964). The ATP required for these reactions was supplied endogenously. Reactions were carried out in the presence of Pb^{++} by forming the droplets from a solution containing 1.4 mM $CaCl_2$, 1 mM $Pb(NO_3)_2$, and 125 mM tris maleate (pH 7.0). The droplets were 50–100 μ in diameter and were applied to a given region either singly or in a series of up to 10 spaced over a period of about a minute. The cycle of contractile activity following each application lasted several seconds and appeared to have the same general characteristics as that elicited by calcium alone; a typical calcium response is shown in Fig. 5 of a report by Podolsky and Costantin (1964).

In control experiments, a solution containing 1 mM $Pb(NO_3)_2$ and 125 mM tris maleate was applied. Although in most cases some movement was elicited by these droplets, in about 25% of the preparations no contractile response at all was seen.

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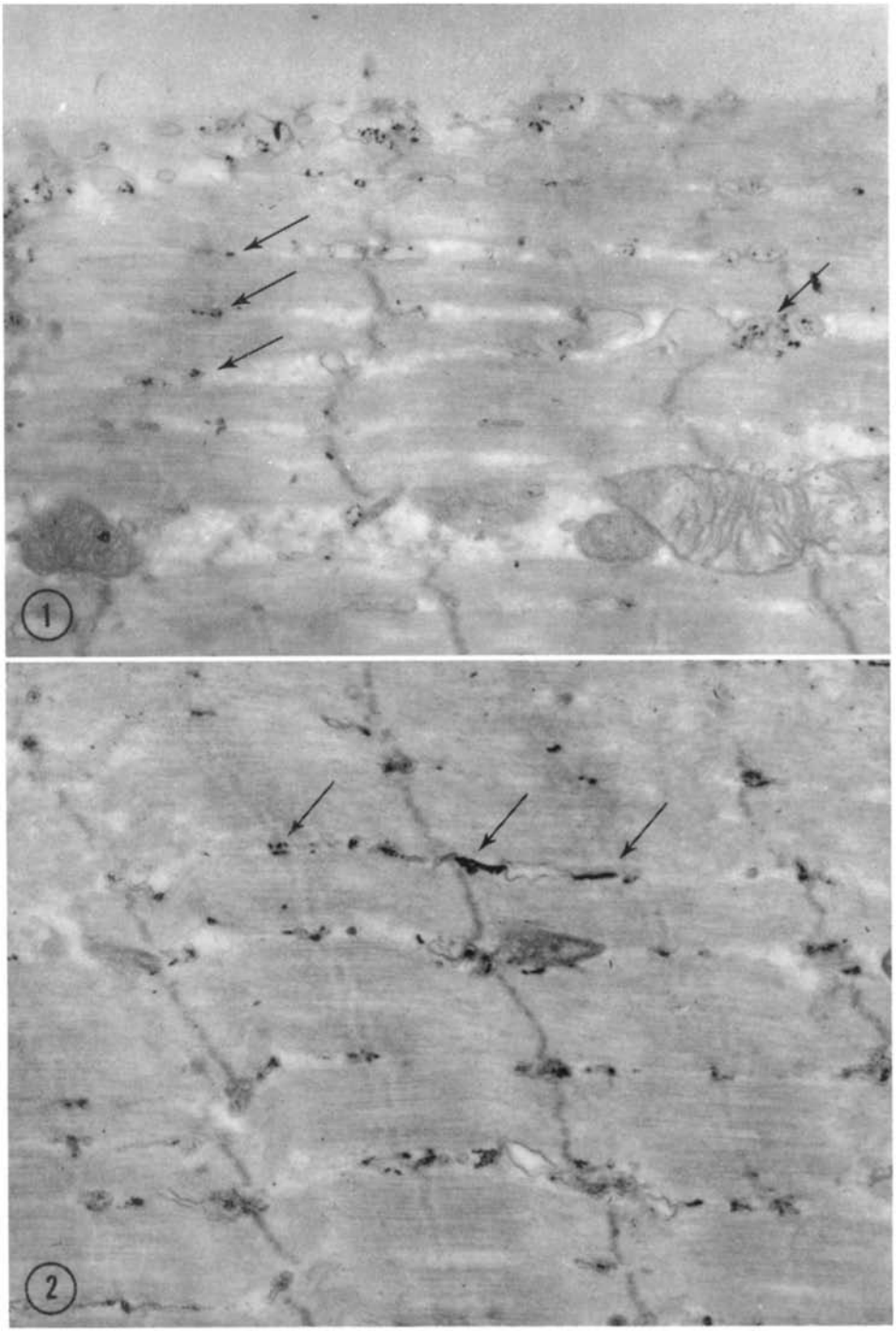

FIGURE 1 Section of skinned fiber showing membrane-associated deposits of electron-opaque material (arrows) resulting from application of calcium, lead, and tris maleate to surface of preparation; strong contractile response was seen. Note that myofibril regions are essentially free of deposits. × 20,000.

FIGURE 2 Section of skinned fiber showing electron-opaque material (arrows) resulting from application of lead and tris maleate; no contractile response was seen. All the deposits appear to be contained within the sarcoplasmic reticulum. The preparation was made from a muscle bathed in a Ringer solution containing 10^{-5} M strophanthidin. × 20,000.

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Electron Microscopy

After application of the test solution, the preparation was fixed with 6% glutaraldehyde in 200 mM cacodylate (pH 7.0), postosmicated, and prepared for electron microscopy (Sabatini et al., 1963). The time between the end of droplet application and the beginning of fixation ranged from 0.5 to 20 min; this variation in interval had no effect on the pattern or extent of deposit formation.

RESULTS

When calcium was added in the presence of lead, sections containing electron-opaque deposits which mark the region of droplet application were found in 12 of 22 preparations; the difficulty of relocating the treated part of the fiber could account for some of the apparently deposit-free preparations. The general distribution of deposits indicates that lead from the droplet penetrated at least several microns into the depth of the fiber (Fig. 1). The deposits were almost always associated with the terminal cisternae and connecting tubules of the sarcoplasmic reticulum and practically never associated with myofibrils or the transverse tubules.

Similar accumulations of density were often present in preparations treated only with the lead nitrate-tris maleate solution, and in which no contractile response was seen (Fig. 2).

DISCUSSION

The nature of the observed deposits is not clear. Although precipitation by lead of the phosphate hydrolyzed from ATP during translocation of the added calcium ions would be expected to produce such deposits, this cannot be the only process at work since similar distributions of density were found in control experiments where only lead nitrate and buffer were added to the fiber. It seems unlikely that the deposits seen in the controls could be due to circulation of endogenous calcium between the internal membranes and the myofilament space, because deposits were formed even when the applied droplet elicited no contractile response at all. Precipitation of phosphate hydrolyzed from ATP during circulation of other ions can probably be ruled out also, since deposit formation was not affected by either the interval between the end of droplet application and the start of fixation or treatment with strophanthidin in concentrations known to inhibit active sodium transport (Horowicz and Gerber, 1965; Costantin and Podolsky, 1967).

The report of Nagai et al. (1965) that microsomes derived from internal membranes of rabbit skeletal muscle accumulate lead as well as calcium raises the possibility that the deposits found in the control experiments are due to the accumulation of lead by the sarcoplasmic reticulum. Thus, since lead has a relatively high electron opacity, the additional density might simply reflect an accumulation of lead, or the deposits could be produced by precipitation of phosphate hydrolyzed from ATP during transport of lead ions.

The unexpected finding that deposits were almost completely absent from myofilament areas known to have contracted for at least several seconds is noteworthy because of the strong evidence that contraction is associated with hydrolysis of ATP in the region of overlap of the two types of myofilaments.[1] Myofilament movement during contraction should not have been a factor in the absence of precipitate, since this movement would be expected to displace longitudinally, rather than eliminate, a deposit.

The question arises as to whether the levels of lead and phosphate required for precipitation could have been exceeded in the contracting region of the fiber. Relevant data on this point are: (*a*) the threshold phosphate concentration for precipitation in the lead nitrate-tris maleate solution is, by direct measurement, close to 0.01 μM/ml, and (*b*) the contraction-associated ("Mg^{++}-activated") ATPase of myofibrils isolated from a gram of frog muscle releases phosphate at a rate of about 1 μM/sec at room temperature[2] (Bendall, 1964;

[1] Important observations in this regard are: (*a*) the myosin ATPase site is located on structures that project from the A filament (Szent-Györgyi, 1953; Rice, 1961; Lowey and Cohen, 1962; Huxley, 1963); (*b*) this ATPase is activated in vitro by actin, the major constituent of the I filament (Szent-Györgyi, 1951; Huxley and Hanson, 1954); and (*c*) both ATPase activity and the ability of muscle fibers to develop force depend on the extent of overlap of the two types of filaments (Infante et al., 1964; Ward et al., 1965; Sandberg and Carlson, 1966; Gordon et al., 1966).

[2] Although the presence of lead nitrate and tris maleate could have inhibited the myofibrillar ATPase activity, the slight effect of these reagents on the contractile response makes it unlikely that the activity was reduced by more than two- or three-fold. The argument is based on (*a*) the empirical relation between ATPase activity and speed of muscle shortening (Bárány, 1967), and (*b*) the observation that the

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Bárány, 1967). Therefore deposit formation would be expected in the myofibrils just under the applied droplet if solutes moved from the droplet into the fiber by simple diffusion and if lead phosphate precipitation were fast. This is apparently not the actual situation, possibly because various interactions of Pb^{++} with structural elements of the fiber (Gillis and Page, 1967; also indicated by the present control experiments) lower the concentration of this ion in the myofilament space below the level required for precipitation.

In conclusion, the fact that the phosphate presumably liberated from ATP in the course of contraction is not precipitated locally by Pb^{++} ions, even though accumulations of density are found elsewhere in the cell, shows that the absence of deposits in an area cannot be taken as evidence for the absence of enzymatic activity in that area. This result, in addition to the observation of Gillis and Page (1967) that when phosphate is added to glycerinated rabbit fibrils incubated in lead the resulting precipitate has a distinct affinity for the A filament projections, makes it appear that deposit localization in the presence of lead is neither necessary nor sufficient evidence for localized phosphatase activity in cells.

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contraction elicited by application of small droplets ($<5\ \mu$ in diameter) of 1.4 mM $CaCl_2$ in the presence of the lead nitrate-tris maleate solution is only slightly slower than that elicited by the same concentration of calcium in the presence of 140 mM KCl.

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