BINDING OF CALCIUM AND MAGNESIUM BY THE CONTRACTILE ELEMENTS

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The observation that brief bathing in solutions of PP[1] or EDTA alters strikingly the properties of extracted muscle fibers, has indicated that the contractile elements contain tightly bound metals which are essential for contractility (1, 2). Because the effect of PP could be reversed by Mg only (1), that of EDTA by Ca (2), it was concluded that the former releases bound Mg and that the latter combines with, and inactivates, bound Ca.

This problem was further studied by determing Ca and Mg in washed muscle fibers chemically under conditions similar to those in the experiments mentioned. The results showed that the fibers actually contain tightly bound Ca and Mg, that PP releases specifically Mg, although only very incompletely, whereas EDTA removes predominantly Ca. It was also found that removal of even a small portion of these metals has a striking effect on the physiocochemical properties of muscle fibers.

Methods

Psoas muscles from the rabbit, extracted in 50 per cent glycerol for 2 or more weeks (3), were used. For the determination of Ca and Mg a few grams of muscle were separated into fine strands (0.2 mm. thick or less) by steel needles. They were washed in 0.16 M KCl and then divided into several nearly equal batches, each containing 0.5 to 0.8 gm. muscle. Each batch was suspended in 25 ml. of an experimental solution. Solutions containing Ca or Mg were washed out by suspending the fibers in 25 ml. 0.16 M KCl and repeating this process at least six times. Since equilibrium between the solution and the fibers is established within a few seconds, this washing is equivalent to very exhaustive dialysis.

Solutions of PP and EDTA were made up in KCl solutions so that the concentration of K ions was about 0.16 M per liter. The disodium salt of EDTA was obtained from Bersworth Company, Framingham, Massachusetts (analytical reagent grade) and was neutralized with KOH. While in these solutions, the fibers were kept at room temperature (about 20°), but during dissection and washing they were immersed in cold solutions (about 10°).

Preparation of Extract.—For analysis the fibers were briefly brought into 0.02 M

[1] The following abbreviations will be used, ATP for adenosinetriphosphate, EDTA for ethylenediamine tetraacetate, PP for inorganic pyrophosphate.

KCl, so as to remove most of the KCl, blotted with filter paper, and weighed. They were extracted by an equal volume of 10 per cent trichloracetic acid for 20 minutes. The coagulated fiber mass was teased with glass needles and washed eight times in 0.5 ml. water. The remaining material yielded only a barely visible amount of ash which gave no visible precipitate with oxalate, even at strongly alkaline reaction.

The extract was centrifuged and the small amount of sediment was discarded. The fluid was then evaporated in a 15 ml. pyrex centrifuge tube in an oven at about 140°. Organic matter was destroyed by adding a drop of 30 per cent hydrogen peroxide and a drop of concentrated nitric acid. By placing the tubes vertically on a thermostatically controlled hotplate (180°) the solutes were concentrated at the bottom of the tubes, because the acid vapor condensed on the walls and carried the solutes downward. The acid then was completely evaporated in an oven at 140°.

Precipitation of Ca.—Into the bottom of each tube were introduced 0.02 ml. 1 M ammonium acetate of pH 6.2 and, while the tubes were in a water bath at 90°, two drops of 2 per cent ammonium oxalate. After standing for 1 or more hours at room temperature, the precipitate was separated by centrifugation and washed twice with 0.3 ml. 0.2 per cent ammonium oxalate containing 0.1 M ammonium hydroxide. The fluids containing Mg were transferred into another centrifuge tube. After drying, oxalate was oxidized by adding a drop of 30 per cent hydrogen peroxide.

Titration.—None of the standard methods seemed adequate to determine the small amounts of Mg present. Therefore, Schwarzenberg's method of titration with EDTA (4), using eriochrome black T as indicator, was adapted for this purpose.

After drying, the content of each tube was dissolved in a drop of 0.2 M HCl and transferred into a small white porcelain crucible for titration. The walls of the centrifuge tube then were rinsed with standardized EDTA reagent (Hach Chemical Company, Ames, Iowa) from a microburet with a capacity of 0.2 ml. (Microchemical Specialties Company, Berkeley, California). This fluid also was transferred to the titration dish. A small amount of indicator solution (Monover, obtained from Hach Chemical Company) was added and, under constant stirring, more reagent until the color was a pure blue. The tube was now rinsed again with reagent from the buret. After transferring this fluid to the titration dish and adding a known amount of $CaCl_2$, the solution was titrated a second time. The two values checked very closely.

Mg was first precipitated as Mg ammonium phosphate in order to remove a substance which bleached the indicator. The precipitate was washed twice with small amounts of 1.5 M ammonium hydroxide and dissolved in a drop of concentrated HNO_3. The residue obtained after evaporating the acid was titrated as described for Ca.

With every series of unknowns, a solution containing 5.10^{-7} M Ca and 5.10^{-7} M Mg was analyzed. The values for Ca were 0 to 6 per cent too high, those for Mg, 0 to 8 per cent too low. The experimental values were corrected for these errors.

After extraction, the muscle fibers were dried at 100° until weight was constant. The results were expressed as millimoles per kilogram wet weight and were calculated on the assumption that dry weight is 18 per cent of wet weight.

RESULTS AND CONCLUSIONS

Factors Influencing Bound Ca and Mg.—Muscle fibers which have been in glycerol solutions for periods of 10 days to several months and washed in 0.16

M KCl for periods up to 2 hours contained on the average in 20 different muscles 0.58 millimole Ca (range 0.46 to 0.7) and 0.55 millimole Mg (range 0.45 to 0.72) per kg. wet weight.

When the fibers were brought into solutions containing $CaCl_2$ or $MgCl_2$ and washed in KCl solution for a few minutes, their content of Ca or Mg at first was considerably elevated, but dropped back nearly to the previous level within about an hour (Figs. 1 and 4). However, if the fibers were washed in distilled water, a large amount of these metals remained, indicating that the

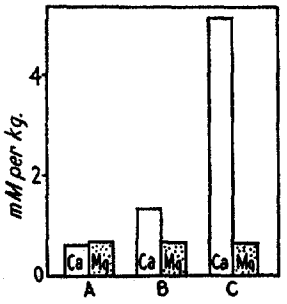

FIG. 1

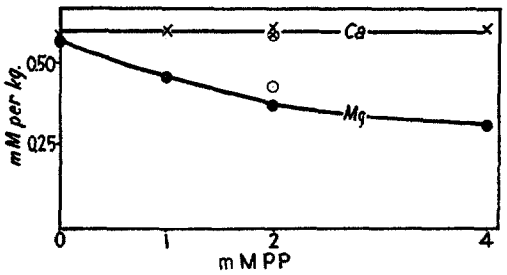

FIG. 2

FIG. 1. Uptake of Ca from 0.05 M $CaCl_2$ and release in KCl solution. Of the three batches of fibers used, batch A was washed only in KCl solutions. B and C were immersed in 0.05 M $CaCl_2$ for 2 minutes. B was then washed in 0.16 M KCl, C in distilled water, both for 20 minutes, by changing solutions eight times. Most of the Ca taken up was washed out by KCl solution, not by water.

FIG. 2. Release of bound Mg by PP. Of five batches of fibers one was brought into 0.16 M KCl, the others for 1 minute into PP solutions (pH 7) of different concentrations. PP was washed out again. This procedure was repeated twice. One batch (crossed circles for Ca, open circles for Mg) was left in 2 mM PP for 20 minutes and washed only once. All batches were extracted at the same time.

excess was bound electrostatically and was released only in exchange with monovalent ions (Fig. 1). This finding confirms previous conclusions based on the effect of ions on tension and volume of muscle fibers (5).

The observation that repeated washing in PP solutions abolishes the softening effect of PP (1) and partly also that of ATP, and that this change in the properties of the fibers is reversed by brief immersion in a solution containing $MgCl_2$ was explained by assuming that bound Mg is essential for the softening action of polyphosphates and that PP partially removed this metal. This conclusion was confirmed by the experiment illustrated in Fig. 2. Muscle fibers were immersed in solutions containing different concentrations of PP for about 1 minute, then washed three times in 20 ml. 0.16 M KCl. This procedure was repeated twice. 4 mM PP removed about half of the bound Mg (range: 45 to 55 per cent in 7 experiments), no Ca. The fibers lost less Mg if they were im-

mersed in PP solution and subsequently washed only once, even if they remained in the PP solution five times longer than the other fibers. PP probably attaches itself to bound Mg and thereby weakens the bond between Mg and protein, allowing the PP-Mg complex to dissociate from protein.

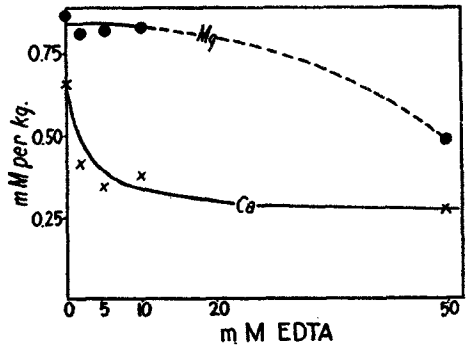

FIG. 3. Release of Ca and Mg as a function of the concentration of EDTA. All fibers were first immersed in 0.16 M KCl containing 2 mM $MgCl_2$ per liter, then washed in 0.16 M KCl. Different batches were immersed in solutions containing different concentrations of EDTA (pH 7) for 15 minutes, then washed again in 0.16 M KCl. Filled circles for Mg, crosses for Ca.

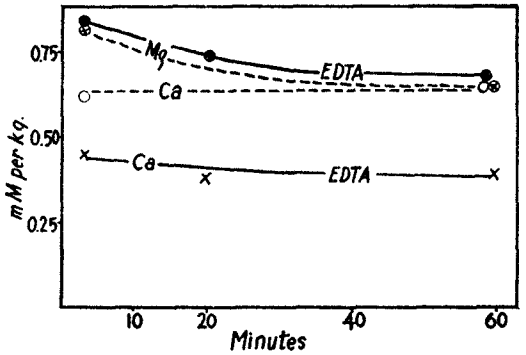

FIG. 4. Release of Ca by EDTA as a function of time. All fibers were first immersed in 0.16 M KCl containing 2 mM $MgCl_2$ per liter. Three batches were bathed in 5 mM EDTA (pH 7) for 2.5, 20, and 60 minutes respectively (filled circles for Mg, crosses for Ca.) Two controls were washed in 0.16 M KCl for 5 and 60 minutes respectively (crossed circles for Mg, open circles for Ca).

In contrast to PP, EDTA mainly removed Ca, producing only small losses in Mg. As shown in Fig. 3 the loss of Ca was almost maximal at a concentration of 5 mM EDTA per liter (range: 35 to 49 per cent in 7 experiments). It occurred within a few minutes (Fig. 4) and it was independent of pH between

pH 6.5 and 8.0 (Fig. 5). Appreciable amounts of Mg were removed only at high concentrations of EDTA and at high pH.

These findings have some interest in connection with the relaxing effect of EDTA. Muscle fibers, contracting in an ATP solution containing Mg, relax when EDTA is added. An excess of Ca subsequently causes contraction. It was concluded that bound Ca is essential for activation of muscle fibers. Still more significant is the fact that brief immersion in an EDTA solution changes the condition of muscle fibers so that ATP causes relaxation even after EDTA

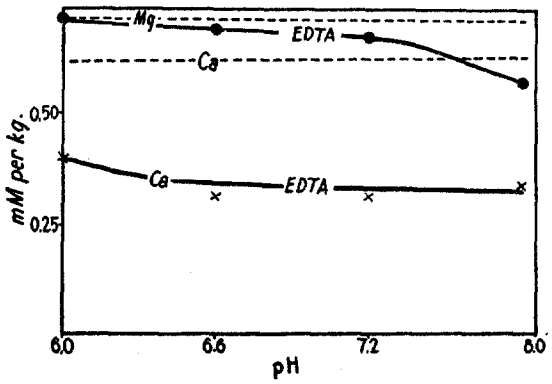

FIG. 5. Release of Ca and Mg by EDTA as a function of pH. All fibers were first immersed in 0.16 M KCl containing 2 mM $MgCl_2$, then washed in 0.16 M KCl for 10 minutes. Four batches were bathed for 20 minutes in 5 mM EDTA adjusted to pH 6, 6.6, 7.2, and 8.0. Interrupted lines indicate the content of Ca and Mg of another batch which was kept in KCl solution also during this period, and served as control. Filled circles for Mg, crosses for Ca.

has been washed out. Because the original state of the fibers is restored by solutions containing $CaCl_2$, it is suggestive to assume that the relaxing action of EDTA is due to the removal of bound Ca, in agreement with the results just described. However, the relaxing action of ATP in EDTA-treated muscle fibers has been found to disappear under certain conditions without the addition of Ca. It appears probable, therefore, that the portion of the metal not removable by EDTA is sufficient for activation and that EDTA acts by firmly combining with this portion.

Physicochemical Properties of Muscle Fibers.—After removing part of the bound Ca or Mg, muscle fibers appeared slightly swollen and more translucent. These effects were already noticeable while the fibers were in 0.16 M KCl, but became much more striking at lower salt concentrations, as shown in the experiments illustrated in Figs. 6 and 7. Tension and weight were measured as described previously (5). When the fibers were brought into distilled water

without previous treatment, tension and weight remained unchanged, but after they were washed in PP solutions three times, as described above, distilled water caused a considerable rise in tension and volume. When the salt concentration was increased again, tension and volume decreased. Brief bathing in solutions containing low concentrations of $MgCl_2$ almost completely restored the original condition of the preparations. Also EDTA induced a tendency to swell, like PP, evidently because it removes bound Ca.

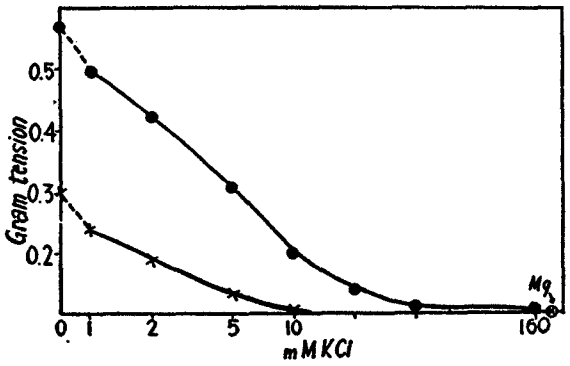

FIG. 6. Contraction in dilute solutions. Originally tension did not rise when fibers were immersed in distilled water. Upper curve, tension after fibers were washed three times in 4 mM PP solution. Crossed circle, after adding 2 mM $MgCl_2$ per liter. Lower curve, the same preparation after being immersed for 2 minutes in solution containing 0.16 M KCl and 2 mM $MgCl_2$ per liter, then washed in 0.16 M KCl for 5 minutes.

ATP has previously been shown to produce effects similar to those described here for PP (5). It is not clear why the fibers used in the earlier experiments swelled and contracted slightly even without any previous treatment, while in the present experiments this occurred only after immersion in solutions of PP, EDTA, or ATP.

The fact that removal of even small amounts of bound Ca or Mg (1 equivalent per 500,000 gm. of actomyosin) strongly increases the swelling and contraction in dilute salt solutions seems remarkable. The number of charges set free seems to be too small to produce this effect. Perhaps the metals form strong cross-links. Polyphosphates may soften the contractile elements by combining with Mg, thereby weakening these bonds.

Swelling, diminution in light scattering, and contraction produced by diminishing salt concentration are expressions of an increase in the charge of protein and, with the exception of contraction, have been studied extensively in protein solutions. Swelling may be explained as the result of a repulsion of protein molecules, like similar phenomena in oriented gels of tobacco mosaic virus (6), or as Donnan phenomenon. Experiments on the effect of pH which

could clarify this problem gave conflicting results when different buffers were used. Contraction is probably also determined by the net charge of protein, because it is always associated with swelling, but it has not been explained adequately. That it is not specific for muscle fibers, is shown by the fact that it was observed also in collagen fibers from the rat's tail. However, PP and EDTA did not increase the effect in this preparation.

The binding of Ca by several simple proteins can be expressed by a single monomolecular dissociation constant of about 10^{-3} (*cf.* reference 7). It seems

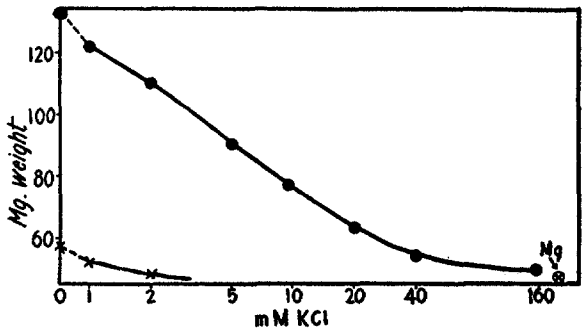

FIG. 7. Swelling in dilute solutions. Freshly prepared fibers did not swell in distilled water. Upper curve obtained after fibers were washed three times in 4 mM PP solution; crossed circle, after adding 2 mM $MgCl_2$ per liter. Lower curve, the same preparation after being immersed for 2 minutes in solution containing 0.16 M KCl and 0.01 M $MgCl_2$ per liter, then washed in 0.16 M KCl for 5 minutes.

unlikely that a single constant would describe the results obtained with muscle fibers. About half of the Ca and 95 per cent of the Mg present in the living muscle fiber are removed by prolonged washing in 50 per cent glycerol and 0.16 M KCl. Such fibers take up Ca and Mg from dilute solutions containing these metals, but the excess is readily released in exchange for monovalent metals. Only about half of the remaining Ca and Mg can be removed by strong chelating agents. Such a strong binding cannot be easily explained at present because aminopolycarboxylic acids, such as EDTA, combine with Ca and Mg more firmly than any other known chemical agents, and the contractile elements bind these metals even more tightly.

After prolonged washing in Ca-free solutions actin was found to contain a constant amount of Ca, 0.215 mg. per gm. actin (8). If the actin in washed muscle fibers contained the same amount of Ca, it would account only for about one-fourth of the amount actually found. Friess, Bowen, and Morales (9) in a brief communication mention that extracted actomyosin after prolonged dialysis contains 0.03 to 0.18 μg. of divalent metals per mg. protein, mostly Mg. Assuming that washed muscle fibers contain 10 per cent actomyosin and that

it binds all the Mg present, it would contain 0.13 μg. per mg. actomyosin, a value close to the upper value for extracted protein.

Mg and Ca must be considered an integral part of the contractile elements. These metals are present only in small amounts, but are bound at strategic locations as shown by their influence on the effects of ATP and the physicochemical properties of muscle fibers. For the understanding of contractility it would be important to know more about where and how these metals are bound.

SUMMARY

Using a technique for determining Ca and Mg based on Schwarzenberg's method of titration with ethylenediamine tetraacetic acid (EDTA), it was found that glycerol-extracted muscle fibers contain on the average 0.58 millimole Ca and 0.55 millimole Mg per kg. muscle. The fibers take up additional Ca or Mg from dilute solutions of these metals, but in KCl solutions, the excess is exchanged for K ions.

Inorganic pyrophosphate (PP) removes part of the bound Mg, no Ca; EDTA removes predominantly Ca, but never more than about one-half the total amount. These results are discussed in relation to previous observations on the effects of PP and EDTA on mechanical properties and contractility of extracted muscle fibers.

After the partial loss of bound divalent metals, muscle fibers swell in dilute salt solutions; they also contract slightly and become more translucent.

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