RELAXATION IN EXTRACTED MUSCLE FIBERS*

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Szent Györgyi (1–3) discovered that ATP[1] produces a strong contraction in muscle fibers which have been preserved in glycerol. Later Bozler (4–7) found that such fibers, which originally are in rigor, can be transformed into a state closely resembling normal relaxation by physiological concentrations of ATP. The fibers then are very extensible and plastic, and give a strong contraction on addition of small amounts of $CaCl_2$. This state can be produced and maintained only in the presence of Mg and only in rather fresh fibers. Following up work by Marsh (8) on the syneresis of muscle homogenates, Bendall (9) has shown that the ability of briefly extracted fibers to relax is due to the presence of a non-dialyzable factor contained in muscle extract, which will be called relaxation factor ("Marsh factor"). The relaxing effect of ATP is further supported by two other substances normally occurring in tissues, PC (10–12) and PP (7, 13, 14).

In the present paper the clarification of the mechanism of action of these factors will be attempted, particularly that of the relaxation factor. It was found that EDTA closely imitates all the effects of this factor. The results indicate that relaxation is caused by the inactivation of bound Ca. Previous evidence that the relaxed state is due to the formation of an enzymatically inactive ATP–protein complex was confirmed.

Technique

Glycerol-extracted psoas muscle from the rabbit (1) was used. Unless stated otherwise, the muscles were extracted for 4 or more weeks. Contractions were recorded by a tension lever writing on a smoked drum (5). The preparations had a cross-sectional area of about 0.2 mm². They were divided into several finer strands and mounted so as to make possible rapid diffusion (6, 7). The effect of chemical agents began almost instantaneously at maximal speed, indicating that diffusion equilibrium was established within a few seconds. At the beginning of every experiment the

* This paper is dedicated to Freiherr W. von Buddenbrock, Professor of Zoology, University of Mainz, Germany, on the occasion of his seventieth birthday.

[1] The following abbreviations will be used: ATP for adenosine triphosphate, EDTA for ethylenediamine tetraacetate, PC for phosphocreatine, PP for inorganic pyrophosphate.

muscle fibers were allowed to shorten about 10 per cent of their length in 8 mM ATP. In some experiments, extension under a constant load was recorded as described previously (15). For reproduction the graphs were drawn from photographic enlargements of the originals (magnified about four times).

All solutions had a total electrolyte concentration equivalent to 0.16 M KCl. They were prepared by mixing stock solutions with 0.16 M KCl. Solutions of ATP (Pabst Laboratories, Milwaukee), EDTA (Analytical Reagent, Bersworth Chemical Co., Framingham, Massachusetts), and PP were brought to pH 7.

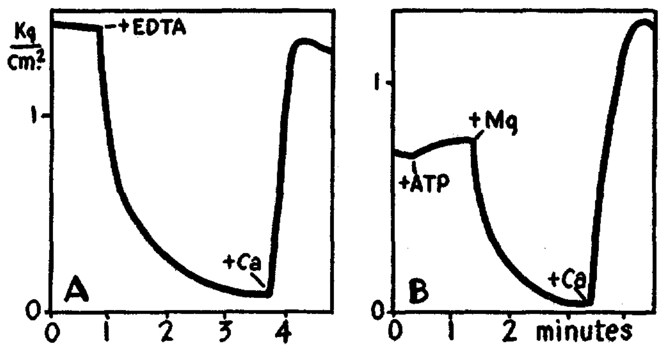

FIG. 1. Relaxing effect of EDTA. A, a contraction was produced by 6 mM ATP containing 2 mM $MgCl_2$ per liter. Addition of EDTA (1 mM per liter) caused relaxation of $CaCl_2$ (2 mM per liter) later caused contraction. B, role of Mg. At first the preparation was in solution of EDTA (1 mM EDTA per liter). The addition of ATP (6 mM per liter) without Mg caused weak contraction, of $MgCl_2$ (2 mM per liter) rapid relaxation, of $CaCl_2$ (2 mM per liter) contraction. 18°

RESULTS

EDTA and the Protein Factor.—As shown by Bendall (9), ATP causes relaxation in old muscle fibers if Mg and the relaxation factor are present. It was found that the effects of this factor are imitated in every detail by EDTA. It seemed probable, therefore, that the factor acts by forming a complex with bound Ca.

EDTA does not noticeably influence contractions produced by ATP, as long as there is an excess of Mg and Ca. In the absence of Ca, however, it has the following effects.

(*a*) Muscle fibers which are brought into a state of contraction by a solution of ATP containing Mg, relax rapidly if EDTA (0.4 mM or more per liter) is added. This effect depends on Mg, like relaxation induced by the relaxation factor, while Ca causes at once a strong contraction in relaxed fiber (Fig. 1).

(*b*) Muscle fibers which have been briefly bathed in 0.16 M KCl containing 5 to 10 mM EDTA per liter and then washed many times in 0.16 M KCl, relax rapidly in a solution containing ATP and $MgCl_2$. Relaxation in such fibers is faster and more complete than that which can be produced by the relaxation factor, and is obtained even with the weakest effective concentrations of ATP (about 0.2 mM per liter). $CaCl_2$ (0.5 mM per liter) elicits a rapid and strong contraction in relaxed fibers (Fig. 2). In short, EDTA-treated fibers behave like briefly extracted fibers, except that the relaxing action of ATP is much stronger.

The effect of EDTA treatment remains undiminished for at least 30 minutes, probably indefinitely, while the fibers are immersed in KCl solution. If they are briefly brought into a solution containing 2 mM $CaCl_2$ and then washed in KCl solution, the previous state of the fibers is restored completely. This cycle, the alternation of states in which ATP causes contraction and relaxation, can be repeated in the same preparation as often as desired. It may be concluded from

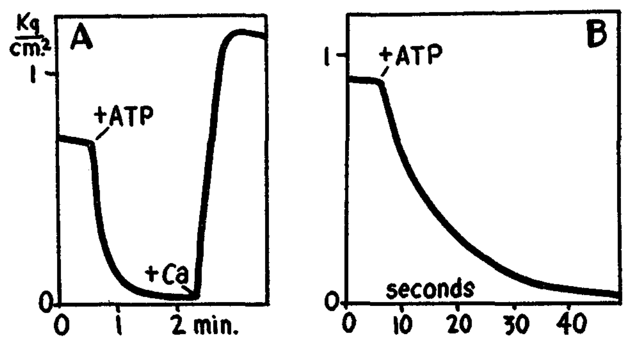

FIG. 2. Effect of ATP on EDTA-treated fibers. A, fibers which had been immersed in 5 mM EDTA, then washed five times in 0.16 M KCl containing 2 mM $MgCl_2$ per liter, relaxed on addition of ATP (6 mM per liter), contracted after the further addition of $CaCl_2$ (1 mM per liter). B, relaxation produced as in A, but recorded on a faster drum. 18°.

these observations that EDTA changes the properties of the fibers by combining with bound Ca, thereby inactivating this metal.

The effect of EDTA disappears under certain conditions in the absence of Ca. After ATP has been washed out from relaxed fibers and rigor has been restored, the subsequent application of ATP causes contraction or a slow and incomplete relaxation. After one or more repetitions of this procedure the original condition is always restored. This behavior again imitates that of briefly extracted fibers (5, 6). The gradual disappearance of the effect of EDTA probably is caused by the removal of EDTA from the fibers in the presence of ATP.

The effect of EDTA is also influenced by pH. If EDTA-treated fibers are washed in a KCl solution to which phosphate or glycylglycine buffer of pH 7 is added, the effect of EDTA disappears gradually and is hardly noticeable after about 15 minutes. At lower pH the effect of EDTA disappears more slowly.

The observation that the effect of EDTA can be abolished without Ca suggests that EDTA does not remove Ca from the contractile elements. In analogous experiments involving Mg (16), on the other hand, the softening action of PP could not be restored, after it had been abolished by washing in solutions of EDTA, or PP, except by immersion in a solution containing Mg, indicating that Mg had been removed.

Role of Mg and Ca.—EDTA-treated fibers are favorable for the study of the significance of Mg and Ca, because EDTA seems to inactivate these metals, even when bound to protein. In the experiment illustrated in Fig. 3, ATP caused only a slight contraction in EDTA-treated fibers. The addition of

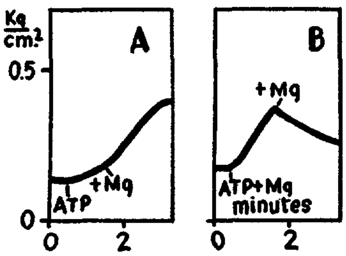

FIG. 3

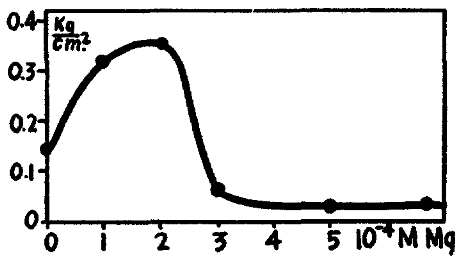

FIG. 4

FIG. 3. Effect of different concentrations of Mg in EDTA-treated fibers. In A ATP (6 mM per liter) gave only a weak contraction. The addition of $MgCl_2$ (0.1 mM per liter) increased contraction. In B the same preparation had been treated with EDTA again. 6 mM ATP containing 0.2 mM $MgCl_2$ per liter caused a strong contraction, followed by relaxation, when more $MgCl_2$ was added (giving a concentration of 0.4 mM per liter). 15°.

FIG. 4. Reversal in the effect of Mg in EDTA-treated fibers. Tension developed after 1 minute plotted against concentration of Mg. The fibers were brought into 6 mM ATP containing different amounts of $MgCl_2$. After every contraction the fibers were immersed in 5 mM EDTA and washed in 0.16 M KCl. 15°.

small amounts of $MgCl_2$ increased this contraction, but above a critical concentration of 0.4 mM per liter it produced relaxation. It is noteworthy that beyond a narrow zone of transition a further increase in the concentration of Mg had no effect on the speed or the completeness of relaxation (Fig. 4).

In most experiments the contraction produced in Mg-free solutions was stronger than in the experiment described. It was diminished by EDTA and by previous washing with PP solutions, but not abolished. Also the concentration required for relaxation varied in different preparations; it was never higher than 0.4 mM, often as low as 0.1 mM per liter.

A reversal in the effect of Mg is demonstrated also in another type of experiment. As mentioned before, EDTA in the presence of ATP and Mg causes relaxation, but beyond a certain concentration, which depends on that of Mg, it causes contraction. In the experiment illustrated in Fig. 5 EDTA-treated fibers relaxed in 6 mM ATP containing 0.2 mM $MgCl_2$ per liter. They contracted after the addition of a rather large amount of EDTA, but relaxed again when more $MgCl_2$ was added. The contraction induced by EDTA developed slowly, but was as strong as that later produced by $CaCl_2$. These observations do not contradict the accepted view that Mg is essential for contraction, because Mg is only incompletely chelated by EDTA at pH 7 (17).

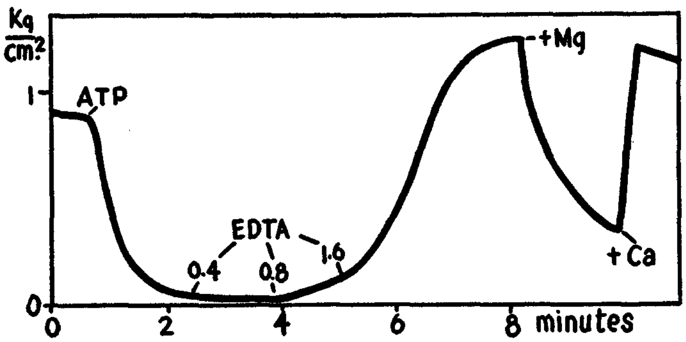

FIG. 5. Contraction induced by high concentrations of EDTA. In EDTA-treated fibers relaxation was produced by 6 mM ATP containing 0.2 mM $MgCl_2$ per liter. EDTA was added, bringing concentration successively to 0.4, 0.8, 1.6 mM per liter. After a strong contraction had been produced, $MgCl_2$ (2 mM per liter) was added, causing relaxation, then $CaCl_2$ (2 mM per liter), causing contraction. 18°.

Contraction produced by lowering the concentration of Mg was first observed in briefly extracted fibers (6). After they were first brought into a relaxed state, they contracted at once, and ATP breakdown increased, when they were immersed into an ATP solution free of Mg. Also recent observations on phosphate liberation in muscle homogenates indicate a reversal in the effect of Mg (9).

The effects of Mg and Ca on the contractile elements described here appear complex, but they may be described briefly as follows. In the presence of ATP, Mg has two distinct effects, contraction and softening. The first of these requires only a very small amount of Mg. At higher concentrations the dissociating effect predominates, resulting in lowered ATPase activity and relaxation. Bound Ca, if not inactivated by a complexing agent, and in the presence of Mg, increases ATPase activity and causes contraction.

Relaxing Action of ATP at 0°.—Szent-Györgyi (1) has shown that ATP produces only a weak contraction at 0° and that it makes the fibers very soft. Mechanically, this state does not differ from relaxation at higher temperatures, but it can be produced in old fibers without the relaxation factor. As shown below, the effects of Mg and Ca are also quite different. This phenomenon was investigated because it challenges the views on the nature of relaxation presented here. In the experiments to be described the rate of extension under a constant load was recorded.

That ATP causes relaxation at 0° without the relaxation factor, can be explained on the assumption that, under conditions of low ATPase activity,

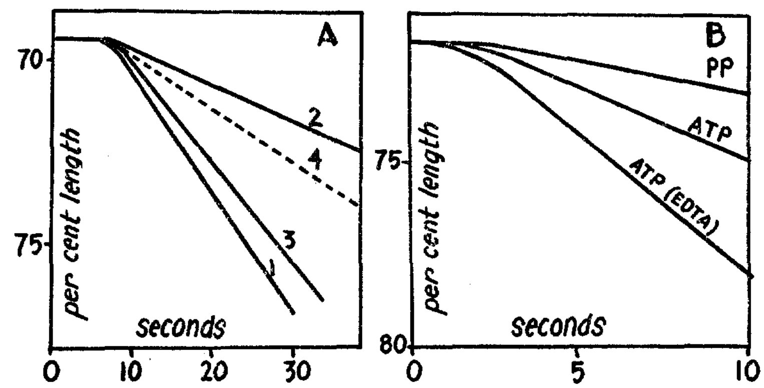

FIG. 6. Factors determining rate of extension under a constant load at 0°. Extension began as soon as ATP or PP was brought into chamber. In A extension by a load of 110 gm. per cm.² 8 mM ATP (without Mg) produced curve 1. After the preparation was alternatively immersed in 2 mM PP and KCl solution three times, extension in ATP became slower (curve 2). The fibers were then immersed in 2 mM $MgCl_2$ and washed several times in KCl solution. ATP then produced curve 3. After adding $MgCl_2$ (2 mM per liter) ATP gave curve 4. In B the preparation was extended by a load of 150 gm. per cm². It was first brought into 8 mM ATP (middle curve). After EDTA treatment the same ATP solution produced the lower curve. Upper curve produced by 8 mM PP containing $MgCl_2$ (2 mM per liter). After every extension the preparations were brought back to their original length by ATP. Ordinate, per cent original length.

it acts like inorganic polyphosphate, which is effective without the factor. Because PP requires the presence of Mg (16), it had to be expected, then, that at 0° Mg would increase the relaxing effect of ATP. Actually, the addition of $MgCl_2$ made extension somewhat slower (Fig. 6 A), undoubtedly because it increased contraction. However, the following experiment demonstrates a relaxing effect of Mg, thus confirming the explanation of relaxation given above.

Muscle fibers were made refractory to PP by previous washing in PP as described previously (16). After this procedure, which presumably removed bound Mg, ATP was much less effective (Fig. 6 A), but brief immersion in

2 mM $MgCl_2$ restored the effect. This cycle of partial refractoriness and restoration of the effect was repeated in the same preparation as often as five times. That the softening effect of ATP cannot be completely abolished, like that of PP, probably is due to the presence of some Mg remaining in the fibers and the stronger action of ATP (Fig. 6 B).

It was found that $CaCl_2$ did not change the rate of extension in these experiments, another difference from relaxation at higher temperatures. This observation, which is related to the fact that the relaxation factor is not necessary, can be explained on the assumption that the factor, as well as low temperatures, produces relaxation primarily by lowering ATPase activity (see below). The contraction produced by ATP at 0°, which is weak because of low ATPase activity, presumably is due to bound Ca. EDTA abolishes it, thereby speeding up extension and revealing the full extent of the softening action of ATP (fig. 6 B). It is interesting that in such fibers the rate of extension increases as temperature is lowered, as had been found previously for the effect of PP (16).

Binding of ATP.—Experiments on briefly extracted muscle fibers have shown that the softening action of ATP is due to the formation of an enzymatically inactive complex with protein (6, 11). This could be demonstrated because the complex is rather stable in the absence of ATP. The conditions for observing this phenomenon were further investigated.

It was found that the effect of different preparations of ATP varied considerably. Relaxation produced by chromatographically purified preparations was usually incomplete, often temporary, but it was rapid and lasting after the addition of a small amount of PP. A maximal relaxing effect usually was produced by PP in concentrations of 0.6 mM per liter. This effect of PP is specific; it could not be obtained by a corresponding, or even much larger, increase in the concentration of ATP. However, low concentrations of PP caused relaxation only in briefly extracted fibers. In old fibers much higher concentrations of PP (more than 4 mM per liter) were required to produce even a slight diminution in contraction. It seems probable that PP increases the relaxing action of ATP by the same mechanisms as EDTA, by complexing bound Ca, because this action of PP is cancelled by $CaCl_2$.

Briefly extracted fibers which have relaxed in a solution of ATP containing Mg and PP, and which are subsequently washed in a KCl solution containing $MgCl_2$, remain in the relaxed state for some time. At 20° rigor usually returns after 3 to 5 minutes (Fig. 7). This period is longer at lower temperature and decreases with increasing age of the fibers. EDTA-treated old fibers also show this phenomenon, but rigor already returns after 1 to 2 minutes (Fig. 7 C).

During the time before the onset of rigor the fibers remain soft and transparent, although no ATP is in the solution. They contract instantly if $CaCl_2$

is added (Fig. 7 B) or if they are immersed in a KCl solution free of Mg, thereby demonstrating that ATP is bound by the contractile elements.

Recently analogous observations were made regarding the effect of PP. It was found that, in the presence of Mg, the effect of PP persisted for some time after the fibers had been washed in KCl solution free of PP, indicating the formation of a rather stable protein–PP–Mg complex. The gradual disappearance of ATP and PP from the fibers is probably, at least in part, due to the enzymatic breakdown of these substances.

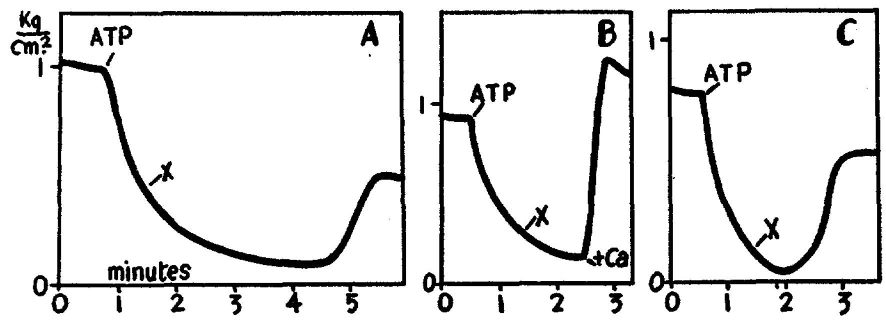

FIG. 7. Effect of washing out ATP from relaxed muscle fibers. Relaxation was produced by a solution containing 6 mM ATP, 0.6 mM PP, and 2 mM $MgCl_2$ per liter. At X the ATP solution was replaced by KCl solution containing 2 mM $MgCl_2$. In B $CaCl_2$ (1 mM per liter) was added later, causing contraction. The fibers used in A and B had been in glycerol for 3 days, those used in C for about 6 weeks. In C the fibers had been briefly immersed in 5 mM EDTA before the application of ATP. 18°.

DISCUSSION

The energy release in extracted, probably also in normal, muscle is controlled chiefly by the interaction of Mg, Ca, and the relaxation factor with the contractile elements. No definite information on the mechanism of this interaction has been available up to now. Bendall (9) believes that the relaxation factor is activated by Mg and inactivated by Ca. This view, however, does not explain the relaxation produced by ATP at 0° and by PP, effects which do not require the factor, but depend on Mg, indicating a direct action of the metals on the contractile protein. According to Weber (18), the factor makes the contractile elements more reactive to ATP and thereby shifts the zone of superoptimal concentrations of ATP to a lower level. This assumption cannot be reconciled with the fact that Ca is ineffective at any concentration of ATP, even in the presence of PC, without the protein factor or a suitable complexing agent.

Goodall and Szent-Györgyi (12) expressed the opinion that the factor is an enzyme. Recently, Lorand (19) found highly purified ATP–creatine transphosphorylase to have a relaxing effect. From the preliminary report available it appears that the relaxation achieved with this material is rather incomplete and requires a pH 6.2 or lower. It seems uncertain, therefore, whether the enzyme has a significant relaxing effect under normal conditions. That rapid transphosphorylation is responsible for relaxation, as assumed by the author, is ruled out by previous observations on the effects of PC (11). In the presence of this substance a nearly maximal contraction is produced by ATP and AMP in concentrations of 10^{-6} to 10^{-5} M per liter, in old as well as briefly extracted muscle fibers, demonstrating the presence of transphosphorylating enzymes. Nevertheless PC does not bring about relaxation in old fibers even at high concentrations of ATP. Thus, rapid transphosphorylation makes ATP effective at very low concentrations, but does not change the character of the response. It is possible, however, that transphosphorylating enzymes produce relaxation by some non-enzymatic action, like EDTA.

At one phase of the present investigation the qualitative similarity of the action of PP to that of the relaxation factor was considered a significant clue to the mechanism of action of this factor. To explain this similarity, it seemed possible that the factor might enhance the softening action of polyphosphates, but it was found not to influence the effect of PP. The possibility that the factor might release PP from ATP was considered, but no evidence for the production of a relaxing agent was found.

A more fruitful attempt to understand the control of muscular activity was based on the view that the effect of ATP depends on a balance between two antagonistic actions, contraction, which is caused by the breakdown of ATP, and a softening action like that caused by PP. Whether contraction or relaxation occurs, then depends on which of these effects predominates.

This line of reasoning accounts for the relaxation produced by ATP at 0° and the closely related depressing effect of high concentrations of ATP. The effect of the relaxation factor can similarly be explained by assuming that it is an ATPase inhibitor, but the main problem, why the factor inhibits only in the absence of Ca, then is still left unanswered. A simple solution of the problem was suggested by the remarkable agreement of the effects of the factor with those of EDTA. The following similarities were found. (1) Relaxation requires the presence of Mg above a critical concentration. (2) $CaCl_2$ produces a strong contraction in relaxed fibers. (3) Both EDTA and the factor are bound to the contractile elements, but slowly released by ATP. (4) In their presence an inactive protein–ATP–Mg complex is formed.

This agreement suggests that the relaxation factor diminishes ATPase activity by forming a complex with bound Ca. According to this hypothesis, represented in Fig. 8, the contractile protein (M) contains Ca to which normally the relaxation factor (R) is attached. The contractile elements then are very rigid, a state called rigor. ATP, in the presence of Mg, firmly combines with the contractile protein and transforms it into an enzymatically inactive complex. The contractile elements now are highly extensible and plastic, in a state of relaxation. They contract when Ca is added. Ca acts by combining with the relaxation factor, thereby exposing bound Ca and increasing enzymatic activity.

It should be noted that the schema presented in Fig. 8 is not intended to represent all observations described here. It does not take into consideration the fact that contraction is produced not only by Ca, but also by a diminution in the concentration of Mg. Ca evidently is not necessary for contraction, but only blocks the inhibitory effect of Mg.

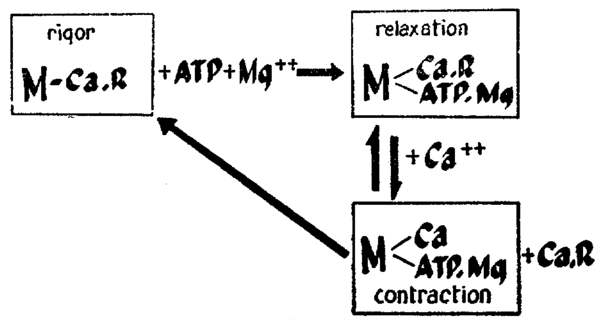

FIG. 8. Explained in text.

That the release of Ca ions initiates normal muscular contraction has been widely accepted (18, 20). This view is supported by the striking effect of Ca in relaxed extracted muscle fibers (6, 7, 9). It should be noted, however, that normal muscle fibers contain so many complexing agents (protein, ATP, etc.) that the formation of Ca ions in effective concentrations appears unlikely. The results reported here indicate, furthermore, that contraction is initiated by bound Ca, not by free Ca ions at all, and that the activity of the contractile elements is governed by the interaction between Mg and Ca. It is uncertain at present how excitation alters suddenly the state of these metals in normal muscle.

SUMMARY

1. Ethylenediamine tetraacetic acid (EDTA) in low concentrations imitates all the known effects of the relaxation factor ("Marsh factor"). In extracted muscle fibers which have contracted in a solution containing adenosinetriphosphate (ATP), the addition of EDTA causes relaxation, the subsequent addition of $CaCl_2$, contraction.

2. In fibers which have been briefly immersed in 5 mM EDTA, ATP causes rapid relaxation if Mg is also present. These fibers have essentially the same properties as briefly extracted fibers. Brief immersion into a solution containing

$CaCl_2$ restores at once the original condition. It is concluded that EDTA produces its action by firmly combining with bound Ca, thereby inactivating it.

3. In relaxed muscle fibers not only Ca, but also lowering the concentration of Mg below a critical level, causes contraction. In such fibers Mg in the lowest effective concentrations increases contraction, but the effect reverses above a certain concentration.

4. At 0° Mg in the presence of ATP has a relaxing effect without the relaxation factor.

5. The results indicate that Mg has two distinct effects in the presence of ATP. It causes contraction at low concentrations, but above a critical concentration its relaxing action prevails. The last of these effects is blocked by bound Ca. If the latter is inactivated by EDTA, Mg in sufficiently high concentrations causes relaxation. The action of the relaxation factor can similarly be explained by assuming that it acts as a complexing agent which inactivates bound Ca.

6. Previous evidence that the relaxed state depends on the formation of an enzymatically inactive ATP–protein complex was confirmed. It was found that PP in low concentrations strongly increases the relaxing effect of ATP in briefly extracted fibers.

REFERENCES

1. Szent-Györgyi, A., *Biol. Bull.*, 1949, **96**, 140.
2. Szent-Györgyi, A., Chemistry of Muscular Contraction, New York, Academic Press Inc., 2nd edition, 1951.
3. Szent-Györgyi, A., Chemical Physiology of Contraction in Body and Heart Muscle, New York, Academic Press, Inc., 1953.
4. Bozler, E., *Fed. Proc.*, 1951, **10**, 19.
5. Bozler, E., *Am. J. Physiol.*, 1951, **167**, 276.
6. Bozler, E., *Am. J. Physiol.*, 1952, **168**, 760.
7. Bozler, E., *J. Gen. Physiol.*, 1953, **37**, 53.
8. Marsh, B. B., *Biochim. et Biophysic. Acta*, 1952, **9**, 247.
9. Bendall, I. R., *J. Physiol.*, 1953, **121**, 232.
10. Bozler, E., *Fed. Proc.*, 1953, **12**, 18.
11. Bozler, E., *J. Gen. Physiol.*, 1953, **37**, 63.
12. Goodall, M. C., and Szent-Györgyi, A. G., *Nature*, 1953, **172**, 84.
13. Bozler, E., *Fed. Proc.*, 1953, **12**, 18.
14. Bendall, I. R., *Nature*, 1953, **172**, 586.
15. Bozler, E., *Am. J. Physiol.*, 1952, **171**, 359.
16. Bozler, E., *J. Gen. Physiol.*, 1953, **38**, 53.
17. The Versenes *Techn. Bull. No. 2*, Framingham, Massachusetts, Bersworth Chemical Co., 5th edition, 1952.
18. Weber, H. H., *Biochim. et Biophysic. Acta*, 1953, **12**, 150.
19. Lorand, L., *Nature*, 1953, **172**, 1181.
20. Sandow, A., *Yale J. Biol. and Med.*, 1952, **25**, 176.