The Nature of the Muscle-Relaxing Factor

II. *Some physicochemical properties*

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ABSTRACT High speed centrifugal fractionation of homogenates of rabbit skeletal muscle has led to the discovery of a soluble muscle-relaxing factor in the homogenate. Assay of the relaxing activity with deoxycholate-treated myofibrils and reconstituted actomyosin systems has established that the activity is not produced by the presence of contaminants. Relaxing activity could be removed or destroyed by charcoal, dialysis, prolonged heating, and treatment with the chelating resin, chelex-100, making it improbable that the effect is due simply to calcium deficiency. Many of the characteristics of this muscle-relaxing factor suggest that it is very similar to or the same as the factor formed by the incubation of muscle granule fractions and ATP. Evidence is presented that some soluble protein component is involved in the stabilization of the factor. The relaxing activity could be separated from the high molecular weight material in the supernatant by the technique of gel filtration. On the basis of the gel used, the molecular weight of the active agent should be less than 4000.

INTRODUCTION

A preliminary communication by Fuchs and Briggs (1) noting that muscle possesses a soluble material capable of inhibiting ATP[1]-induced tension generation in glycerinated muscle fibers has added considerable weight to the supposition that the Marsh muscle-relaxing system (2), by the formation of such an inhibitor (3), is responsible for relaxing contracted muscle and keeping it in the resting state. Acceptance of this hypothesis requires, however, demonstration that the relaxing activity attributed to this soluble factor, isolated directly from muscle, is not due to contamination of the assay systems or the soluble system by the particulate portion of Marsh extract; for studies by Hasselbach (4) have indicated the persistence of remnants of endoplasmic reticulum in glycerinated muscle fibers and myofibrils, materials used for the assay of soluble relaxing factor in previous studies (1, 3, 5, 6). Uncontami-

[1] Abbreviations: ATP, adenosinetriphosphate; EDTA, ethylenediaminetetraacetate.

nated assay systems were, therefore, developed in the preceding paper (7) so that it would be possible to determine whether the activity found in the soluble portion of the muscle homogenate is authentic. Acceptance of the soluble inhibitor hypothesis also requires that the relaxing effects produced by preparations purported to contain such a factor are actually due to the *presence* of some substance therein rather than the *abscence* of calcium as suggested by Ebashi (8) and A. Weber (9, 10). By directed study of the physicochemical properties of the soluble relaxing factor a decision between these two possibilities has been achieved.

MATERIALS AND METHODS

The compositions of extraction solution and contraction solution, as well as the method of preparation of crude Marsh extract, granule fraction, supernatant fraction, glycerinated muscle fibers, and myofibrils, are presented in the preceding communication (7).

Limitations on the possible molecular size of soluble relaxing factor were achieved by the technique of gel filtration described by Porath and Flodin (11). The gels used in these studies, sephadex G-50 and G-25 (obtained from Pharmacia, Box 100, Rochester, Minnesota), were washed with 2 mM EDTA to remove contaminating metals. They were then equilibrated with contraction solution and packed into 20 × 200 mm columns. Void volumes were determined with hemoglobin. The volume of the supernatant fraction placed on the column was equal to two-thirds the void volume. The columns were eluted with contraction solution and 5 ml fractions were collected for analysis of protein and relaxing activity. All procedures were carried out in a cold room at 2–5°.

Chelex-100, 200 to 400 mesh, was purchased from the California Corporation for Biochemical Research, Los Angeles, California. Chelex-100 is prepared by chemically attaching iminodiacetate exchange groups to a styrene-divinylbenzene lattice. This gives a resin with complexing properties similar to those displayed by EDTA in solution; *i.e.*, a high affinity for divalent cations. Before use, an aqueous suspension of the resin was neutralized to pH 7 with HCl, and then mixed with a 5 mM solution of EDTA to remove contaminating divalent metals. The mixture was filtered and the resin washed exhaustively with double glass-distilled water.

Purified actin and myosin were prepared according to the procedures given by Mommaerts (12). G-actin was converted to F-actin with KCl (100 mM) and $MgCl_2$ (1 mM). Reconstituted actomyosin was obtained by mixing 4 parts by weight of myosin, dissolved in 0.6 M KCl with 1 part F-actin. The mixture was diluted with 10 volumes of distilled water and the precipitate resuspended in extraction solution.

Syneresis of synthetic actomyosin and myofibrils was measured by a procedure similar to that of Mueller (13). A predetermined amount of protein was pipetted into graduated Kolmer type centrifuge tubes containing contraction solution or supernatant solutions containing 5 mM ATP which had been allowed to come to room temperature (22–24°C). The tubes were mixed by inverting them several times

during a period of about 30 seconds and the volume of precipitate was measured after centrifugation for 3 minutes at 1500 RPM in a refrigerated centrifuge.

The activated charcoal (norit A) used in the adsorption experiments was treated with 10 mM EDTA, then with 0.01 M HCl, thoroughly washed with double glass-distilled water, and dried.

Dialysis was carried out with ordinary cellophane dialysis tubing which had been washed with a solution of 5 mM EDTA and then rinsed with double glass-distilled water.

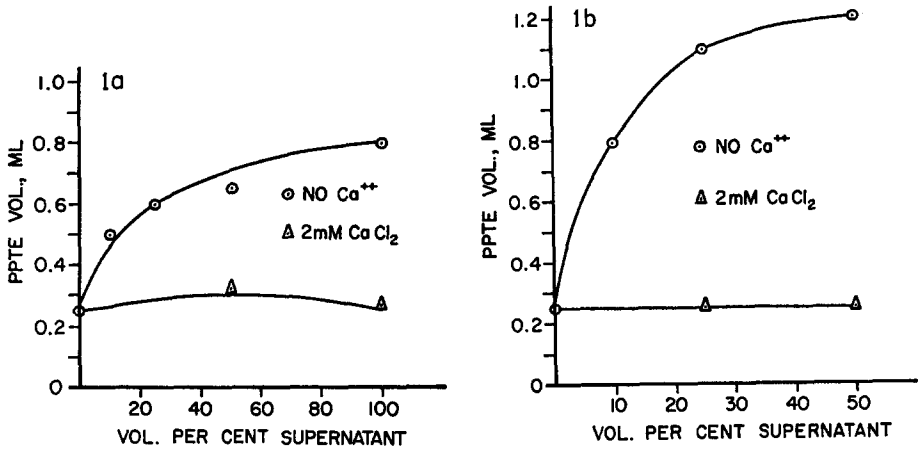

FIGURE 1. Supernatant inhibition of ATP-induced syneresis of uncontaminated actomyosin preparations. Myofibril and actomyosin syneresis were measured as described in text. (*a*) Reconstituted actomyosin, 11.2 mg in a final volume of 8 ml. (*b*) Myofibrils treated with deoxycholate as described in (7). Myofibrillar protein, 30 mg in a final volume of 8 ml: Ppte, volume of myofibril or actomyosin precipitate (syneresis volume). Volume per cent indicates the percentage of the volume of the solution occupied by supernatant. The reversibility of inhibition was tested by adding calcium (16 μmoles).

RESULTS

A. *Demonstration of the Relaxing Activity of Supernatant Fraction*

Proof that the relazing activity of the supernatant fraction from muscle does not depend upon any contaminant in the assay system has been achieved through the use of two types of actomyosin preparations: reconstituted actomyosin, established to be free of endoplasmic reticular material by electron microscopy (4), and deoxycholate-treated myofibrils (7). The results are presented in Fig. 1. As indicated in this figure, dilution of the supernatant fraction as much as four to five times with contraction solution reduced only slightly the capacity to inhibit syneresis. Dilution beyond that point, however, quickly led to complete loss of relaxing activity. The reversibility of the effect of supernatant was demonstrated by adding 16 μmoles of $CaCl_2$ to 8 ml of test solution.

To establish that the component of the supernatant fraction responsible for its relaxing activity is truly a soluble, *i.e.* non-particulate material, the relaxing activity of the supernatant fraction was separated from all large molecular weight components of the supernatant by the technique of gel filtration (11). Columns of sephadex G-50 and G-25 were used for this purpose. The structure of the gel, G-50, is such that it excludes material of molecular weight greater than 10,000 from the water phase within the gel. As shown in Fig. 2, when supernatant was subjected to such a procedure, none of the relaxing activity was associated with the protein material nor

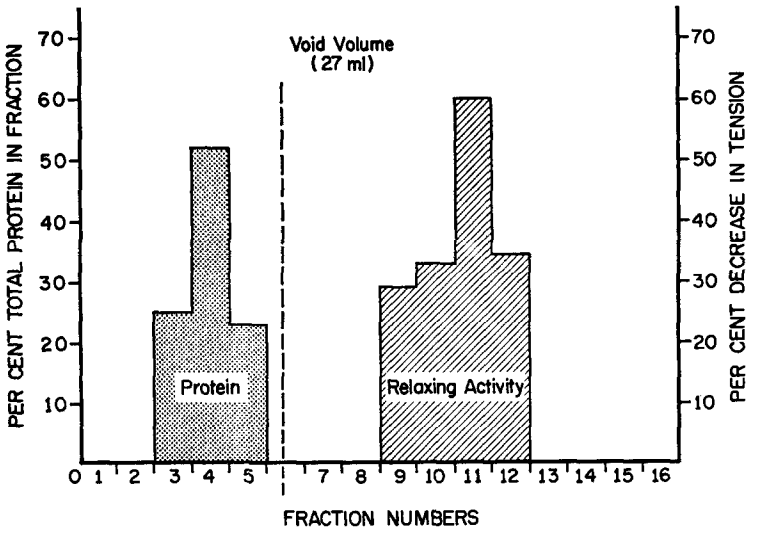

FIGURE 2. Separation of soluble relaxing factor by gel filtration. Relaxing activity was assayed by inhibition of tension generation in single glycerinated muscle fibers. Five ml fractions were collected from a sephadex, G-50 column and numbered sequentially as indicated on the abscissa. Other details of the experiment are given in the text.

did it appear within the void volume of the column, indicating that it had equilibrated with the internal water of the gel and hence, is of molecular weight less than 10,000. Experiments with sephadex G-25 yielded similar results; *i.e.*, the relaxing activity appeared after the void volume had been eluted, hence, the molecular weight can be set as less than 4,000. Unfortunately, the relaxing activity so isolated was highly unstable and could be kept at 0° for no longer than 24 hours. This was in contrast to the relaxing activity in the unfractionated supernatant which could be stored at 0° for 4 to 6 days. These observations suggest that the protein portion of supernatant stabilizes the soluble relaxing factor, a conclusion reached earlier by Fuchs and Briggs (1).

Further evidence that no particulate substance is involved in the relaxing

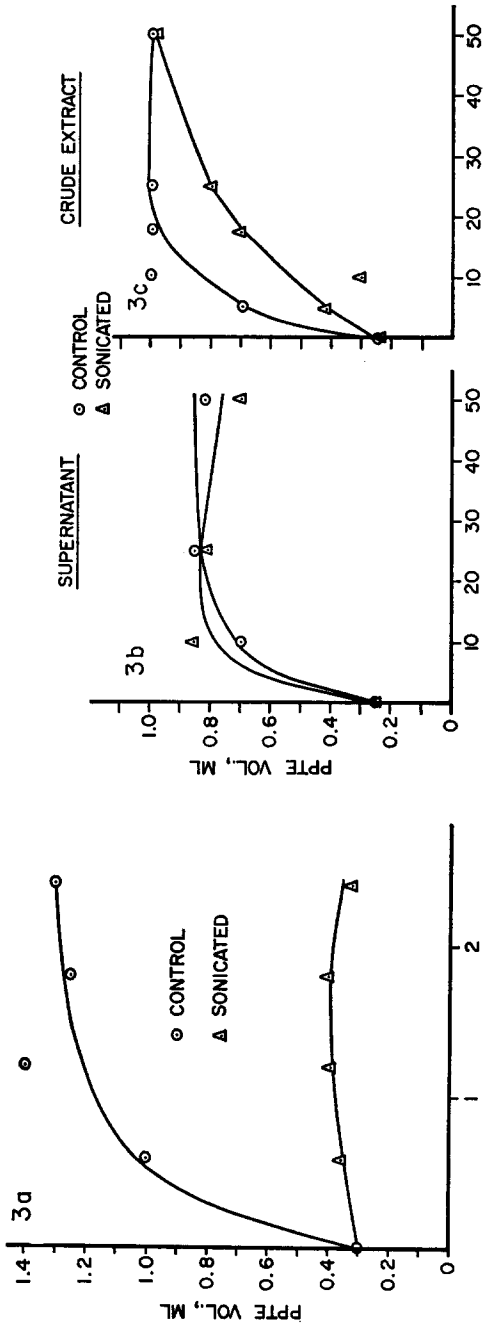

FIGURE 3. Effect of sonic vibration on various relaxing preparations. Myofibril syneresis was measured as described in text. Sonic vibration was performed as indicated in the text. (*a*) Activity of granule preparation after sonic vibration. (*b*) Activity of supernatant after sonic vibration. (*c*) Activity of crude Marsh extract after sonic vibration. PPTE, volume of myofibril precipitate (syneresis volume). Myofibrillar protein concentration, 30 mg in a final volume of 8 ml.

activity of the supernatant fraction was provided by experiments in which the sensitivities of crude Marsh extract, granules, and supernatant fraction to sonic vibration were investigated. Gergely *et al.* (14) have shown that the granule fraction is sensitive to sonic vibration; presumably the crude Marsh extract and supernatant fraction would also be sensitive to sonic vibration if they depended upon any particulates therein for their relaxing activity. The effect of 20 minutes of sonic vibration at 9 kc is illustrated in Fig. 3. As noted by Gergely *et al.* the granule fraction was completely inactivated. In contrast, the supernatant fraction was completely insensitive to sonic vibration. The crude Marsh extract was partially inactivated. These results are not unexpected if it is assumed that the relaxing activity of the crude Marsh extract is derived from the presence of both a soluble relaxing factor and a particulate relaxing factor.

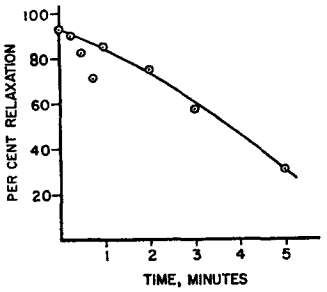

FIGURE 4. Time-course of thermal inactivation of supernatant. The supernatant was heated at 100°. Activity was assayed with single glycerinated fibers. Supernatant concentration, 100 volumes per cent.

B. *Physicochemical Properties of the Supernatant Fraction in Relation to Relaxing Activity*

1. THERMAL STABILITY

The relaxing activity of a supernatant preparation after heating at 100° for various intervals of time is reported in Fig. 4. Before the supernatant was assayed, coagulated protein was removed by centrifugation. Quite clearly there was a steady loss of activity as the interval of heating was lengthened.

The stability of the heat-deproteinized supernatant fraction was also investigated. Fig. 5 compares the relaxing activity remaining in a heat-deproteinized preparation and a control preparation after 1, 2, and 3 days of storage at 2°. The relaxing activity of the heat-denatured supernatant was much more labile than that of the non-treated preparation, very little activity remaining at the end of 3 days. The control preparation lost about 60 per cent of its activity in 3 days' time.

2. REMOVAL OF SUPERNATANT RELAXING ACTIVITY BY ADSORPTION

The inactivation of supernatant by the addition of 30 mg of charcoal per ml of supernatant is shown in Fig. 6 *a*. The supernatant was exposed to the

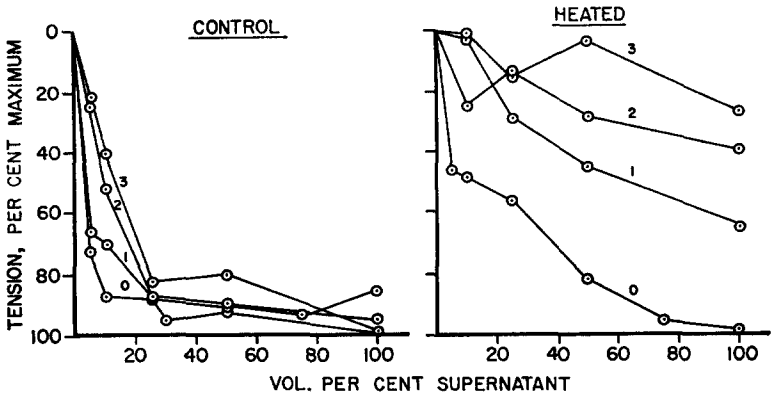

FIGURE 5. Effect of heating on stability of supernatant. Supernatant was heated at 100° for 3 minutes. Denatured protein was removed by centrifugation. Relaxing activity was assayed with glycerinated psoas fibers. The numbers on the curves represent the age (in days) of the preparation after the heat treatment.

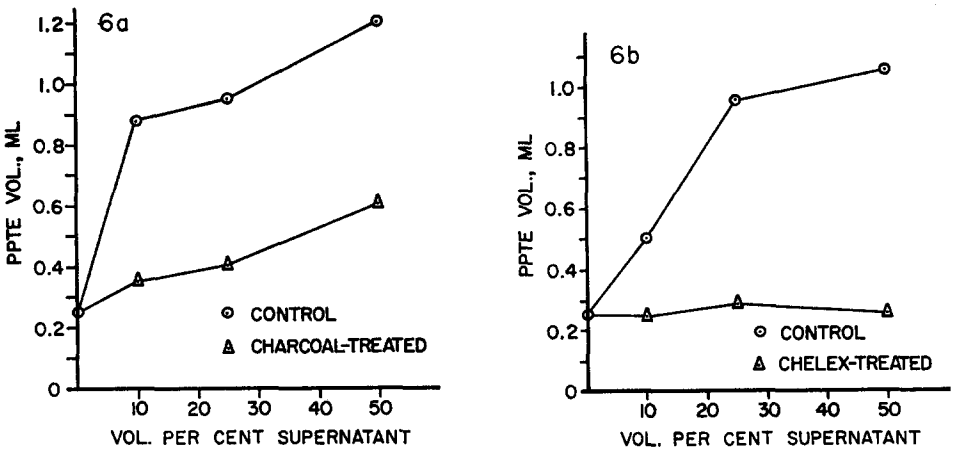

FIGURE 6. Adsorption of supernatant relaxing activity. Myofibril syneresis was measured as described in text. (*a*) Supernatant treated with charcoal as described in text. Myofibrillar protein, 30 mg in final volume of 8 ml. (*b*) Supernatant treated with chelex-100 as described in text. Myofibrillar protein, 30 mg in final volume of 8 ml. PPTE, myofibril precipitate volume (syneresis volume).

charcoal for 10 minutes at 0°. Then the charcoal was removed from the supernatant by 1 hour of centrifugation at 78,000 *g*. Inasmuch as the charcoal was treated with both acid and EDTA, the likelihood of calcium contamination is quite remote. Therefore, it seems likely that the loss of activity was due to charcoal adsorption of the ingredient responsible for producing relaxation. The possible adsorption of relaxing activity by chelex-100 was also investigated because its ability to complex calcium makes it unlikely that

any loss of relaxing activity attendant upon its use could possibly occur as a consequence of surreptitious addition of calcium. Before the chelex-100–treated supernatant was assayed, sufficient magnesium was added to replace that removed by the resin. As indicated in Fig. 6 *b*, there was a total loss of relaxing activity as a consequence of exposure of supernatant to this resin.

3. DIALYSIS

Evidence that the relaxing activity of soluble preparations is not due to calcium deficiency, but to the presence of some agent, is obtained from the pair of dialysis experiments shown in Fig. 7. In the first of this pair of experi-

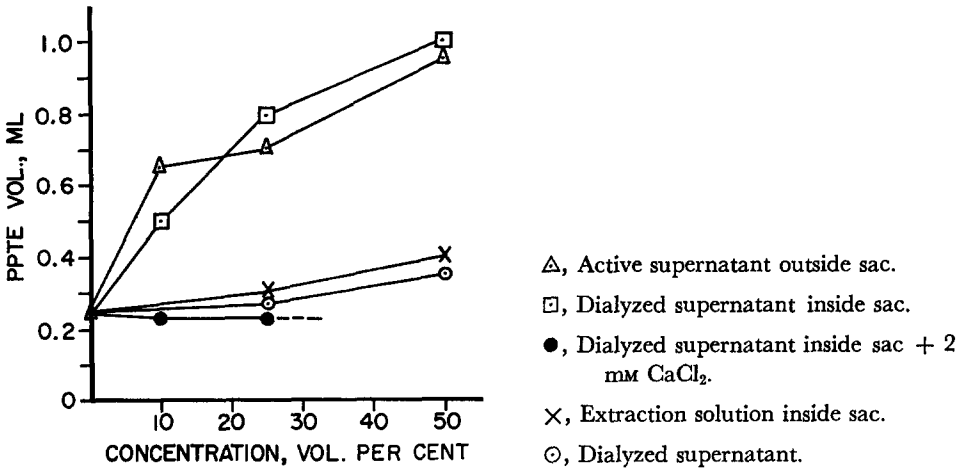

FIGURE 7. The dialyzability of the relaxing factor activity of supernatant. Myofibrillar syneresis was measured as described in text. Dialyzed supernatant was prepared by dialyzing 1 volume of active supernatant for 40 hours at 2–4°C against 40 volumes of chelex-treated extraction solution. For second dialysis, 1 volume each of dialyzed supernatant and extraction solution was dialyzed for 18 hours at 2–4°C against 10 volumes of active supernatant. Myofibrillar protein, 30 mg in final volume of 8 ml.

ments, 10 volumes of Marsh supernatant were dialyzed for 18 hours against 1 volume of extraction solution. At the end of the dialysis period, the Marsh supernatant possesses relaxing activity. The extraction solution possessed no relaxing activity. The second part of the experiment consisted of dialyzing a similar 10 volumes of Marsh supernatant against 1 volume of dialyzed supernatant. This experimental procedure was suggested by studies (1) which had indicated that soluble muscle protein somehow stabilizes relaxing activity. It was felt that the inability to demonstrate relaxing activity in the extraction solution might be due to rapid loss of activity in a non-protein environment. In order to provide such an environment, a fraction of the Marsh supernatant was inactivated by dialysis (dialyzed supernatant). As indicated in Fig. 7,

when active Marsh supernatant was dialyzed against the inactive supernatant, relaxing activity was transferred to the inactive supernatant. If Marsh supernatant depended upon a calcium deficiency for its relaxing activity, then that deficiency should have equilibrated with extraction solution as readily as with dialyzed supernatant.

DISCUSSION

Though it originally appeared that the muscle-relaxing activity of the muscle homogenates described by Marsh is attributable to the microsomal fraction thereof (15, 16), it now seems justifiable to conclude that a soluble relaxing factor also exists in such homogenates. In support of this, the soluble relaxing activity of the homogenate has been shown to be fully active on reconstituted as well as deoxycholate-treated actomysin systems. The relaxing activity of the supernatant is thus totally independent of any intrinsic relaxing agents in the assay system.

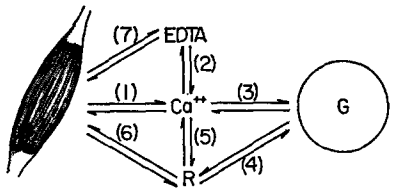

FIGURE 8. Schematic representation of the various hypotheses proposed to explain how granules (*G*) inhibit actomyosin.

Fig. 8 indicates schematically the various mechanisms which have been proposed for the granule inhibition of actomyosin systems; i.e., "relaxation." The central role of calcium in this scheme is based on the ease with which calcium reverses all relaxation systems. Reaction 3 is well substantiated by the works of Ebashi (8), Parker and Gergely (5), and Hasselbach (17). However, since the uptake of calcium is an active process, it is doubtful whether an equilibrium constant can justifiably be applied to this reaction (8). If the relaxation produced by granules is due merely to the withdrawal of calcium, then the level of calcium which determines actomyosin activity must be measured (reaction 1) and the levels so established must be achieved by the granules (reaction 3). Ebashi (8) and A. Weber (9, 10), through the use of EDTA and other calcium-chelating agents, have studied reaction 2 and have shown that actomyosin systems relax at predictable values—though they do not agree on a particular value of ionized calcium—and conclude, therefore, that reaction 1 is of prime importance. On the other hand, a comparative study of the influence of a particulate chelator, chelex-100, and granules on calcium levels and actomyosin activity by Briggs and King (18) does not support the predictability of calcium levels and actomyosin activity. An important defect in the hypothesis of Weber and Ebashi is the lack of

evidence that reaction 3 can lead to the levels of calcium required by analysis of reaction 2.

The alternative mechanism for the relaxation of actomyosin, *i.e.* the formation of a soluble inhibitor R, reaction 4, is favored by the results presented in this communication. The evidence to be considered first excludes reaction 3 as the basis for relaxing activity. The possibility that the relaxing activity of the supernatant is due to calcium deficiency rather than to the postulated inhibitor R has been considered. However, the physicochemical characteristics of the supernatant relaxing factor completely exclude this explanation of its relaxing activity. Heating destroyed relaxing activity but could not reverse a calcium deficiency. Charcoal and chelex-100 adsorbed relaxing activity but could not adsorb a calcium deficiency. It is also impossible, within the framework of the calcium deficiency hypothesis, to explain the dialysis experiments. Thus, when equal volumes of inactive supernatant and extraction solution were dialyzed against a large volume of active supernatant, relaxing activity reappeared in the inactive supernatant, but never in the extraction solution. Whatever the calcium concentration in the supernatant, it should have equilibrated equally with the inactive supernatant and extraction solution. The restoration of activity only to the dialyzed supernatant must, therefore, be ascribed to the inward movement of the small but labile inhibitor designated R in this scheme.

The mechanism by which R inhibits the actomyosin–ATP interaction cannot at the present time be designated. It is possible that R is a chelator and inhibits through a reduction of ionic calcium (reaction 5). The possibility that it interacts directly with actomyosin as indicated by reaction 6 must also be considered. There is no evidence available which would allow a distinction to be made between these two possibilities.

A question of considerable importance is whether this soluble supernatant relaxing factor isolated directly from muscle is identical with the soluble relaxing factors obtained by incubation of granules and ATP (3, 5). Although not enough information is available for a definitive answer to this question, it is of significance that all these preparations have certain common properties. The soluble factor described here is similar to that of Briggs and Fuchs (3) and Parker and Gergely (5) in its response to charcoal treatment and dialysis. For example, Parker and Gergely were unable to prepare an active protein-free dialysate. Briggs and Fuchs were able to demonstrate the dialyzability of their preparation, but only with considerable loss of activity. The data available on thermal stability do not allow a quantitative comparison of the various preparations. Parker and Gergely report that upon heating their material at 100°C there was approximately a 15 per cent loss in activity after 2 minutes and a 50 per cent loss after 15 minutes. Similarly, Nagai *et al.* (6) state that there was only a slight loss of activity after 2 minutes at

100°C. The soluble factor of Briggs and Fuchs showed a 50 per cent loss of activity after heating for 5 minutes at 70°C, whereas the preparation reported here lost on the average about 30 per cent of its activity after heating for 2 minutes at 100°C. However, different preparations varied considerably in their stability. The data do suggest, however, that these different groups of workers may be dealing with similar, if not identical, chemical compounds. It seems possible, therefore, to tentatively conclude that the soluble relaxing factor in the supernatant was biosynthesized in the muscle and is a natural component of muscle.

The existence of a soluble inhibitor has been suggested previously. What is of particular physiological importance in the present studies is the discovery that the soluble inhibitor exists in muscle. Heretofore it had only been produced by *in vitro* systems. Before this soluble relaxing factor can be given a physiological role in the regulation of muscle activity, however, it will be necessary to show that its concentration actually varies with the activity of muscle. The demonstration here, that the relaxing activity of soluble fractions of muscle can be readily assayed and that the relaxing activity of the soluble fraction is sufficiently stable to give reliable assays, makes such a project entirely feasible.

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