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# PARTICIPATION OF A DIALYZABLE COFACTOR IN THE RELAXING FACTOR SYSTEM OF MUSCLE

## II. STUDIES WITH MYOFIBRILLAR ATP-ASE

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SUMMARY

Inhibition of myofibrillar ATPase by granules sedimented at 35,000 × *g* often did not account for the inhibitory activity of the crude extract from which they were prepared. In such cases full inhibition could be obtained by combining the granules with a dialysate prepared from the 35,000 × *g* supernatant. The requirement for the dialyzable cofactor was particularly pronounced at low concentrations of granules. In those cases where the granules manifested no requirement for added cofactor, incubation of the granules at 37° for 60 min produced a requirement for this cofactor. Both calcium and carnosine were found to antagonize the inhibitory effect of the relaxing factor system.

The following abbreviations are used: ATP, adenosinetriphosphate; ATPase, adenosinetriphosphatase; ADP, adenosinediphosphate; RFS, relaxing factor system; TCA, trichloroacetic acid.

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## INTRODUCTION

One manifestation of the relaxing factor system (RFS) observed by MARSH[3] was the inhibition of myofibrillar ATPase activity. PORTZEHL[4], using this criterion, concluded that the relaxing activity of a crude muscle extract could be fully recovered in the granules sedimented from the extract. This conclusion is at variance with our results reported in the preceding paper[5], where we have shown that the granules do not account for the tension-decreasing activity of the parent crude extract and that a dialyzable cofactor present in the crude extract is required for full activity.

In reinvestigating the myofibrillar ATPase system our experiments with some RFS preparations bore out PORTZEHL's[4] conclusions, but with others the requirement for the dialyzable cofactor could clearly be demonstrated. Although no obvious explanation is available for the two types of RFS, some light may be thrown on the relationship between granules and cofactor by our observation that moderate heating of the active granules converts them into the type requiring the cofactor.

## METHODS AND MATERIALS

Crude *extract*, *granules*, *supernatant* and *dialysate* were prepared as described in the preceding paper[5]. In the absence of sucrose the ATPase activity of the granules is 4–5 times higher, making it difficult to observe their effect on the myofibrillar ATPase. Hence, the material used in this study was always prepared with sucrose.

*Myofibrillar suspensions* were prepared by a modification of PERRY's method[6]. All operations were carried out at 2°. Ground rabbit skeletal muscle was homogenized in a Waring blendor for 60 sec with 3 vol. of 65 m*M* succinate buffer, pH 7.5. The homogenate was centrifuged at 600 × *g* for 15 min. The supernatant was discarded, the precipitate, except for the bottom layer containing connective tissue and debris, rehomogenized in a Potter-Elvehjem homogenizer and centrifuged as before. This process was repeated 4 to 5 times. The last precipitate was taken up in the smallest possible volume of succinate buffer, and rehomogenized in a Waring blendor for 30 sec. Two volumes of 50 % glycerol were added to the suspension and it was stored at — 15°.

Before use, an aliquot of the myofibril suspension was centrifuged at 10,000 × *g* for 15 min in order to remove glycerol. The myofibrils were then washed by resuspension in 50 vol. of 65 m*M* succinate buffer, pH 7.5 and centrifuged at 3,600 × *g*. This was followed by two more washings. The myofibrils were finally homogenized in a small volume of buffer with a loose-fitting Potter-Elvehjem homogenizer. At this stage the protein concentration was 25–30 mg/ml. These myofibrils liberated 0.2–0.3 μmole of phosphate per mg of protein per min under the usual conditions of the tests.

ATPase determinations were carried out at room temperature (23–25°) in 50 m*M* KCl, 20 m*M* histidine, 2.5 m*M* oxalate, 320 m*M* sucrose, 5 m*M* $Mg^{++}$ and 5 m*M* ATP, final volume 1 ml, pH 7.5, unless otherwise indicated, by measuring the amount of inorganic phosphate liberated in 5 min. Proper corrections were made for the ATPase of the granules. The reaction was stopped by adding 5 % TCA, the mixture filtered and an aliquot taken for the determination of inorganic phosphate according to FISKE AND SUBBAROW[7].

Crystalline di-sodium ATP was obtained from Pabst Laboratories, Milwaukee, Wis., carnosine from the California Foundation for Biochemical Research, Los Angeles, Calif.

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## RESULTS

In comparing the ATPase-inhibiting activity of the 35,000 × *g*-granules with that of the crude extract two different patterns were observed.

With some preparations the effect of varying amounts of granules, crude extract, and reconstituted system was as shown in Fig. 1. At high concentrations the three systems inhibited myofibrillar ATPase equally well; but as the concentration was reduced, the granules were much less active than the crude extract. At about one third of the concentration producing maximal inhibition the granules had no activity, while the crude extract still produced 50 % inhibition. Addition, however, of the dialysate of the 35,000 × *g*-supernatant to the granules rendered them as active in inhibiting the myofibrillar ATPase as the crude extract. These observations suggest that the dialyzable cofactor introduced in the preceding paper[5] is also involved in the inhibition of myofibrillar ATPase.

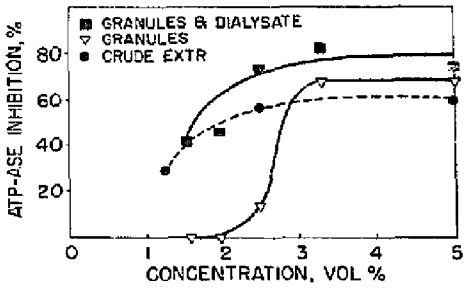


Fig. 1. Inhibition of myofibrillar ATPase by granules requiring cofactor. The concentration of crude extract is expressed in vol. %. The concentration of granules or granules and dialysate (vol. %) is expressed in terms of the volume of crude extract from which they were obtained. ●, crude extract; ▽, granules; ■, granules and dialysate, mixed in the proportion in which they were present in the crude extract. For other details see text.

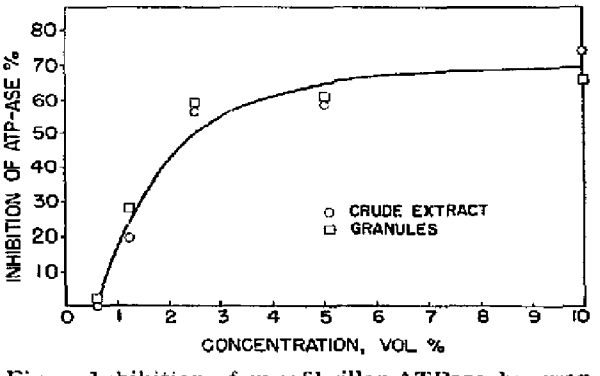


Fig. 2. Inhibition of myofibrillar ATPase by granules not requiring cofactor. For the meaning of concentration see Fig. 1. □, granules; ○, crude extract. For other details see text.

Other preparations behaved in a different way. In these cases, there was no difference between the inhibitory activity of the crude extract and that of the granules (Fig. 2), and addition of the dialyzable cofactor to such granules had no effect.

If increasing amounts of dialysate were added to granules which were less active than the crude extract, the degree of activation of the granules depended on the amount of dialysate added (Fig. 3). When the reconstituted system contained the original ratio of dialysate to granules, the inhibitory activity was equivalent to that of the crude extract. Increasing this ratio further did not produce more inhibition of ATPase.

In those preparations where the activity of the granules was essentially equal to that of the crude extract, incubation of the granules at 37° for 60 min considerably reduced their activity. Heated granules in large amounts could, however, produce as much ATPase inhibition as those granules that had not been heated (Fig. 4). The addition of the dialyzable cofactor restored the inhibitory activity of the heated granules to that of the nonheated granules. It was also found that heating at 37°

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increased the ATPase activity of the granules by 90–100 %, suggesting a change in their structure.

Various experiments were performed in order to characterize both the granules and the dialyzable cofactor. Table I summarizes the results of various treatments of the dialysate. Exposure to acid or alkali lead to a considerable loss of activity. At neutral pH the cofactor was fairly resistant to heating at 60°, but boiling destroyed most of the activity. Treatment with charcoal or Dowex-1 removed cofactor activity. These properties may be useful in the purification of the cofactor.

The activity of the granules was lost by treatment with sonic vibration (10 kc) for 20 min, and they could not be reactivated by addition of cofactor. Similarly, an acetone powder, prepared by adding 4 vol. of acetone at 0° to a suspension of granules, when taken up in the original volume had no activity, nor could it be reactivated by cofactor. The presence of a few thymol crystals almost completely inactivated the granules. Incubation with 1 % desoxycholate destroyed the activity.

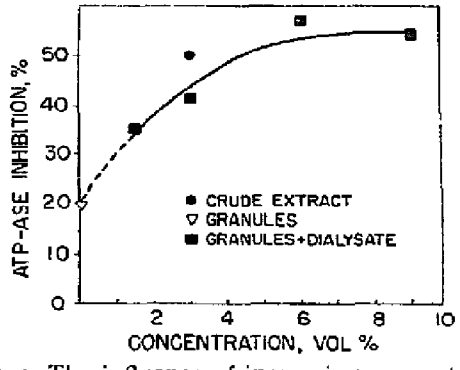


Fig. 3. The influence of increasing amounts of dialysate on the inhibition by granules of myofibrillar ATPase. For the meaning of concentration see Fig. 1. ●, crude extract; ▽, granules; ■, granules and dialysate. For other details see text.

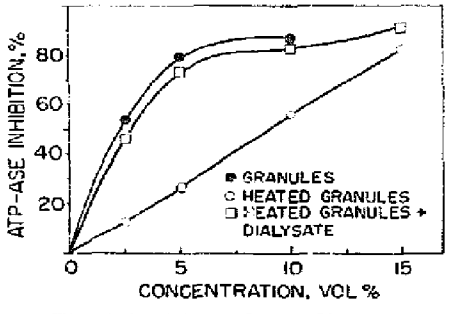


Fig. 4. The inhibition of myofibrillar ATPase by heated granules. For the meaning of concentration see Fig. 1. ●, granules; ○, granules incubated at 37° for 60 min; □, heated granules and dialysate mixed in the proportion in which they were present in the crude extract. For other details see text.

TABLE I

THE EFFECT OF VARIOUS TREATMENTS ON DIALYSATE ACTIVITY

| *Experiment* | *Inhibitions of myofibrillar ATPase, %* |
|---|---|
| Granules, incubated at 37° for 60 min | 5 |
| 1. plus nontreated dialysate | 69 |
| 2. plus acid-treated dialysate (pH 3) | 10 |
| 3. plus alkali-treated dialysate (pH 10) | 20 |
| 4. plus Dowex-1-treated dialysate | 22 |
| 5. plus charcoal-treated dialysate | 0 |
| 6. plus dialysate heated at 60° for 10 min | 44 |
| 7. plus dialysate boiled for 10 min | 21 |
| Dialysate | 0 |

The amount of dialysate used was just enough to produce maximal inhibition of the myofibrillar ATPase when added to granules that had been treated at 37° for 60 min. The acid and alkali-treated dialysates were kept at the pH indicated for 30 min at 0°, and were neutralized with a small volume of 0.25 *N* alkali or acid before testing. Dowex-1 was in the chloride form; no change of pH of the dialysate was observed as a result of the treatment with it. The charcoal was thoroughly washed with HCl and water. For other details of the assay see METHODS.

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The activity of the granules was not lost by repeated freezing and thawing. There was, however, no solubilization of their active component, and all the activity could be recovered in the sediment after centrifugation at 35,000 × *g*.

The inhibition of myofibrillar ATPase could be antagonized by $Ca^{++}$, and, as already shown with the single fibre system[5], by carnosine, in concentrations exceeding 5 m*M*.

## DISCUSSION

The evidence presented in this and the preceding paper[5] make it clear that the so-called relaxing factor of muscle can be resolved into at least two components. One component is presumably identical with the granular fraction previously described by PORTZEHL[4], BENDALL[8], LORAND, MOLNAR AND MOOS[9] and EBASHI[10], and whose involvement in muscular relaxation was first suggested by KUMAGAI, EBASHI AND TAKEDA[11]; the other is a dialyzable cofactor.

The identity of our granular component with the granules studied in these papers is somewhat obscured by several circumstances. First, these authors appear to have employed fibre bundles for tension measurements, a system in which there is an apparent requirement for some transphosphorylating system. Second, as shown by the work reported here, some granule preparations, although as active as the crude extract when tested on myofibrils, when tested on the tension of single fibres always showed a cofactor requirement. It should, perhaps, be mentioned at this point that PORTZEHL[4] concluded that the relaxing activity almost completely resides in the granules, although a close inspection of her published results (*cf.* Fig. 2, ref. [4]) reveals that the granules were in fact only about half as active as the crude extract*, a situation, in essence, similar to that encountered by us with a large number of our preparations. In such cases addition of the dialyzable cofactor contained in the 35,000 × *g*-supernatant restored the activity of the granules to the level of the crude RFS.

We have indeed found some granule preparations that were, at all concentrations, just as active as the crude extract. In those preparations in which the ATPase-inhibiting activity of the granules did not account for the activity of the crude RFS, the activity of the granules could be restored by the addition of a second component, a dialysate of the 35,000 × *g*-supernatant. Such stimulation by the dialysate occurred only at low granule concentrations, high concentrations of granules, even in the absence of added dialysate, inhibited the myofibrillar ATPase. The requirement for added cofactor was always observed in the tension experiments with single fibres, and no preparation of granules was found which was as active as the crude extract, provided the comparison was made at concentrations suitable for assaying relaxing activity.

According to LORAND, MOLNAR AND MOOS[9] the granules which potentiate the phosphoenolpyruvate relaxation of fibres contain 35 % lipid and are inactivated by acetone and desoxycholate. The granules used in the present work were also sensitive to acetone and desoxycholate; moreover, thymol, another lipid-soluble material, destroyed the activity. The sedimentation properties and the procedures of preparation of the granules make it unlikely that they are intact mitochondria: their identifi-

* In terms of equivalent concentrations, it took about twice as much of granules as of the crude extract to produce the same inhibition.

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cation with any specific structural component of the muscle cell requires further study. The finding that the granules are destroyed by sonic vibration suggests that a certain organizational integrity is required for their activity.

The dialyzable cofactor is not identical with the substrates creatine phosphate or phosphoenolpyruvate which are involved in the phosphorylation of ADP. Neither of these substances could replace the dialyzable cofactor. It is difficult to be certain that this dialyzable cofactor is not related to Fraction B of KUMAGAI *et al.*[11]. It would seem that their fraction contained besides myokinase other components necessary to cause a reversal of the shortening of their fibre bundles; for a crude myokinase preparation could not replace Fraction B except in poorly washed fibres. Since KUMAGAI *et al.* carried out their fractionation with ammonium sulfate, the fractions obtained were presumably dialyzed. At first glance this would rule out the presence of our dialyzable cofactor in their fraction. However, considering the difficulty we encountered in trying to reduce the activity of the crude extract by dialysis[5], it may be that the cofactor was attached to some protein component of Fraction B in a non-dialyzable form.

WEBER[13] has suggested that the granules, when in contact with the myofibrils, in the presence of ATP produce a substance that, by dissociating actomyosin, inhibits ATPase and reduces tension. The participation of a substance produced by the RFS, myofibrils, and ATP might be indicated by the inhibition of myofibrillar ATPase and syneresis by pyridoxalphosphate[12], and its reversal by carnosine, since carnosine also inhibits the RFS. Furthermore, according to WEBER this substance does not appear free in solution but is present only in the myofibrils, and the role of the cofactor is to prevent the destruction by the myofibrils of this postulated substance. One obvious difficulty with this theory is the fact that some granule preparations are as active as the crude extract at all concentrations, and cannot be made more active by adding the cofactor. For on WEBER's hypothesis, the prevention of the destruction of the "relaxing substance" by the cofactor should increase the activity of the granules when they are present in sub-optimal concentrations.

The possibility that the cofactor requirement is the result of the isolation procedure of the granules must be considered. The following observations are in support of this possibility. Some granule preparations have been found which require no added cofactor in order to inhibit myofibrillar ATPase. Heating such granules at 37° produces a cofactor requirement. Two possible interpretations come to one's mind. First, the granules, in their physiological state do not need a cofactor, but owing to the various preparative procedures suffer some structural change leading to reduced activity. This activity can then be increased to the original level by adding a substance that, properly speaking, would not be part of RFS. As an alternative the granules may contain a tightly bound cofactor, which, owing to structural changes, again arising from preparative procedures, becomes dissociated and as a consequence is washed out from the granules, thereby a requirement for the cofactor being produced. The effect of heating the granules could be interpreted on either of these hypotheses as an exaggeration of the sort of change that may occur during their isolation.

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*References p. 224.*

tions of America, Inc., The Life Insurance Medical Research Fund and the Massachusetts Heart Association. Preliminary reports of this work have been presented[1,2].

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# PARTICIPATION OF A DIALYZABLE COFACTOR IN THE RELAXING FACTOR SYSTEM OF MUSCLE

## III. SUBSTITUTION OF PYROPHOSPHATE FOR THE COFACTOR

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## SUMMARY

2 m*M* pyrophosphate restores the capacity of muscle granules sedimented at 35,000 × *g* to relax muscle fibres and inhibit myofibrillar adenosinetriphosphatase. These effects are reversed by $Ca^{++}$; pyrophosphate, thus, can replace the dialyzable cofactor described in the preceding two communications[7,8]. Pyrophosphatase destroys the activating effect of pyrophosphate but not that of the cofactor present in the 35,000 × *g*-supernatant, rendering it unlikely that the cofactor is identical with pyrophosphate.

The following abbreviations are used: ATP, adenosinetriphosphate; PP, pyrophosphate; PPase, pyrophosphatase; ATPase, adenosinetriphosphatase; RFS, relaxing factor system; Tris, tris(hydroxymethyl)aminomethane.

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