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

I. STUDIES WITH SINGLE GLYCERINATED FIBRES

F. NORMAN BRIGGS*

Department of Pharmacology, Harvard Medical School,

AND G. KALDOR** AND J. GERGELY

Cardiac Biochemistry Research Laboratory, Departments of Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, Mass. (U.S.A.)

(Received October 4th, 1958)

SUMMARY

Granules sedimented in 2 h at 35,000 × *g* do not account for the relaxing activity of a crude muscle extract on the ATP-induced tension of glycerinated single muscle fibres. Large amounts of granules, however, are able to produce maximal relaxation. The combination of the granules and the 35,000 × *g* supernatant, or its dialysate, reproduces the relaxation brought about by an equivalent amount of crude extract. This suggests the participation of a dialyzable cofactor in the mechanism of relaxation. The cofactor is not replaceable by phosphoenolpyruvate or creatine phosphate, even in the presence of the appropriate kinases. Carnosine inhibits relaxing activity.

INTRODUCTION

MARSH[3] has observed that an extract of fresh muscle inhibits the ATP-induced syneresis and the ATPase activity of myofibrils. Subsequently, BENDALL[4] showed that in the presence of ATP this extract (Marsh factor)*** brings about marked lengthening of loaded glycerinated fibre bundles. Following these initial observations various attempts have been made to purify and characterize this factor. BENDALL[5] presented evidence that the relaxing factor is identical with myokinase and, in view of his observations on the relaxing effect of PP in the presence of ATP, postulated that PP might somehow be formed by the enzyme.

The following abbreviations are used: ATP, adenosinetriphosphate; PP, pyrophosphate; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; FMN, flavin-mononucleotide; FAD, flavin-adenine dinucleotide; C, creatine; CP, creatine phosphate; PEP, phosphoenolpyruvate; RFS, relaxing factor system; Tris, tris(hydroxymethyl)aminomethane.

* Present address: Department of Physiology, Tufts University School of Medicine, Boston, Mass.

** Present address: McArdle Memorial Laboratory, University of Wisconsin, Madison, Wis.

*** Several names have been suggested for the active principle or principles in a fresh muscle extract that causes relaxation, in addition to this one coined by BENDALL. They include GOODALL AND SZENT-GYÖRGYI's X-factor and relaxing factor[6], the MB (MARSH-BENDALL) factor of the WEBER school[14], and BOZLER's relaxation factor[8]. In view of the by now apparent complex nature of the factor, and in view of the fact that the above listed names have been applied to rather different substances, we prefer to use the more general term relaxing factor system and abbreviate it RFS.

References p. 218.

The studies initiated by GOODALL AND SZENT-GYÖRGYI[6], and continued by LORAND[7], led LORAND to the conclusion that the CP–creatine kinase system is involved in the process of muscular relaxation, a conclusion adumbrated by BOZLER's observations[8] on the effect of CP on the ATP-induced tension of fresh glycerinated fibre bundles. LORAND[7] suggested that the Marsh factor acts by rephosphorylating bound ADP and that myokinase might serve the same function. What was taken to be further evidence for this hypothesis was furnished by the observation of MOOS AND LORAND[9] that the PEP–pyruvate kinase system could relax glycerinated fibres. KUMAGAI, EBASHI AND TAKEDA[10], however, showed that myokinase or creatine-phosphokinase and CP were unable to relax muscle fibre bundles, and they found that a particulate fraction obtained by ammonium sulfate fractionation, presumably identical with or similar to the KIELLEY-MEYERHOF[11] granular ATPase, was necessary for relaxation together with myokinase and, in the case of well washed fibres, another protein fraction. PORTZEHL[12] and BRIGGS AND PORTZEHL[13] made a systematic study of this problem using well washed myofibrils and single muscle fibres and concluded that transphosphorylating systems are not, properly speaking, relaxing factors, and suggested that, in the case of fibre bundles, these systems cause relaxation only by maintaining within the fibres an ATP concentration sufficiently high to activate traces of relaxing factor which had not been washed out. HASSELBACH AND WEBER[14] had previously shown that the factor is not active at low ATP concentrations.

The requirement for transphosphorylating systems having been ruled out, PORTZEHL[15] proceeded to show that granules sedimented at 35,000 $\times$ g inhibited myofibrillar ATPase without any further addition. In view of the parallelism between the effect of the original Marsh factor on contraction and on ATPase this was taken as a demonstration of the association of the relaxing factor with the granules. BENDALL[16] has recently confirmed PORTZEHL's findings and has shown that the granules inhibit myofibrillar syneresis, thus duplicating the effect of the original Marsh factor.

LORAND, MOLNAR AND MOOS[17] and EBASHI[18] investigated the relaxing activity of granules isolated by centrifugation from a crude muscle extract and found that such granules had a limited capacity to relax glycerinated muscle bundles. They further observed that this relaxation was greatly increased by the addition of PEP[17] and myokinase or CP and creatine kinase[18].

This somewhat complicated picture prompted us to reinvestigate the effect of the granular relaxing factor on single glycerinated muscle fibres. These studies led us to the conclusion that a dialyzable cofactor is involved in the RFS. The cofactor does not appear to be identical with C, CP, or PEP. The requirement for the cofactor could also be shown in the myofibrillar ATPase system (see the following paper). Finally it was found that PP could replace the cofactor in both tension relaxation and ATPase inhibition, but as shown in the third paper, PP is not identical with the cofactor.

METHODS

Psoas fibres were obtained from a rabbit, killed by fracturing the cervical vertebrae, in which rigor had been allowed to develop at 7°. Fibre bundles were then removed and extracted and stored at −18° in 50% (v/v) glycerol containing 0.01 M phosphate buffer, pH 6.9. The extraction medium was changed on three successive days and the fibre bundles were divided into progressively smaller bundles in order to facilitate extraction. A few more changes of the medium were made subsequently at irregular

References p. 218.

intervals. At the time of the experiment the fibres had been stored for 40 to 300 days.

Before each experiment a single muscle fibre was separated with the aid of a dissecting microscope from a fibre bundle. The single fibre was then mounted on a sensitive isometric tensiometer, placed in 160 mM KCl and its length adjusted to a point where any further increase would just produce a measurable tension (100 % rest length). After 2 min equilibration at room temperature (24°) the fibre was placed in 5 mM ATP, 2 mM $MgCl_2$, 20 mM Tris, 70 mM KCl, pH 7.5. Tension measurements were made at frequent intervals until equilibrium was reached. After the addition of any substance to the bath, a new series of recordings was made until equilibrium was again reached.

The relaxing factor was usually prepared by homogenizing ground rabbit muscle in a Waring blendor for 1 min with 3 vol. of 50 mM KCl, 20 mM histidine, 2.5 mM potassium oxalate, and 320 mM sucrose. The homogenate was spun at 3600 × g for 45 min. The turbid supernatant, referred to in this and the following two papers as crude extract, was filtered through 6 layers of cheesecloth and was centrifuged at 35,000 × g for 120 min. The precipitate was taken up in 320 mM sucrose in 1/10 of the volume of the original extract. This material will be referred to as granules. The method of PORTZEHL[15], which omits sucrose, was also used to prepare crude extract and granules. Crystalline di-sodium ATP, DPN, TPN, FMN and FAD were obtained from Sigma Chemical Co., St. Louis, Mo.; CP, PEP, and carnosine from California Foundation for Biochemical Research, Los Angeles, Calif.

RESULTS

The relaxing activity of a crude extract and that of the granules prepared from the same extract are compared in Fig. 1a. The crude extract was much more active. This was particularly striking when an amount of crude extract just sufficient to produce a maximal decrease in tension was used. A concentration of 20 to 25 volume % of the crude extract was usually required to produce maximum relaxation, while the granules in an equivalent amount produced only an 8 % decrease in tension. Addition of granules, up to an amount corresponding to 3 times the crude extract necessary to

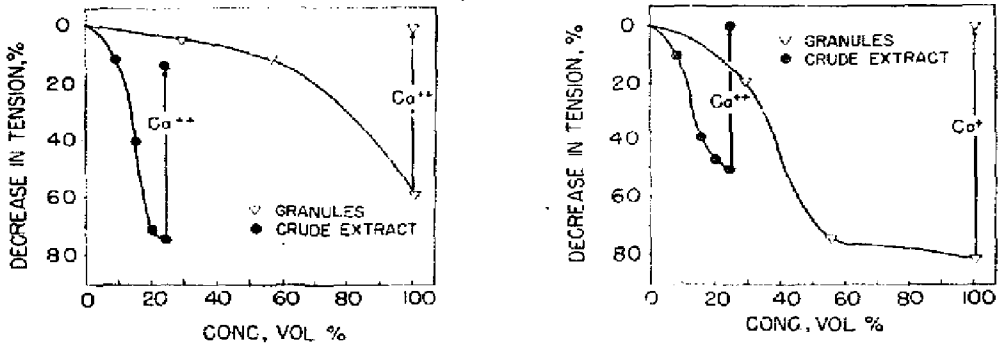

Fig. 1. Influence of crude extract and granules on tension of single glycerinated muscle fibres. (a) Crude extract prepared with sucrose-salt solution. (b) Crude extract prepared according to PORTZEHL[15]. The concentration of the extract is expressed in vol. %. The concentration of granules (vol. %) is expressed in terms of the volume of the crude extract from which they were obtained. ●, Crude extract; ▽, granules. Muscle fibres had been extracted for 51 days. ATP, 5 mM; Mg, 2 mM; μ, 0.16, room temperature. For other details see text.

References p. 218.

produce maximum relaxation, produced a full—about 70 to 80 %—drop in tension. In some preparations the amount of granules required for maximum relaxation corresponded to as much as 8–10 times the amount of crude extract required for an optimal effect.

The crude extract and granules whose relaxing activities are compared in Fig. 1b were prepared according to PORTZEHL[15]. The results obtained with these preparations agreed qualitatively with those found with the sucrose-extracted material. The maximal relaxation, however, was less than with sucrose extracts, and the difference between the activity of the crude extract and that of the granules was reduced. For these and reasons[19] to be mentioned in the next paper, sucrose was used routinely in the preparation of the crude extract and of the granules.

The greater activity of the crude extract, in comparison with the granules, appears to be due to the presence of some substance in the crude extract not sedimented in two hours by a force of 35,000 × *g* (Fig. 2). The addition of the 35,000 × *g*-supernatant subsequent to the addition of granules produced a decrease in tension comparable to that obtained by an equivalent amount of the crude extract (Fig. 2a). Addition of Ca^{++} at the end of the experiment almost completely reversed the relaxation. In the experiment illustrated in Fig. 2b the sequence of additions was reversed. The supernatant alone produced a negligible drop in tension, but further addition of granules produced good relaxation. It will be noted that in the first experiment, where the granules were added first, the supernatant produced a very rapid drop in tension. If the addition of the supernatant preceded that of the granules, the relaxation occurred more slowly. This suggests that the reaction of the granules with the fibres is the rate-limiting step for relaxation.

The influence of increasing amounts of supernatant on the relaxation brought about by an amount of granules equivalent to the amount of crude extract which just produces maximal relaxation is shown in Fig. 3. It can be seen that relaxation increased with increasing volumes of supernatant, and it became maximal when the amount of supernatant was about one half the amount of crude extract just required to produce maximal relaxation. A dialysate prepared from the supernatant was equally effective in bringing the activity of the granules up to that of the crude extract*. The reversal by calcium of the relaxation produced by granules plus supernatant was not complete, but the relaxation produced by granules plus dialysate could be completely reversed by the addition of calcium. Fig. 3 also shows the influence of both supernatant and dialysate on tension in the absence of granules. The supernatant seemed to have a slight relaxing effect which was only partly reversed by calcium. The dialysate produced no decrease in tension.

Table I summarizes results obtained with eight different preparations of relaxing factor. The tension-decreasing activities of the various fractions and their combinations were compared with equivalent amounts of crude extract. It is obvious that the relaxing activities of the combinations, granules and supernatant or granules and dialysate, greatly exceed the sum of the activities of the individual fractions and are comparable with the activity of the parent crude extract. Fig. 4 shows that when the granules were mixed with supernatant or with dialysate in the proportion in which

* If instead of the crude extract, the supernatant or the dialysate, the solution used for preparing relaxing factor, and containing 2.5 m*M* oxalate, was added no activation of the granules occurred.

References p. 218.

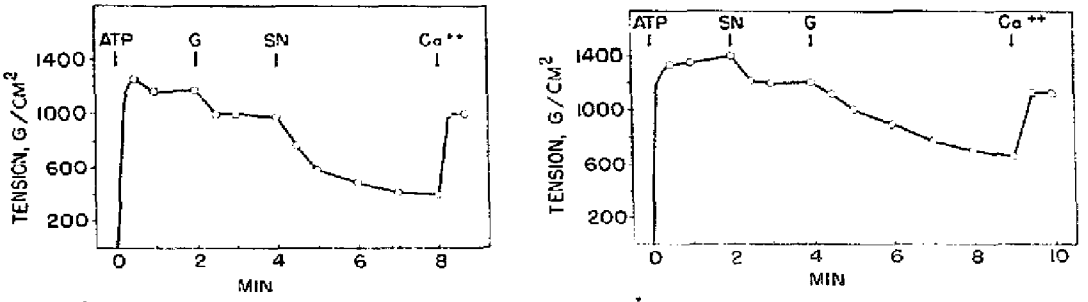

Fig. 2. Influence of granules and supernatant, prepared from crude extract by centrifugation for 120 min at 35,000 × g, on tension. Fibres had been extracted for approximately 300 days. (a) At 0 time the fibre was placed in the bath containing 5 m*M* ATP (for other components see METHODS). G indicates the addition of granules (final volume concentration, in terms of crude extract, 22%); SN indicates the addition of supernatant (final volume concentration, in terms of crude extract, 22%). At 10 min Ca^{++} was added, final concentration 2 m*M*. (b) Additions as in (a), except that the supernatant and granules were added in reverse order. For details see legend of Fig. 1.

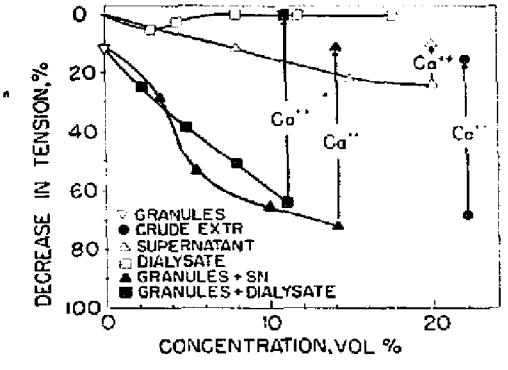

Fig. 3. Influence of supernatant and its dialysate with and without granules on tension. Abscissa: Concentration of supernatant or dialysate in terms of the volume of the crude extract from which they were obtained. Without added granules: △, supernatant; □, dialysate. With added granules: ▲, supernatant; ■, dialysate. The granules were added in a concentration equivalent to 22 vol. % of crude extract. ● indicates the effect of crude extract, 22 vol. %. Arrows indicate reversal by 2 m*M* Ca^{++}. Fibres had been extracted for 71 days. For other details see the legend of Fig. 1.

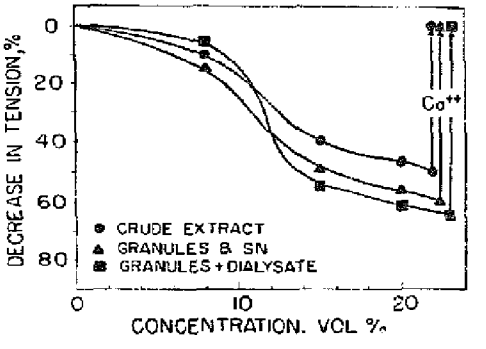

Fig. 4. Relaxing activity of reconstituted systems. ●, crude extract; ▲, granules and supernatant mixed in the proportion in which they occur in the crude extract; ■ granules and dialysate mixed in the proportion in which they occur in the crude extract. Concentrations as defined in the legend of Figs. 1 and 3. Fibres had been extracted for 54 days. For other details see the legend of Fig. 1.

they occur in the crude extract, their relaxing activity was equivalent to that of the crude extract at all concentrations tested. Thus, the relaxing activity of crude Marsh factor can be accounted for only by combining the granules with a dialyzable cofactor.

Some preliminary work has been done to characterize the particulate granules and the dialyzable component. The granules* could be stored in the frozen state for as long as three weeks without loss of activity. Treatment of the granules with 1% desoxycholate completely destroyed relaxing activity. The cofactor** at pH 2.5 or 9.0

* The activity of the granules was tested in the presence of added cofactor, in an amount sufficient to produce maximal relaxation with untreated granules.

** Cofactor activity was tested in the presence of an amount of granules that, when fully activated, would produce maximal relaxation.

References p. 218.

TABLE I

RELAXING ACTIVITY OF VARIOUS MUSCLE FRACTIONS

Experiment	Per cent decrease in tension with					
	Crude extract	35,000 × g-granules	35,000 × g-supernatant	Dialysate of supernatant	Granules and supernatant	Granules and dialysate
1	54	4	4	5	38	47
2	53	8	14	5	56	64
3	70	—	—	—	61	56
4	64	0	2	—	64	—
5	50	8	6	—	42	—
6	14	—	5	—	—	—
7	40	9	—	11	—	48
8	53	14	14	0	59	50

The relaxing activity of each fraction was tested at a concentration equivalent to that of the crude extract. The granules and supernatant or granules and dialysate were combined in the proportion in which they occur in the crude extract. The decrease in tension was taken as the difference between the per cent decrease obtained 4 to 8 min after the addition of the fraction under investigation and the per cent decrease remaining after reversal by 2 m*M* calcium. The fibres used in these experiments had been extracted for about 300 days. Other conditions as described in the legend of Fig. 1.

lost 50 % of its activity in 1 h at 0°. At pH 7 it was stable at 0°, but lost 1/3 of its activity in 15 min at 60°. The cofactor could be adsorbed to charcoal. Work is now in progress to utilize this property for the purification of the cofactor. A number of substances have been tested as substitutes for the cofactor. These included FAD, FMN, TPN, DPN, PEP, PC and thiamine pyrophosphate, all of which were ineffective. Inorganic pyrophosphate could replace the cofactor. However, the instability of the cofactor at pH 9 and its adsorption to charcoal rule out its identity with pyrophosphate. Studies with pyrophosphate as a cofactor are the subject of another communication[20]. Addition of myokinase, CP and creatine kinase, PEP and pyruvate kinase to granules did not result in relaxation. Carnosine in concentrations exceeding 6 m*M* was found to completely antagonize relaxing factor activity.

In view of the fact that the dialysate of the supernatant could act as a cofactor for the granules we compared the activities of dialyzed and nondialyzed crude extract. No appreciable loss of activity occurred on extensive dialysis. The 35,000 × *g*-supernatant completely lost its cofactor activity on dialysis. However, after dialysis against a small volume, cofactor activity could be demonstrated in the dialysate.

DISCUSSION

Our results show that although granules can relax glycerin-extracted muscle fibres, they do not account for the relaxing activity of crude extract and in order to obtain maximal relaxation with granules one has to add 3 to 5 times as much as would be present in the amount of crude extract sufficient to produce the same effect. These observations appear to contradict PORTZEHL's[15] conclusion — based on the study of the inhibition of the myofibrillar ATPase by granules — that the relaxing activity of the crude extract is entirely accounted for by the granules in the extract. Examination of PORTZEHL's data, however, suggests that her conclusion is not borne out by them, since in fact the granules appear to be only about half as active as the original extract.

References p. 218.

The activity of the granules could be brought to that of the parent crude extract by adding dialysate prepared from the 35,000 × *g*-supernatant. The reconstituted system consisting of granules and dialyzable cofactor behaved in all aspects examined exactly as the crude extract. Calcium and, as shown the first time in this paper, carnosine reversed the relaxation brought about by the crude extract or reconstituted system. If the ATP concentration in the bath was allowed to drop below a critical level, owing to the ATPase activity of the granules the relaxation was reversed. This phenomenon is the counterpart of the observation made by HASSELBACH AND WEBER[11] that no relaxation occurs upon addition of Marsh factor at low ATP concentrations.

The dialyzable cofactor is not identical with CP or PEP, since addition of CP or PEP does not reactivate the granules. This is in agreement with the conclusions of PORTZEHL[12] and BRIGGS AND PORTZEHL[13], *viz.* that ADP phosphorylating enzyme systems are not required for relaxing activity. In the light of our data it is more difficult to interpret the reports of LORAND, MOLNAR AND MOOS[17] and EBASHI[18] showing that the relaxing activity of a particulate fraction, tested on glycerinated muscle fibres, is stimulated by phosphokinase systems. In view of the results of BRIGGS AND PORTZEHL[13] and PORTZEHL[12] the simplest explanation of the above reports would be that the fibres still contained small amounts of the RFS and their thickness and ATPase activity was such that, in the absence of kinase activity, the ATP concentration fell below that required for relaxation.

Our finding that the dialysis of the crude extract led to no appreciable loss of relaxing activity—in spite of the dialysability of the cofactor, and loss of cofactor activity of the 35,000 × *g*-supernatant on dialysis—is puzzling. The relationship between granules and cofactor will be discussed after the presentation of our work dealing with the inhibition of myofibrillar ATPase by the relaxing factor.

NOTE ADDED IN PROOF

Since this communication was submitted for publication the relaxing activity of the RFS—crude extract or granules or granules and cofactor—has been reinvestigated in the presence of 2.5 m*M* oxalate. Under these conditions, essentially identical with those used in the work described in the following paper, the amount of RFS required for relaxation is 4–5 less than under the conditions which prevailed in the experiments described in this report. It will be noted that in the presence of 2.5 m*M* oxalate the concentration of the RFS required to relax a single fibre is the same as that required for the inhibition of the myofibrillar ATPase (*cf.* the following paper, p. 218).

(Received March 13th, 1959)

ACKNOWLEDGEMENTS

This work was supported by grants from the National Heart Institute, U. S. Public Health Service (H-1166 (C5) (C6), H-2205, H-3250), The Muscular Dystrophy Associations of America, Inc., The Life Insurance Medical Research Fund and the Massachusetts Heart Association. Preliminary reports of this work have been presented[1,2].

This work was carried out during the tenure by J. G. of an Established Investigatorship of the American Heart Association, Inc.

References p. 218.

REFERENCES

1 J. GERGELY, *Ann. N.Y. Acad. Sci.*, 72 (1959) 538.
2 J. GERGELY, F. N. BRIGGS AND G. KALDOR, *4th Intern. Congr. Biochem., Vienna, 1958, Abstr. Communs.*, p. 83.
3 B. B. MARSH, *Nature*, 167 (1951) 1065; *Biochim. Biophys. Acta*, 14 (1952) 247.
4 J. R. BENDALL, *Nature*, 170 (1952) 1058; *J. Physiol.*, 121 (1953) 232.
5 J. R. BENDALL, *Nature*, 173 (1954) 548; *Proc. Roy. Soc. (London), B.*, 142 (1954) 409.
6 M. C. GOODALL AND A. G. SZENT-GYÖRGYI, *Nature*, 172 (1953) 84.
7 L. LORAND, *Nature*, 172 (1953) 1181.
8 E. BOZLER, *J. Gen. Physiol.*, 38 (1954) 149.
9 C. MOOS AND L. LORAND, *Biochim. Biophys. Acta*, 24 (1957) 461.
10 H. KUMAGAI, S. EBASHI AND F. TAKEDA, *Nature*, 176 (1955) 166.
11 W. W. KIELLEY AND O. MEYERHOF, *J. Biol. Chem.*, 176 (1948) 591.
12 H. PORTZEHL, *Biochim. Biophys. Acta*, 24 (1957) 474.
13 F. N. BRIGGS AND H. PORTZEHL, *Biochim. Biophys. Acta*, 24 (1957) 488.
14 W. HASSELBACH AND H. H. WEBER, *Biochim. Biophys. Acta*, 11 (1953) 160.
15 H. PORTZEHL, *Biochim. Biophys. Acta*, 26 (1957) 373.
16 J. R. BENDALL, *Nature*, 181 (1958) 1188.
17 L. LORAND, J. MOLNAR AND C. MOOS, *Conference on the Chemistry of Muscular Contraction, Tokyo, 1957*, Igaku Shoin Ltd., p. 85; *Biol. Bull.*, 113 (1957) 323.
18 S. EBASHI, *Arch. Biochem. Biophys.*, 76 (1958) 410.
19 J. GERGELY, G. KALDOR AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 34 (1959) 218.
20 G. KALDOR, J. GERGELY AND F. N. BRIGGS, *Biochim. Biophys. Acta*, 34 (1959) 224.

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

II. STUDIES WITH MYOFIBRILLAR ATP-ASE

J. GERGELY AND G. KALDOR*

Cardiac Biochemistry Research Laboratory, Departments of Medicine, Massachusetts General Hospital and Harvard Medical School,

AND F. NORMAN BRIGGS**

Department of Pharmacology, Harvard Medical School, Boston, Mass. (U.S.A.)

(Received October 4th, 1958)

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.

* Present address: McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisc.
** Present address: Dept. of Physiology, Tufts University School of Medicine, Boston, Mass.

References p. 224.