

# The Nature of the Muscle-Relaxing Factor

## I. *An improved assay system*

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**ABSTRACT** Analysis of the effects of deoxycholate and benzalkonium chloride on granule relaxing activity, myofibril and glycerinated fiber responses to ATP, and the sensitivity of these actomyosin-containing systems to relaxing factors have established that it is possible to differentially inactivate intrinsic granule relaxing factor. It is therefore possible to use these agents to treat myofibrils and glycerinated muscle fibers so that they may be used for the assay of muscle-relaxing factor preparations. The destruction of intrinsic relaxing factor can be produced by treating actomyosin with 1 mM deoxycholate or 0.005 per cent benzalkonium chloride for 5 minutes at room temperature.

### INTRODUCTION

Engelhardt (1) first noted that if contraction is the result of a reaction between ATP<sup>1</sup> and actomyosin it is necessary to assume that some mechanism exists whereby this reaction is prevented from taking place in resting muscle. Evidence that such a mechanism actually exists has been presented by Bozler (2), who deduced that there was some substance in muscle which was inhibiting the contraction of "fresh" glycerinated muscle fibers and by Marsh (3), who established that muscle homogenates inhibit ATP-induced syneresis. The inhibitory action was originally presumed to be produced by some substance in the homogenate termed "relaxing factor" and was subsequently shown by Bendall (4) to produce inhibition (relaxation) of ATP-induced tension generation and ATPase activity of glycerol-extracted fiber bundles.

Identification of the active constituent in the homogenate indicated that the activity was associated with a particulate (granule) fraction of the homogenate (5-7), most likely the microsomal fraction (7-9). In order to explain how such granules, with a diameter of approximately 1000 Å (9, 10), could produce relaxation in a glycerinated fiber or myofibril with intermyofilament

<sup>1</sup> Abbreviations: ATP, adenosinetriphosphate; ATPase, adenosinetriphosphatase; EDTA, ethylenediaminetetraacetate; TCA, trichloroacetic acid; DOC, deoxycholate.

spacings of approximately 140 Å (11) it is, however, necessary to postulate (8, 12) that they produce relaxation either by the removal of some component essential for actomyosin activity or by synthesizing and/or releasing some soluble, low molecular weight substance. Evidence in favor of each of these mechanisms has appeared. It has been argued that relaxation is solely the consequence of the uptake of calcium by the granules (13–15). This hypothesis assumes that actomyosin contractility is dependent upon the presence of a small amount of calcium. Evidence for such calcium dependence has been presented by A. Weber and Winicur (16), A. Weber and Herz (17), and Ebashi (13). According to the other postulate, based on the experiments of Briggs and Fuchs (18), Parker and Gergely (19), and Nagai (20), the inhibition of actomyosin contractility requires the presence of a soluble substance produced through an interaction between granules and ATP. In terms of this formulation the granule is considered to be an agent for the formation and/or release of the true soluble relaxing factor. If this is the case, physiologically it should be possible to find the soluble inhibitor in muscle *per se*. Fuchs and Briggs (21) thus reexamined the soluble supernatant fraction of muscle homogenates and found what appears to be the hypothetical soluble inhibitor. Unfortunately, most of the investigations cited in favor of both hypotheses are somewhat ambiguous because of possible contamination of the actomyosin preparations by remnants of endoplasmic reticulum (granules). According to Hasselbach (22) such remnants of sarcoplasmic reticulum can be seen in electron micrographs of glycerinated fibers, myofibrils, and natural actomyosin although reconstituted actomyosin appears to be free of such contamination. Whether these remnants of sarcoplasmic reticulum are functional relaxing systems was not determined. Ulbrecht (23), has however, shown that these vesicles catalyze an ATP-ADP<sup>32</sup> exchange and hence it may be that they also possess some relaxing activity.

The experiments which form the following communications were undertaken to determine the nature of the material in the soluble supernatant which inhibits actomyosin contractility. Attention was first directed toward the development of unambiguous assays systems; *i.e.*, actomyosin preparations free of functional vesicular material. Experiments were then designed to establish a number of physicochemical properties of the supernatant relaxing system in order to decide whether or not the properties of that system could be accommodated by a mere lack of calcium therein or whether the properties of the system are such that an inhibitor substance must be postulated.

## METHODS AND MATERIALS

A. *Solutions*

*Extraction solution* was of the following composition: 30 mM potassium chloride, 10 mM potassium phosphate, 10 mM histidine hydrochloride, 5 mM magnesium chloride, 5 mM potassium oxalate, pH 6.7. *Contraction solution* was exactly equivalent to extraction solution except that sodium adenosinetriphosphate, pH 6.7, was included to give a concentration of 5 mM.

B. *Preparation of Relaxing Systems*

All preparations were derived from the skeletal muscle of 4 to 5 kg white rabbits maintained on standard rabbit chow. The animals were killed by intravenous injection of sodium pentobarbital. The back and leg muscles were excised as rapidly as possible and chilled in ice. All subsequent operations were carried out in a cold room at 2–4°C. One volume of chilled minced muscle was homogenized with 3 volumes of extraction solution for 40 seconds in a Waring blender. The homogenate was centrifuged for 25 minutes at 11,000 *g* to remove myofibrils, mitochondria, and connective tissue fragments. The supernatant so obtained is referred to as *crude Marsh extract*. Crude Marsh extract was passed through several layers of cheesecloth and centrifuged for 2 hours at 78,000 *g*. The sediment containing vesicular material referred to as *granules* was washed once with extraction solution and with the aid of a Potter-Elvehjem homogenizer, was resuspended in a volume of extraction solution equal to  $\frac{1}{10}$  the volume of crude extract. Thus, 1 ml of granular fraction contained material derived from 2.5 gm of muscle. The 78,000 *g* supernatant containing the soluble relaxing activity is referred to simply as the *supernatant fraction*. All preparations were stored in polyethylene bottles in the cold room and were generally not used longer than a week.

C. *Assay Systems*

Single, glycerol-extracted fibers were prepared from rabbit psoas muscle according to the method of Szent-Györgyi (24). When it was desired to have fibers contaminated with active intrinsic relaxing factor, the glycerol extraction was carried out for only 2 to 4 days (2), otherwise the fibers were extracted for more than 90 days. Tension generation was induced in these fibers with contraction solution and recorded isometrically with the aid of a RCA 5734 transducer tube or with a Cahn electrobalance adapted for the purpose.

Myofibrils were prepared by a modification of the method of Perry (25). One volume of minced rabbit muscle was homogenized for 1 minute in a Waring blender with 3 volumes of 70 mM tris, pH 7.0, containing 5 mM EDTA. EDTA was included to minimize spontaneous shortening of the myofibrils during isolation. The homogenate was centrifuged at 1,500 *g* for 15 minutes at 0–2°C. The supernatant was discarded and the sediment resuspended in 70 mM tris by means of a Waring blender. The suspension was filtered through several layers of cheesecloth and subjected to

6 or 7 cycles of centrifugation and resuspension. After the final washing the myofibril sediment was suspended in 70 mM tris to give a protein concentration of approximately 20 to 25 mg/ml. The myofibril suspension was stored in a polyethylene bottle at 2–4°C and was discarded after 7 to 10 days.

Myofibrillar and actomyosin ATPase were measured in a constant temperature bath at 25°C, using a magnetic stirring device to insure rapid mixing. The enzyme suspension was pipetted into the reaction vessel containing contraction solution. Aliquots of 2 ml were removed at 10 seconds and 5 minutes and mixed with 2 ml of ice cold 10 per cent TCA. Inorganic phosphate was measured according to the procedure of King (26) and the difference between the 10 second and 5 minute values was taken to represent the phosphate liberated in 5 minutes. Since the relaxing factor preparations often possessed some ATPase activity, separate determinations of phosphate liberation were made in the absence of actomyosin and suitable corrections were made.

Protein was estimated by the method of Lowry *et al.* (27).

## RESULTS

A number of surface-active agents have previously been used to destroy the intrinsic relaxing activity contaminating glycerinated fibers and myofibrils. Ebashi (28) employed deoxycholate and benzalkonium chloride while Weber and Herz (17) used saturated thymol solution. The use of these agents was based on their reported ability to destroy relaxing activity of isolated granules. Almost no information has been reported on the effects of these agents on such properties of the actomyosin system as contractility or sensitivity to EDTA or other relaxing agents. Without this information the use of these agents for the purpose of differentially inactivating the vesicular material contaminating actomyosin preparations is highly empirical.

To preferentially destroy any granular relaxing material contaminating myofibrils or glycerinated muscle fibers it is necessary to find a concentration of an agent which will inactivate granules without modifying the activity of actomyosin. The first agent to be studied for such properties was deoxycholate. The influence of various concentrations of deoxycholate on the ability of granules to inhibit myofibrillar ATPase was initially investigated. Deoxycholate was added to extraction solution in concentrations between 0.5 and 10.0 mM. Ten volumes of this solution was then added to 1 volume of the concentrated granule preparation and allowed to stand at room temperature for 5 minutes. The mixture was then immediately chilled in ice water and subjected to centrifugation to separate the granules from the deoxycholate. The granules were washed twice with extraction solution, suspended in extraction solution, and tested for their myofibrillar ATPase-inhibiting activity. The control granules were treated in a comparable manner. The results of these experiments, presented in Fig. 1, show that

0.5 mM deoxycholate was relatively ineffective in destroying granule relaxing activity while 1, 5, and 10 mM deoxycholate were highly effective.

A quantitative assessment of the ability of deoxycholate to destroy the relaxing activity of granules was then undertaken. The granule fraction was

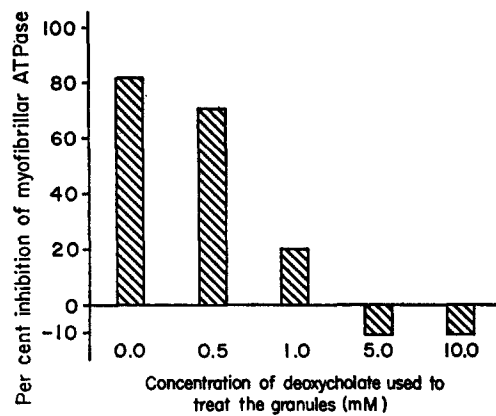


FIGURE 1. Influence of deoxycholate treatment on granule relaxing activity. ATPase activity was measured as described in the text. Myofibrillar protein concentration, 5 mg in a final volume of 10 ml.

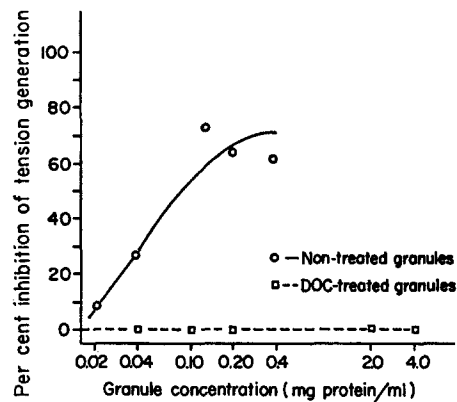


FIGURE 2. Comparison of relaxing activities of deoxycholate-treated and control granule preparations. Granule relaxing activity assayed on glycerinated fibers as described in text.

exposed to 1 mM deoxycholate for 5 minutes at room temperature and then washed free of deoxycholate. The influences of various concentrations of treated granules and control granules on the tension generation of glycerinated fibers were then compared (Fig. 2). Clearly, 4 mg of granules treated with deoxycholate were less effective than 0.04 mg of non-treated granules;

actually, there was no evidence of any relaxing activity even at the highest granule concentration.

To determine whether deoxycholate has an effect on actomyosin the ATPase activity of deoxycholate-treated myofibrils was measured. Exposure to deoxycholate was for a period of 5 minutes at room temperature. As indicated in Fig. 3, if ATP were not present, concentrations of deoxycholate up to 5 mM were without influence on myofibrillar ATPase. Ten mM deoxycholate was definitely deleterious to myofibrillar ATPase. If ATP were present, then the myofibrils were much more sensitive to the inhibiting influence of deoxycholate.

To determine the influence of deoxycholate on the sensitivity of actomyosin preparations to the inhibitory effects of granules, myofibrils were treated

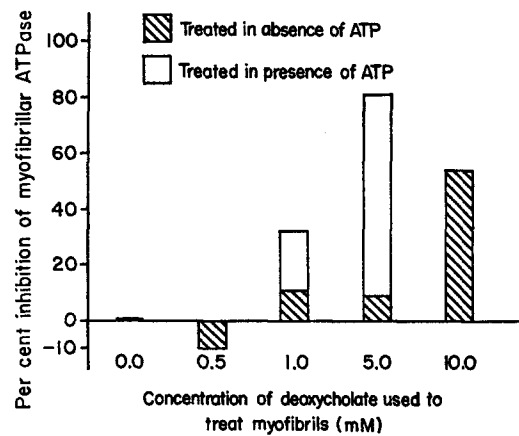


FIGURE 3. Influence of deoxycholate on myofibrillar ATPase. Myofibrillar ATPase measured as described in text. Myofibril protein, 5 mg in final volume of 10 ml.

with either 1 or 5 mM deoxycholate for 5 minutes at room temperature, then washed three times with 70 mM tris. The capacity of various granule concentrations to inhibit the ATPase activity of these deoxycholate-treated myofibrils relative to a control myofibril preparation is shown in Fig. 4. Clearly the myofibrils treated with 5 mM deoxycholate have become almost completely resistant to the inhibitory effects of granules, while the myofibrils treated with 1 mM deoxycholate showed nearly normal sensitivity to the inhibitory effects of granules. At 100 per cent activity the non-treated myofibrillar ATPase liberated 0.26  $\mu$ mole phosphate per minute per milligram myofibrillar protein, the myofibrils treated with 1 mM DOC, 0.23  $\mu$ mole phosphate per minute per milligram protein, and the myofibrils treated with 5 mM DOC, 0.24  $\mu$ mole phosphate per minute per milligram protein. The sensitivity of glycerinated fibers to EDTA after treatment with 1 or 5 mM deoxycholate was also investigated. In a manner similar to the observations just noted, the muscle

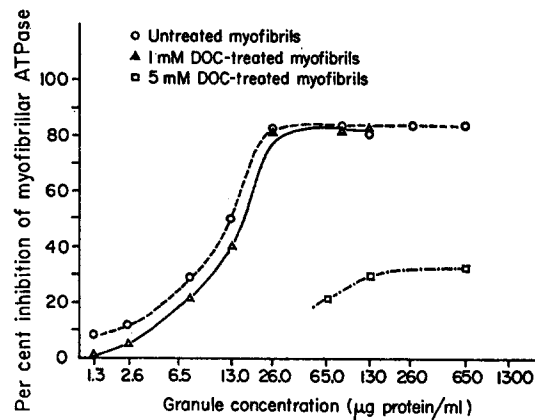


FIGURE 4. Influence of deoxycholate treatment of myofibrils on their sensitivity to granule relaxing activity. Myofibrillar ATPase measured as described in text. Myofibril protein, 5 mg in final volume of 10 ml.

fibers treated with 5 mM deoxycholate showed only a minimal response to 2 mM EDTA while the fibers pretreated with 1 mM deoxycholate responded in a normal manner.

TABLE I  
SURVEY OF THE EFFECTS OF BENZALKONIUM CHLORIDE  
ON CONTRACTION OF GLYCERINATED FIBERS, GRANULE  
RELAXING FACTOR, AND THEIR INTERACTION

Benzalkonium chloride* (w/v)	Parameter investigated			
	(a) Tension generation	(b) Response to granules†	(c) Capacity of granules to inhibit tension generation‡	(d) Activity of intrinsic factor
<i>per cent</i>				
$5 \times 10^{-4}$	—	—	—	—
$1 \times 10^{-3}$	—	—	+	±
$5 \times 10^{-3}$	—	—	+	+
$1 \times 10^{-2}$	—	±	+	+

+, indicates destruction of normal activity; —, indicates no effect on normal activity; ±, indicates less than normal activity.

(a) Untreated fibers. (b) Benzalkonium-treated fibers tested with untreated granules. (c) Untreated fibers tested with benzalkonium-treated granules. (d) Fibers extracted 2 to 4 days, see Methods.

\* Exposure to benzalkonium chloride was for 5 minutes at room temperature.

† The response of glycerinated fibers to granules and the influence of benzalkonium chloride on granules were examined with fibers extracted for more than 90 days (see) Methods.

Similar, but not so exhaustive, exploration of the effects of benzalkonium chloride on glycerinated fibers, granules, and the interaction between the fibers and granules was undertaken. The results are summarized in Table I. As with deoxycholate, it appears that benzalkonium chloride can be used to preferentially destroy intrinsic relaxing factor. The data indicate that there is, as with deoxycholate, only a narrow range of concentrations where benzalkonium chloride can destroy the intrinsic relaxing factor without altering the sensitivity of the fiber to the granule relaxing factor.

#### DISCUSSION

On the basis of the above evidence, it appears that the actomyosin systems, glycerinated muscle fibers or myofibrils, treated with 1 mM deoxycholate or 0.005 per cent benzalkonium chloride at room temperature for 5 minutes, are free of any functional granule relaxing factor. This means that such preparations, if properly pretreated, may be used for the assay of relaxing factors. The ease with which these preparations are obtained compared to the very tedious preparation of purified F-actin and myosin makes them very desirable for such assays.

The data indicate that there is not a large difference between the concentration of the surface agent which completely destroys the relaxing system and the concentration of the agent which begins to alter the sensitivity of the actomyosin system to the relaxing agent. The uncritical use of surface-active agents for the preferential destruction of the vesicular system would seem to be unjustified. For example, Ebashi (28) suggests that exposure of glycerinated bundles of muscle fibers to 0.5 mM deoxycholate at room temperature for 3 minutes is sufficient to inactivate the granule relaxing factor. The studies presented here do not support those findings. Ebashi (28) also recommends 0.15 per cent benzalkonium chloride; though the current investigation suggests that this is sufficient to destroy the vesicular system, it was found that such a concentration does decrease the sensitivity of the actomyosin system to relaxing agents.

It is also clear that sensitivity to relaxing agents is lost at lower concentrations of the surface-active agents than is the capacity of the actomyosin systems to undergo syneresis or develop tension or catalyze the hydrolysis of ATP. The preferential loss of sensitivity to relaxing agents has also been noted by other investigators (16) and suggests that a high degree of structural specificity is involved in the interaction between actomyosin and relaxing agents.

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