SOME FURTHER PROPERTIES OF THE MUSCLE RELAXING-FACTOR SYSTEM AND THE SEPARATION OF THE EFFECTIVE SUBSTANCE

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SUMMARY

1. Caffeine, Ca^{2+}, dinitrophenol, cyanide and nicotine inactivate the activity of the inhibitory action of microsomes on the myofibrillar ATPase. This inactivation became less effective after the incubation of microsomes with ATP, Mg^{2+} and oxalate.

2. The relaxing action of microsomes removed once by the addition of the agents, was again recovered by the continuance of further incubation.

3. The immune globulin prepared from the serum of a rabbit injected with the microsomes of chicken skeletal muscle inhibited the relaxing action of the chicken microsomes not preincubated with ATP, but had no effect on that of microsomes preincubated with it.

4. A protein-free relaxing substance was separated from microsomes by means of the chromatography on cellulose column followed by the elution with an ATP solution.

5. The relaxing substance separated was inactivated by contracture-producing agents and remained more stable under a 2-min heat treatment at 100° than that in the mixture containing microsomes.

INTRODUCTION

In the previous papers, it was reported that the inhibitory action of the muscle granules, *i.e.*, microsomes, on the myofibrillar ATPase is intensified by preincubation with ATP, oxalate and Mg^{2+} (see ref. 1) and that some contracture-producing agents evidently inhibit the relaxing action of the crude extract containing microsomes on the single glycerol-extracted muscle fibers contracted with ATP[2]. Furthermore, it was observed that some types of agents, for example, caffeine and DNP become less effective for the action of the crude extract preincubated with ATP and Mg^{2+} (see ref. 2).

These facts are considered to be in support of the possibility that a relaxing substance is produced from microsomes in the presence of ATP and others. Recently, BRIGGS AND FUCHS[3] reported on the formation of a dialyzable relaxing substance by interaction of ATP and microsomes. PARKER AND GERGELY[4] also reported their successful acquisition of a granule-free soluble relaxing substance by

means of centrifugation in the wake of incubation of the granules and ATP in the presence of cofactor.

The present report will deal with a detailed study of the effects of some contracture-producing agents and the antibody against microsomes having an effect on the activity of microsomes inhibiting the myofibrillar ATPase, as well as with the recent results on the separation of a microsome-free relaxing substance and some of its properties.

MATERIALS AND METHODS

Materials

Myofibrils and microsomes of the skeletal muscles of the rabbit and chicken were prepared as described in the previous reports[1,2]. Immune globulin was prepared in the following manner: a rabbit was injected intravenously with a dose of 40 mg protein of chicken microsomes once every day for 5 consecutive days. The rabbit was bled 10 days after the last injection. The precipitin test was made to examine the antibody content of the rabbit serum. The globulin fraction was obtained from the serum by ammonium sulphate fractionation. The control globulin was prepared in the same manner from the serum of a rabbit not injected with microsomes. Each globulin preparation was dialyzed with Tris-buffer (pH 7.0) for 48 h at 0°. ATP is a crystalline disodium salt prepared by the Sigma Chemical Company. Cellulose powder is a commercial preparation by the Toyo Roshi Company.

Determination of ATPase activity

The determination of ATPase activity was made by a reaction mixture of the following composition; 2 m*M* ATP, 2 m*M* $MgCl_2$, 2 m*M* K-oxalate, 0.07 *M* KCl, 0.07 *M* Tris buffer (pH 7.0), 1–1.5 mg of myofibrils and if necessary, a contracture-producing agent, microsomes and antibody. The final volume was brought to 3.0 ml with the addition of deionized water. The reaction mixture was incubated in the bath at 20° and next the reaction was started by an addition of ATP, or myofibrils when microsomes were preincubated with ATP. The reactions were run for 2 and 4 min respectively and were stopped with 2 ml of a 5% trichloroacetic acid. The amount of inorganic phosphate liberated was analyzed by the Fiske-Subbarow method. The ATPase activity was determined by the difference between the amounts of the inorganic phosphate liberated in 2 and 4 min respectively. A correction was made of the hydrolysis of ATP due to the microsomal ATPase.

Separation of the relaxing substance

Centrifugation: The following incubation mixture was centrifuged at 90000 × *g* for 50 min after a 10-min incubation at room temperature (about 18°). The mixture contained 3 m*M* ATP, 3 m*M* Mg^{2+}, 3 m*M* oxalate, 0.07 *M* KCl, 0.07 *M* Tris buffer (pH 7.0) and 10–20 mg of microsomes. The total volume was 9.0 ml.

Chromatography: The chromatography on cellulose column (1 cm × 7 cm, 300 mesh) was applied and a protein-free solution was separated from microsomes by being eluted with a mixture containing 2 m*M* ATP, 2 m*M* Mg^{2+}, 2 m*M* K-oxalate, 0.07 *M* KCl and 0.07 *M* Tris buffer (pH 7.0). First, the cellulose column was well washed with deionized water, and next with the eluent. Microsomes adsorbed were equivalent to 1–2 mg of protein. The elution velocity of the mixture was 0.8 ml/min.

Determination of the protein

A quantitative determination was made on the protein by the micro-Kjeldahl method. The protein in the eluate obtained through the column was tested in the following manner: the eluate was concentrated in vacuum, mounted on a filter paper and treated with a 2% acetic acid solution containing 0.05% bromphenol blue and 1% $HgCl_2$.

RESULTS

Elimination of the inhibiting action of microsomes

The rate of the ATP hydrolysis by the action of myofibrils prepared from a rabbit muscle was 0.2–0.3 μmoleP/min/mg of protein.

As indicated in Fig. 1, caffeine ranging in concentration from 1 m*M* to 12 m*M* had no effect on the activity of myofibrillar ATPase. Microsomes in amounts of 1.92, 2.7 and 5.4% of myofibrillar protein lowered the activity of myofibrillar ATPase to 53, 31 and 18% of the original value, respectively. This corresponds approximately with the results in the previous paper[1]. Microsomes equivalent to 1.92% of the myofibrillar protein was enough to reduce the activity of myofibrillar ATPase to 39% of the control, when preincubated with ATP, Mg^{2+} and oxalate. Caffeine eliminated the inhibitory effect of microsomes on the ATPase activity and its effectiveness increased with increase in its concentration. The inhibitory effect of microsomes

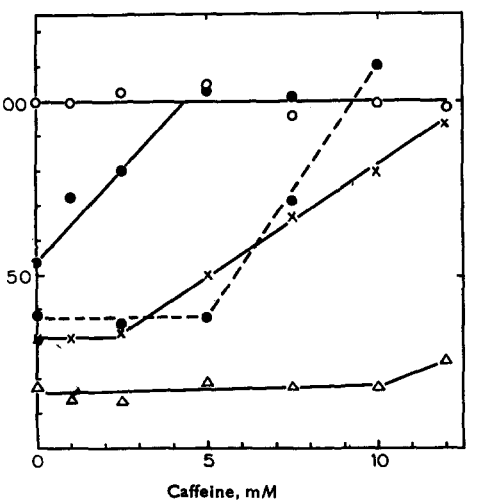

Fig. 1. Effect of caffeine on the inhibitory action of microsomes with and without preincubation by ATP. Ordinate: the rate of the ATP hydrolysis by myofibrils alone was taken as 100% activity. Abscissa: concentration of caffeine in m*M*. Myofibrils. 0.41 mg of protein/ml. ATP = Mg^{2+} = oxalate = 2 m*M*. KCl = Tris buffer (pH 7.0) = 0.07 *M*. Microsomal protein concentrations; O—O, microsomes nil; ●—●, 0.0078 mg/ml; ×—×, 0.011 mg/ml; △—△, 0.022 mg/ml. Solid lines, microsomes without preincubation. Broken line, microsomes preincubated with ATP for 6 min. Temperature 20°C.

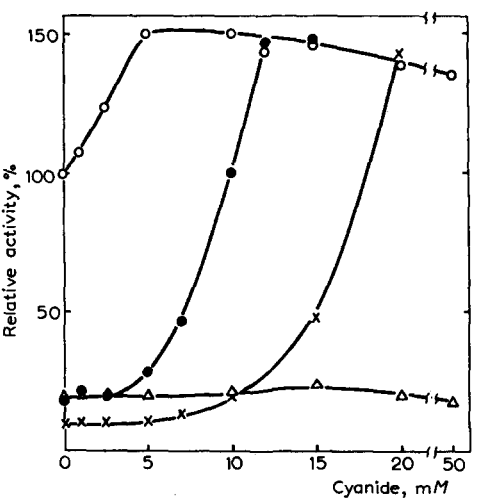

Fig. 2. Effect of cyanide on the inhibitory actions of microsomes and of EDTA. Myofibrils, 0.34 mg of protein/ml. Microsomes, 0.014 mg of protein/ml. O—O, myofibrils alone; ●—●, with microsomes; ×—×, with microsomes preincubated with ATP for 6 min; △—△, with 0.1 m*M* EDTA. Conditions otherwise as in Fig. 1.

preincubated with ATP was eliminated for the first time by caffeine of higher concentration than in the case of experiment without preincubation.

The similarity of actions of nicotine, cyanide and Ca^{2+} as well as DNP to that of caffeine was shown in Table I. As a whole, the action of the agents at the time of preincubation of microsomes with ATP became less effective.

TABLE I

EFFECT OF AGENTS ON THE MYOFIBRILLAR ATPASE AND THE INHIBITORY ACTION OF MICROSOMES

Relative activity: the rat of ATP hydrolysis by myofibrils alone was taken as 100. Inhibition: 100 (ATPase activity without microsomes—ATPase activity with microsomes)/ATPase activity without microsomes. Myofibrils, 0.41 mg of protein/ml. Microsomes, 0.016 mg of protein/ml. Other experimental conditions were the same as stated for Fig. 1.

Agents	*Relative acitvity of myofibrils (%)*	*Inhibition with microsomes (%)*	*Inhibition with incubated microsomes (%)*
	100	69	85
Caffeine 5 m*M*	99	36	82.2
DNP 5 m*M*	70	6.3	67.2
7.5 m*M* *	60	0	70
Cyanide 5 m*M*	160	29	85
Nicotine 5 m*M*	172	0	73.3
Ca^{2+} 0.2 m*M*	170	0	0
0.02 m*M* *	140	16	82

* A different preparation of microsomes was used.

On Ca^{2+} contamination

Table I shows that the efficacy of Ca^{2+} was most strong. Accordingly, the following experiment was performed so as to examine whether or not the action of the agents used is attributable to a minute quantity of Ca^{2+} in each solution.

The ATPase activity of myofibrils was lowered to 16% of the control by 0.1 m*M* EDTA. The remaining activity was the same as that induced by microsomes equivalent to 4% of myofibrillar protein. The inhibitory effect of microsomes was eliminated completely by 0.02 m*M* Ca^{2+} and that of EDTA was also removed by 0.075 m*M* Ca^{2+}. As shown in Fig. 2, 12 m*M* cyanide completely recovered the ATPase activity reduced by microsomes, but 50 m*M* cyanide had no effect on the activity lowered by EDTA. This result indicates that the eliminating action of cyanide on the relaxing activity is not due to the contamination with Ca^{2+}.

Treatment with Amberlite IR 120 did not give rise to changes in the action of caffeine.

Production of relaxing substance after addition of agent

If the actions of caffeine and DNP are attributable to their combination with the relaxing substance produced by preincubation of microsomes and if those do not alter strongly the microsomal ability to produce the relaxing substance, the relaxing activity of microsomes which was once lost by the addition of the agents is considered to be recovered by means of further incubation with ATP and others.

Fig. 3 shows the relation between the relaxing activity of microsomes and the incubation time after the addition of caffeine. Microsomes preincubated for 10 min produced a 77% inhibitory effect on the myofibrillar ATPase. Addition of 4 m*M* and 8 m*M* of caffeine reduced the above effect to 56 and 16%, respectively. However, the

microsomal inhibition was almost completely recovered by further incubation and thereafter disappeared gradually 60–70 min later. Similar results were obtained with DNP.

The experiment with Ca^{2+} is shown in Fig. 4. The process after the addition of 0.02 m*M* Ca^{2+} was almost analogous to that after the addition of caffeine, but the repeated inhibitory effect on the myofibrillar ATPase originating from a 5-min incubation after the addition of 0.04 m*M* Ca^{2+} disappeared more rapidly than after the addition of caffeine. This result may reveal that a large amount of Ca^{2+} changes the ability of microsomes to produce the relaxing substance. Except in the case of 0.04 m*M* Ca^{2+}, these facts will indicate that even after the addition of the agent, microsomes still continue to produce the relaxing substance in the presence of ATP and others.

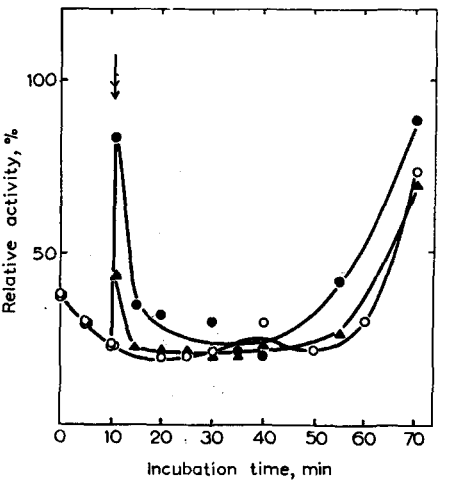

Fig. 3. Inhibitory action of microsomes in dependence on the time of incubation with ATP and caffeine. Abscissa: the incubation time after addition of ATP. Arrow indicates when caffeine was added to incubation mixture. Myofibrils, 0.45 mg of protein/ml. Microsomes, 0.013 mg of protein/ml. ○—○, without caffeine; ●—●, 8 m*M* caffeine; ▲—▲, 4 m*M* caffeine. Conditions otherwise as in Fig. 1.

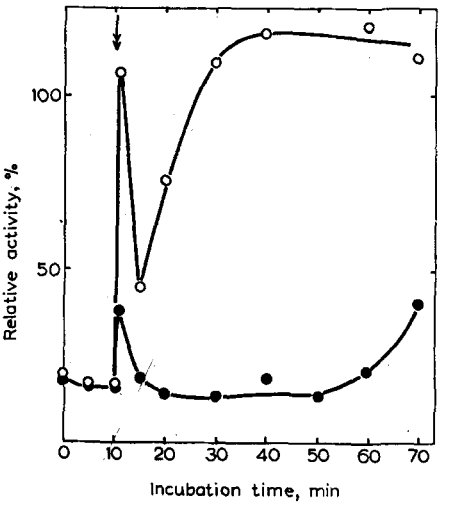

Fig. 4. Inhibitory action of microsomes in dependence on the time of incubation with ATP and Ca^{2+}. Arrow indicates when Ca^{2+} was added to the incubation mixture. Ordinate: when Ca^{2+} was added, the rates of ATP hydrolysis by myofibrils in the presence of 0.02 m*M* and 0.04 m*M* Ca^{2+} respectively, were taken as 100 % activity. Myofibrils, 0.45 mg of protein/ml. Microsomes, 0.018 mg of protein/ml. Symbols: ○—○, 0.04 m*M* Ca^{2+}; ●—●, 0.02 m*M* Ca^{2+}. Conditions otherwise as in Fig. 3.

In the previous paper[2], it was pointed out that the tension of the glycerol extracted muscle fiber developed by the addition of caffeine or nicotine in the presence of the crude extract decreased again with the passage of time. The supposition that the phenomenon may be compared with that shown in Fig. 3, was confirmed by the observations that the single glycerol-extracted fiber previously relaxed with a fresh crude extract contracted on the addition of 5 m*M* and 7 m*M* nicotine, and caused a rapid re-relaxation (Fig. 5).

Effects of antibody on the inhibiting action of microsomes

In contrast with the above agents considered to inactivate the relaxing substance itself, the antibody against the microsomes of chicken skeletal muscle was taken up as an inactivator which affected the microsomes themselves.

Both the myofibrillar and microsomal ATPases of chicken skeletal muscle were not influenced by the control and immune globulin prepared from the rabbit serum under the present experimental conditions. As shown in Table II, the ATPase activity of the myofibrils is inhibited by the non-preincubated microsomes equivalent to 6%

TABLE II

EFFECT OF CONTROL AND IMMUNE GLOBULIN ON THE INHIBITORY ACTION OF CHICKEN MICROSOMES

Each material was added in the orders as shown in the first column into the reaction mixture. Microsomes were incubated with globulin for 20 min. Myofibrils, 0.43 mg of protein/ml. Microsomes, 0.026 mg of protein/ml. Control globulin and immune globulin, respectively, 0.28 mg/ml. Inhibition was calculated as described in Table I. Other conditions were the same as stated for Fig. 1.

System	*Preincubation time with ATP, min*	*Inhibition (%)*
Myofibrils + ATP	—	0
Microsomes + myofibrils + ATP	—	45
Microsomes + ATP + myofibrils	5	81
Microsomes + gluoblin* + ATP + myofibrils	—	70
Microsomes + ATP + globulin* + myofibrils	5	83
Microsomes + antibody + ATP + myofibrils	—	8
Microsomes + ATP + antibody + myofibrils	5	78

* Control globulin.

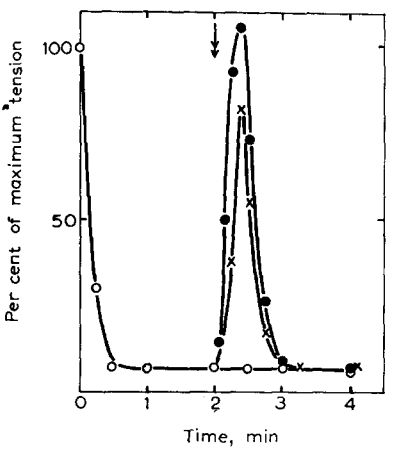

Fig. 5. Effect of nicotine on the single glycerol-extracted fiber relaxed with fresh crude extract. Arrow indicates when the relaxing solution containing 2 m*M* ATP, 2 m*M* oxalate, 2 m*M* Mg^{2+}, 0.07 *M* KCl, 0.07 *M* Tris buffer of pH 7.0 and 10 volume percent of crude extract was replaced with another ATP solution containing 10% of crude extract and nicotine. ○—○; nicotine nil; ×—×, 5 m*M* nicotine; ●—●, 7 m*M* nicotine. Temperature 16°. The preparations of the single glycerol-extracted muscle fiber and crude extract and the measurement of tension were performed as described in the previous paper[2].

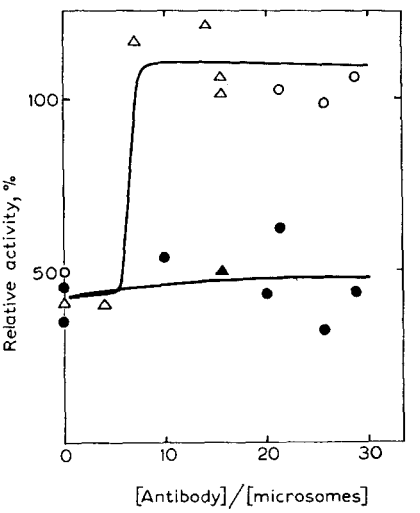

Fig. 6. Effect of antibody on the inhibitory action of chicken microsomes. Abscissa: the concentration ratios of antibody to microsomes. The myofibrils prepared from both the rabbit, ○—○, and chicken, △—△, muscles were used. Microsomes were incubated with antibody for 20 min. Myofibrils, 0.43 mg of protein/ml. Microsomes, 0.028 mg of protein/ml. Full symbols microsomes preincubated with ATP for 5 min. Open symbols; microsomes without preincubation. Conditions otherwise as in Fig. 1.

of the myofibrils and the degree of inhibition was stronger by the preincubated ones. The control globulin did not exert effects on the inhibiting action of the microsomes. On the other hand, the immune globulin almost eliminated the inhibiting action of the microsomes not incubated with ATP, but did not remove that of the microsomes preincubated with ATP. For the purpose of appearance of sufficient efficacy of the antibody against the inhibiting action of the microsomes, it was necessary to incubate for 20 min the antibody and microsomes in a mixture free of ATP and myofibrils.

Fig. 6 displays the relationship between the effectiveness of antibody and the concentration ratio of antibody to microsomes. An experiment was made using the myofibrils prepared from both the chicken and rabbit muscles. An amount of the antibody 4 times as large as that of microsomes had no effect on the microsomal inhibiting activity regardless of presence or absence of preincubation with ATP. However, an amount of the antibody 7–28 times as large completely removed the action of the non-preincubated microsomes and did not eliminate that of the preincubated microsomes. Furthermore, it can be seen that the chicken microsomes inhibited the ATPase of myofibrils from the rabbit muscle.

In order to compare with the efficacy of caffeine for the relaxing activity, the microsomes were subjected to further incubation after the addition of antibody. In contrast with the case of caffeine, etc., even when the microsomes preincubated with ATP were added with the amount of antibody 15 times as large, there was no immediate disappearance of their inhibiting action (Fig. 7). The inhibiting action of the microsomes in the presence of antibody disappeared completely 57 min afterwards.

These results may suggest that the microsomes were placed by the antibody

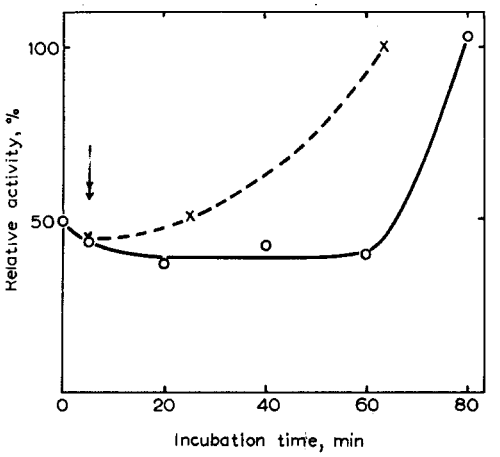

Fig. 7. Inhibitory action of chicken microsomes in dependence on the time of incubation with ATP and antibody. Arrow indicates when antibody was added to incubation mixture. O—O, without antibody; ×—×, with antibody. Chicken myofibrils, 0.43 mg of protein/ml. Microsomes, 0.028 mg of protein/ml. Antibody, 0.42 mg/ml. Conditions otherwise as in Fig. 3.

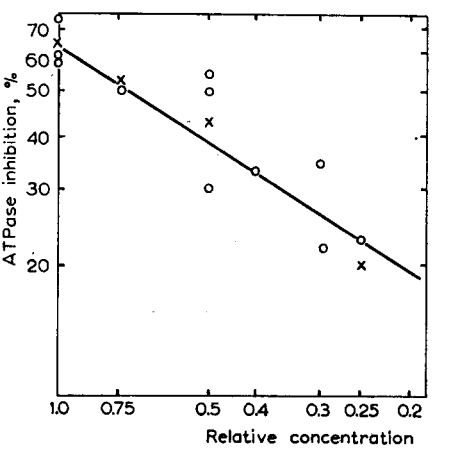

Fig. 8. Relationship between the inhibitory actions and the relative concentrations of the supernatant and the eluate. Ordinate: ATPase inhibition, 100 (ATPase activity of myofibrils alone—ATPase activity with supernatant or eluate)/(ATPase activity of myofibrils alone). Abscissa: the relative concentrations of supernatant and eluate. O—O, Supernatant; ×—×, eluate. Myofibrils, 0.45 mg of protein/ml. Other conditions were the same as stated for Fig. 1.

beyond the scope of the reaction system and that the antibody has no effect on the relaxing substance itself.

As stated by BAIRD AND PERRY[5], it was observed that the mitochondrial fraction of pigeon-breast muscle also had an inhibitory action on the myofibrillar ATPase. Accordingly, a precipitin test was performed on the mitochondria and the antibody of the microsomes, and it led to the observation that the antibody of microsomes reacts also with the mitochondria and inhibits the relaxing activity of the mitochondrial fraction.

Separation of the relaxing substance

The preceding experimental results suggest that the relaxing substance differs from the microsomes and is produced by the incubation of the latter with ATP. Therefore, if the substance is not so labile, there will be a possibility for its separation from the microsomes.

Centrifugation: A supernatant with the inhibitory action on the ATPase activity of the myofibrils was obtained by centrifugation at 90000 $\times$ g for 50 min of the pre-

TABLE III

CHROMATOGRAPHY ON THE CELLULOSE COLUMN

A column of 1 $\times$ 7 cm was used, the cellulose was 300 mesh. The eluent contains 2 mM ATP, 2 mM Mg^{2+}, 2 mM oxalate, 0.07 M KCl, 0.07 M Tris buffer of pH 7.0. Microsomes adsorbed 1–2 mg of protein. Elution velocity, 0.8 ml/min.

Fraction No.	*Volume of eluate (ml)*	*Inhibition (%)*	*Protein*
1	20	10, 8, 0,	
2	20	28, 25, 55,	Nil
3	20	76, 48, 56,	Nil
4	20	49, 8, 51,	Nil
5	20	5, 0, 0,	
6	20	0, 0, 0,	

incubated mixture containing microsomes of 1–2 mg protein/ml. Fig. 8 shows the relationship between the activity of the supernatant and its relative concentration. The relative concentration was determined, of the 5 supernatants obtained from different microsomes, by changing the respective volumes in the reaction mixture.

The relation shown in the figure coincided with that indicated by BRIGGS AND FUCHS[3]. Furthermore, the inhibitory action of the supernatant was also eliminated by the addition of the above-mentioned agents. However, this supernatant contained a large amount of protein, and dialysis of the relaxing substance in the supernatant was not successful. For dialysis, an ordinary cellophane membrane washed with water for 50 h was used.

The above-mentioned result was different from that presented in the previous paper[1]. The cause of this differerence is at present unknown.

Chromatography: The relaxing activity was found in the eluate passing through the cellulose column with microsomes adsorbed. Three results of the study made of the relaxing activity and protein in the eluate were shown in Table III. The peak of the relaxing activity always stood at the third fraction. The amount of protein in

the eluate was, even though it was present, less than 0.5–1.0 μg/3 ml. Such amount of the microsomal protein has no significant effect on the ATPase activity of 1–1.5 mg myofibrillar protein[1]. Needless to say, this eluate did not contain materials which were either precipitated by trichloroacetic acid or coagulated by heat. This eluate is considered to contain a microsome-free relaxing substance.

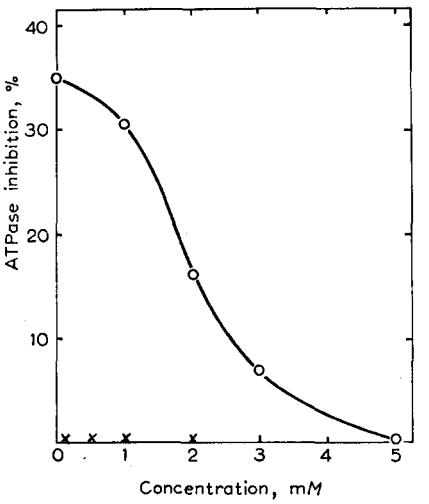

Fig. 9. Effect of caffeine and Ca^{2+} on the inhibitory action of the eluate. Ordinate: ATPase inhibition was calculated as shown in Fig. 8. Myofibrils, 0.35 mg of protein/ml. O—O, caffeine; ×—×, Ca^{2+}. Conditions otherwise as in Fig. 8.

Nature of the protein-free relaxing substance

As indicated in Fig. 8, the relationship between the relative concentration of the protein-free relaxing substance and its activity was the same as that of the supernatant prepared by centrifugation. The initial mixture showing a 65% inhibition was obtained by dilution of the original eluate with the ATP solution so as to correct the concentration of ATP hydrolyzed by the microsomal ATPase. The mixture showing a 35% inhibition was completely deprived of its activity by 5 m*M* caffeine (Fig. 9).

TABLE IV

EFFECTS OF AGENTS ON THE INHIBITORY ACTIVITY OF THE RELAXING SUBSTANCE SEPARATED BY CHROMATOGRAPHY

The solution showing 50.2 % inhibition was obtained by dilution of the original eluate with the ATP solution. Other experimental conditions were the same as those of Fig. 9.

Agent and treatment	*Inhibition (%)*
Relaxing substance	50.2
+ 2.5 m*M* caffeine	15.2
+ 2.5 m*M* DNP	10.5
+ 2.5 m*M* cyanide	8.6
+ 2.5 m*M* nicotine	18.6
+ 0.02 m*M* Ca^{2+}	2
After heating for 2 min at 100°	48.0
After standing for 1 h at 20°	39.6
After standing for 24 h at 2°	34.6

Table IV shows the efficacies of the other agents on the inhibiting action of the substance as well as the stability of the substance. There was little or no change in the activity of the relaxing substance, even when subjected to a heating in boiling water at pH 7.0 for 2 min. This makes a contrast with the marked instability of the mixture containing microsomes. Moreover, there was no spontaneous recovery of the inhibitory activity of the relaxing substance as a result of the further incubation after the addition of caffeine. The results thus obtained will demonstrate that the contracture-producing agents interacted with the relaxing substance.

DISCUSSION

Although a complete exclusion can not be made of the inhibiting effect of the agents on the reaction producing the relaxing substance, the results shown in the present paper will reveal distinctly that the action of the above-mentioned contracture-producing agents is mainly attributable to the interaction between the agents and the relaxing substance. Referring to the results shown in Fig. 3, Fig. 4 and in this paper, those on Ca^{2+} agree generally with the most recent results reported by HASSELBACH AND MAKINOSE[6] in which the relaxing activity of microsomes was inhibited by the addition of Ca^{2+} and was spontaneously reactivated several minutes later. According to them, the spontaneous disappearance of Ca efficacy is due to the ability of microsomes to incorporate Ca^{2+} ion. However, there is no evidence that microsomes incorporate large amounts of caffeine and DNP. Furthermore, caffeine and DNP did not give rise to the accelerated hydrolysis of ATP by the microsomal ATPase[7] Accordingly, for an adequate explanation of the results shown in Fig. 3, it must be concluded that the microsomes continue to produce the relaxing substance for some time even when an agent is present in the incubation mixture.

The results with the antibody show that the antibody reacts with microsomes and mitochondria, but not with the relaxing substance produced. The above findings may suggest that the common protein participating in the production of the relaxing substance is contained in both the microsomes and mitochondrial fraction. However, as BAIRD AND PERRY[5] suggested, the possibility of the mitochondrial fraction being contaminated with an element containing the relaxing factor can not be excluded.

The experimental results about the action of antibody will be of help in eliminating the microsomes from the incubation mixture. An attempt to elute the protein free relaxing substance from the cellulose column adsorbing microsomes was successful. Such a microsome-free preparation having an effect on the tension development of the single glycerol-extracted muscle fiber and the myofibrillar ATPase was independently reported by BRIGGS AND FUCHS[3] as well as by PARKER AND GERGELY[4]. The preparations so far reported were acquired, by means of centrifugation, from the mixture containing microsomes. The separation of the protein-free relaxing substance strongly suggests that the relaxation of muscle is caused by interaction between the contractile element and the protein-free relaxing substance originating from the microsomes. Some contracture in the muscle will result from a decline in concentration of the substance due to its combination with the agent.

The relaxing substance separated from microsomes remained more stable to the effect of heat than that in the presence of microsomes. This fact may suggest that

some substance released from the microsomes because of thermal denaturation inactivated the relaxing substance.

The separation of the relaxing substance by means of chromatography is considered to give an important clue to the further purification and identification of the protein-free substance.

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