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The Adenosine-Triphosphatase Activity of Desensitized Actomyosin

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1. A simple procedure involving repeated washings of actomyosin, extracted as the complex from myofibrils (natural actomyosin) at ionic strength less than 0·002, is described for the preparation of a desensitized actomyosin. 2. The Mg^{2+}-activated adenosine triphosphatase of natural actomyosin was markedly inhibited by ethylenedioxybis(ethyleneamino)tetra-acetic acid, whereas that of the desensitized actomyosin was unaffected. 3. The activity of the Ca^{2+}-activated adenosine triphosphatase of natural actomyosin was generally lower than that of the Mg^{2+}-activated adenosine triphosphatase, whereas in the desensitized actomyosin the difference between the activities was considerably less. In both natural and desensitized actomyosin the adenosine-triphosphatase activities in the presence of Mg^{2+} were similar. 4. The conversion of the natural into the desensitized actomyosin was accompanied by the removal of a protein fraction containing the factors responsible for the sensitivity to ethylenedioxybis(ethyleneamino)tetra-acetic acid and for modifying the Ca^{2+}-activated adenosine triphosphatase. When added to a desensitized actomyosin this fraction effected a reversal to the natural form. The recombination was facilitated by increasing the ionic strength of the medium. The two factors showed different stabilities to heat and tryptic digestion.

Since the original report of the difference in sensitivity to chelating agents of the Mg^{2+}-activated ATPase* of actomyosin extracted as the complex NAM and actomyosin prepared from separately purified actin and myosin (Perry & Grey, 1956), evidence for the presence of additional factors in the crude actomyosin complex has accumulated. These factors are probably concerned in modifying the enzymic and physical characteristics of the actomysin complex so that they can be controlled by the movement of Ca^{2+} to and from the vesicles of the sarcotubular system during the contraction–relaxation cycle in muscle. The most clearly defined component so far studied is the EGTA-sensitizing factor, a protein fraction that restores EGTA-sensitivity to the Mg^{2+}-activated ATPase of synthetic actomyosin or trypsin-treated preparations of NAM (Ebashi, 1963). During studies on the relation of the EGTA-sensitizing factor to tropomyosin (Perry, Davies & Hayter, 1966), a simple method was developed for converting natural actomyosin into a form the ATPase activity of which was no longer sensitive to EGTA. This preparation has been named desensitized actomyosin (DAM).

The present paper describes the changes in properties that accompany the conversion of actomyosin from the natural into the desensitized form and presents preliminary observations on the nature of the factors removed from natural actomyosin during the process.

* Abbreviations: ATPase, adenosine triphosphatase; NAM and DAM, respectively 'natural' and 'desensitized' (see the text) actomyosin; EGTA, ethylenedioxybis-(ethyleneamino)tetra-acetic acid.

METHODS

Natural actomyosin. Natural actomyosin was extracted as described by Perry & Corsi (1958) from rabbit myofibrils prepared by the method of Perry & Zydowo (1959). After two precipitations by the dilution method the actomyosin was usually washed twice by centrifugation for 15min. at 1200*g* in 50mM-KCl, finally dispersed in 3–4vol. of this solution and stored at 0°. When NAM suspensions were required at lower ionic strength for subsequent conversion into DAM, washing was carried out twice with 5vol. of water rather than 50mM-KCl. As a result of this procedure the EGTA-sensitivity was somewhat decreased (see Table 1).

Desensitized actomyosin. Natural actomyosin suspended in 2mM-tris–HCl buffer, pH7·6, was washed several times with water by centrifugation for 20min. at 33000*g* and the sedimented actomyosin gently redispersed in a hand-operated homogenizer. Washing was continued (usually two to five times) until the Mg^{2+}-activated ATPase of the final preparation, resuspended in about the original volume

of 2mM-tris–HCl buffer, was no longer inhibited by the addition of EGTA. The main aim of the procedure was to lower the ionic strength to a minimum, when the EGTA-sensitizing and other factors were readily washed out of the actomyosin. If the suspensions were not readily sedimented after the second or third step, further washings were carried out in 2mM-tris–HCl buffer, pH 7·6, instead of water. This decreased the hydration of the protein suspension so that it became easier to handle. The ATPase activity of these preparations in the presence of Ca^{2+} or Mg^{2+} was retained for up to 3 weeks at 0°. All preparations were used within this period. Some preparations were prepared by the method originally described by Perry *et al.* (1966) which involved treating NAM at pH 8·6, but the more rapid method described above was adopted for most of the experiments reported in this paper.

Soluble protein fraction of myofibrils. This is the fraction described by Perry (1953) and Perry & Corsi (1958), and was prepared by diluting 1 vol. of myofibril suspension in the original preparation medium (0·1M-KCl–39mM-borate buffer, pH 7·0) with an equal volume of 5mM-tris–HCl buffer, pH 8·6, and dialysing the whole suspension against 20 vol. of the tris–HCl buffer for 4–5 days at 0° with daily changes of dialysis medium. The viscous suspension was centrifuged for 40 min. at 100000*g* and the clear supernatant used. This extract is referred to below as the 'soluble fraction'.

Myosin. Myosin was prepared by the standard procedure used in these Laboratories (Perry, 1955).

Estimation of protein concentration. The protein content of the actomyosin preparations was determined by the biuret reaction (Layne, 1957), which was standardized by micro-Kjeldahl nitrogen estimations. In the clear supernatant after centrifugation the $E_{280}^{1\,cm.}$ value was used as an approximate indication of protein concentration.

Enzymic assays. The Mg^{2+}- or Ca^{2+}-activated ATPase assays were in general carried out on about 0·6mg. of actomyosin in 2ml. at 25° as described by Perry (1960). All specific activities, unless otherwise indicated, were determined in 2·5mM-tris–ATP–25mM-tris–HCl buffer, pH 7·6, containing $MgCl_2$ (2·5mM) or $CaCl_2$ (2·5mM) as appropriate. Activities were expressed as μg. of phosphorus in inorganic phosphate liberated by 1mg. of actomyosin in 5min. under these conditions. After all additions, less the substrate, the samples were equilibrated at 25° for 5min. and the reaction was started by addition of substrate. For determining the EGTA-sensitivity of a preparation the Mg^{2+}-activated ATPase was measured in the presence and absence of 1mM-sodium EGTA, pH 7·6.

Viscosity measurements. These were carried out as described by Perry & Cotterill (1964).

Reagents. Tris–ATP was prepared by the method of Schwartz, Bachelard & McIlwain (1962) from the disodium salt (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany). Twice-crystallized trypsin and thrice-crystallized soya-bean trypsin inhibitor were supplied by the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Tris was supplied by C. F. Boehringer und Soehne G.m.b.H. EGTA was supplied by Fisons Ltd., Loughborough, Leics. All other reagents were of analytical grade and distilled deionized water was used throughout.

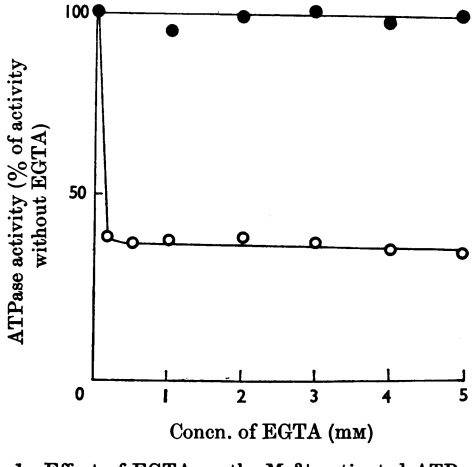

Fig. 1. Effect of EGTA on the Mg^{2+}-activated ATPase of DAM and NAM. Standard assay conditions were used with 2·5mM-$MgCl_2$ and approx. 0·6mg. of protein per assay. ●, DAM; ○, NAM.

RESULTS

Ethylenedioxybis(ethyleneamino)tetra-acetic acid and the biological activity of actomyosin. The most striking difference in the biological properties of NAM and DAM was the complete insensitivity of the Mg^{2+}-activated ATPase of the latter to 1–5mM-EGTA (Fig. 1). A similar difference in superprecipitation behaviour of the two actomyosins was also observed with Mg^{2+} as the added bivalent cation (Fig. 2). In this respect DAM resembled 'synthetic' actomyosin (Perry & Grey, 1956) or NAM subjected to mild treatment with trypsin (Ebashi, 1963).

According to the binding constants given by Sillén & Martell (1964), 1mM-EGTA in the incubation medium would decrease the concentration of free Ca^{2+} to considerably less than 0·1μM, the critical threshold concentration for the myofibrillar ATPase (Weber & Winicur, 1961) and contractile activity *in vivo* (Portzehl, Caldwell & Rüegg, 1964). This implies that significant concentrations of this bivalent cation are not essential either for the Mg^{2+}-activated ATPase or for the superprecipitation of DAM.

Adenosine-triphosphatase activities of desensitized and natural actomyosin. Under the standard assay conditions but in the absence of added bivalent cations, the specific ATPase activities of both actomyosin preparations were low. The activity of the DAM was slightly higher and about 20–30% of that obtained at maximal activation by bivalent cations. The Mg^{2+}-activated ATPases of DAM and NAM behaved similarly to ionic strength and pH changes, although under the standard assay conditions the specific activity of DAM was usually

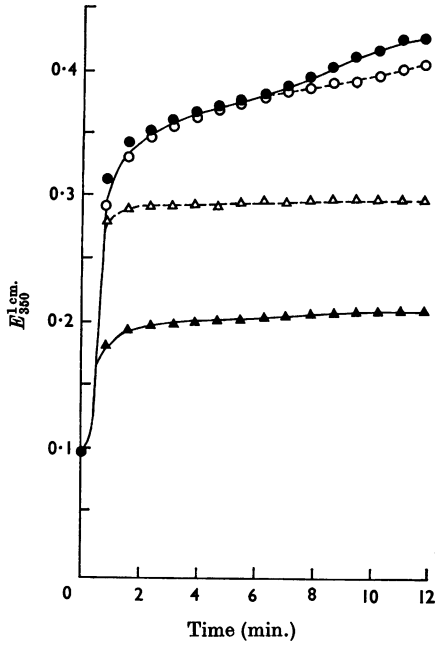

Fig. 2. Effect of EGTA on the superprecipitation of DAM and NAM. Superprecipitation was followed by measuring E_{350} with a Gilford spectrophotometer by using cuvettes of 1cm. light-path containing actomyosin (0·8mg.), tris–HCl buffer, pH7·6 (25mM), $MgCl_2$ (1·7mM) and tris–ATP (1·7mM). When present, the EGTA concentration was 0·1mM. ATP was added at zero time. The total volume was 3ml. DAM and NAM were obtained from the same myofibril preparation. ○, DAM; ●, DAM+EGTA; △, NAM; ▲, NAM+EGTA.

10–20% higher (Table 1). On the other hand, the Ca^{2+}-activated ATPase was invariably significantly higher in preparations of DAM (Table 1). The EGTA-sensitivity of the NAM varied according to the precise procedure followed in its preparation. It was most sensitive when a low concentration of potassium chloride or tris–hydrochloric acid buffer, pH7·6, e.g. 30–50mM, was present during the washing procedures. In general the greater the

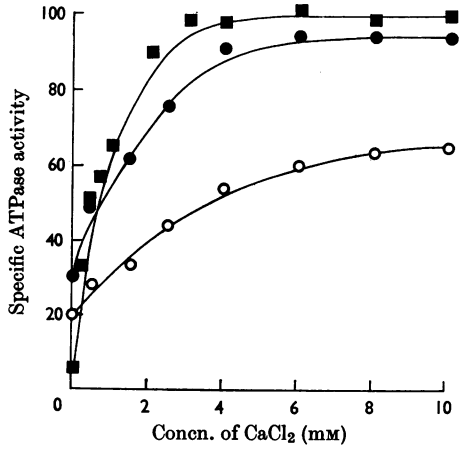

Fig. 3. Specific ATPase activities at different Ca^{2+} concentrations of DAM, NAM and myosin. Standard assay conditions were used. NAM and DAM were obtained from the same myofibril preparation. ■, Myosin; ●, DAM; ○, NAM.

Table 1. *Adenosine-triphosphatase activities of natural and desensitized actomyosin preparations*

ATPase was assayed as described in the Methods section, in the presence of $MgCl_2$ (2·5mM) or $CaCl_2$ (2·5mM) as shown. NAM preparations 14, 15 and 20 were standard (see the Methods section) and washed in KCl or buffer; NAM preparations 10, 11 and 12 were washed finally twice with water.

Preparation	Mg^{2+}-activated ATPase (mg. of P/mg./5min.)	Inhibition of Mg^{2+}-activated ATPase by 1mM-EGTA (%)	Ca^{2+}-activated ATPase (mg. of P/mg./5min.)	Mg^{2+}/Ca^{2+} ATPase activity ratio
14 NAM	43·3	78	24·0	1·81
DAM	48·4	0	50·9	0·95
15 NAM	57·0	72	28·0	2·04
DAM	67·3	0	95·0	0·71
20 NAM	75·6	70	37·6	2·01
DAM	82·9	0	101·0	0·82
10 NAM	63·0	39	41·8	1·51
DAM	61·2	0	69·9	0·88
11 NAM	84·4	50	54·4	1·55
DAM	89·5	0	97·8	0·92
12 NAM	70·5	34	55·5	1·27
DAM	89·9	0	102·0	0·88

sensitivity of the Mg^{2+}-activated ATPase to EGTA the greater was the difference between the Ca^{2+}-activated ATPase of the NAM and the DAM prepared from it.

In view of the similarity of the specific Mg^{2+}-activated ATPase of the two types of actomyosin preparations it seemed unlikely that appreciable amounts of actin were selectively removed from NAM when it was converted into DAM, but rather that another factor (or factors) responsible for the differences in properties was removed.

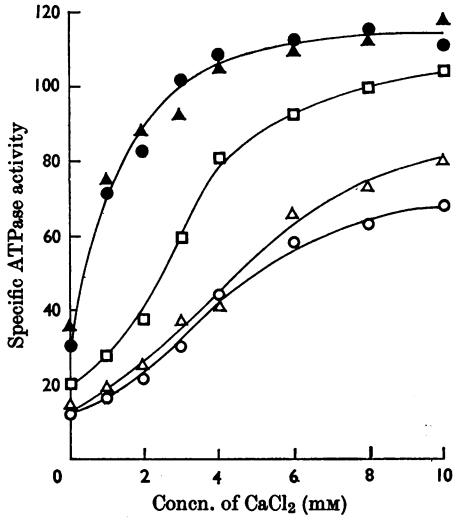

Fig. 4. Effect of washing NAM suspension with water on the response of ATPase to various concentrations of Ca^{2+}. Standard assay conditions were used. Actomyosin suspensions in 50mM-KCl–2mM-tris–HCl buffer, pH7·6, were centrifuged for 20min. at 33000*g*, and the clear supernatant was discarded and replaced by water. Ionic conditions of washed NAM suspensions were as indicated in Table 2. ○, Original NAM suspension; △, after first washing; □, after second washing; ▲, after third washing; ●, after fourth washing.

When the Ca^{2+}-activation curves of DAM and NAM were compared it was apparent that the difference in the specific ATPase activities under the conditions of measurement was due to greater sensitivity of the ATPase activity of DAM to changes in the concentration of Ca^{2+} (Fig. 3). At higher Ca^{2+} concentrations (above 10mM) the specific ATPase activity of NAM approached that of DAM. The response of the ATPase of DAM to Ca^{2+} resembled that of myosin itself (Fig. 3).

Conversion of natural into desensitized actomyosin. As the difference in the activating effects of Ca^{2+} over the range 0–10mM on the ATPase was a consistent observation and appeared to be correlated with the EGTA-sensitivity, it provided an alternative index of the conversion of NAM into DAM. The changes in Ca^{2+}-activated ATPase activity that occurred on washing a suspension of NAM in 50mM-potassium chloride with water are illustrated in Fig. 4. Table 2, which summarizes other aspects of the conversion, shows that Mg^{2+}-activated ATPase specific-activity rose only slightly whereas the marked increases in the specific activity of the Ca^{2+}-activated ATPase were paralleled by a loss of EGTA-sensitivity.

The E_{280} and E_{260} values of the water washings obtained in the experiment illustrated in Fig. 4 and Table 2 indicated that significant amounts of material, which was, in the later stages at least, probably protein, was being removed from the NAM. Conversion of NAM into DAM by this procedure involved on average the removal of up to about 10% of the total protein present in the original actomyosin suspension. This is an approximate

Table 2. *Effect of washing natural actomyosin suspension with water on the* Mg^{2+}*-activated adenosine triphosphatase sensitivity to ethylenedioxybis(ethyleneamino)tetra-acetic acid and the composition of the supernatant*

Conditions were as for Fig. 4.

	Whole suspension		Supernatant		
System	Mg^{2+}-activated ATPase (μg. of P/5min./mg.)	Inhibition of Mg^{2+} activated ATPase by 1mM-EGTA (%)	Protein removed (% of whole suspension)*	E_{280}/E_{260} ratio	Concn. of KCl in suspension (mM)
Original NAM	60·1	53	6·1	0·45	50
Washed 1×	59·9	40	2·1	0·69	8
Washed 2×	70·8	26	2·2	1·39	1
Washed 3×	67·3	0	0·85	1·44	0·4
Washed 4×	74·7	0	—	—	0·14

* Based on the assumption that $E_{280}^{1\,cm.}$ of supernatant containing 1mg. of protein/ml. was 1·0.

Table 3. *Effect of washing natural actomyosin with various concentrations of tris buffer*

In each case approx. 20ml. of suspension of NAM in water at pH7·0 was centrifuged for 15min. at 30000*g*, the supernatant discarded and replaced with an equal volume of appropriate buffer. Tris buffers were pH7·6. The washing was repeated twice with the medium indicated.

System	Mg^{2+}-activated ATPase (μg. of P/mg./5min.)	Inhibition of Mg^{2+}-activated ATPase by 1mM-EGTA (%)	Ca^{2+}-activated ATPase (μg. of P/mg./5min.)	$E_{280}^{1\,cm.}$ of first supernatant
Original NAM	85·0	58	55·0	—
Washed with 50mM-tris	89·5	38	57·7	0·356
Washed with 20mM-tris	84·0	29	58·6	0·470
Washed with 10mM-tris	80·8	6	79·6	0·660
Washed with water	93·8	0	109	1·02

value based on E_{280} measurements and the assumption that on average at a protein concentration of 1mg./ml. the $E_{280}^{1\,cm.}$ value of the supernatant was 1·0, owing to the presence of small amounts of nucleotide.

An essential part of the procedure for effective conversion of NAM into DAM was to decrease the ionic strength of the actomyosin system. If washing was carried out with 50mM-potassium chloride, but otherwise under the conditions illustrated in Fig. 4, no conversion occurred.

If NAM prepared in a solution of low ionic strength was centrifuged for 20min. at 33000*g* a clear supernatant was obtained that when added to a DAM preparation restored the enzymic properties associated with NAM. The effect was not obtained with the supernatant from suspension of a NAM preparation in 50mM-potassium chloride. This result implies that the NAM suspensions in water or media of low ionic strength, i.e. 1–2mM-buffer or potassium chloride, contain in a soluble form the factors responsible for the difference in properties of the two actomyosins. Table 3 illustrates that these factors were readily washed out by water but that in the presence of buffer or salt solutions this did not occur. It follows that the factors associated with NAM and which are responsible for the differences in enzymic properties from DAM can be reversibly bound or released depending on the ionic conditions.

Increasing the pH to 8–9 in media of low ionic strength also facilitated the conversion of NAM into DAM (Table 4). It has been reported (Perry *et al.* 1966) that a protein fraction extracted from myofibrils under these conditions, the 'soluble fraction' (see the Methods section), contains appreciable amounts of EGTA-sensitizing factor. Assays also showed that the 'soluble fraction' was extremely effective in decreasing the Ca^{2+}-activated ATPase activity of DAM. Thus the 'soluble fraction' extracted from whole myofibrils had similar properties to the supernatant obtained from NAM as described above.

Table 4. *Removal of ethylenedioxybis(ethyleneamino)-tetra-acetic acid-sensitizing factor from natural actomyosin at different* pH *values*

NAM suspension was in 12mM-tris–HCl buffer monitored at pH values indicated. After standing for 40hr. at 0° the suspension was centrifuged for 20min. at 33000*g* and the pellet suspended in the original volume of 12mM-tris–HCl buffer, pH7·6. The EGTA-sensitivity of the NAM was assayed as indicated in the Methods section before and after centrifuging. The supernatant obtained in each case was freeze-dried, dialysed against 10mM-tris–HCl buffer, pH7·6, and tested for ability to confer EGTA-sensitivity on a DAM preparation. A 1mg. sample of extracted protein was used per assay.

pH of extraction medium	Inhibition of Mg^{2+}-activated ATPase by 1mM-EGTA (%): NAM before centrifuging	NAM after centrifuging	DAM in the presence of supernatant removed by centrifugation
5·4	38	37	0
6·2	35	23	8
7·2	37	18	12
8·1	42	17	28
9·0	30	2	43

Viscosity of solutions of NAM and DAM. Both NAM and DAM suspensions dissolved readily in 0·5M-potassium chloride and gave viscous solutions, the viscosities of which fell on addition of ATP. At the same total nitrogen concentration NAM solutions usually had a slightly higher relative viscosity, and the fall obtained on addition of ATP was somewhat greater than that obtained with DAM (Table 5).

Nature of factors. From comparison of the properties of NAM and DAM and the effects on the two actomyosin systems of extracts obtained from NAM and from whole myofibrils, two effects can be

Table 5. *Effect of ATP on the relative viscosity of normal and desensitized actomyosin*

Viscosity was measured at 0° in 0·5M-KCl–50mM-tris–HCl buffer, pH 7·6, containing $MgCl_2$ (2·5mM). The protein concentration was 0·4mg./ml. in all cases, and the final ATP concentration was 0·5mM. DAM preparations 18 and 19 were prepared by the method of Perry *et al.* (1966). Preparation 20 was made as described in the Methods section.

Preparation		Relative viscosity		
		−ATP	+ATP	Difference
18	DAM	3·47	1·47	2·00
		3·40	1·44	1·96
	NAM	3·97	1·54	2·43
		3·89	1·49	2·40
19	DAM	3·56	1·51	2·05
		3·51	1·53	1·98
	NAM	4·32	1·65	2·67
		4·23	1·62	2·61
20	DAM	3·74	1·60	2·14
		3·69	1·57	2·12
	NAM	4·58	1·77	2·81
		4·50	1·72	2·78

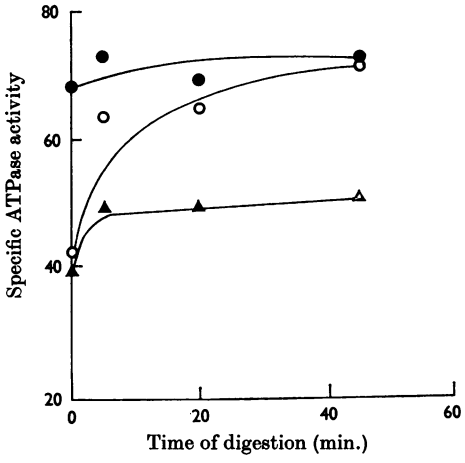

Fig. 5. Effect of trypsin on the enzymic properties of NAM. NAM suspension (7·5mg./ml.) was digested with 1mg. of trypsin/100mg. of actomyosin at 0° in 80mM-tris–HCl buffer, pH 7·6, for the times indicated. Digestion was stopped with 2mg. of trypsin inhibitor/mg. of trypsin and ATPase assays were carried out immediately under standard conditions. ●, Mg^{2+}-activated ATPase; ○, Mg^{2+}-activated ATPase in the presence of 1mM-EGTA; ▲, Ca^{2+}-activated ATPase.

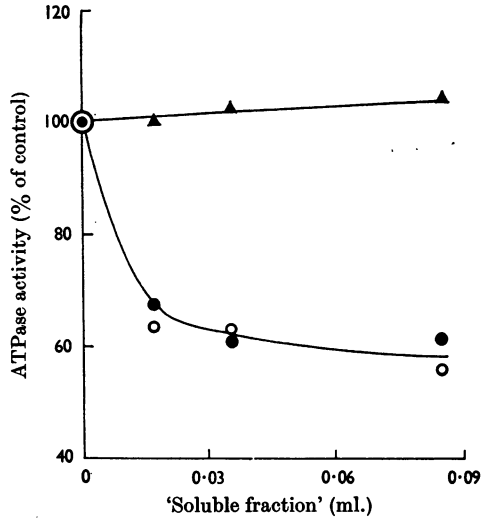

Fig. 6. Effect of trypsin on the factor modifying the Ca^{2+}-activated ATPase. A 1ml. sample of 'soluble fraction' ($E^{1cm.}_{280}$ 2·8) in 10mM-tris–HCl buffer, pH 7·6, was digested with 50μg. of trypsin and the reaction was stopped with 100μg. of inhibitor. The digest was immediately assayed at the concentrations indicated under standard conditions on DAM. ▲, 30min. digestion at 25°; ●, 30min. digestion at 0°; ○, undigested control sample of 'soluble fraction'.

clearly recognized. As yet it cannot be decided finally how many factors are responsible for these effects, but provisionally they can be divided into (1) the EGTA-sensitizing factor (Ebashi, 1963), and (2) the factor that decreases the effect of Ca^{2+} on the ATPase activity of DAM.

The factors appeared to be protein in nature, for in all cases the activities were not lost on dialysis and were precipitated by ammonium sulphate. The EGTA-sensitizing activity was destroyed on heating at pH 7·6 for 15min. at 100°, whereas the factor modifying the Ca^{2+}-activated ATPase was completely stable under these conditions.

Further evidence of differences in stability was obtained by studying the effect of trypsin on the enzymic properties of NAM. A typical experiment is illustrated in Fig. 5, which shows that over the period of digestion the Mg^{2+}-activated ATPase activity changed very little, whereas the EGTA-sensitivity was completely destroyed. During the period over which the EGTA-sensitivity disappeared, the Ca^{2+}-activated ATPase activity rose only slightly and even at the end of the digestion period was less than that of the Mg^{2+}-activated ATPase. The conditions of digestion for the experiment illustrated in Fig. 5 were such that there was apparently little significant digestion of the myosin, the Ca^{2+}-activated ATPase activity of which rises during tryptic digestion (Perry, 1951). The digestions illustrated in Fig. 5 were carried out at 0°, but the effect on the Ca^{2+}-activated ATPase could be destroyed by digestion of the 'soluble

fraction' at higher temperatures, e.g. 25° (Fig. 6). This suggests that the latter effect was due to a protein factor considerably more stable to trypsin than the EGTA-sensitizing factor.

DISCUSSION

Up to now studies of the EGTA-sensitizing factor have been carried out by using as an assay system either actomyosin prepared from separately purified actin and myosin, or actomyosin desensitized to EGTA by previous treatment with trypsin (Ebashi, 1963). The procedure described in the present paper enables natural actomyosin to be desensitized rapidly and efficiently without the disadvantages associated with the other methods. Initially this preparation was developed for the study of the EGTA-sensitizing factor, but it has also proved useful for the investigation of the factor modifying the Ca^{2+}-activated ATPase of actomyosin, which cannot be demonstrated with NAM. DAM can also be used to assay the inhibitory factor described elsewhere (Hartshorne, Perry & Davies, 1966), the properties of which are different from those of the factors described in the present paper.

The experiments with EGTA confirm that significant amounts of Ca^{2+} are not required either for Mg^{2+}-activated ATPase or for the contractile response so far as it is represented by the superprecipitation of actomyosin. Therefore, if a bridge of the type suggested by Davies (1963) is involved in contraction or in the Mg^{2+}-catalysed hydrolysis of ATP by actomyosin systems, Ca^{2+} does not appear to be involved.

Evidence is now good that Ca^{2+} has a role at some stage in the contraction–relaxation cycle, but it would seem more likely that it is concerned in the mechanism of action of the protein factors that are associated with the control of the enzymic activity, which is closely related to the contractile response (Perry, 1967).

In this respect it is noteworthy that the two differences in the enzymic properties of DAM and NAM described in the present paper involve Ca^{2+} and that the factors responsible can be readily removed or replaced in an actomyosin system. Both effects involve the interaction of ATP with actomyosin and, although the centres of enzymic catalysis are not present in actin, this protein is an essential requirement for all the effects observed.

It is possible that the effects observed represent different aspects of the modification of the actin–myosin interaction by a complex protein system, through which the biological activity of actomyosin is regulated by changes in the concentration of free Ca^{2+}. Whatever the nature of the system present in natural actomyosin, the preliminary studies suggest that factors of different stabilities to heat and to trypsin may be responsible for the individual effects, and that the system which regulates the function of actomyosin *in situ* may be of a complexity not previously appreciated.

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REFERENCES

Davies, R. E. (1963). *Nature, Lond.*, **199**, 1068.
Ebashi, S. (1963). *Nature, Lond.*, **200**, 1010.
Hartshorne, D. J., Perry, S. V. & Davies, V. (1966). *Nature, Lond.*, **209**, 1352.
Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 447. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Perry, S. V. (1951). *Biochem. J.* **48**, 257.
Perry, S. V. (1953). *Biochem. J.* **55**, 114.
Perry, S. V. (1955). In *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Perry, S. V. (1960). *Biochem. J.* **74**, 94.
Perry, S. V. (1967). *Progr. Biophys. molec. Biol.* **17**, 327.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V. & Cotterill, J. (1964). *Biochem. J.* **92**, 603.
Perry, S. V., Davies, V. & Hayter, D. (1966). *Biochem. J.* **99**, 1c.
Perry, S. V. & Grey, T. C. (1956). *Biochem. J.* **64**, 5p.
Perry, S. V. & Zydowo, M. (1959). *Biochem. J.* **71**, 220.
Portzehl, H., Caldwell, P. C. & Rüegg, J. C. (1964). *Biochim. biophys. Acta*, **79**, 581.
Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
Sillén, L. G. & Martell, A. E. (1964). *Spec. Publ. chem. Soc. no.* 17: *Stability Constants of Metal-ion Complexes, Section II: Organic Ligands*, p. 697.
Weber, A. & Winicur, S. (1961). *J. biol. Chem.* **236**, 3198.