Biochem. J. (1969) **115**, 993
Printed in Great Britain

The Relaxing Protein System of Striated Muscle

RESOLUTION OF THE TROPONIN COMPLEX INTO INHIBITORY AND CALCIUM ION-SENSITIZING FACTORS AND THEIR RELATIONSHIP TO TROPOMYOSIN

BY M. C. SCHAUB AND S. V. PERRY
Department of Biochemistry, University of Birmingham, P.O. Box 363, *Birmingham* 15

(*Received* 22 *July* 1969)

1. A method involving isoelectric precipitation and chromatography on SE-Sephadex (sulphoethyl-Sephadex) is described for the preparation of the troponin complex free of tropomyosin from low-ionic-strength extracts of natural actomyosin and myofibrils. 2. Purified troponin complex required tropomyosin to inhibit the Mg^{2+}-stimulated adenosine triphosphatase activity and superprecipitation of desensitized actomyosin in the presence of ethanedioxybis(ethylamine)tetra-acetate. An upper limit of 35000 for the 'molecular weight' of the troponin complex was derived from the amounts required to bring about 50% of the maximum inhibition of the Mg^{2+}-stimulated adenosine triphosphatase activity of desensitized actomyosin of known concentration. 3. In the presence of dissociating reagents the troponin complex could be dissociated into inhibitory and Ca^{2+}-sensitizing factors, which could be isolated separately on SE-Sephadex. The inhibitory factor inhibited the Mg^{2+}-stimulated adenosine triphosphatase activity and superprecipitation of desensitized actomyosin independently of the concentration of free Ca^{2+} in the medium. 4. The Ca^{2+}-sensitizing factor changed its electrophoretic mobility on polyacrylamide gel in the presence of ethanedioxybis(ethylamine)tetra-acetate. It formed a complex with the inhibitory factor at low ionic strength and the original biological activity of the troponin complex could be restored on mixing the inhibitory factor with the Ca^{2+}-sensitizing factor in the ratio of about 3:2. 5. Evidence is presented indicating that the ability of tropomyosin preparations to restore relaxing-protein-system activity to the troponin complex and their inhibitory effect on the Ca^{2+}-stimulated adenosine triphosphatase activity of desensitized actomyosin are two properties of different stability to preparative procedures and tryptic digestion. This suggests that the relaxing protein system of muscle may contain another as yet uncharacterized component.

The inhibition of the Mg^{2+}-stimulated ATPase* activity of myofibrils or natural actomyosin by Ca^{2+}-chelating agents (Perry & Grey, 1956) has been shown by Ebashi and collaborators (Ebashi, 1963; Ebashi & Ebashi, 1964; Ebashi & Kodama, 1965, 1966) to be mediated by a complex involving tropomyosin B and the newly discovered myofibrillar protein troponin. More recently it has been shown (Hartshorne & Mueller, 1968; Hartshorne, Theiner & Mueller, 1969) that troponin itself can be dissociated into two components, troponin B, which inhibits the Mg^{2+}-stimulated ATPase activity of DAM in the absence of EGTA, and troponin A, which, although inactive on the ATPase itself, when added to the inhibitor renders it effective only in the presence of EGTA. The inhibitor component obtained from troponin is similar in properties to the inhibitory factor isolated earlier from myofibril preparations (Perry, Davies & Hayter, 1966; Hartshorne, Perry & Schaub, 1967).

The protein complex of troponin and tropomyosin, the activity of which is studied *in vitro* with the use of EGTA, is the system that regulates the myofibrillar ATPase and thus contractile activity in response to changes in the intracellular Ca^{2+} concentration (Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962; Weber & Herz, 1963). The lack of precise definition of nature of the complex is clear from the various names that have been

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxy-bis(ethylamine)tetra-acetate; NAM, natural actomyosin; DAM, desensitized actomyosin; RPS, relaxing protein system; SE-Sephadex, sulphoethyl-Sephadex.

used for its description, e.g. 'native' tropomyosin, EGTA-sensitizing factor, relaxing-factor protein system etc. To avoid any assumptions about the nature of mechanism of the system and thus to facilitate the description of the individual components of the system as their mechanism of action is elucidated, it is proposed in this and future communications to refer to the complete complex involved in the control of the Mg^{2+}-activated ATPase of the myofibril or NAM as the 'relaxing protein system' (RPS). This system is taken to include the components, i.e. troponin and tropomyosin B, currently reported as essential to confer inhibition by EGTA on the Mg^{2+}-stimulated ATPase activity of DAM, and the activity of the system is measured by the extent of this inhibition under specified conditions (see the Methods section).

The present paper is concerned with the resolution of the troponin complex into its components, the relationship of one of them to the inhibitory factor described previously (Hartshorne *et al.* 1967) and the relationship of tropomyosin to the activity of the troponin complex in the RPS.

METHODS

All preparations and chromatographic separations were carried out at 0–5°.

DAM. NAM was extracted as described by Perry & Corsi (1958) from myofibrils prepared from the back and hind-leg muscles of New Zealand white rabbits by the method of Perry & Zydowo (1959). After precipitation by dilution with 9 vol. of water the actomyosin was washed with water by centrifugation for 20–40 min. at 55000*g* until its Mg^{2+}-stimulated ATPase activity was no longer inhibited by Ca^{2+}-chelating reagents and its ATPase activity was similar in the presence of Ca^{2+} or Mg^{2+} (Schaub, Hartshorne & Perry, 1967*b*). Usually six or seven washing steps were needed and finally the DAM was suspended in 5 mM-tris–HCl buffer, pH 7·6. Preparations could be stored for up to several months in 50% glycerol at −20° without significant change in enzymic activity. After storage in this way preparations were washed 3 times with 5 mM-tris buffer, pH 7·6, to remove the glycerol before use.

Tropomyosin. Tropomyosin B was prepared by the method of Bailey (1948) to the stage just before crystallization and then isoelectrically precipitated twice with 100 mM-sodium acetate buffer, pH 4·6, in the presence of 1 M-KCl.

Extraction of RPS from NAM or myofibrils. Suspensions of NAM, extracted from myofibrils and subsequently precipitated in the presence of 15 mM-2-mercaptoethanol, or myofibrils that had been washed twice with 2 mM-tris-HCl buffer, pH 7·4, containing 15 mM-2-mercaptoethanol, were dialysed against 10 vol. of the same solution. The dialysis fluid was changed once and after 24 hr. the viscous suspension was diluted with an equal volume of fresh dialysis fluid before centrifugation for 30 min. at 55000*g*. Occasionally the residues were diluted by the addition of their own volume of dialysis fluid and centrifuged again and the combined supernatants freeze-dried and stored at −20°. By this procedure 6–8% of the total protein of NAM was extracted. Extraction was virtually completed after 15–24 hr., provided the concentration of the buffer was less than 3 mM (Schaub, *et al.* 1967*b*).

Troponin complex. Freeze-dried samples of low-ionic-strength extracts (5–15 mg./ml.) were suspended in 1 M-KCl–2 mM-dithiothreitol–0·1 mM-$CaCl_2$ for 1–2 hr. About 30–40% of the material did not dissolve and was centrifuged off. The pH of the supernatant (approx. pH 8) was then lowered slowly to 4·6 by addition of 0·1 M-HCl and left for 1 hr., the precipitate that formed was centrifuged off and discarded, and the pH value of the supernatant adjusted back to 7 with some 2 M-tris. Solid $(NH_4)_2SO_4$ was then added to 70% saturation, the suspension left for 1 hr. and the precipitate collected by centrifugation. The precipitate was subsequently dialysed against 100 vol. of 15 mM-tris–HCl buffer, pH 7·4, containing 0·5 mM-dithiothreitol and 0·1 mM-$CaCl_2$ and the dialysis fluid changed once or twice. If a slight precipitate was formed it was centrifuged off. The samples were concentrated by freeze-drying and redissolved in dialysis fluid for enzymic and electrophoretic analyses. If the analyses revealed that tropomyosin was still present the isoelectric precipitation step was repeated. When an excess of the slowly migrating material was apparent from the electrophoretic pattern a further precipitation step at 10 mM-$MgCl_2$, in 15 mM-tris–HCl buffer, pH 7·4, containing 2 mM-dithiothreitol was performed to decrease the amount of this material.

Chromatography of troponin complex and its components. SE-Sephadex C-50 (Na^+ form) was equilibrated with 33 mM-sodium citrate buffer, pH 6·0, containing 1 mM-dithiothreitol. About 2–3 ml. containing 15–25 mg. of troponin complex previously equilibrated with the column buffer was loaded/g. of SE-Sephadex and a linear 0–0·8 M-KCl gradient applied in a total volume of 400 ml. of buffer solution for a 2–2·5 g. column of SE-Sephadex. For dissociation into its components, troponin complex that had been previously chromatographed on SE-Sephadex was rechromatographed under the same conditions except that 6 M-urea was included in all buffers. In earlier experiments purified troponin complex was similarly chromatographed on Whatman CM-cellulose (H^+ form) that had been previously washed with 20 mM-tris–HCl buffer, pH 6·0, containing 0·5 mM-dithiothreitol and to which a linear gradient of 0–0·8 M-KCl was applied. Under these conditions, where dissociation of the troponin complex also occurred, the tris–HCl solution had not enough buffering capacity and consequently pH values of 3·4–3·8 were observed in the eluent. Columns (18–22 cm. × 2 cm.) were run at 0–4° with a flow rate of 15–20 ml./hr. and 5 ml. fractions were collected. The fractions were dialysed against 15 mM-tris–HCl buffer, pH 7·4, containing 0·5 mM-dithiothreitol and 0·1 mM-$CaCl_2$ and freeze-dried before analysis.

Disc electrophoresis on polyacrylamide gel. Unless otherwise stated in the Results section the final concentrations in the gels were 8% acrylamide and 0·2% bisacrylamidomethane, 0·4% dimethylaminoproprionitrile, 0·005% potassium ferricyanide, 0·12% ammonium persulphate, 6 M-urea, 24 mM-tris and 160 mM-glycine, giving pH 8·5. In some gels the urea was replaced by 10–20% glycerol. The gels in glass tubes (6 mm. × 80 mm.), usually set within 20 min. at 20°, were then inserted in the upper electrode vessel of an apparatus built as described by Davis (1964). The tank buffer contained 25 mM-tris–160 mM-glycine, pH 8·5, and 15 mM-2-mercaptoethanol. A pre-run of 1 hr., without samples, was sufficient to let the 2-mercaptoethanol

in the tank buffer migrate through the gel and to remove the excess of ammonium persulphate (Bennick, 1968). The samples in a volume of 0·05–0·15 ml. usually containing 200–300 μg. of protein in 6 M-urea, 20–30% sucrose and 15 mM-2-mercaptoethanol were then layered directly on to the top of the gels underneath the tank buffer solution. The use of the spacer sample gel on top of the columns was not found to improve the resolution of proteins (Schaub & Strauch, 1968). Electrophoresis was performed at 2 mA and 15 v/tube at 20° for 2–4 hr. until the marker dye, Bromophenol Blue, had migrated through the gels. After electrophoresis the gels were immediately stained with a solution of 1% Naphthalene Black 10B in water–ethanol–acetic acid (5:3:1, by vol.) and the excess of dye was removed by soaking them in 7% acetic acid for several days. To evaluate the migration patterns the light-absorption by the stained gels was traced by scanning with the Chromoscan J 312 (Joyce and Loebl Co.) with a red filter. Electrophoresis was also performed by using riboflavin as catalyst for gel formation instead of ammonium persulphate. The staining patterns were the same. Similarly the patterns were not affected by the absence of 2-mercaptoethanol from the tank buffer.

Determination of protein concentration. The protein was determined by an ultra-micro method that involved nesslerization after digestion (Strauch, 1965), assuming an N content of 16%.

Assay of ATPase activity. The assays were carried out on 0·3–0·6 mg. of actomyosin at 25° in a volume of 2 ml. containing 2·5 mM-tris ATP, 25 mM-tris–HCl buffer, pH 7·6, with 2·5 mM-$MgCl_2$ or -$CaCl_2$. For the assessment of RPS activity 1 mM-EGTA was included in the $MgCl_2$ medium. The incubation mixture (1·9 ml.), complete except for ATP, was equilibrated for 5 min. at 25° and the reaction started by the addition of 0·1 ml. of 50 mM-tris ATP. P_i liberated after 5 min. of incubation was determined by the method of Fiske & SubbaRow (1925). Specific activities of the DAM preparations, expressed as μg. of P_i released/5 min./mg. of protein in the presence of $MgCl_2$, varied between 70 and 90.

Tryptic digestion of tropomyosin. Tropomyosin preparations were digested with trypsin in a ratio of 50–100:1 (w/w) at 0° for various times in 50 mM-tris–HCl buffer, pH 7·6. Digestion was stopped with 2 mg. of trypsin inhibitor/mg. of trypsin and the samples were used for enzymic assays under standard conditions.

Reagents. Tris ATP was prepared from the disodium salt by the method of Schwartz, Bachelard & McIlwain (1962). Twice-crystallized trypsin and thrice-crystallized soya-bean trypsin inhibitor were supplied by the Sigma Chemical Co. (St Louis, Mo., U.S.A.). All reagents were A.R. grade when obtainable. Distilled deionized water was used throughout.

RESULTS

Preparation and chromatography. High RPS activity was always associated with low-ionic-strength extracts from NAM or myofibrils. Such extracts had a higher E_{280}/E_{260} ratio than extracts prepared in 20–100 mM-tris–HCl buffer, pH 7·4 (Fig. 1), which possessed little RPS activity. After the precipitation steps (see the Methods section) necessary for removal of the tropomyosin, yields of troponin tended to be low since part of the troponin complex was co-precipitated. Attempts to improve the yield of troponin complex in the supernatant by carrying out isoelectric precipitation in the presence of urea (2–4 M), lithium chloride (0·5–1·5 M) or EDTA (5–10 mM) resulted in a significant increase of contamination by tropomyosin in the troponin-complex fraction.

Chromatography of troponin complex on Sephadex G-200 in the presence of 0·7–1·5 M-potassium chloride and on DEAE-cellulose in 25 mM-tris–HCl buffer, pH 7·4, with an increasing salt gradient, did not achieve reproducible further

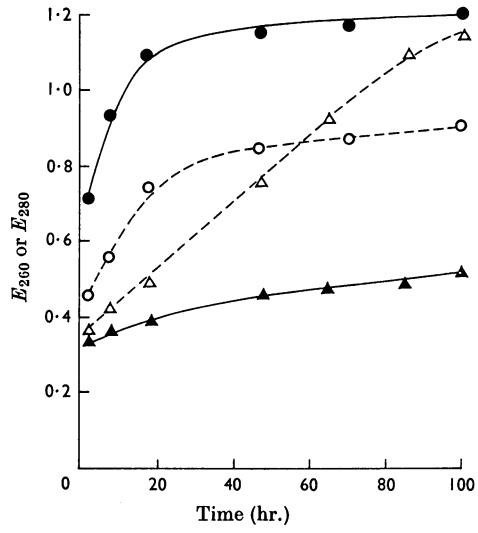


Fig. 1. Time-course of protein extraction from NAM suspensions at low ionic strength. Extraction was carried out at 10 mg./ml., 0° and pH 7·4, and was followed by measurement of the E_{260} and E_{280} of the supernatant after centrifugation. ●, 2 mM-tris–HCl, E_{280}; ○, 2 mM-tris–HCl, E_{260}; ▲, 50 mM-tris–HCl, E_{280}; △, 50 mM-tris–HCl, E_{260}.

Table 1. *Ratio of extinctions at* 280 *and* 260 *nm. of low-ionic-strength extracts, troponin complex and column-purified troponin complex.*

Values in parentheses are standard deviations.

	No. of preparations	E_{280}/E_{260}
Low-ionic-strength extract	9	1·46 (±0·09)
Troponin complex before column chromatography	15	1·32 (±0·12)
Troponin complex after column chromatography	7	1·69 (±0·05)
Material eluted at low ionic strength from SE-Sephadex	6	1·11 (±0·16)

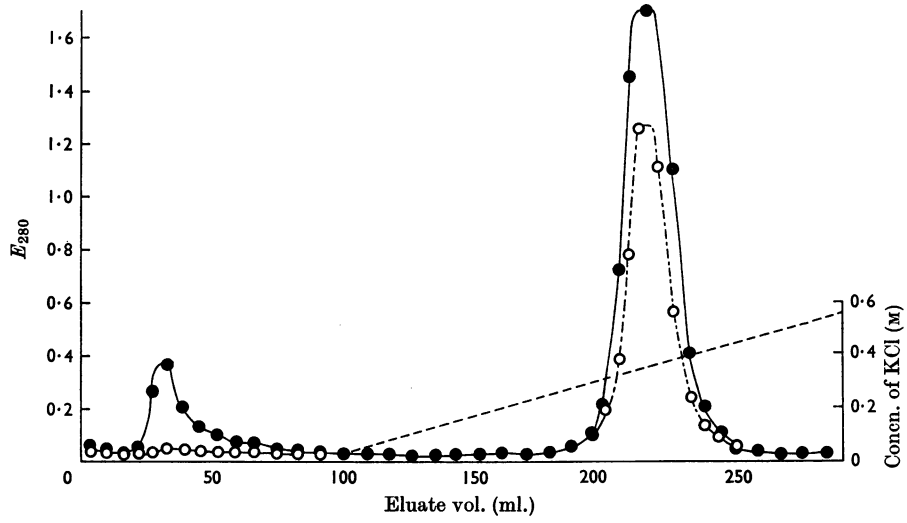


Fig. 2. Chromatography of preparations of troponin complex on SE-Sephadex. About 40–50 mg. of protein was loaded on a column (18 cm. × 2 cm.) of SE-Sephadex C-50 equilibrated against 33 mM-sodium citrate buffer, pH 6·0, containing 1 mM-dithiothreitol. Elution was by the KCl gradient indicated by the broken line. ●, Purified troponin complex (see the Methods section); ○, rechromatography of the main peak.

purification. In 33 mM-sodium citrate buffer, pH 6·0, however, the troponin complex was held on SE-Sephadex, and inactive material of relatively low E_{280}/E_{260} ratio (Table 1) could be eluted with this buffer. The troponin complex was eluted from the column at about 0·3 M-potassium chloride (Fig. 2) and had an E_{280}/E_{260} ratio of 1·7. When rechromatographed under the same conditions the troponin complex was eluted as a single peak at the same ionic strength as in the original run.

Disc electrophoresis on polyacrylamide gel of troponin complex. The chromatographically purified troponin complex gave a single band on disc electrophoresis at pH 8·5 in 4–10% polyacrylamide gels containing 10% of glycerol (Fig. 3). If the glycerol was replaced by urea the single troponin band began to dissociate into two components at urea concentrations of 2·0–3·0 M. Concentrations of urea up to 10 M did not further change the electrophoretic pattern. The double-band pattern could also be demonstrated in the presence of urea buffered with 25 mM-tris–HCl, pH 7·5, 25 mM-tris–acetate, pH 6·0, or 50 mM-sodium veronal buffer, pH 8·6. The slowly migrating constituent of dissociated troponin varied somewhat in its appearance. It diffused over the first third of the distance of the fast band or sometimes was confined to a sharp slowly migrating band that entered only a few millimetres into the gel. The latter appearance was more usual when small amounts of troponin complex (20–40 μg. of protein) were loaded on to the gel.

Tropomyosin purified as outlined in the Methods section migrated in the presence of urea as a single band whether prepared in the presence or absence of thiol-protecting reagents, as found by Woods (1967) (but cf. Parsons, Parsons, Blanshard & Lawrie, 1969). When it was present in troponin preparations, tropomyosin could be identified as a third band in the electrophoretic pattern migrating somewhat slower than the faster component of dissociated troponin (Fig. 4). Fig. 4(*c*) demonstrates that the three main bands present in the low-ionic-strength extract of NAM can be attributed to the dissociated troponin complex and to tropomyosin. Troponin that still contained tropomyosin did not develop a clear band pattern in the absence of urea, as reported by Arai & Watanabe (1968*a*).

When 2–5 mM-EGTA or -EDTA was added to the sample before application on to the gel, the fast band of the troponin complex was replaced by a very faint double band and a new band appeared with an R_F value of about 1·5 relative to its original mobility (Fig. 5). The new band stained less intensely than the original, e.g. although 20–40 μg. of troponin complex gave a distinct fast band in the absence of EGTA the corresponding band obtained in the presence of EGTA was not visible after the standard staining procedure. 2–3 mM-EGTA or -EDTA was required to bring about complete change in mobility because the troponin complex was prepared as a routine in solutions containing calcium chloride. No change of the original electrophoretic pattern occurred when calcium chloride alone or equimolar amounts of chelating agent and calcium chloride were

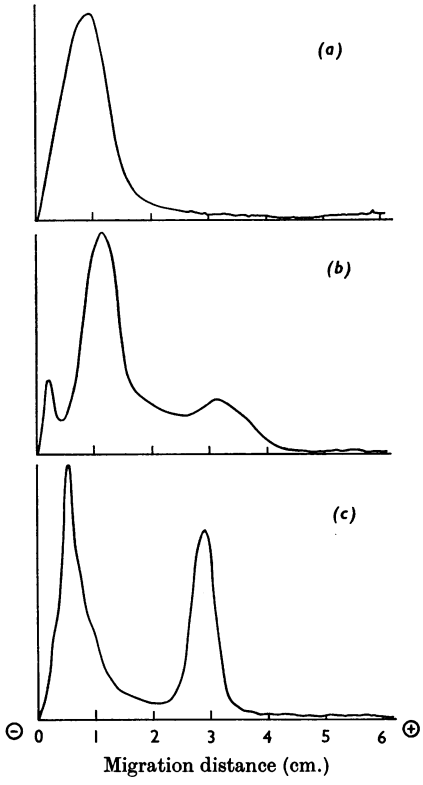


Fig. 3. Densitometric traces of electrophoretograms of column-purified troponin complex. About 200–250 μg. of protein was applied to 8% polyacrylamide gels in tris–glycine buffer, pH 8·5, containing 15 mM-2-mercaptoethanol as indicated in the Methods section. (*a*) Gel containing 10% of glycerol; (*b*) gel containing 2·5 M-urea; (*c*) gel containing 6 M-urea.

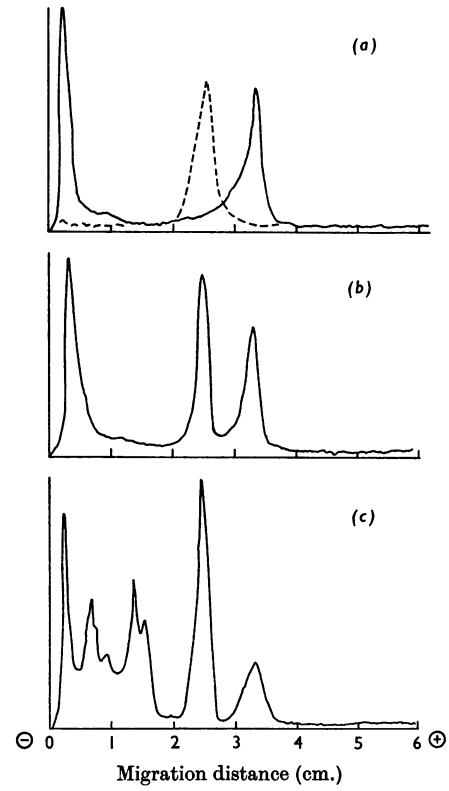


Fig. 4. Densitometric traces of electrophoretograms of troponin complex and tropomyosin. About 200–300 μg. of protein was applied to polyacrylamide gels of standard composition containing 6 M-urea. (*a*) Troponin complex (——) and tropomyosin (----); (*b*) mixture of troponin complex and tropomyosin; (*c*) low-ionic-strength extract from myofibrils.

added to the sample. When 5 mM-magnesium chloride and 5 mM-EGTA were added the same change occurred as with EGTA alone. The mobilities of the slowly migrating constituent of tropomyosin and of other bands observed in the initial low-ionic-strength extract were not affected by EGTA.

When EDTA was used during preparation of troponin complex, part of the electrophoretically fast-migrating constituent irreversibly changed to the modified form. A similar effect was observed when RPS was extracted initially with 1 M-potassium chloride either from ethanol- and ether-treated muscle powder or from NAM as described by Hartshorne & Mueller (1969). This spontaneous partial transformation, presumably due to loss of Ca^{2+}, also occurred in troponin samples that had been left for longer periods, e.g. overnight, at high salt concentrations, particularly when no calcium chloride was included in the solutions used during preparation.

Effect of troponin complex on the ATPase activities of DAM. With purification of the troponin complex a decreased amount of protein was required to bring about maximum inhibition (60–65%) of the Mg^{2+}-stimulated ATPase activity of DAM in the presence of EGTA and optimum amounts of tropomyosin (Fig. 6). For a given preparation the minimum amount of troponin complex required for maximal inhibition in the Mg–EGTA medium was proportional to the amount of DAM in the test over a wide range of concentrations (0·1–0·9 mg./assay). On average 59 μg. of purified troponin complex produced half of the maximum inhibition of the Mg^{2+}-stimulated ATPase activity of 1 mg. of DAM under the standard assay conditions in the presence of EGTA (Table 2).

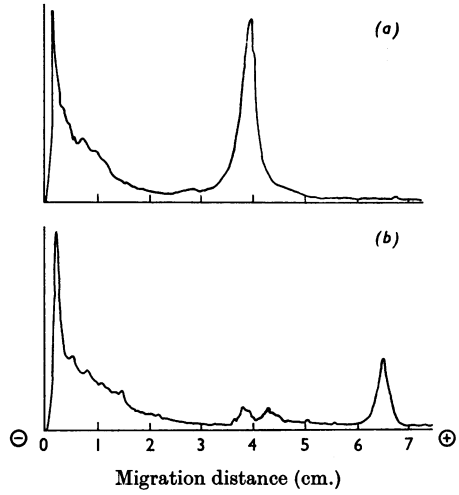


Fig. 5. Densitometric traces of electrophoretograms of troponin complex in the presence of EGTA. Polyacrylamide gels of standard composition with 6M-urea were used. (*a*) About 200μg. of troponin complex without EGTA; (*b*) about 250μg. of troponin complex containing 2mM-EGTA.

Purified troponin-complex preparations required additional tropomyosin, amounting to 0·4–1·0 of the troponin N added, for maximal effect. However, a large excess of tropomyosin over troponin decreased the inhibitory effect of the troponin complex on the Mg^{2+}-stimulated ATPase activity (Fig. 7) (cf. Yasui, Fuchs & Briggs, 1968). Little activation of the Mg^{2+}-stimulated ATPase activity of DAM was observed when tested with tropomyosin and troponin in the absence of EGTA (cf. Hartshorne & Mueller, 1968, 1969).

Fig. 8 shows the effect of the same troponin preparations as in Fig. 6 on the Ca^{2+}-stimulated ATPase activity of DAM. As would be expected from its tropomyosin content, the original low-ionic-strength extract inhibited the Ca^{2+}-stimulated system strongly (Schaub, Hartshorne & Perry, 1967*a*), whereas the purified preparations did not. In addition, the material in the first peak eluted on SE-Sephadex chromatography of troponin complex (Fig. 2) and material with the same electrophoretic mobility as tropomyosin (Fig. 9) inhibited the Ca^{2+}-stimulated ATPase activity of DAM. This latter material was present in the troponin complex preparation before column chromatography at too

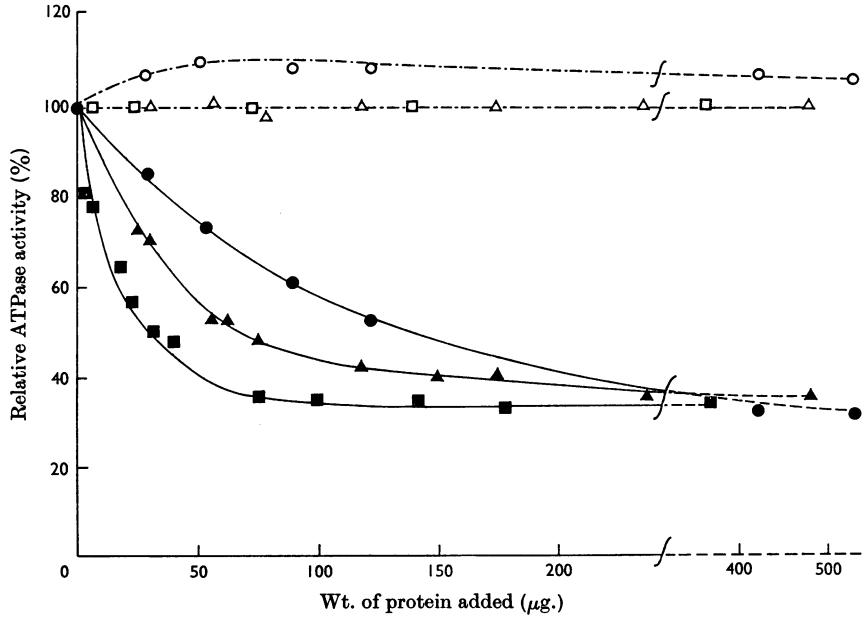


Fig. 6. Effect of low-ionic-strength extract from NAM and preparations of troponin complex isolated from it on the Mg^{2+}-stimulated ATPase activity of DAM. Assays were carried out under standard conditions as indicated in the Methods section, with 0·53mg. of DAM. ●, ▲ and ■, tested in the presence of 1mM-EGTA; ○, △ and □, tested in the absence of EGTA. ● and ○, Low-ionic-strength extract; ▲ and △, troponin complex before column chromatography plus optimum concentration of tropomyosin; ■ and □, column-purified troponin complex plus optimum concentration of tropomyosin.

Table 2. *Amounts of troponin complex required for maximal and half-maximal inhibition of the* Mg^{2+}*-stimulated ATPase activity of DAM in the presence of EGTA*

The results were obtained by plotting percentage inhibition of the Mg^{2+}-stimulated ATPase activity against the amount of troponin complex added to a given DAM preparation. Assays were carried out in each case in the presence of optimum tropomyosin concentration.

DAM preparation	Amount of troponin complex required (μg./mg. of DAM) Maximal inhibition	Half-maximal inhibition
1	180	42
2	280	80
3	210	66
4	200	72
5	200	45
6	160	40
7	200	53
8	210	72
Mean	205	59

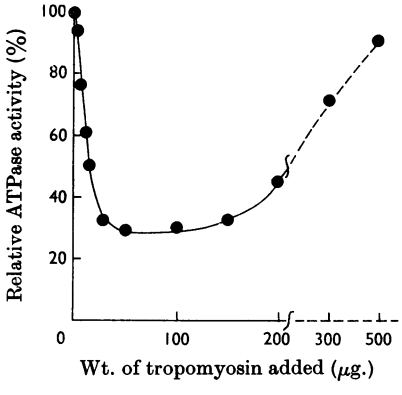


Fig. 7. Effect of tropomyosin on the RPS activity of 45 μg. of troponin complex on 0·38 mg. of DAM. Assays were carried out under standard conditions in the presence of EGTA (see the Methods section).

low a concentration to be detected in the enzymic test.

Separation of the two troponin components by chromatography and their effects on the ATPase activity of DAM. The RPS activity of the troponin complex was not decreased by treatment with urea up to a concentration of 6 M and subsequent removal of the urea by exhaustive dialysis (Fig. 10). Troponin preparations could also be precipitated by 7% (w/v) trichloroacetic acid without loss in activity.

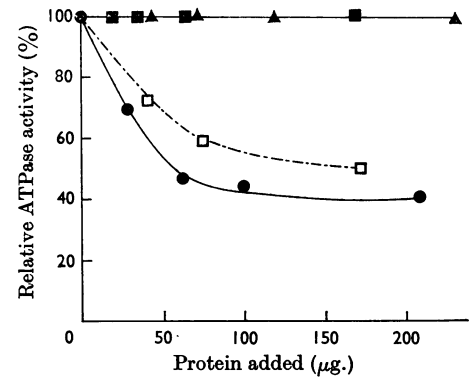


Fig. 8. Effect of low-ionic-strength extract from NAM and preparations of troponin complex isolated from it on the Ca^{2+}-stimulated ATPase activity of 0·53 mg. of DAM. Assays were carried out under standard conditions with $CaCl_2$ as activator. ●, Low-ionic-strength extract; ▲, troponin complex before column chromatography; ■, column-purified troponin complex; □, first peak eluted at low ionic strength from SE-Sephadex loaded with troponin complex.

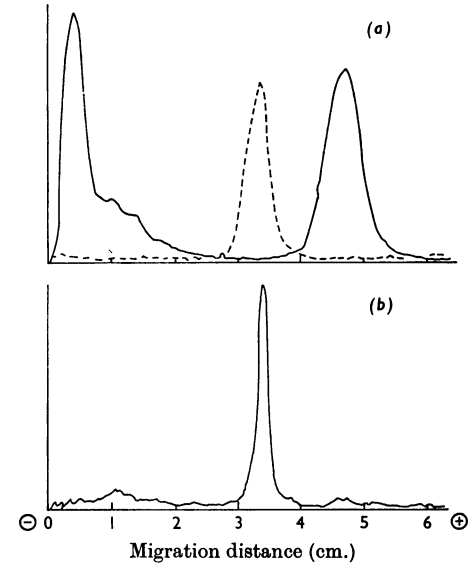


Fig. 9. Densitometric traces of electrophoretograms of fraction eluted at low ionic strength from SE-Sephadex. About 200–300 μg. of protein was applied to polyacrylamide gels of standard composition containing 6 M-urea. (*a*) Troponin complex (——) and tropomyosin (– – – –); (*b*) first peak eluted at low ionic strength from SE-Sephadex loaded with troponin complex.

When troponin complex that had been previously purified on SE-Sephadex was rechromatographed under the same ionic conditions but with the addi-

tion of 6M-urea, the protein was eluted in two main peaks (Fig. 11). The first was eluted at low ionic strength and corresponded in the gel electrophoresis to the fast troponin component and the second one, eluted at 0·2M-potassium chloride, represented the slowly migrating electrophoretic band (Fig. 12c). Similar results were obtained by chromatography of the troponin complex that was free of tropomyosin on CM-cellulose in the absence of urea at pH 3·5.

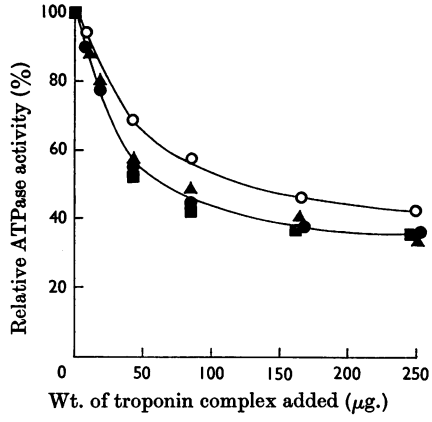


Fig. 10. RPS activity of troponin complex after treatment with urea. Troponin complex was kept at 0° for 7 hr. in urea and subsequently exhaustively dialysed against 15mM-tris–HCl buffer, pH 7·4, containing 0·5mM-dithiothreitol before the enzymic assays were carried out under standard conditions on 0·48mg. of DAM. ●, Control; ▲, 2M-urea; ■, 4M-urea; ○, 6M-urea.

Under these conditions the material corresponding to the slowly migrating band in disc electrophoresis was eluted in the range 0·5–0·7M-potassium chloride. In this latter chromatographic system, however, the elution pattern with increasing salt concentration was more diffuse than that obtained with the SE-Sephadex and urea.

Both methods lead to a preparation of the component migrating slowly on electrophoresis that after exhaustive dialysis against 15mM-tris–HCl buffer, pH 7·4, strongly inhibited the Mg^{2+}-stimulated ATPase activity of DAM in the absence as well as in the presence of EGTA. Its inhibitory effect was enhanced significantly by the addition of tropomyosin, but in the absence of tropomyosin higher amounts of the inhibitor protein alone brought about almost the same maximum inhibition (Fig. 13). It had no effect on the Ca^{2+}-stimulated ATPase activity of DAM in the absence of tropomyosin.

The electrophoretically fast-moving troponin component eluted in the SE-Sephadex–urea system had no effect on the Mg^{2+}- or Ca^{2+}-stimulated ATPase activities of DAM. At low relative concentrations it decreased the inhibitory effect of the other troponin component in the Mg^{2+}-stimulated system and abolished it completely when the ratio of fast component to inhibitor was 2:3 based on nitrogen content. Full RPS activity was regained by such a mixture of the two troponin constituents in the presence of tropomyosin, i.e. the Mg^{2+}-stimulated ATPase activity of DAM was inhibited up to 60% in the presence of EGTA but unaffected in its absence.

Preparations of troponin complex that were

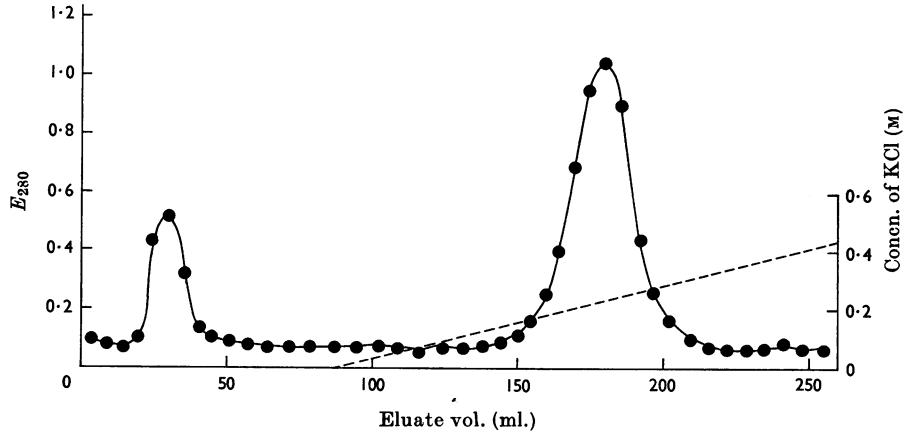


Fig. 11. Chromatography of column-purified troponin complex on SE-Sephadex in 6M-urea. About 40–50mg. of protein was loaded on a column (18cm. × 2cm.) of SE-Sephadex C-50 equilibrated against 33mM-sodium citrate buffer, pH 6·0, containing 1mM-dithiothreitol and 6M-urea. Elution was by the KCl gradient indicated by the broken line.

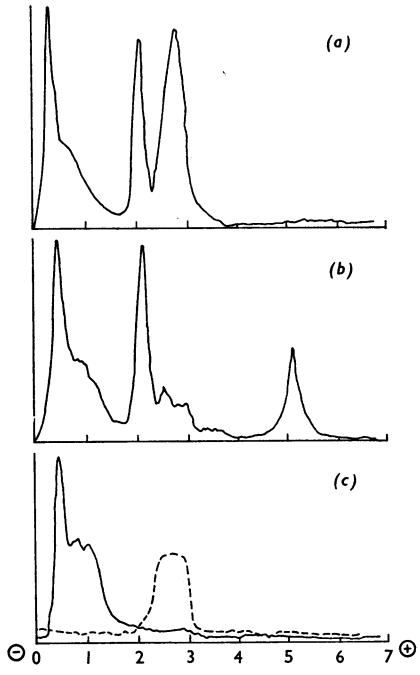


Fig. 12. Densitometric traces of electrophoretograms of fractions isolated from troponin-complex preparations. About 200 μg. of protein was applied to polyacrylamide gels of standard composition containing 6 M-urea. (*a*) Material of the first peak eluted at low ionic strength from SE-Sephadex loaded with a thixotropic troponin preparation; (*b*) same sample containing 5 mM-EDTA; (*c*) components of column-purified troponin complex obtained by rechromatography on SE-Sephadex in the presence of 6 M-urea; inhibitory factor of the troponin complex eluted at a KCl concentration of 0·2 M (——) and the calcium-sensitizing factor eluted at low ionic strength (– – – –) are shown.

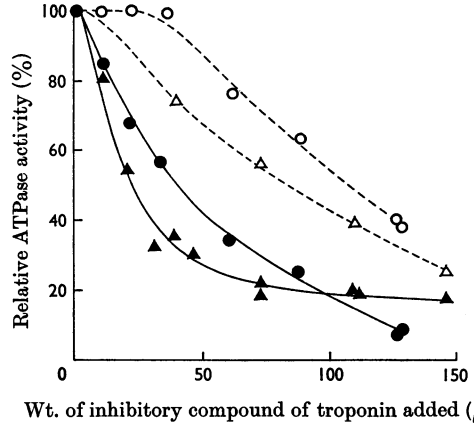


Fig. 13. Effect of the inhibitory component of troponin on the Mg^{2+}-stimulated ATPase activity of DAM. Assays were carried out under standard conditions on 0·4 mg. of DAM: ● and ▲, in the presence of optimum concentration of tropomyosin; ○ and △, in the absence of tropomyosin. ● and ○, Inhibitory component prepared by chromatography of troponin complex on SE-Sephadex in 6 M-urea; ▲ and △, inhibitory component prepared by chromatography of troponin complex on CM-cellulose at pH 3·5.

extracted at high salt concentrations or that were left to sediment for longer periods of time, e.g. overnight, in 60–80% ammonium sulphate saturation often had some general inhibitory effect on the Mg^{2+}-stimulated ATPase activity of DAM in the absence of EGTA, in addition to their RPS activity. Correspondingly they also showed a predominance of the slowly migrating material on electrophoresis. The excess of inhibitory component in these preparations could be precipitated by 10 mM-magnesium chloride.

Possible heterogeneity of the tropomyosin. Occasionally troponin complex preparations that were highly thixotropic were obtained. This was particularly the case when isoelectric precipitation was carried out in the presence of more powerful dissociating reagents than 1 M-potassium chloride, such as 3 M-urea or 1 M-lithium chloride over the pH range 4·0–5·0 and at protein concentrations of 5–25 mg./ml. A peak of normal troponin complex was eluted at 0·3 M-potassium chloride on SE-Sephadex chromatography of such thixotropic preparations in the absence of urea. The peak eluted by the starting buffer was larger than the peak obtained from the usual troponin-complex preparations and was also highly thixotropic. From disc electrophoresis it was apparent that a considerable amount of troponin complex was carried over in this peak with the material presumed to be tropomyosin (Fig. 12). In the presence of EGTA the mobility of the fast troponin band was changed in the normal manner. This readily eluted fraction inhibited the Ca^{2+}-stimulated ATPase activity of DAM. From the latter property and the electrophoretic pattern it would be assumed that the fraction was saturated with tropomyosin and hence would inhibit the Mg^{2+}-stimulated ATPase activity of DAM in the presence of EGTA. Surprisingly these preparations had very little RPS activity alone, but with the further addition of a Bailey-type tropomyosin they sensitized the Mg^{2+}-stimulated ATPase activity of DAM to EGTA.

The contaminating material of standard preparations of troponin complex that was eluted from SE-Sephadex at low ionic strength and that migrated with the same electrophoretic mobility as tropomyosin (Fig. 9) failed to produce EGTA-sensitizing

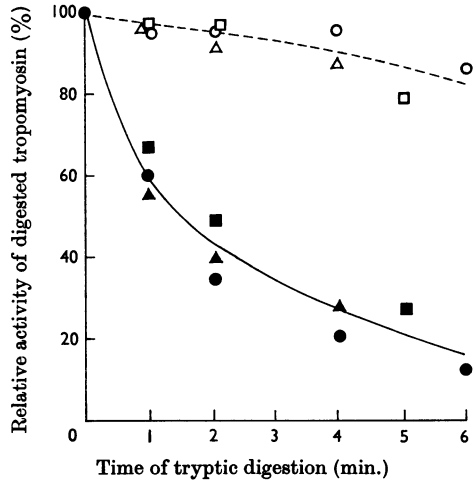


Fig. 14. Effect of tryptic digestion of Bailey-type tropomyosin preparations on their ability to promote RPS activity of troponin complex and on their inhibition of the Ca^{2+}-stimulated ATPase activity of DAM. Different tropomyosin preparations were digested by trypsin as indicated in the Methods section and subsequently tested on 0·4–0·5 mg. of DAM. The effects of trypsin-treated tropomyosin preparations are plotted as percentage of the untreated controls. ●, ▲ and ■, 100–150 μg. of trypomyosin digested for different periods of time as indicated were assayed with an equal amount of troponin complex under standard conditions in the presence of 2·5 mM-$MgCl_2$ and 1 mM-EGTA. ○, △ and □, 50–80 μg. of the same tropomyosin samples were assayed in the presence of 2·5 mM-$CaCl_2$.

activity when mixed with troponin-complex preparations inactive in the absence of tropomyosin.

Further evidence of possible heterogeneity of the usual tropomyosin preparations was apparent when their ability to restore RPS activity to the troponin complex was compared with their activity on the Ca^{2+}-stimulated ATPase activity of DAM after digestions with trypsin (Fig. 14). Mild tryptic digestion of tropomyosin preparations decreased their ability to restore RPS activity by up to 90% when tested with an intact troponin complex, whereas their inhibitory effect on the Ca^{2+}-stimulated system was virtually unchanged.

DISCUSSION

The purified troponin preparation, which we prefer to describe as a complex because of its composite nature, possesses the following properties: (1) on addition to DAM the Mg^{2+}-stimulated ATPase activity and superprecipitation are inhibited by low concentration of EGTA only in the presence of Bailey-type tropomyosin, which itself has no RPS activity; (2) no effect on Mg^{2+}- and Ca^{2+}-stimulated ATPase activities of DAM or NAM.

From the average amounts of troponin complex required for 50% of the maximum inhibition of the Mg^{2+}-stimulated ATPase activity of DAM in the presence of EGTA (Table 2) it is possible, if certain simple assumptions are made, to estimate the upper limits of the amount of troponin complex required to be effective per active centre involved. It is assumed that the DAM contains 15% of actin on average, which value is supported by 3-methylhistidine analysis (S. V. Perry & C. I. Harris, unpublished work); it follows that myosin and actin are in a 1:2 molar ratio. Further, it is assumed that the affinity of troponin complex for the active centre is infinitely high, so that at 50% of the maximum inhibition of the ATPase all the troponin complex present will have interacted with 50% of the centres involved. Therefore if myosin has two such centres per molecule it follows that the weight of the troponin complex combining with each centre, i.e. the 'molecular weight', is about 35000. The same value is also obtained if the complex acts on the actin possessing one active centre per monomer of 45000. In both cases the deduced values are upper limits as they would be lowered if the affinity of troponin for the active site under the assay conditions was less than assumed and if the troponin preparations contained impurity. It would be further lowered if the complex did not contain the optimum proportions of the two components (see below). This value is somewhat lower than the estimations of 50000 (Ebashi, Kodama & Ebashi, 1968) and 44000 (Arai & Watanabe, 1968*b*) based on physicochemical properties of the troponin complex.

The results presented agree with the findings of Hartshorne *et al.* (1969) that troponin consists of two components, which we suggest might be named the 'calcium-sensitizing factor' and the 'inhibitory factor', corresponding to the troponin A and troponin B respectively described by Hartshorne *et al.* (1969). This nomenclature is in our view preferable in that it gives some indication of function and avoids the confusion resulting from use of troponin A and troponin B for different preparations by different workers (cf. Hartshorne *et al.* 1969; Arai & Watanabe, 1968*b*). Although the troponin complex moved as a single band on gel electrophoresis at low ionic strength in the absence of urea, the two components became apparent as separately migrating species when electrophoresis was carried out in urea. Other workers (Arai & Watanabe, 1968*a*,*b*; Wakabayashi & Ebashi, 1968; Yasui *et al.* 1968) have failed to appreciate that dissociation takes place on gel electrophoresis in urea and have identified the fast-moving component with troponin itself, when in reality it represents only part of this complex, namely the calcium-sensitizing factor.

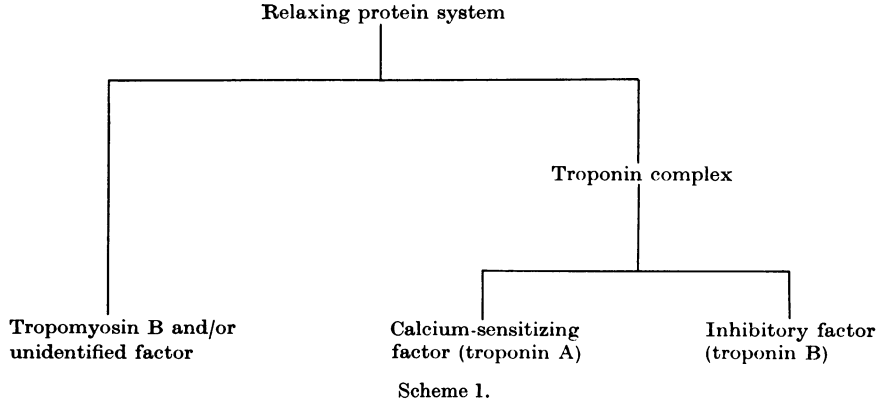
Wakabayashi & Ebashi (1968) first reported that the electrophoretic pattern of troponin depended on the Ca^{2+} concentration, but of its two components the calcium-sensitizing factor clearly has some special relationship to Ca^{2+} since its electrophoretic mobility alone is modified in the presence of EGTA (Figs. 5 and 12). As suggested by Hartshorne *et al.* (1969) the role of the calcium-sensitizing factor is presumably to confer Ca^{2+}-dependence on the action of the inhibitory factor.

The troponin component of lower electrophoretic mobility appears to be identical with the inhibitory factor reported earlier (Perry *et al.* 1966; Hartshorne *et al.* 1967). Conditions of high ionic strength favoured the spontaneous appearance of the inhibitory factor in preparations of the troponin complex; likewise high ionic strength was earlier reported as essential for its extraction from the myofibril (Hartshorne *et al.* 1967). A further similarity is the precipitation of both preparations by 5–10mM-magnesium chloride.

The possibility that the inhibitory factor is a modified form of troponin has been suggested earlier (Perry, 1967*a*) and was supported by observation that inhibitory activity developed in preparations of RPS on storage and after heating under certain conditions (Perry, 1967*b*).

However, the precise relationship of these two components in the troponin complex is not clear. We have no evidence as to whether the inhibitory and the calcium-sensitizing factors exist in a fixed stoicheiometric ratio in troponin-complex preparations or whether the relative proportions vary in each troponin sample and depend on the method of preparation. At present we consider that the latter may be the case and explain the variation in specific activity of troponin preparations as being due to differences in the relative amounts of the two components present. Certainly activity is not lost on dissociation and indeed the two components are remarkably stable, surviving treatment with 6M-urea and with trichloroacetic acid, properties that are more likely to be associated with low-molecular-weight proteins. These properties are compatible with a maximum 'molecular weight' of 35000 for the troponin complex.

Although the purified troponin-complex preparations described all require Bailey-type tropomyosin for full RPS activity, some caution must be exercised in making conclusions about the precise role of tropomyosin B in the system. Evidence has been provided elsewhere indicating that protein fractions apparently free of tropomyosin B are able to confer RPS on DAM (Perry *et al.* 1966; Perry, 1967*c*). It has also been shown that Bailey-type tropomyosin alone can modify the enzymic properties of the actomyosin complex (Schaub & Ermini, 1969). The present study provides evidence that preparations can be obtained from tropomyosin by selective tryptic digestion and from troponin preparations by SE-Sephadex chromatography that can inhibit the Ca^{2+}-stimulated ATPase activity of DAM but do not restore RPS activity to troponin preparations. All past evidence suggests that the ability to inhibit the Ca^{2+}-stimulated ATPase activity of DAM is a property of tropomyosin (Schaub, Perry & Hartshorne, 1967). If this is so then some additional component, not yet characterized, that is present in tropomyosin preparations is required together with troponin complex to sensitize the Mg^{2+}-stimulated ATPase activity of DAM to EGTA. Another possibility might be that tropomyosin B has two properties, namely the ability to inhibit the Ca^{2+}-stimulated ATPase activity of DAM and to promote RPS activity of the troponin complex. In this event the procedures described modify the tropomyosin so that the latter property is selectively destroyed. A third possibility, which cannot be completely excluded at this stage, is that tropomyosin B itself is essential for the RPS system



Scheme 1.

and that inhibition of the Ca^{2+}-stimulated ATPase activity of DAM is brought about by an uncharacterized component of the tropomyosin preparations.

In summary, our present view of the system that regulates the Mg^{2+}-stimulated ATPase activity of the myofibril in contraction and relaxation contains at least three components related as indicated in Scheme 1.

This work was in part supported by a research grant from the Medical Research Council.

REFERENCES

Arai, K. & Watanabe, S. (1968*a*). *J. Biochem., Tokyo*, **64**, 69.
Arai, K. & Watanabe, S. (1968*b*). *J. biol. Chem.* **234**, 5670.
Bailey, K. (1948). *Biochem. J.* **43**, 271.
Bennick, A. (1968). *Analyt. Biochem.* **26**, 453.
Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.
Ebashi, S. (1963). *Nature, Lond.*, **200**, 1010.
Ebashi, S. & Ebashi, F. (1964). *J. Biochem., Tokyo*, **55**, 604.
Ebashi, S. & Kodama, A. (1965). *J. Biochem., Tokyo*, **58**, 107.
Ebashi, S. & Kodama, A. (1966). *J. Biochem., Tokyo*, **60**, 733.
Ebashi, S., Kodama, A. & Ebashi, F. (1968). *J. Biochem., Tokyo*, **64**, 465.
Ebashi, S. & Lipmann, F. (1962). *J. Cell Biol.* **14**, 389.
Fiske, C. H. & SubbaRow, Y. (1925). *J. biol. Chem.* **66**, 375.
Hartshorne, D. J. & Mueller, H. (1968). *Biochem. biophys. Res. Commun.* **31**, 647.
Hartshorne, D. J. & Mueller, H. (1969). *Biochem. biophys. Acta*, **175**, 301.
Hartshorne, D. J., Perry, S. V. & Schaub, M. C. (1967). *Biochem. J.* **104**, 907.
Hartshorne, D. J., Theiner, M. & Mueller, H. (1969). *Biochim. biophys. Acta*, **175**, 320.
Hasselbach, W. & Makinose, M. (1961). *Biochem. Z.* **333**, 518.
Parsons, A. L., Parsons, J. L., Blanshard, J. M. & Lawrie, R. A. (1969). *Biochem. J.* **112**, 673.
Perry, S. V. (1967*a*). *Progr. Biophys. molec. Biol.* **17**, 325.
Perry, S. V. (1967*b*). *Abstr. 7th int. Congr. Biochem., Tokyo*, p. 331.
Perry, S. V. (1967*c*). In *Symposium on Fibrous Proteins, Canberra*, p. 154. Ed. by Crewther, W. G. Sydney: Butterworths.
Perry, S. V. & Corsi, A. (1958). *Biochem. J.* **68**, 5.
Perry, S. V., Davies, V. & Hayter, D. (1966). *Biochem. J.* **100**, 289.
Perry, S. V. & Grey, T. C. (1956). *Biochem. J.* **64**, 5P.
Perry, S. V. & Zydowo, M. (1959). *Biochem. J.* **71**, 220.
Schaub, M. C. & Ermini, M. (1969). *Biochem. J.* **111**, 777.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*a*). *Nature, Lond.*, **215**, 635.
Schaub, M. C., Hartshorne, D. J. & Perry, S. V. (1967*b*). *Biochem. J.* **104**, 263.
Schaub, M. C., Perry, S. V. & Hartshorne, D. J. (1967). *Biochem. J.* **105**, 1235.
Schaub, M. C. & Strauch, L. (1968). *Hoppe-Seyl. Z.* **349**, 809.
Schwartz, A., Bachelard, H. S. & McIlwain, H. (1962). *Biochem. J.* **84**, 626.
Strauch, L. (1965). *Z. klin. Chem.* **3**, 165.
Wakabayashi, T. & Ebashi, S. (1968). *J. Biochem., Tokyo*, **64**, 731.
Weber, A. & Herz, R. (1963). *J. biol. Chem.* **238**, 599.
Woods, E. F. (1967). *J. biol. Chem.* **242**, 2859.
Yasui, B., Fuchs, F. & Briggs, F. N. (1968). *J. biol. Chem.* **243**, 735.