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TENSION DEVELOPMENT IN SKINNED GLYCERINATED RABBIT PSOAS FIBER SEGMENTS IRRIGATED WITH SOLUBLE MYOSIN FRAGMENTS

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SUMMARY

Single glycerinated rabbit psoas muscle fibers were skinned by splitting them lengthwise. The fiber segments thus obtained were more easily accessible to solutes in the surrounding medium than the intact fibers. Using such segments, active tension could be fully abolished by adding *N*-ethylmaleimide under conditions which lead to inhibition of actin activation of the ATPase activity of myosin. Such muscles could, however, develop tension after irrigation with myosin or with the water-soluble active myosin fragments heavy meromyosin (HMM) or its subfragment 1 (HMM-S1). The induced tensions increased with increasing protein concentration in the irrigating solution. At any given protein concentration, the tension generated by myosin was larger than that produced by HMM which was, in turn, greater than that induced by HMM-S1 e.g. at 15 mg/ml protein the tensions produced by these three myosin moieties were 44.0, 14.0 and 2.8 g/cm^2, respectively. The tension was found to be intimately associated with ATP splitting; thus, HMM and HMM-S1 which have been treated with reagents abolishing actin-activated ATPase failed to induce tension development. A contractile force may thus be generated through the interaction with actin of the water-soluble, enzymatically active, myosin subfragments involving the splitting of ATP.

INTRODUCTION

We have recently demonstrated that myofibrils in which myosin has been extracted so as to make them incapable of contraction upon adding MgATP, contracted after irrigation with heavy myosin (HMM) or heavy myosin subfragment 1 (HMM-S1) [1–4]. These results lent support to the idea that the formation of a continuous three-dimensional structure may not be obligatory for mechanochemical transduction in actomyosin systems. It was most important at this stage to ascertain that our experimental results indeed reflected a mechanochemical interaction of the soluble myosin fragments with actin and were not due to the action of residual active

Abbreviations: HMM, heavy meromyosin; HMM-S1, heavy meromyosin subfragment 1.

filamentous myosin. To this end, we investigated skinned muscle fiber segments in which myosin has been completely inactivated by the combined action of an extracting medium and of a chemical poison, *N*-ethylmaleimide [5].

MATERIALS AND METHODS

Preparation of skinned segments of single glycerinated muscle fibers

Fresh strips of rabbit psoas muscle about 5 cm long and 2 mm wide, were glycerinated according to Szent-Györgyi [6]. Before each experiment, the fiber bundle was equilibrated with rigor solution (for composition see Table I) for 5 min and a single fiber, 0.4–0.6 mm long, was isolated. The fiber was then carefully split in two all along its length by means of a pair of sharpened jewellers' tweezers. By pulling apart the two segments, the sarcolemma rolled down along the split (Fig. 1), thus exposing the contractile fibrils of both segments directly to the external solution.

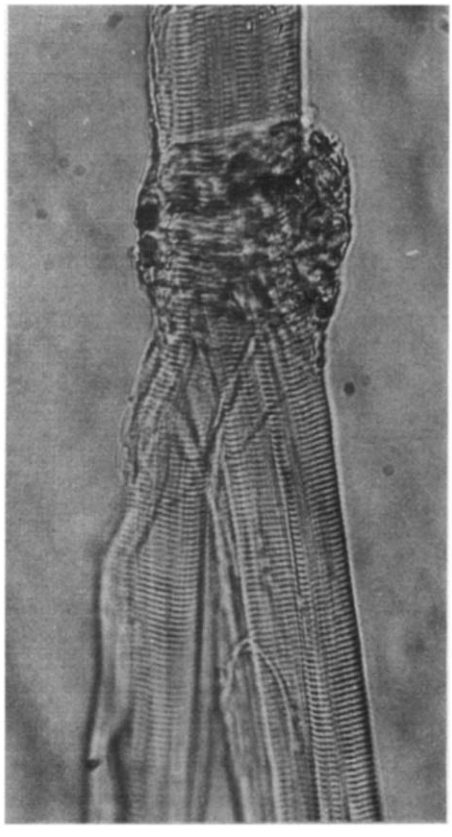

Fig. 1. Photomicrograph of a single glycerinated fiber of rabbit psoas being split lengthwise, thus producing two skinned fiber segments. The rolled sarcolema gives rise to a bulge visible at a point where the two segments join. (Magnification × 378).

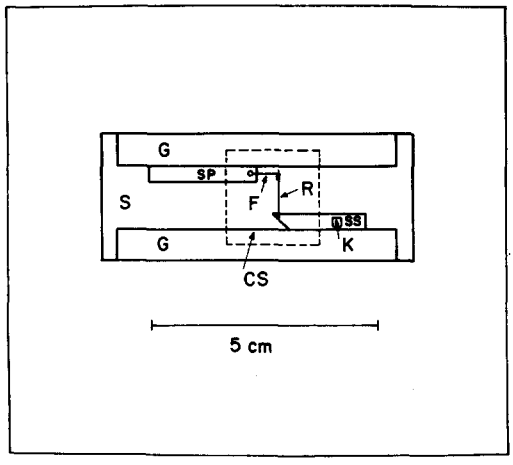

Fig. 2. Apparatus used for the study of the mechanical properties of skinned fiber segments. The diameter of the reed R was either 17 or 33 μm. The deflection of the reed was measured with a calibrated ocular graticule using $\times$ 250 magnification.

Set-up for tension measurements

We constructed an apparatus similar to the one described by Weis-Fogh and Amos [7] for studying the properties of microscopic preparations (Fig. 2). It consisted of a glass support (SP) and a glass reed (R) attached to a sliding slip (SS) mounted on the microscope slide (S). The sideways deflection of the reed from its resting position was calibrated in terms of force by means of microscopic weights suspended at the point of fiber attachment and was found to be proportional to the force in the range of contractile stresses developed by the fiber segments used. The length of the fiber segment (F) could be adjusted by moving the sliding slip with the help of knob (K) on a silicone-greased surface along the guide G. The fiber segment was mounted in the apparatus by transferring it from the preparative petri dish onto the slide and covering it rapidly with a drop of rigor solution. One end of the segment was then rigidly affixed with plasticine to the glass support while the other end was tied to the glass reed. The apparatus was then covered with a coverslip (CS) and transferred under the

TABLE I

COMPOSITION OF SOLUTIONS

Concentrations in mM. Imidazole buffer, pH 7.0 was used except in the poisoning solution where Tris buffer, pH 7.9, was used.

Solution	ATP	$MgCl_2$	Pyrophosphate	KCl	$CaCl_2$	Buffer	Protein (mg/ml)	pH
Rigor	–	–	–	50	0.1	10	–	7.0
Activating	2.5	2.5	–	50	0.1	10	–	7.0
"Poisoning"	–	5	10	500	–	10	–	7.9
Myosin irrigating	–	–	–	400	–	10	0–16.3	7.0
HMM irrigating	–	–	–	50	0.1	10	0–20.2	7.0
HMM-S1 irrigating	–	–	–	50	0.1	10	0–19.8	7.0

microscope (Leitz Orthomat) for observation. The volume of an experimental chamber thus formed was 0.2 ml. The medium was changed by applying the appropriate solution at one end of the coverslip and sucking with a pipette at the opposite end. Activation was achieved by adding 10 μl of a MgATP solution (final concentration 2.5 mM). The composition of the various solutions used is given in Table I. All experiments were carried out at room temperature (approx. 25 °C).

Mechanical characteristics of muscle preparations

The maximal isometric tension at rest length for a number of segment preparations (P_0) was determined using a glass reed with a compliance of 24 μm/mg and varied between 1.0 and 1.5 kg/cm^2; the cross-section was assumed to be circular. The strain vs. stress relationship of a skinned segment is compared with the corresponding relation for a single glycerinated fiber in Fig. 3. The muscles were bathed in a myosin-extracting medium (a modified Hasselbach-Schneider solution containing 0.6 M KCl, 10 mM pyrophosphate, 2 mM $MgCl_2$, 0.1 M phosphate buffer, pH 6.4) in order to minimize the stretch resistance associated with the interaction between thin and thick filaments. The static modulus of elasticity (resistance to stretch) measured between 0 and 6 % strain was 0.004 and 0.28 kg/cm^2 for skinned and intact fibers, respectively. Skinned segments were capable of over 95 % shortening even when contracting auxotonically, i.e. against a spring with a compliance of 0.3 μm/μg. In an unloaded contraction they shortened by 97–98 %, an amount comparable to that of myofibrils [4].

Chemical inactivation of fiber segments was carried out by adding 24 mol excess *N*-ethylmaleimide over muscle myosin (taken as 10 % of the wet muscle weight) to the "poisoning" solution (see Table I) filling the experimental chamber. The value of the maximum tension developed by the segments in an activating solution decreased

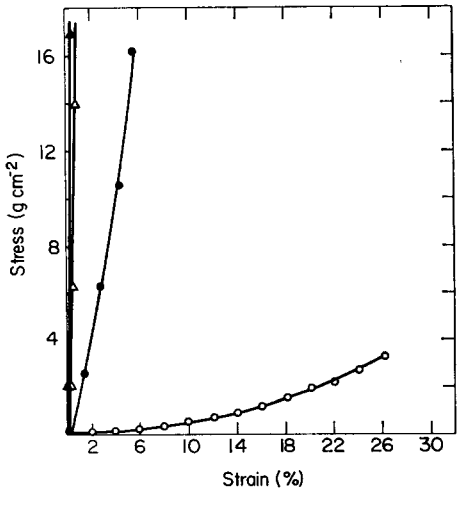

Fig. 3. Stress vs. strain relationships of skinned fiber segments (empty symbols) and of single glycerinated muscle fibers (filled symbols). Circles, in Hasselbach-Schneider solution; triangles, in rigor solution.

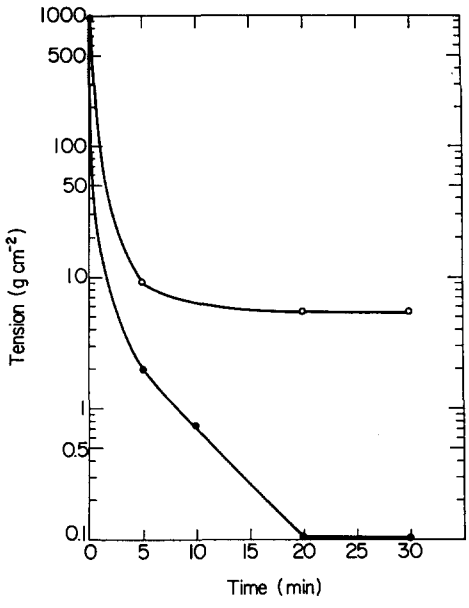

Fig. 4. Maximum isometric tension developed by skinned fiber segments (●) and by intact, glycerinated, single fibers (○) as function of time of incubation in 0.004 mM *N*-ethylmaleimide. 5-min treatment of a skinned segment with "poisoning" solution alone, i.e. without the addition of *N*-ethylmaleimide, reduced maximum tension to 4 g/cm², due to extraction of myosin.

sharply with time of poisoning (Fig. 4). The above amount of *N*-ethylmaleimide was found to be enough for complete inhibition of contraction after 20 min. Lowering the KCl concentration in the poisoning solution to 100 mM resulted in an incomplete inhibition. For comparison we present in Fig. 4 the corresponding curve for intact glycerinated single muscle fiber under the same conditions. As can be seen from the figure, the tension-generating capability of the skinned segments was practically abolished after 20 min while, at the same time, the intact fibers still exhibited a residual tension of about 1 % P_0. For complete poisoning of unskinned fibers, about 10 times more *N*-ethylmaleimide was required.

In all experiments employing poisoned segments, the preparations were treated with *N*-ethylmaleimide for 30 min and tension development in activating solution was found to be fully abolished. Irrigation with myosin or with myosin fragment was performed by replacing rigor solution with the proper protein irrigating solution (see Table I) for a period ranging from 3 to 5 min.

In order to measure the small tensions developed by poisoned segments after irrigation with myosin species, glass reeds of larger compliance were used: 12.5 μm/μg for HMM or HMM-S1 and 0.3 μm/μg for myosin; the largest deflection observed amounted to 10–15 % decrease in segment length.

Myofibrils and protein preparations

Myofibrils were prepared from a mixture of rabbit back and psoas muscles [8] and kept at −18 °C in 50 % glycerol for not longer than 6 months. Shortly before the

experiment, the fibrils were centrifuged for 15 min at 5000 × *g*, resuspended in rigor solution, centrifuged and resuspended three more times. They were poisoned by treatment with *N*-ethylmaleimide in a solution containing 3 mg/ml myofibrils, 0.1 M KCl, 5 mM Tris buffer, pH 7.9, 3 mM $MgCl_2$ and 3 mM sodium pyrophosphate for 0.5 h. The molar excess of *N*-ethylmaleimide over the myofibrillar myosin was 30. The reaction was stopped by the addition of 1 mM β-mercaptoethanol, followed by repetitive sedimentation and resuspension of the myofibrils in rigor solution.

Myosin was prepared from white back muscles of New Zealand rabbits [9]. The procedures described by Lowey and Cohen [10] and by Lowey et al. [11] were followed for the preparation of HMM and HMM-S1, respectively. The myosin fragments were further purified by passing through a Sepharose-adipic dihydrazide-ATP affinity chromatography column [12]. Purity was checked by sodium dodecyl sulphate gel electrophoresis, in an analytical ultracentrifuge and by adsorption on a DEAE-cellulose as previously described [2, 4] as well as by the absence of super-precipitation in the presence of actin and MgATP. The amount of myosin impurity in all fragment preparations was found to be less than 1 %.

The following procedure was employed for the preparation of chemically modified HMM: (a) *N*-ethylmaleimide-HMM: to a 2 ml solution of 10 mg HMM in 0.5 M KCl, 50 mM Tris buffer, pH 7.9, were added 70 μl of a solution containing 35 μg *N*-ethylmaleimide. After 15 min incubation at 4 °C in the presence of 1 mM MgADP the reaction was stopped by 1 mM β-mercaptoethanol and the preparation was dialyzed against rigor solution; (b) salyrgan (mersalyl)-HMM: salyrgan treatment was carried out at 0 °C by adding 32 molar excess of reagent and dialyzing against rigor solution; (c) trinitrophenylated ($N_3Bz_3SO_3$)-HMM: trinitrophenylation of HMM by trinitrobenzene sulfonate ($N_3Bz_3SO_3$) was performed essentially as described by Fabián and Muhlrad [13], with the difference that excess reagent was removed by dialysis against a solution containing 50 mM KCl, 5 mM Tris buffer, pH 7.6.

Protein concentrations were determined by the method of Lowry et al. [14] or the Biuret method. Ca^{2+}, Mg^{2+}-EDTA- and actin-activated ATPase activities were

TABLE II

THE ATPase ACTIVITIES OF HMM, HMM-S1 AND CHEMICALLY MODIFIED HMM

In all ATPase activity measurements, the concentration of HMM or HMM-S1 was 0.1 mg/ml. The assay solution contained: Mg^{2+}-ATPase: 50 mM KCl, 2.5 Mm ATP, 4 mM $MgCl_2$; Ca^{2+}-ATPase: 50 mM KCl, 2.5 mM ATP, 4 mM $CaCl_2$; EDTA-ATPase: 500 mM KCl, 2 mM EDTA, 2 mM ATP; actin-activated ATPase: as in Mg^{2+}-ATPase plus 0.66 mg/ml actin. Activities were measured at pH = 7.6 except actin activation which was measured at pH 7.2. All measurements were done at room temperature (approx. 25 °C).

Preparation	ATPase (μmol P_i/mg per min)			
	Mg^{2+}	Ca^{2+}	EDTA	Actin
HMM	0.02	1.05	1.22	0.15
HMM-S 1	0.02	1.90	1.95	0.20
N-Ethylmaleimide-HMM	0.05	0.05	0.00	0.03
Salyrgan-HMM	0.00	0.00	0.00	0.00
$N_3Bz_3SO_3$-HMM	0.13	0.25	0.24	0.12

measured by following H^+ liberation in a pH-stat [15]. The ATPase activities of HMM, HMM-S1 and modified HMM's are presented in Table II. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was carried out according to Weber and Osborn [16] (7.5% polyacrylamide, 1% sodium dodecyl sulfate, 0.1 M phosphate buffer, pH 7.0, the length of the gel was 5 cm and the current passed through each tube was 5 mA).

Standard chemicals were obtained from B.D.H.; ATP and salyrgan from Sigma Chemical and *N*-ethylmaleimide from Mann Laboratories.

RESULTS

Tension development in poisoned fiber segments irrigated with myosin, HMM or HMM-S1

As described above, conditions were found under which full abolishment of

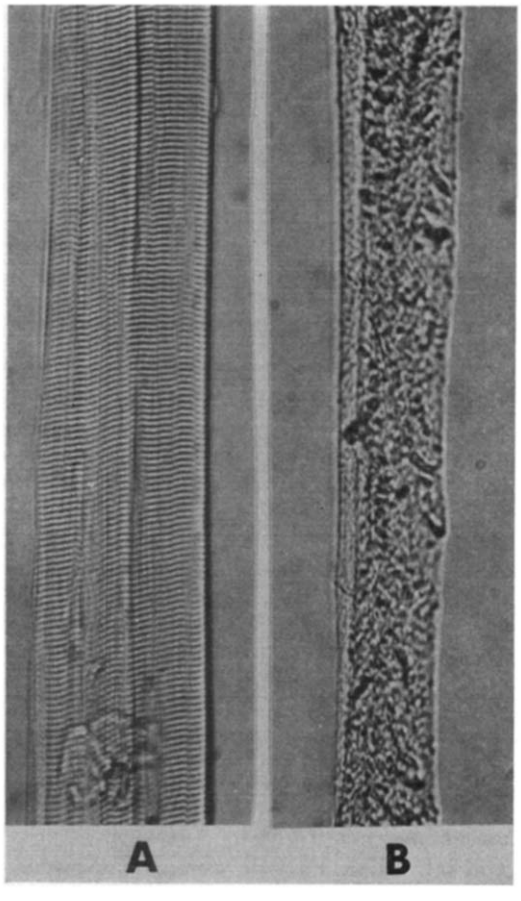

Fig. 5. Photomicrographs of a native (unpoisoned) fiber segment before and after contraction by MgATP. A, fiber segment in rigor solution, sarcomere length: 2.27 μm; B, 3 min after adding 2.5 mM MgATP × 378 magnification.

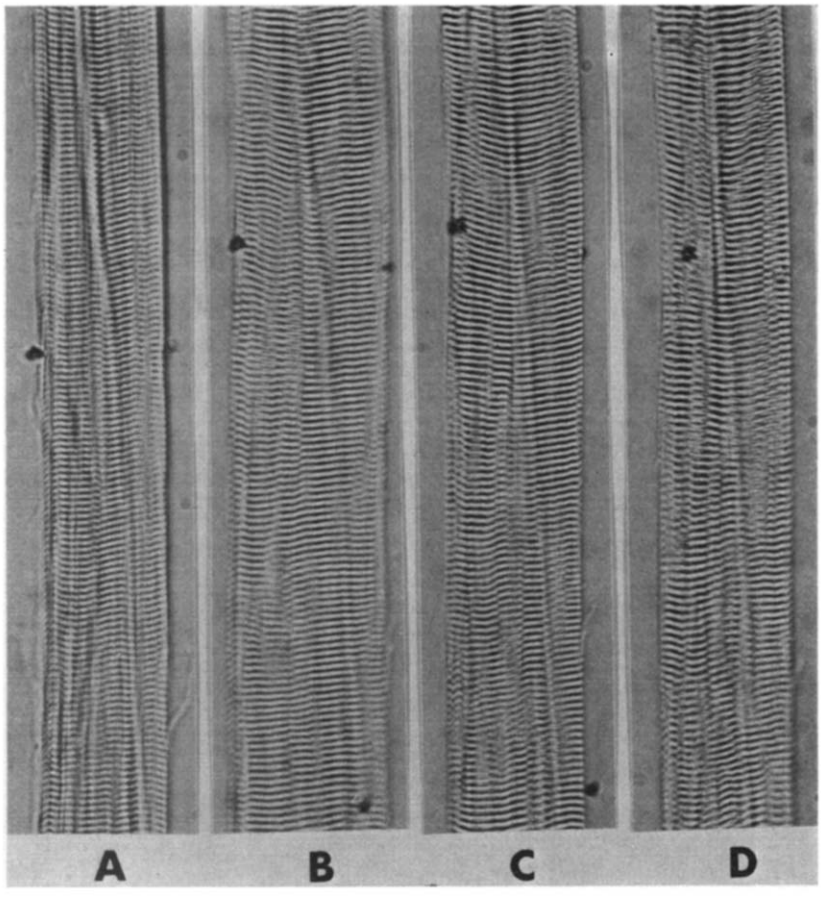

Fig. 6. Sequence of photomicrographs showing the absence of an effect of MgATP on the banding pattern of a poisoned fiber segment. A, fiber segment in rigor solution; B, in poisoning solution; C, in rigor solution following poisoning; D, 10 min after the addition of 2.5 mM MgATP. Such treatment did not lead to the generation of any tension. Sarcomere length in A–D, 2.35 μm. × 378 magnification. Note swelling of the segment in poisoning (high ionic strength) solution.

tension generation by glycerinated, skinned, muscle fiber segments could be achieved. Thus, in the experiments to be reported involving irrigation of such segments with the various myosin moieties, there was no contribution to measured tension from the original muscle myosin. Moreover, while the contraction of unpoisoned fiber segments was accompanied by the disappearance, under the microscope, of the banding pattern (Fig. 5), no such change could be observed in poisoned segments (Fig. 6).

Following the sequence described by Fig. 6, MgATP was removed by repetitive washings with rigor solution, and a solution containing 0.4 M KCl, 10 mM imidazole buffer, pH 7.0, was added. Myosin was now introduced into the segments by applying a high ionic-strength myosin-irrigating solution (Table I) in which the myosin was in a dissociated form. After 3 min irrigation (Fig. 7A), the segments were first washed with 0.4 M KCl, 10 mM imidazole and then with rigor solution. (The 0.4 M KCl solu-

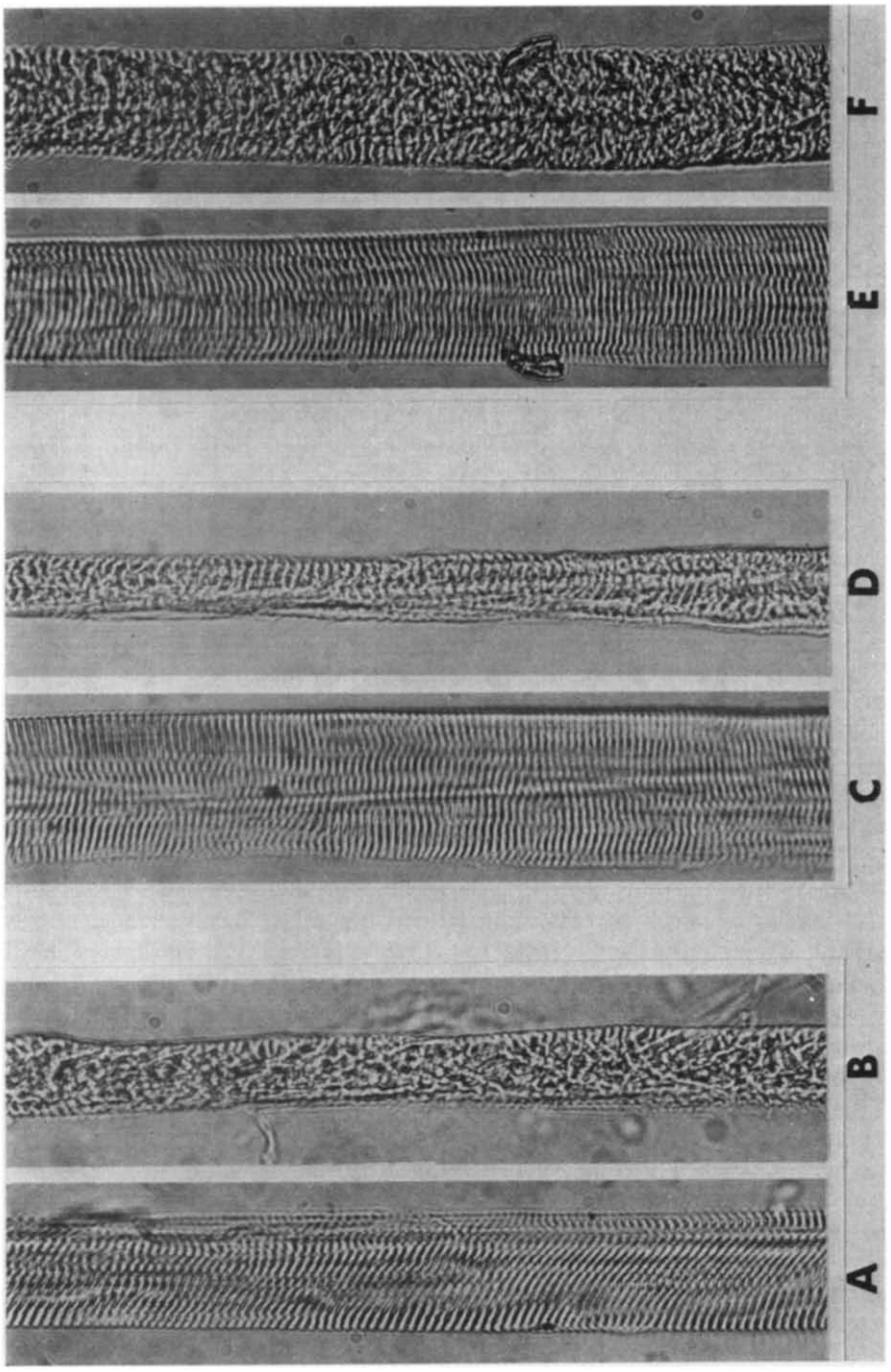

Fig. 7. Photomicrographs showing the effect of MgATP on poisoned fiber segments irrigated with myosin, HMM or HMM-S1. (A) After irrigation with 4.1 mg/ml myosin. (B) 10 min after the addition of 2.5 mM MgATP to the irrigated segment. Sarcomere length in A, as well as preceding steps (corresponding to Figs. 6A–6D), 2.49 μm. (In all experiments, $\times$ 378). (C) During irrigation of the segment used in Fig. 6 with 20.2 mg/ml HMM. Note swelling of the segment. Sarcomere length, 2.35 μm. (D) 10 min after the addition of 2.5 mM MgATP to the HMM-irrigated segment. (E) Fiber segment during irrigation with 11.1 mg/ml HMM-S1. Sarcomere length, 2.49 μm (same as in the preceding steps). (F) 10 min after the addition of 2.5 mM MgATP to the HMM-S1 irrigated segment.

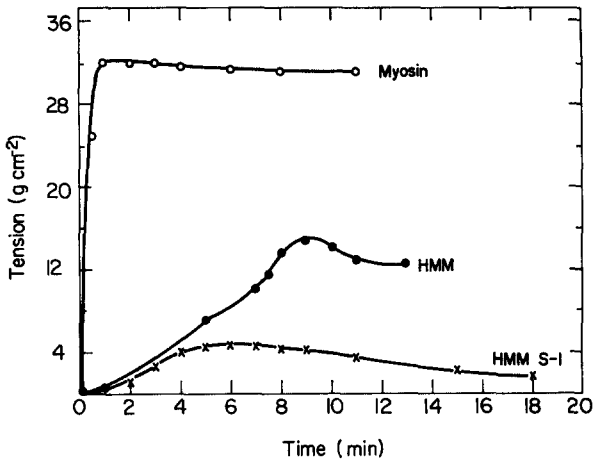

Fig. 8. The time course of tension development by poisoned fiber segments irrigated with 4.1 mg/ml myosin (○–○), 14.5 mg/ml HMM (●–●) and 19.8 mg/ml HMM-S1 (×–×). 2.5 mM MgATP were added at time zero. Contraction was followed by measuring at selected time intervals the deflection of the glass reed from its resting position.

tion was used in order to prevent precipitation of myosin in the experimental chamber.) MgATP was now added; this led to a gradual disappearance of the banding pattern (Fig. 7B) which closely resembled the situation prevailing during the contraction of unpoisoned fiber segments (Fig. 5). Simultaneously, the segments developed tension, the time course of which is shown in Fig. 8. Maximal tension was reached within the first 60 s and remained practically constant during the next 20 min.

Similar experiments have been performed, using HMM or HMM-S1 instead of myosin. Following poisoning (Fig. 6B), a routine check of behavior in activating solution was carried out; this invariably showed complete lack of tension generation and no change in sarcomere length or appearance (Fig. 6D). The muscle segment was washed with rigor solution and HMM (or HMM-S1)-irrigating solution (Table I) introduced (Fig. 7C). After 3 min, MgATP was added and changes in the banding pattern were followed. As can be seen from Fig. 7D, the appearance of the segments was now similar to that of a contracted unpoisoned segment (Fig. 5B) or of a contracted myosin-irrigated poisoned segment (Fig. 7B). Soon after the addition of MgATP, the segments developed a contractile force which reached a maximum after a time period ranging from 6 to 9 min and began to decline thereafter (Fig. 8). By the time the tension reached its peak, the disappearance of the banding pattern (Fig. 7D) was complete.

Irrigation with HMM-S1 gave similar results with respect to changes in sarcomere banding pattern (compare Figs. 7E and 7F to Figs. 7C and 7D). Tension generation (Fig. 8) was followed by a more pronounced relaxation which was nearly complete after 20 min. Relaxation could be reversed and tension redeveloped by the readdition of MgATP (Fig. 9B).

It is interesting to note that the segments visibly swelled in high ionic-strength solution as well as in the presence of added protein. In this respect, the behavior of the poisoned fiber segments resembled that of myofibrils under similar conditions.

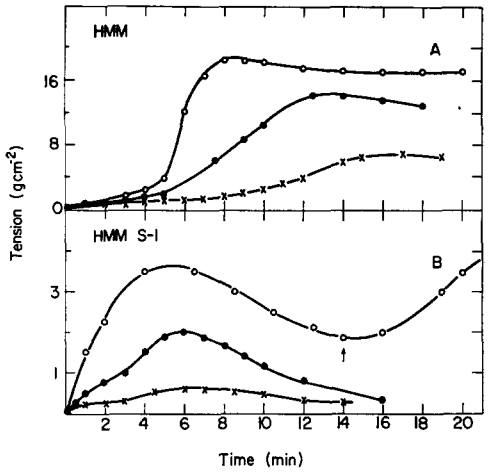

Fig. 9. The time course of tension development by poisoned fiber segments irrigated with various concentrations of HMM and HMM-S1. HMM: ○–○, 20.2 mg/ml; ●–●, 14.5 mg/ml; ×–×, 7.2 mg/ml. HMM-S1: ○–○, 19.8 mg/ml; ●–●, 11. 1 mg/ml; ×–×, 5.5 mg/ml. 2.5 mM MgATP was added at time zero. The arrow marks time of readdition of 2.5 mM MgATP.

The time course, as well as the final value, of the tension developed by the poisoned fiber segments in the presence of HMM or HMM-S1 depended on the concentration of protein in the irrigating solution. Fig. 9 shows that, as the protein concentration was increased, the maximal tension was attained earlier and its value became larger. No change in pH could be detected during the course of such experiments. In order to establish the effect of protein concentration on the value of the maximal tension developed, several fiber preparations were studied at each concentration. This procedure was necessary in view of the irreproducibility of the value of the maximum tension developed when a given fiber segment was irrigated with the same protein concentration more than once (the tension in successive contractions decreased continuously; this effect may be associated with the fact that the mechanical relaxation achieved with Hasselbach-Schneider solution applied between successive irrigations in order to relax the tension and remove applied protein was not accompanied by the reappearance of a regular sarcomere banding pattern, thus reflecting on some irreversible structural damage). Fig. 10 summarizes the results of experiments carried out on completely poisoned fiber segments irrigated with myosin, HMM and HMM-S1. For any given protein concentration (w/v), tension generated decreased in the order: myosin > HMM > HMM-S1; thus, at 16.1 mg/ml protein, the tension developed by myosin was 3.1 times larger than that produced by HMM which was, in turn, 4.5 times larger than that generated by HMM-S1.

The very development of tension after irrigation with a soluble active myosin fragment depended on whether or not the addition of MgATP was preceded by washing the irrigated fibers with the rigor solution. In the former case, no tension could be detected. This was probably due to a critical decrease in the HMM (or HMM-S1) concentration in the myofibrillar space.

When either myosin fragment, together with 2.5 mM MgATP, was introduced

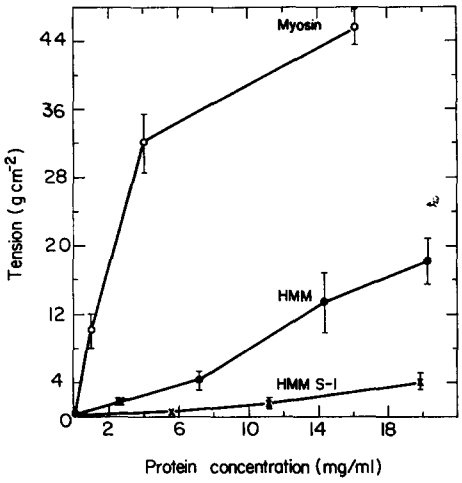

Fig. 10. The relationship between the maximum tension developed by poisoned fiber segments irrigated with myosin (○–○), HMM (●–●) and HMM-S1 (×–×) and the protein concentration in the irrigating solution. The points are means of three experiments, the vertical bars indicate S.E. of measurement.

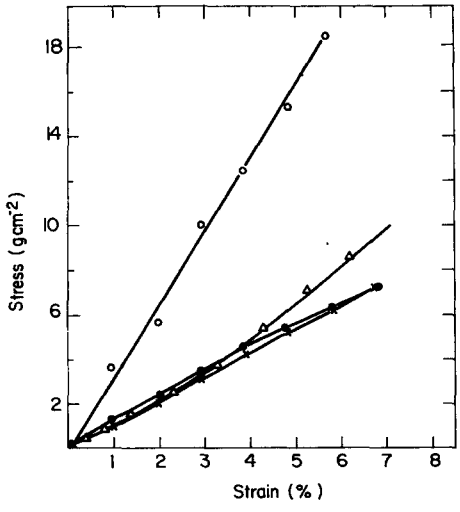

Fig. 11. The stress vs. strain relationship of poisoned skinned fiber segments irrigated with different proteins. The modulus of elasticity of this segment up to 4 % strain is given in brackets. △–△, poisoned fiber segment in rigor solution (0.124 kg/cm^2); ○–○, segment after irrigation with 16.3 mg/ml myosin (0.324 kg/cm^2); ●–●, segment in HMM-irrigating solution containing 20.2 mg/ml HMM (0.124 kg/cm^2); ×–×, segment in HMM-S1-irrigating solution containing 19.8 mg/ml HMM-S1 (0.110 kg/cm^2).

to a poisoned fiber segment in activating solution, immediate contraction took place. However, for 20.2 mg/ml HMM, the maximal tension developed in such contraction was only about 50% that developed during the usual procedure, i.e. when MgATP was added after irrigation with protein in rigor solution.

The stress vs. strain relationship (under rigor conditions) for poisoned fiber segments following irrigation with myosin or in the presence of either HMM or HMM-S1 is shown in Fig. 11. While, in the case of myosin, the stiffness increased

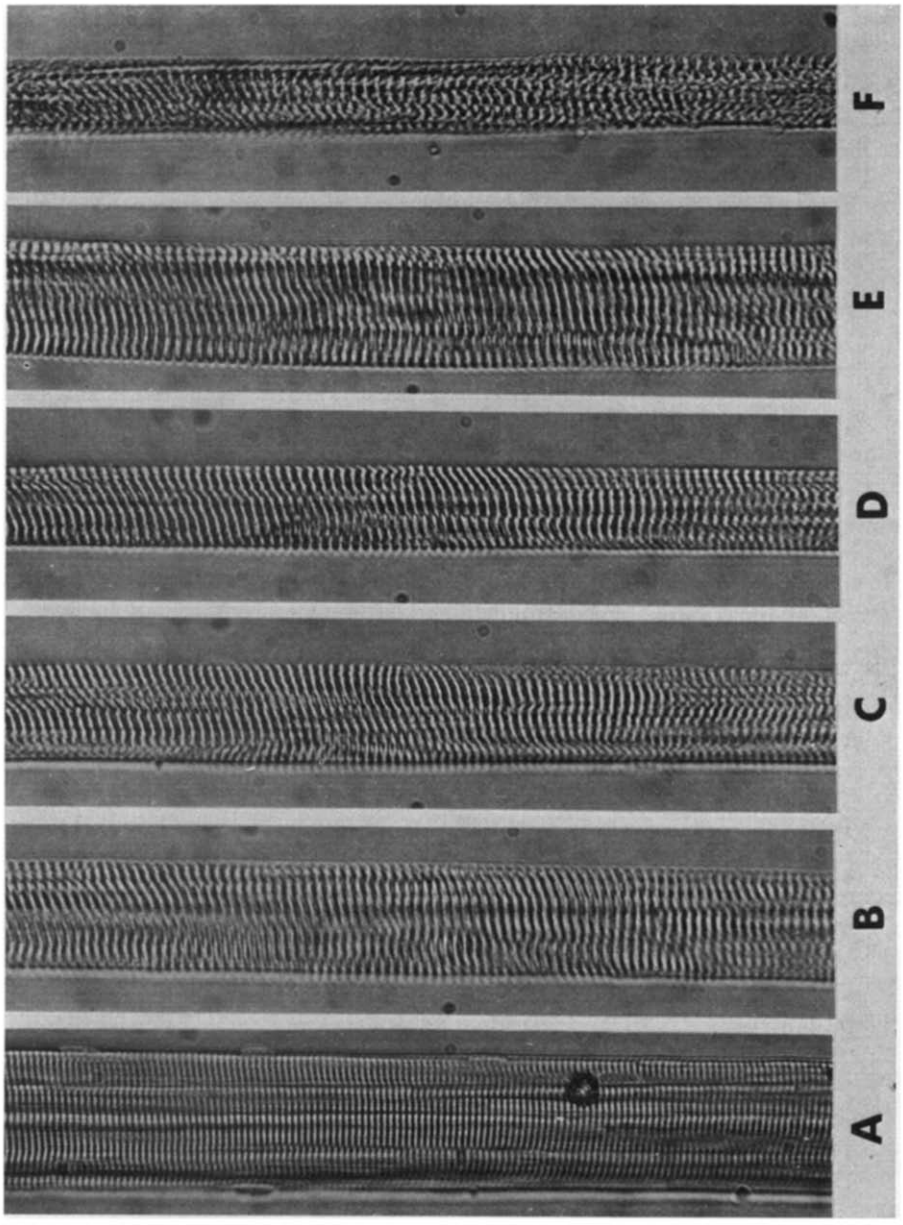

Fig. 12. Photomicrographs of a stretched fiber segment in an HMM-irrigation experiment. (A) Fiber segment in rigor solution; sarcomere length (s.l.), 2.20 μm. (B) In Hasselbach-Schneider solution after stretch; s.l., 3.38 μm. (C) In rigor solution after stretch; s.l., 3.38 μm. (D) 10 min after adding 2.5 mM MgATP; s.l. between 3.28 and 3.56 μm. (E) In HMM-irrigating solution containing 20.2 mg/ml HMM; s.l., 3.66 μm. Note swelling of segment. (F) 10 min after adding 2.5 mM MgATP to irrigated stretched segment. $\times$ 378 magnification.

from 0.124 to 0.324 kg/cm^2, suggesting an increase in the degree of cross-linking of myosin and actin, neither HMM nor HMM-S1 appeared to contribute to the stiffness of the fiber segments.

Tension development in stretched fibers

An alternative method for abolishing the tension resulting from interaction between actin and native muscle myosin was to stretch the fiber segment to a point where the two sets of filaments no longer overlap. Stretching was best accomplished in Hasselbach-Schneider solution, where the static stiffness of the fibers was minimal. Under these conditions, a 70 % strain could be applied while producing a stress of only 20–24 g/cm^2. With Hasselbach-Schneider solution replaced with rigor solution containing 5 mM magnesium pyrophosphate or with relaxing solution, resting stress at 70 % strain was so large that any effect due to HMM was shadowed. Fig. 12 illustrates a typical experiment in which a fiber segment (Fig. 12A) was stretched in Hasselbach-Schneider solution to a sarcomere length of 3.38 μm (Fig. 12B). When the fiber was washed out with rigor solution (Fig. 12C) and MgATP added (Fig. 12D) no tension was developed. However, after irrigation with 20.2 mg/ml HMM (Fig. 12E), MgATP produced tension which in this experiment amounted to 3.5 g/cm^2. The development of tension was accompanied by the loss of the organized structure of the sarcomeres (Fig. 12F). The value of the maximum tension was in this case smaller than that produced in a poisoned muscle at the same HMM concentration (cf. Fig. 10). This might be due to damage inflicted on the muscle segments by stretching, or to a partial masking of the HMM-induced force by the large resting tension.

Correlation between tension generation by soluble myosin fragments and actin-activated ATP splitting

In order to establish a correlation between the tension generation observed and ATP splitting by the soluble myosin fragments in combination with muscle actin the following experiments were carried out:

(1) Poisoned glycerinated skinned fiber segments were irrigated with various types of chemically modified HMM, the Mg^{2+}-activated ATPase activity of which was not enhanced by actin anymore. HMM was treated with *N*-ethylmaleimide, salyrgan or $N_3Bz_3SO_3$ (see Table II for the ATPase activities of the preparations). Under the same conditions at which intact HMM caused tension development, none of the inactivated species was effective. Particularly interesting is the case of trinitrophenylated HMM, the Mg^{2+}-activated ATPase activity of which is much higher than that of untreated HMM; however, as in the case of *N*-ethylmaleimide- or salyrgan-HMM which are practically poisoned with respect to their EDTA- and the Ca^{2+}-activated ATPase activities, the addition of actin to $N_3Bz_3SO_3$-treated HMM in the presence of Mg^{2+} does not cause any increase in activity.

(2) After irrigating a *N*-ethylmaleimide-poisoned muscle segment with 14.5 mg/ml HMM, 5 mM sodium pyrophosphate and 2.5 mM $MgCl_2$ were added instead of MgATP. No tension development could be detected in this case.

(3) Another useful test for the existence of mechanochemical effects is to verify the absence of HMM- or HMM-S1-induced tension under the conditions where the ATP splitting is greatly reduced by the inhibitory effect of the troponin-tropomyosin system in the absence of Ca^{2+}. Such a test could not be carried out on

intact fibers because these were not 100 % Ca^{2+} sensitive and residual tension developed in relaxing solution was enough to mask small forces associated with HMM. Poisoned fiber segments, on the other hand, lost their Ca^{2+} sensitivity as a result of *N*-ethylmaleimide treatment: when a poisoned fiber was irrigated with myosin in the virtual absence of Ca^{2+} (adjusted with ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA) to a concentration of 10^{-9} M) the addition of MgATP caused the development of a contractile force the value of which was practically the same as that observed for the same myosin concentration in the presence of Ca^{2+}. The test was therefore carried out on stretched muscles: after applying 60 % stretch the fiber segment was introduced into a relaxing solution and the absence of tension generation was noted. The fiber was then irrigated with a solution containing 20.2 mg/ml HMM and 2 mM EGTA and MgATP added. No tension development could be detected within 15 min. When the same fiber was later placed in HMM-irrigating solution (containing 0.1 mM Ca^{2+} and 20.2 mg/ml HMM), 2.8 g/cm^2 tension was generated within 5 min, after which the fiber segment broke.

(4) The most direct way of correlating contractility to ATP splitting is the parallel measurements of ATP hydrolysis and the mechanical response. Since ATPase activity measurements of glycerinated skinned fiber segments might be complicated by diffusional effects and because of the technical difficulty associated with measuring ATPase in our experimental chamber, we investigated the effect of HMM and of HMM-S1 on the contractility and ATPase activity of poisoned myofibrils (see Materials and Methods). Contraction was followed by measuring the drop in absorbance at 350 nm of myofibrillar suspensions upon adding MgATP; such changes have been shown to be associated with myofibrillar contraction [17]. The absorbance of a 1 mg/ml suspension of native myofibrils dropped from a value of 2.20 to 2.01 upon adding 2 mM MgATP (final concentration) (Fig. 13A). The absorbance of *N*-ethylmaleimide-treated myofibrils at the same concentration was not affected by 2 mM MgATP (Fig. 13B). The Mg^{2+}- and Ca^{2+}-activated ATPase activities of the

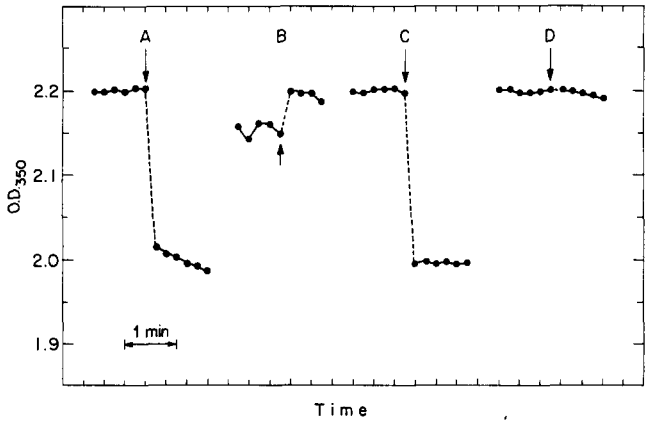

Fig. 13. Turbidity changes at 350 nm following addition (marked by arrows) of 2.0 mM MgATP. A, native myofibrils; B, *N*-ethylmaleimide-poisoned myofibrils; C, *N*-ethylmaleimide-poisoned myofibrils irrigated with 2.5 mg/ml HMM; D, *N*-ethylmaleimide-poisoned myofibrils irrigated by salyrgan-poisoned HMM.

poisoned myofibrils were 0.018 and 0.003 μmol P_i/mg myofibrillar protein per min, respectively, as compared to 0.5 and 0.3 μmol P_i/mg protein per min in the case of the native myofibrils. When the poisoned myofibrils were irrigated with 2.5 mg/ml HMM, a change in absorbance from 2.20 to 1.99 took place after the addition of MgATP (Fig. 13C) while myofibrils irrigated with the same concentration of salyrgan-inactivated HMM were insensitive to MgATP (Fig. 13D). The Mg^{2+}-activated ATPase activity of the HMM-contracted system was 0.1 μmol P_i/mg myofibrillar protein per min.

The following experiment was carried out in order to check the possibility that tension generation was not due to aggregates formed by the added myosin fragments through binding along the poisoned myosin filaments. To a suspension of *N*-ethylmaleimide-poisoned myosin filaments (5 mg/ml), the ATPase activity of which has been practically abolished, HMM was added. The weight ratio of the proteins was 1 : 1. Myosin was sedimented at 13 000 $\times g$, washed three times with rigor solution and examined by sodium dodecyl sulfate gel electrophoresis. No bands corresponding to the heavy chains of HMM could be detected.

DISCUSSION

After irrigation with myosin, poisoned skinned glycerinated muscle fiber segments were capable of generating tension. The contraction was associated with a loss of the banding pattern which was reminiscent of that observed in a normal contraction of unpoisoned segments. These results are in line with those of Hanson and Huxley [18] who succeeded in restoring the contractility of ghost myofibrils after irrigation with myosin under similar conditions. The tension measured in our experiments increased with increasing myosin concentration and assumed a value of 44 g/cm^2 at a protein concentration of 16.1 mg/ml, which amounts to 3.0–4.4 % P_0. The fact that the tension developed after irrigation with myosin is only a small fraction of P_0 is probably associated with the formation, in the middle of the sarcomeres after the addition of MgATP, of very short and/or a relatively small number of myosin filaments.

Irrigation of inactivated muscle segments with the myosin fragments HMM and HMM-S1 could also lead to the development of contractile force. Just as in the case of intact myosin, the generation of force was accompanied by a loss of the organized banding pattern which is characteristic of a contracting unmodified muscle segment. Tension production appears to be intimately associated with the splitting of ATP by the combined action of the soluble myosin species and the actin filaments of the muscle. Thus, no tension development could be observed under conditions where ATP splitting by acto-HMM is not supposed to take place, e.g. when inactive HMM was employed or when MgATP was substituted by magnesium pyrophosphate. Furthermore, as demonstrated with HMM-S1 (Fig. 9B), tension development could be re-established by a second addition of ATP during relaxation which was probably due to ATP depletion.

The fact that HMM does not adsorb on *N*-ethylmaleimide-treated myosin filaments rules out the possibility that the myosin filaments in poisoned muscle segments serve as a backbone for aggregate formation by HMM. This conclusion is further supported by our observation that the modulus of elasticity of poisoned fiber segments under rigor conditions was not affected by the presence of either fragment (Fig. 11).

For a given protein concentration, HMM is less efficient in generating tension than myosin. Thus, at a concentration of 16.1 mg/ml the tension produced by HMM is only 32 % of that developed by myosin. On a molar basis this value reduces to 23 %. The higher efficiency of myosin may possibly be partly due to an increase in effective concentration due to filament formation. Heavy meromyosin subfragment 1 is even less effective than HMM (Fig. 10): the ratio of tensions generated at any given concentration varied between 4 and 5. A myosin head is thus 6–7.5 times more effective when forming part of the double-headed HMM than when acting alone. This fact may be partly accounted for if one assumes that the probability of attachment (or detachment) of one HMM head is affected by the binding of its twin head. It should be recognized, however, that energetic cooperative or anti-cooperative effects may be superimposed on the purely statistical factors [19].

In summary: the efficiency of tension generation decreases in the order of myosin > HMM > HMM-S1.

The present experiments are in line with our earlier finding that HMM as well as HMM-S1 can cause the contraction of "ghost" myofibrils [1–4] and the shrinking of actomyosin threads [20, 21] or gels [22] in which the myosin component has been inactivated, under conditions which give rise to ATP splitting by acto-HMM or acto-HMM-S1.

In view of our results it is not impossible that monomeric myosin may also participate in mechanochemical energy transductions in vivo, particularly in non-muscle cells.

An hydrodynamic model, common to muscle contraction and cytoplasmic streaming, has previously been proposed in order to explain tension generation in systems in which actin and myosin do not form a continuous three-dimensional network [1, 20, 23].

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