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REACTIVITY OF MYOSIN TO ANTIBODIES IN CROSS-STRIATED CHICK MYOFIBRILS

I. MUSCLE AT REST LENGTH*

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SUMMARY

The amount of myosin which reacted in striated chick myofibrils at rest length with antibodies, prepared against myosin, light meromyosin, heavy meromyosin and actin was quantitatively determined. This was done by taking advantage of the relative insolubility of the antibody–antigen complex in a solvent which extracts myosin readily from the myofibril. Extraction of myosin was followed by measuring ATP phosphohydrolase (EC 3.6.1.3) activity in the soluble and insoluble fractions with and without antibody treatment, by making total protein balance, and by determining the antibody concentration from fluorescence measurements. Antibodies prepared against myosin, light and heavy meromyosin reacted extensively with myosin in the myofibril. Antibody prepared against actin fixed only small amounts of myosin. There are more antigenic sites against anti-myosin than against anti-light and less against anti-heavy, as determined from the amount of antibody bound by the myofibrils. The sum of the bound anti-light and anti-heavy exceeded the value of bound anti-myosin only by about 10–20%, indicating that these preparations contained antibodies which reacted at different sites. The experiments rule out the possibility that steric hindrance, or blocked antigenic sites significantly influence the results obtained when the myosin in a myofibril at rest length reacts with antibodies prepared against myosin, light and heavy meromyosin. The distribution of myosin in the A-band is discontinuous. At rest length a narrow central region does not react with anti-myosin or anti-light meromyosin. The changes in the sarcomere pattern in various conditions suggest that migration of myosin within the A-band is possible.

Abbreviations: LMM, light meromyosin; HMM, heavy meromyosin; anti-M, antibody against myosin; anti-L, antibody against light meromyosin; anti-H, antibody against heavy meromyosin; anti-Ac, antibody against actin.

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INTRODUCTION

Fluorescein-labeled antibodies were successfully used for the detection of small amounts of myosin in individual cells[1]. It has been also shown that antibodies prepared against myosin, actin, HMM and LMM stain myofibrils in a pattern characteristic for each antibody preparation[2]. The distribution of antibodies depends on the state of contraction[3]. Some of the problems in correlating the staining pattern with the localization of particular proteins within the myofibril have been discussed previously[4]. These include questions of purity of antigens, whether the antibody reacts against the antigen which is being localized, the effects of steric hindrance and blocked antigenic sites on the staining pattern. An accurate interpretation of the characteristic staining pattern in terms of the localization of myosin requires the quantitative determination of what fraction of the myosin present in the myofibril has reacted.

In this study the reactivity of myosin was measured. The measurements were based on the observation that those portions of the myofibrils, where antibody deposition took place, became resistant to solvents which remove these sarcomere fragments from an untreated myofibril[5]. This approach has been utilized by HOLTZER who found that pretreatment of myofibrils with anti-myosin prevents extraction of material by potassium iodide which gives a precipitin reaction with anti-myosin[4]. The binding of the different antibody preparations by the myofibrils and the effect of these antibodies on the extractability of myosin is reported in this paper.

PREPARATIONS AND PROCEDURES

Myofibrils

From the musculus pectoralis minor of a chick, bundles were tied *in situ*, at approximately rest length, to applicator sticks and placed in glycerol–water (1:1) for 24 h at 0°, and for 3 weeks at −25°. The bundles were then Waring-blendered for 1 min in 0.04 M NaCl, containing 0.01 M imidazole–HCl buffer (pH 7.0). The myofibrils were centrifuged down and resuspended in a medium consisting of 50 % glycerol, 0.02 M NaCl and 0.005 M imidazole (pH 7.0) and stored at −25°. The experiments reported here were made on fibrils stored for 2–6 months. Storage did not seem to influence the measurements.

Antibodies

Anti-L and anti-H, and anti-Ac were the same lot with which the original localization studies have been performed[1,2]. Two lots of myosin antibodies were used. Their labeling and some other properties have been described previously[1,2]. The antibodies were stored at −25° and were centrifuged at 8000 rev./min for 15 min before use to remove insoluble matter. They had a protein concentration of 1.0–1.5 % and their fluorescence was equivalent to a $1.3–2.2 \cdot 10^{-4}$ M fluorescein solution with the exception of one of the anti-M (M-11) which had a fluorescence equivalent to a $2.5 \cdot 10^{-5}$ M fluorescein solution.

Determination of ATPase activity (EC 3.6.1.3)

Reaction was started by addition of protein, in 1 ml 0.04 M NaCl, 0.01 M imidazole buffer (pH 7.0) to 2 ml solution containing 0.001 M ATP, 0.0075 M $CaCl_2$, 0.04 M

NaCl, 0.05 M imidazole–HCl buffer (pH 7.0). After shaking for 5 min at 23°, the reaction was stopped with 1 ml silico tungstate reagent and inorganic phosphate determined according to MARTIN AND DOTY[6], with slight modifications. To the sample 5 ml benzene–isobutanol and 1 ml of ammoniummolybdate reagent was added. After shaking and separation of the phases 2 ml of the benzene–isobutanol layer was measured into a 5-ml volumetric flask, washed after and made up to volume, after addition of 0.2 ml stannous chloride reagent, with the sulfuric acid–ethanol solution. Color was read in a Beckman DU spectrophotometer at 675 mμ. 0.1 μmole phosphate gave an extinction of 0.127. ATPase runs were made at two different protein concentrations and the phosphate liberated by the unfractionated myofibrils was around 0.1 and 0.2 μmole.

Fluorescence determinations

A Farrand fluorometer was used for the measurements of fluorescence employing standards of $5 \cdot 10^{-8}$ and $5 \cdot 10^{-7}$ M fluorescein in solvent containing 0.1 N NaOH and 2 % Na_2CO_3.

Protein determinations

The method of LOWRY *et al.*[7] was used. For each determination 4–60 μg protein at five different concentrations was introduced in a volume of 0.5 ml to which 2.5 ml copper–alkali and 0.25 ml Folin–Ciocalteu reagent was added and color read in a Beckman spectrophotometer at 750 mμ. Bovine serum albumin, myofibrils and myosin all gave extinction of 0.050 for 10 μg of protein with the lot of Folin–Ciocalteu reagent used. The myofibrils were allowed to stand in 0.1 N NaOH containing 2 % Na_2CO_3 overnight, to insure complete solubilization, before fluorescence and protein determinations were made. Standards were based on Kjehldahl nitrogen determinations, using a conversion factor of 6.2 to obtain the quantity of protein.

Determination of antibody uptake by myofibrils

To a series of 0.05 ml of myofibril, containing 80–90 μg protein suspended in 25 % glycerol, 0.04 M NaCl and 0.01 M imidazole buffer (pH 7.0), increasing amounts of antibody were added (0.01–0.3 ml) and incubated with occasional stirring for 16–20 h. The volume was made up to 1 ml with 0.04 M NaCl and 0.01 M imidazole buffer (pH 7.0) and stirred by sucking up in a Pasteur pipette and centrifuged in 1-ml tubes of a Misco centrifuge at 8000 rev./min for 7 min in the cold room (+1° to −2°). Excess antibody was removed by rinsing and centrifuging the sediment with 1 ml salt solution twice. The sedimented myofibrils were resuspended again with capillary in 1 ml salt solution centrifuged and rinsed and centrifuged twice again. The bound antibody was independently obtained from protein and fluorescence determinations.

A stirring rod made of 1.1 mm inner diameter polyethylene tubing pulled over a dissection needle and sealed over flame was used in place of the Pasteur pipette in later stages of the experiments. Losses were reduced during resuspensions using this device.

Determination of the myosin reacted

General considerations: A loss of extractability of myosin was taken as a measure

of its interaction with antibody. The distribution of myosin between the soluble fraction and insoluble residue, under various conditions, was obtained by measuring the ATPase activity of these fractions. This procedure seems to be a reliable one for the following reasons: Myosin is the only ATPase present in a washed myofibrillar suspension in significant amounts. We have also found, in exploratory experiments, that treatment of myofibrils with the antibodies used here had no effect on the ATPase activity of the myofibrils. This is in line with the observation that anti-M did not influence the ATPase activity of extracted myosin[8]. Independently from the ATPase studies, the myosin in the soluble and insoluble fraction was also obtained by making a total protein balance. With this method the myosin content of the myofibril had to be known and it was taken to be 55 % of the myofibril, a value obtained for a similarly treated myofibril preparation of rabbit psoas[9]. The amount of antibody in the various fractions had to be determined separately from the myofibrillar proteins. This was done by fluorescence measurements. The determination of myosin from protein balance is subject to greater errors and is more ambiguous than the ATPase studies. For instance, one has to assume that the antibody did not interfere in the solubilization of proteins, other than myosin, in significant amounts. This assumption is certainly not true when anti-Ac was used in which case myosin was determined only from ATPase runs. Still, the protein balance studies were not only a useful check to test the validity of the ATPase determinations, but also served to test for the presence of an ATPase with a markedly smaller molecular weight than myosin.

To centrifuged myofibrils containing 1 mg protein, the respective antibodies or, to the controls the same volume of 0.067 M neutral phosphate buffer, in 20 % glycerol was added. The myofibrils were resuspended and occasionally stirred for 16–20 h. At the end of the incubation the myofibrils were centrifuged and the excess antibody and phosphate were removed by rinsing and centrifuging the sediment twice with 1 ml 0.04 M NaCl and 0.01 M imidazole buffer (pH 7.0) for 7 min at 5000 rev./min in the cold room. The myofibrils were resuspended in 1 ml salt solution, centrifuged, rinsed and centrifuged twice. This procedure involves a loss of about 10–30 %.

The washed myofibrils were resuspended in 1 ml salt solution. 0.2 ml aliquot was introduced into 2.0 ml 0.04 M NaCl containing 0.01 M imidazole buffer for ATPase, protein determinations and microscopic studies. 0.1-ml aliquot was placed into 1.7 ml 0.1 M NaOH, 2 % Na_2CO_3 solution for fluorescence and protein measurements. This fraction is denoted as "unextracted". The remainder was centrifuged down. The fluorescence of the supernate was determined to obtain the unbound antibody not removed by washing, to make correction for the antibody bound in the "unextracted" fraction. It amounted to 0.2–0.3 % of the antibody initially added and about 1–3 % of the antibody which was bound. The sediment was resuspended in 0.7 ml of extracting solution which consisted of: 0.55 M NaCl, 0.02 M imidazole buffer (pH 7.0), 0.01 M neutralized sodium-pyrophosphate and 0.001 M $MgCl_2$. Extraction was done in an ice-water bath in the cold room. Extraction at higher temperatures led to a considerable inactivation of ATPase. In this way inactivation was kept below 20 %. Aliquots were taken for ATPase, fluorescence, protein and microscopic measurements, as described above, and this fraction is denoted as "extracted". The remainder was centrifuged down. Aliquots taken from the supernatant fraction are called "soluble" fraction. The sediment was resuspended in 0.4 ml 0.04 M NaCl, 0.01 M imidazole buffer (pH 7.0) and aliquots taken again. These

aliquots are called "insoluble" fraction. The precipitate usually was more sticky and did not resuspend readily after extraction, especially in cases where significant amounts of myosin were removed. A considerable amount of protein was lost by sticking to the walls of the capillary. The ATPase activity of this fraction had to be multiplied by a factor for the missing proteins obtained from protein and fluorescence measurements. The factor varied from 1.2 to 1.7. In certain cases, like when low concentrations of antibody were used, the ATPase activity in the precipitate was not determined. It may be seen that only a centrifugation is the difference between the "extracted" and "soluble" fractions, thus the myosin solubilized is obtained in a straightforward manner.

Microscopy: A Zeiss microscope, equipped with phase and fluorescent accessories, was used. The fibrils were centrifuged and resuspended in 0.067 M phosphate buffer (pH 8.0) in 20% glycerol and photographed under oil immersion at about 1000 × magnification, using Kodak M plates for the phase, and Kodak AG spectroscopic plates for the fluorescent pictures. Sarcomere lengths were obtained by measuring four successive sarcomeres from the same straight portion of the myofibril directly from the phase and fluorescent plates with the aid of a Gaertner travelling microscope. The width of the A bands was obtained by averaging the measurements of four A bands of the same sarcomeres of each myofibril.

RESULTS

Antibody uptake of the myofibrils

Fig. 1 shows the anti-M uptake of myofibrils at a wide range of antimyosin concentration. Fig. 2 compares the uptake of different antibodies by myofibrils at the lower range of antibody concentrations. The following points may be worth noting:

1. With a great excess of anti-M added the antigenic sites of the myofibrils become saturated. The antibody in the anti-M preparation, which is specific to sites on the myofibril, is high, and calculated from the initial portion of the curve; it amounts to about 20% of the total γ-globulin fraction. The amount of antibody

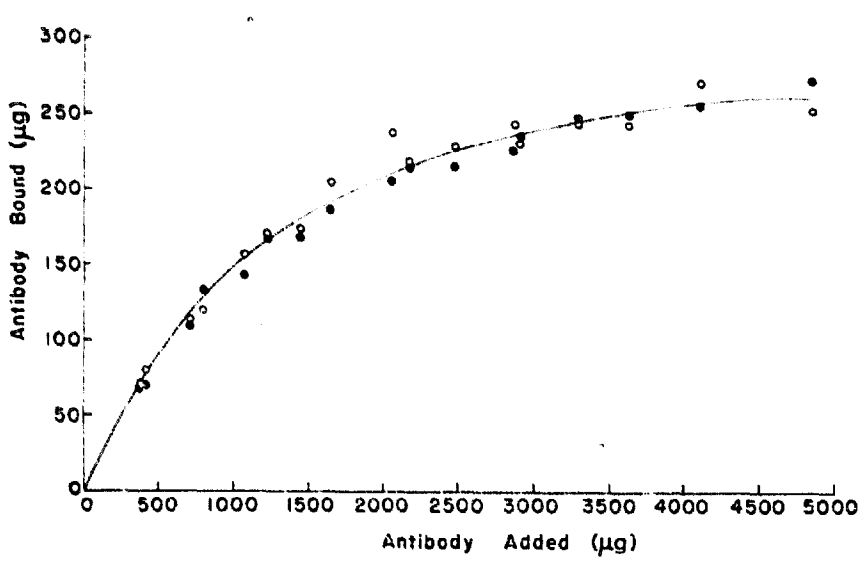

Fig. 1. Anti-myosin uptake of 80 μg myofibril. Open symbols, protein determinations; solid symbols, fluorescence determinations. Abscissa, anti-M added (μg); ordinate, anti-M bound (μg).

bound by the myofibrils is considerable. At saturation point 1 g myofibril reacts with about 3.2 g anti-M. Taking the molecular weight of myosin as 450 000, the myosin content of the myofibril as 55 %, the molecular weight of the antibody as 160 000, and assuming that the anti-M was bound predominantly by myosin, 1 mole of myosin in the myofibril can react with about 16 moles of antibody. This ratio is in the range of the value obtained with purified myosin[8].

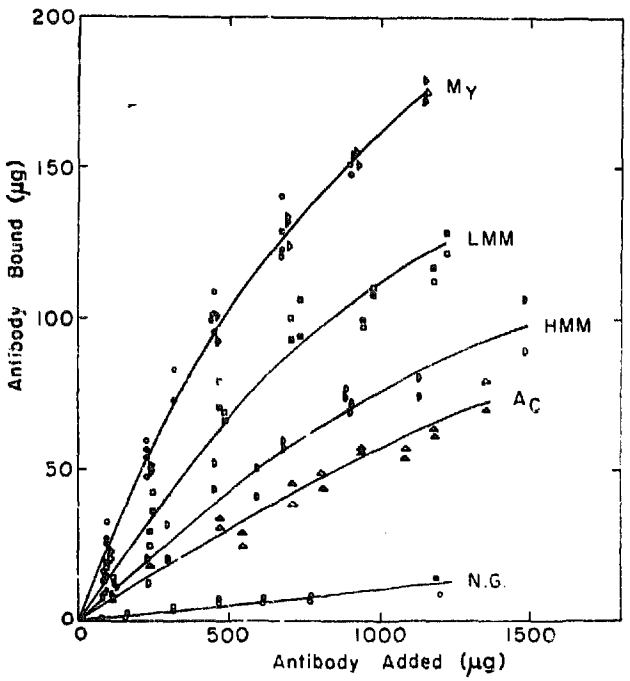

Fig. 2. Uptake of different antibodies by 80 μg myofibril. Open symbols, protein determinations; solid symbols, fluorescence determinations. Abscissa, antibody added (μg); ordinate, antibody bound (μg).

2. The amount of antibody bound depends on the type of antibody added. This variation may be partly due to differences in the antigenecity of the various protein fractions. More likely, it is connected with and roughly proportional to the concentration of antigenic sites in the myofibril which interact with the antibody used. At comparable concentrations the bound anti-L is 70 %, the anti-H 45 % of the bound anti-M. There is overlap between the anti-L and anti-H but they are also bound at different sites in the A-band.

The interpretation of the results with anti-Ac is more difficult. It is of some interest, though, that the ratio of uptake of anti-M and anti-Ac approximates, closely, the ratio of the concentration of myosin and actin in the myofibril. Only a small amount of normal globulin is bound by the myofibrils.

The amounts of antibodies used in the solubilization studies were kept well below saturation levels, and not more than about 15 times the weight of the myofibrils were added for two reasons: to avoid the interfering action of possible impurities, also to conserve the limited stores of antibody available.

Effect of antibodies on the solubilization of myosin

When control myofibrils, to which no antibody was added, are extracted with the solvent used, most of the ATPase and a considerable amount of protein is extracted.

25 measurements yielded, for the soluble ATPase, an average of 87 ± 4 %, the greatest deviations observed were 75 and 99 %. The total protein extracted averaged 82 ± 8 %. The protein extracted was considerably more than usually obtained using Hasselbach–Schneider solution. The reason was traced to the omission of phosphate from the extraction medium. In the presence of phosphate buffer, both at pH 6.5 and at pH 7.0, the protein extracted averaged 62–67 %, a value similar to the one found using myofibrils prepared from glycerinated rabbit psoas muscle[9]. It appeared reasonable to assume that the myosin content of the chick myofibrils used here did not differ greatly from the myosin content of rabbit psoas and was taken to be 55 % of the proteins of the washed myofibrils. It is clear, also, that the solvent which did not contain phosphate, removed a considerable amount of protein other than myosin from the myofibril. The protein extracted, which was not myosin, is then 27 % and had to be taken into consideration when the amount of myosin extracted in various conditions was calculated from protein determinations alone.

A representative experiment is shown in Table I, which shows the effect of anti-M on the solubilization of myosin, the calculations used to obtain the quantity of myosin, based on protein and fluorescence determinations of the antibody, also the balance of the distribution of ATPase.

It can be seen that the quantity of bound antibody agrees with the value which is expected from the curve for anti-M of Fig. 1, at the antibody myofibril ratio used. A good agreement was obtained in all the experiments which are reported here. Anti-M leads to an extensive fixation of the myosin in the myofibril. The soluble ATPase is down to 20 % of the total. The decrease is not due to inactivation in any extent since the remainder of ATPase was measured in the insoluble fraction. The value of myosin extracted, calculated from protein balance data, agrees with the ATPase results.

It may be pointed out again that the most straightforward procedure is the comparison of the ATPase activity of the "soluble" and "extracted" fractions. In

TABLE I

EFFECT OF ANTI-M ON THE EXTRACTION OF MYOSIN

725 μg myofibril contains 398 μg myosin (55 %); non-myosin extracted, 196 μg (27 %); myosin extracted, 265 − 196 = 69 μg = 17 % of 398 μg.

*Anti-myosin**	*Protein (μg)*	*Antibody (μg)*	*Protein − antibody (μg)*	*μmoles P/5 min at 23°*
Unextracted	2005	1270	735	1.39
Extracted	1980	1255	725	1.29
Insoluble**	1695	1137	558	1.0 (78 %)
Soluble	364	99	265	0.26 (20 %)
Control				
Unextracted	840			1.84
Extracted	794			1.55
Soluble	671			1.49 (96 %)

* 7900 μg antibody to 1000 μg myofibril.
** The values were multiplied by a factor of 1.43 to correct for the loss in resuspension.

the "insoluble" fraction only about 70 % of the expected protein and fluorescence was recovered and the ATPase value of the "insoluble" fraction was multiplied by a factor of 1.43.

In the experiment shown on Table I, at least 80 % of the myosin in the myofibril was available for antibody and reacted with antibody to an extent that it became insoluble to the solvent used. The 80 % value can be considered a minimum value for the following reasons: the ratio of antibody to myofibril was such that the antibody present was still in limiting and far from saturating amounts. The soluble myosin

TABLE II

EFFECT OF ANTIBODIES ON THE EXTRACTION OF MYOSIN

Antibody was added to 1000 μg myofibril. Antibody bound and antibody extracted was also calculated for 1000 μg myofibril and corrected for the 10–30 % loss incurred during the washing to remove the excess antibody.

	Antibody added (μg)	*Antibody bound (μg)*	*Antibody extracted (μg)*	*ATPase soluble (%)*	*ATPase insoluble (%)*	*Myosin calculated from protein soluble (%)*
Rest-length myofibrils:						
1 Control	0			87		
2 N-globulin	8022	112	17	82		90
3 Anti-myosin I	2240	416	70	47		45
4 Anti-myosin I	7900	1752	137	20	78	17
5 Anti-myosin II	8780	1760	247	23	79	24
6 Anti-myosin II	15 850	2300	203	<5	97	<5
7 Anti-LMM	7200	1144	66	15	82	23
8 Anti-LMM	8840	1424	117	21	80	27
9 Anti-HMM	7270	789	125	35	60	40
10 Anti-HMM	8420	812	159	37	60	38
11 Anti-HMM	12 000	940	96	15	72	6

does not all represent unreacted myosin. A considerable amount of antibody, about 8 % of the total antibody bound, was extracted in the soluble fraction. Its presence there may derive from a higher solubility product of the antibody–antigen complex at the high ionic strength of the extraction medium ($I = 0.6$). This may be especially true for those myosin molecules where the antigenic sites are only partly occupied with antibody. There appears to be, then, no compelling reason to consider steric hindrance as a major factor in considering the reaction of myosin in the myofibril with anti-M.

Table II summarizes the experiments with the various antibodies at different concentrations.

Anti-M, anti-L and anti-H were all effective and fixed myosin extensively though in the case of anti-H somewhat more antibody was required for the same effect. Within experimental error all myosin reacted if antibody was added at a sufficiently high concentration. These preparations, thus, all contain antibodies which will fix myosin in the myofibril. Measurements using lower concentrations of antibodies indicate that there is an inverse proportionality between antibody uptake and solubilization of myosin. The amount of antibody bound, when a comparable fraction of myosin is fixed, follows the same order as Fig. 2 and is largest with anti-M and smallest with anti-H. Neither steric hindrance nor blocked antigenic sites of myosin in the myofibril can be invoked thus to explain the characteristic staining patterns

using these antibodies. The results with actin are tentative only. For some reasons unknown, ATPase was inactivated by 40–50% when the extracting medium was added. The results still indicate that anti-Ac influences but little the solubilization of myosin and that anti-Ac does not contain antibodies against myosin in large quantities. N-globulin does not interfere with the extractability of myosin.

The myosin solubilized as calculated from protein balance agrees within experimental error with the values obtained from ATPase runs, not only for anti-M but for anti-L and anti-H also. If these antibodies would fix only one portion of each myosin molecule, leaving the other half soluble, a type of result one would expect if the meromyosins were dissociated in muscle, the calculated quantity of myosin from protein determinations should be greatly in excess of the values of the ATPase determinations. In this case antibody prepared against light meromyosin, the portion of myosin which does not carry the ATPase centers, should not greatly influence solubilization of ATPase. A concept of the meromyosins present in the myofibril, dissociated and separated from each other, is not supported by these experiments.

Effect of extraction on the sarcomere band pattern of antibody-treated myofibrils

Plate I shows the phase and fluorescent photographs of antibody-treated myofibrils, from "unextracted" and "extracted" lots. The patterns of unextracted fibrils is the same as reported previously by MARSHALL *et al.*[3].

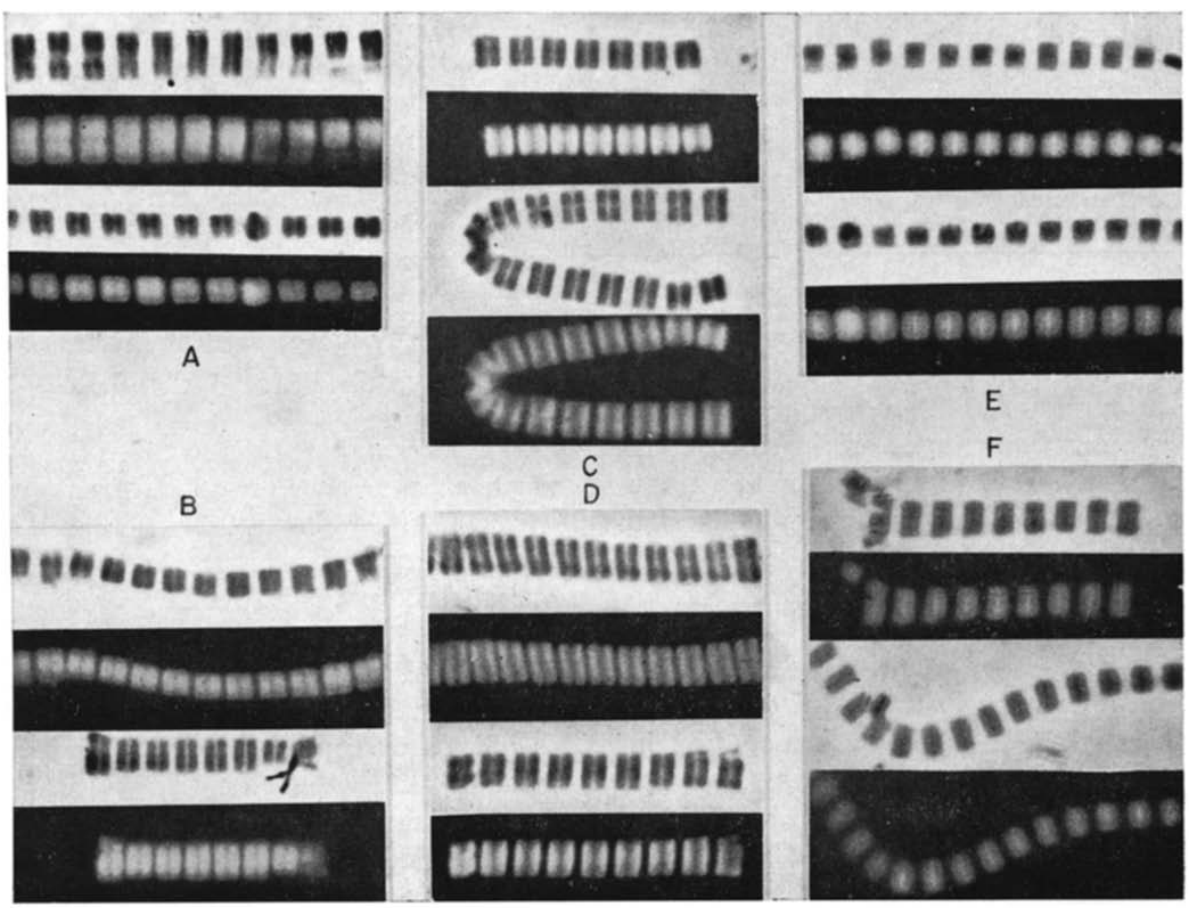

Plate I. Phase and fluorescent photographs of antibody-treated myofibrils. Anti-M treated: A, unextracted; B, extracted. Anti-L treated: C, unextracted; D, extracted. Anti-H treated: E, unextracted; F, extracted.

On extraction, the pattern of anti-M-treated myofibrils did not change much except the total sarcomere length was reduced by about 10%. Since the width of the A-band stayed constant the change occurred mostly in the I-band (Table III).

TABLE III

SARCOMERE LENGTH OF MYOFIBRILS

Same myofibrils as shown in Table II. Numbers in brackets show which of the preparations on Table II were used for the measurements. The measurement was determined with a microscope equipped with phase and fluorescent accessories.

	No. of fibrils	*Sarcomere (μ)*			
		Phase	*Fluorescent*	*Phase*	*Fluorescent*
Control					
Unextracted	9	2.22 ± 0.16		1.54 ± 0.03	
Anti-myosin (5)					
Unextracted	12	2.14 ± 0.12	2.16 ± 0.15	1.57 ± 0.05	1.68 ± 0.04
Extracted	14	2.03 ± 0.09	2.02 ± 0.11	1.60 ± 0.07	1.60 ± 0.07
Anti-LMM (8)					
Unextracted	13	2.08 ± 0.17	2.07 ± 0.14	1.61 ± 0.04	1.67 ± 0.07
Extracted	14	2.00 ± 0.19	1.99 ± 0.19	1.48 ± 0.09	1.49 ± 0.09
Anti-HMM (10)					
Unextracted	16	2.18 ± 0.17	2.18 ± 0.16	1.56 ± 0.03	1.62 ± 0.05
Extracted	17	1.87 ± 0.11	1.85 ± 0.11	1.17 ± 0.08	1.12 ± 0.10
Anti-HMM (11)					
Unextracted	24	2.28 ± 0.19	2.28 ± 0.19	1.54 ± 0.06	1.55 ± 0.07
Extracted	30	2.12 ± 0.12	2.11 ± 0.12	1.31 ± 0.07	1.31 ± 0.08

There is some change in the width of the A-band when anti-L-treated fibrils are extracted. This may be due to a collapse of the central portions. The density in the center of the A-band appears to be reduced, as if material was removed either by extraction or by migrating towards the edges. The clearing up of the center of the A-band is not as drastic as found previously using 0.6 M potassium iodide as solvent[5] and is hard to quantitate. Especially, since the width of the clear central zone depends on the length of the sarcomere, even with unextracted myofibrils in agreement with previous reports[3]. The shortening of the sarcomere is due partly to the shortening of the I-band.

Extraction of anti-H-treated myofibrils leads to a shortening in the sarcomere and a decrease in the width of the A-band. In phase, the M-band and the lateral edges of the A-band appear more prominent. There is some shortening in the I-band also. In experiments where 15% of the myosin and 8% of the bound antibody was extracted, the A-band width was reduced to 1.35 μ. In myofibrils from which 35% of the myosin and 17% of the bound antibody was extracted, the A-band was reduced to 1.17 μ.

The decrease in the A-band may be explained by specific extraction of myosin from the lateral portions of the A-band. There is no particular reason to assume such

a localized extraction and we believe the results are more in agreement by assuming a migration of material within the A-band as a result of extraction procedures. If there is a continuity of the myosin molecules, or myosin-containing structures from the edges to near the center of the A-band at which regions the molecules, or their organization, is fixed, movement may occur to either direction, depending on which of these anchor points are released. Continuity may derive from the stretching of the molecule from the edge to the central regions of the A-band, or the myosin may be

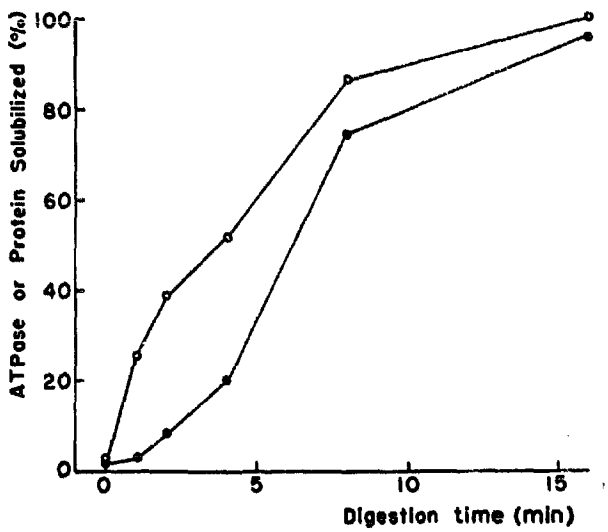

Fig. 3. Solubilization of ATPase and protein at low ionic strength at various time of tryptic digestion. 8400 μg myofibril was digested with 840 μg freshly dissolved 2 × crystallized salt-free trypsin (Worthington) at 23° in 25 ml 0.04 M NaCl and 0.02 M imidazole buffer (pH 7.0). At various times aliquots were taken out, the reaction was stopped with soybean trypsin inhibitor, double the weight of trypsin. ATPase and protein determinations were made on the total sample and on the supernatants of the samples which were centrifuged in 0.04 M NaCl, 0.02 M imidazole (pH 7.0). No inactivation of ATPase took place during the digestion. Abscissa, time of digestion; ordinate, percent of ATPase (solid symbols) and of protein (open symbols) solubilized as expressed in percent of total.

organized in the filaments in such a way that they act as a unit from the edge to the center. Using antibodies, the continuity may be reinforced by the precipitin reaction. The findings are interpreted to mean that extraction under conditions where little myosin is removed from the sarcomere, leads to movement of myosin towards the center of the A-band after anti-H treatment, to movement towards the edges of the A-band after anti-L treatment. However, an increase of concentration of proteins or fluorescence within these portions of the A-band as a result of extraction should be quantitatively demonstrated.

There are other observations pointing to some movement of myosin within the A-band. Controlled digestion of the myofibrils with trypsin at low ionic strength solubilizes significant quantities of myofibrillar proteins before ATPase passes into solution (Fig. 3). Among the solubilized proteins, fragments of myosin may also be present, nevertheless, the ATPase centers of the myosin were retained by the fibrils. At this stage the sarcomere pattern is altered, the Z-band has disappeared, both the H-band and I-band are wide and empty and narrow doublets of denser regions can be seen laterally of the erstwhile H-band. This is the only region which

is stained by anti-M, anti-L and anti-H. Since no ATPase had been removed, this observation is in line of the movement of material into the denser regions.

DISCUSSION

The characteristic staining pattern obtained with the different antibodies may have a number of explanations. (a) The staining reflects accurately the localization of the protein against which the antibody has been prepared, and is due to the particular localization of the antigen involved. (b) The pattern is due to steric hindrance. Steric hindrance may arise from the partial or total inability of the antibody to penetrate the myofibrils, a penetration restricted more at a denser region of the sarcomere. (c) The antigenic sites may be blocked by interacting with other proteins of muscle. Thus, heavy meromyosin may be able to react only when it is not combined with actin, *i.e.*, at those portions of the sarcomere where actin is not present. Such a region would be the H-band which is devoid of actin according to present concepts. (d) The antigens, against which the antibodies have been prepared, could easily contain highly antigenic impurities. The antibodies prepared thus could contain mostly, or partly, antibodies against impurities and not against actin, myosin or meromyosins, and the staining pattern may reflect the localization of these "impurities".

On the basis of the quantitative studies reported here, the following statements can be made with confidence: (a) Antibodies prepared against myosin, light and heavy meromyosin, fix myosin in the myofibril. (b) There is little or no resistance, or steric hindrance to the penetration of the antibody into the resting myofibril. (c) Antigenic sites for anti-L and anti-H are not blocked to an extent to interfere with the fixation of myosin. (d) It is very unlikely that the meromyosins are present in the myofibril in a dissociated form. (e) While it is not necessary that all the staining arises from the presence of myosin, the lack of staining, in conditions when myosin is fixed indicates the lack of myosin, or at least its specific antigenic portions in the sarcomere segments which do not stain. (f) The thick filaments in the A-band, or other myosin-carrying structures, are discontinuous as far as myosin is concerned at the center of the A-band. The lack of staining of the central region with anti-L and the weaker staining against anti-M shows the absence of myosin or absence of those segments of myosin which carry the antigenic sites against these antibodies.

The results suggest not only that the arrangement of myosin in the A-band has a center of symmetry but also that movement of myosin may be achieved towards the center, or towards the edges of the A-band. The conditions for such movement are artificial ones here.

The results described here do not decide whether all the staining observed with anti-H is due to the presence of heavy meromyosin. They only show that anti-H sera contain antibodies which render myosin insoluble. On the basis of evidence presented elsewhere[3] it is likely that the M-line staining with anti-H is due to an unknown antigen. It is possible that by fixing this unknown antigen *in situ*, myosin, in turn, is fixed. Another possibility is that the weaker lateral staining of the A-band with anti-H shows the position of heavy-meromyosin antigen and leads to the fixation of myosin. Resolution of this problem requires further studies.

Little can be said about anti-Ac from these studies, except that the preparation does not contain considerable amounts of antibodies which insolubilize myosin.

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REFERENCES

1 H. HOLTZER, J. M. MARSHALL, Jr. AND H. FINCK, *J. Biophys. Biochem. Cytol.*, 3 (1957) 705.
2 J. M. MARSHALL, Jr., H. HOLTZER, H. FINCK AND F. PEPE, *Exptl. Cell Res. Suppl.*, 7 (1959) 219.
3 B. TUNIK AND H. HOLTZER, *J. Biophys. Biochem. Cytol.*, 11 (1961) 67.
4 H. HOLTZER, in D. RUDNICK, *Molecular and Cellular Synthesis, 19th Growth Symposium*, Ronald Press, New York, 1961, p. 35.
5 A. G. SZENT-GYÖRGYI AND H. HOLTZER, *Biochim. Biophys. Acta*, 41 (1960) 14.
6 J. B. MARTIN AND D. M. DOTY, *Anal. Chem.*, 21 (1949) 965.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 A. SAMUELS, *Arch. Biochem. Biophys.*, 92 (1961) 497.
9 W. HASSELBACH AND G. SCHNEIDER, *Biochem. Z.*, 321 (1951) 462.