Clin. exp. Immunol. (1977) 28, 27–34.

Myosin autoantibodies detected by immunofluorescence

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(*Received* 10 *November* 1976)

SUMMARY

Autoantibodies to striated and smooth muscle myosins were detected by indirect immunofluorescence and confirmed by absorption with purified contractile proteins extracted from human, rabbit and chicken muscle.

Myosin antibodies were rare: of fifty-five sera examined from patients with various skeletal and cardiac muscle disorders, only one serum, from a case of Coxsackie viral pericarditis, had anti-myosin activity. It reacted with cardiac muscle and type 1 fibres of skeletal muscle, staining the 'A' band of the sarcomere only. The antibody was absorbed by skeletal myosin and by skeletal heavy meromyosin fragments, but not by smooth muscle myosin.

Two types of smooth muscle myosin autoantibodies are described. One is restricted to smooth muscle myosin and examples were found in polyclonal and monoclonal SMA sera. The second type of smooth muscle myosin antibody cross-reacted with skeletal and cardiac muscle and with cytoplasmic myosin in liver, kidney and thyroid cells. It was completely absorbed using either smooth or skeletal myosin and by heavy meromyosin fragments.

The different types of myosin autoantibodies reflect the variety of myosins found in mammalian tissues. Cross-reacting myosin antibodies indicate epitopes on the heavy meromyosin fragment which are common to several different tissue myosins.

INTRODUCTION

Myosin and actin are the two major contractile proteins found in all mammalian cells capable of independent movement. Human autoantibodies to actin were first detected over 10 years ago when Johnson, Holborow & Glynn (1965) described the immunofluorescent staining of smooth muscle by sera from patients with chronic active hepatitis. Identification of the antigen was only later confirmed by absorption of these sera with F-actin (Gabbiani *et al.*, 1973; Lidman *et al.*, 1976; Bottazzo *et al.*, 1976). Actin of similar or identical structure and composition occurs in many tissues and this explains why the antibodies react with the renal glomeruli and tubules, with hepatocytes, with smooth, skeletal and cardiac muscle, and with cardiac conducting tissue (Fairfax & Doniach, 1976).

This study was initiated to see whether autoantibodies occur to myosin. Myosins form a class of filamentous proteins which have in common the ability to bind reversibly with actin, and to catalyse the breakdown of ATP during muscle contraction. The myosins from various muscular and non-muscular tissues are all different. Purified myosin has been extracted from smooth and striated muscle (Pollard & Weihing, 1974). This study was therefore performed using smooth and striated muscle for indirect immunofluorescence to detect myosin antibodies: the results were confirmed by absorption of the sera with myosin and other contractile proteins.

MATERIALS AND METHODS

Patient's sera. Fifty-five sera from patients with skeletal or cardiac muscle disorders, including myasthenia gravis, myocardial infarction and Dressler's syndrome, were tested for antibodies to myosin by immunofluorescence. One hundred and

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sixteen sera with polyclonal smooth muscle titres of 1/40 or more were also studied. These had been selected from a large number tested for SMA and were mainly derived from patients with liver disorders. One hundred and thirteen of these had been classified by their appearance on rat kidney as SMA-G and SMA-T, as described by Bottazzo *et al.* (1976) since they also stained glomeruli and tubules, and three were classified as SMA-V since they reacted with vessel walls only. Four monoclonal sera, obtained from patients with myeloma and Waldenström's macroglobulinaemia which had been shown to react with smooth muscle giving a SMA-V pattern on kidney were tested for antimyosin activity by absorption. All the sera were tested fresh, or were stored at −20°C for up to 5 years. The fluorescence patterns were compared with those given by a rabbit antiserum to chicken gizzard myosin (smooth muscle) described elsewhere (Gröschel-Stewart *et al.*, 1976).

Serological methods. Unfixed 5 μM cryostat sections were prepared from rat liver and kidney, human thyrotoxic thyroid gland and from striated muscle from rat gastrocnemius, soleus, diaphragm and heart, and from ox skeletal and cardiac muscle and ox Purkinje tissue. The tissues were obtained within 4 hr of death, snap-frozen in isopentane, and stored at −70°C. The standard sandwich technique was employed with FITC-conjugated antihuman Fab and monospecific conjugates of antisera to the three main immunoglobulin classes. The sera were tested at 1/10 dilution and titrated to endpoint. Fluorescein-conjugated goat antirabbit IgG was used to study the immunofluorescence given by the rabbit antimyosin serum on tissue sections.

The identification of the different fibre types in skeletal muscle was made using the myosin ATPase reaction at pH 9·4 (Padykula & Herman, 1955) on serial skeletal muscle sections. Stretched, glycerinated rabbit thigh muscle, stored at −20°C for at least 3 weeks prior to use after the method of Rome (1967), was minced and homogenized to obtain a preparation of myofibrils. These were washed twice in Coons' buffer and stained by indirect immunofluorescence in suspension to identify A-band staining. A Leitz Ortholux microscope was used to view the same portion of the sarcomere alternately by phase contrast and fluorescence with epi-illumination without moving the microscope stage.

Muscle protein extraction. Skeletal muscle myosin from human leg muscle, and smooth muscle myosin obtained from human uterus and from chicken gizzard were used to absorb the sera. Skeletal muscle myosin was prepared and purified according to the method of Richards *et al.* (1967). The smooth muscle myosins were purified by ammonium sulphate precipitation and chromatography on Sepharose 4 B(Gröschel-Stewart *et al.*, 1976). Tropomyosin was extracted from acetone-dried human muscle and purified to homogeneity by ammonium sulphate precipitation. F actin was prepared by standard methods from chicken gizzard (Spudich & Watt, 1971). The purified fractions were stored at −20°C in 50% glycerol and were dialysed free of glycerol prior to use.

The absorption experiments were all repeated using purified contractile proteins prepared from rabbit skeletal muscle kindly donated by Dr G. Offer. Column purified myosin in 50% glycerol and phosphate buffer (5 mg/ml) and freshly-prepared C-protein in phosphate buffer (1·8 mg/ml) were prepared as described by Offer, Moos & Starr (1973). Antibodies which were absorbed by skeletal myosin were subsequently tested for absorption with heavy meromyosin (HMM) and light meromyosin (LMM) fragments produced by tryptic digestion of purified rabbit skeletal myosin. The crude fractions used were preserved in 50% glycerol and 0·05 M KCl (10 mg/ml).

Absorption of sera. Sera were diluted according to titre (Tables 1–3) in Coons' buffer. 0·06 ml of the diluted serum was incubated with an equal volume of protein solution starting with an excess (5 mg/ml) and then decreasing concentrations of

TABLE 1. Absorption of skeletal myosin autoantibody using purified human muscle myosins

Serum DRA (IgG-titre 1/80): 0·06 ml serum at 1/10 dilution		
Quantity of myosin added (mg)	IFL on skeletal muscle (A bands) after absorption	
	Smooth muscle myosin	Skeletal myosin
0·5	++	−
0·25	++	−
0·125	++	−
0·063	++	−
0·031	++	−
0·016	++	−
0·008	++	+
0·004	++	++

antigen. The mixtures were incubated with constant shaking for 1 hr at 37°C and then for 24 hr at 4°C. After centrifugation at 20,000 *g* for 30 min at 4°C, the supernatant was tested by immunofluorescence on the composite blocks. Controls were prepared from identically treated sera diluted with Coons' buffer.

TABLE 2. Absorption of broad-reacting myosin autoantibody with human muscle myosins

Serum WEN. (IgG-titre 1/320): 0·06 ml serum at 1/20 dilution		
Quantity of myosin added (mg)	IFL on kidney and liver cytoplasm, smooth muscle and skeletal muscle after absorption	
	Smooth muscle myosin	Skeletal myosin
0·5	−	−
0·25	−	−
0·125	−	+
0·063	+	+++
0·031	+	+++
0·016	+	+++
0·008	++	+++
0 004	+++	+++

TABLE 3. Absorption of monoclonal smooth muscle antibody with human muscle myosins

Serum BERN (IgM-K-titre 1/10,000): 0·06 ml serum at 1/1200 dilution		
Quantity of myosin added (mg)	IFL on kidney vessels (smooth muscle) after absorption	
	Smooth muscle myosin	Skeletal myosin
0·5	−	++
0·25	−	++
0·125	−	++
0·063	−	++
0·031	−	++
0·016	−	++
0·008	+	++
0·004	++	++

RESULTS

Autoantibodies to skeletal and cardiac myosin

Two sera from the fifty-five patients with muscle disorders tested stained type 1 fibres of skeletal muscle selectively giving a 'red-zebra' pattern (Fig. 1) as originally described by Feltkamp and Feltkamp-Vroom (1965). Both gave a titre of 1/80 using anti-IgG conjugate. Neither sera stained smooth muscle, kidney, liver or thyroid. Only one of these sera reacted equally strongly with cardiac and ox Purkinje tissue and on longitudinal sections of striated muscle this gave a striated appearance. This serum (DRA) was shown to react with the 'A' band of the sarcomere of isolated myofibrils. Absorption was performed

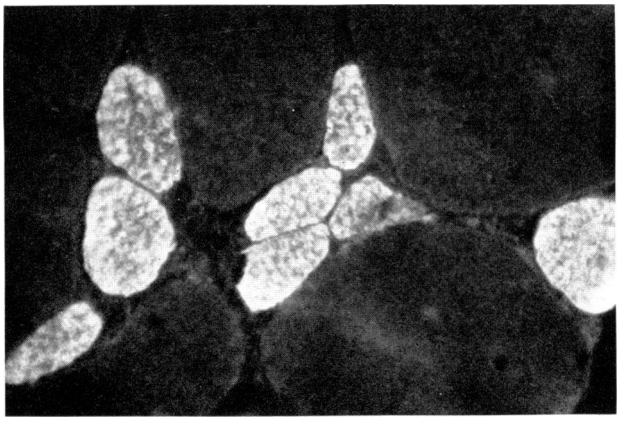

FIG. 1. Autoantibody to striated muscle myosin (serum DRA) showing selective staining of type 1 fibres of skeletal muscle, but no staining of vessel walls.

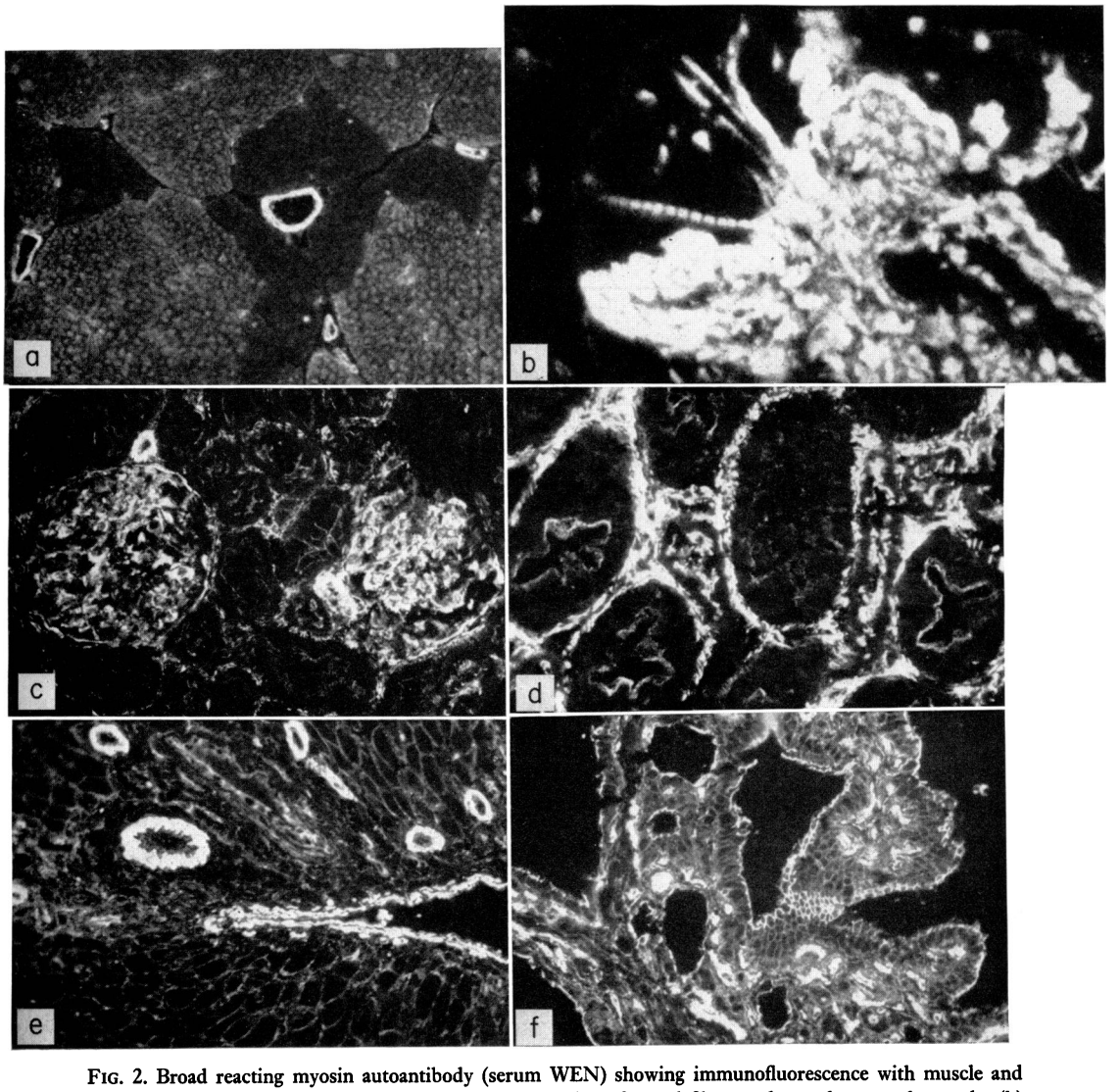

FIG. 2. Broad reacting myosin autoantibody (serum WEN) showing immunofluorescence with muscle and cytoplasmic myosins: (a) rat soleus muscle showing staining of type 1 fibres and vascular smooth muscle; (b) ox conducting tissue showing 'A' band immunofluorescence of the myofibrils. The cytoplasm is unstained; (c) rat kidney showing staining of the blood vessels, glomeruli and proximal tubules; (d) rat kidney medulla with strong positive staining of myosin filaments around the distal tubules and weaker IFL at the luminal edge; (e) polygonal staining of rat liver hepatocytes; (f) human thyrotoxic showing a pericellular immunofluorescence pattern.

with myosin from smooth and skeletal muscle and with actin, tropomyosin and C-protein: the antibody was completely absorbed by myosin from human and rabbit skeletal muscle (Table 1) but the titre was unchanged after absorption with smooth muscle myosin and with the other muscle proteins. The antibody was completely absorbed by heavy meromyosin from skeletal muscle but not absorbed by light meromyosin. The antibody had been detected in a 62-year-old man with a Coxsackie B_4 viral pleuropericarditis. Cardiac damage was evident from raised LDH enzyme levels, electrocardiographic abnormalities and radiological cardiomegaly. After recovery the patient was lost to follow-up, but a blood sample obtained five years later was negative by immunofluorescence.

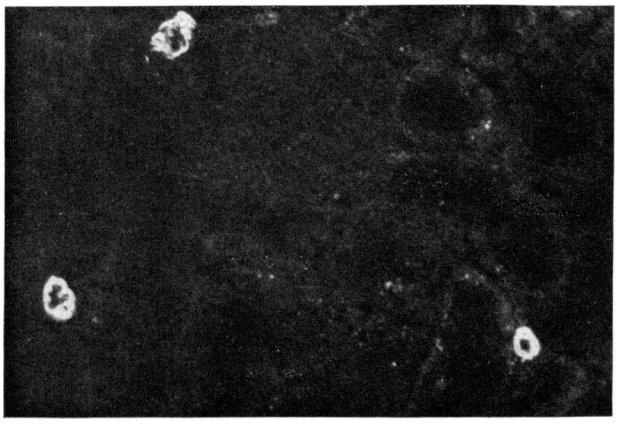

FIG. 3. Anti-smooth muscle myosin (serum BERN) on rat kidney section showing staining of vessel walls only (SMA-V pattern).

Smooth muscle antibodies to myosin

One hundred and sixteen SMA positive polyclonal sera were tested on transversely-cut skeletal muscle sections: two sera gave selective staining of type 1 fibres as described above, one weakly at 1/10, and one (Serum WEN) to a titre of 1/160 with-IgG on skeletal muscle and 1/320 on smooth muscle (Fig. 2a). This latter serum was from a 68-year-old man with bronchiectasis. The serum stained the myofibrils of type 1 fibres of skeletal muscle, the myocardium and Purkinje myofibrils (Fig. 2b). 'A'- band staining was seen on rabbit isolated myofibrils. On rat kidney the smooth muscle, glomeruli and scattered clumps of filaments in the proximal and distal tubules (Figs. 2c and d) gave a strong positive reaction. In the distal tubule the peripheral fibres appeared thicker than those seen with anti-actin sera. Polygonal staining was seen on rat hepatocytes (Fig. 2e). Peripheral staining also occurred on human thyroid epithelium (Fig. 2f). Complete absorption of all these staining patterns occurred with either smooth muscle myosin or skeletal muscle myosin and with HMM fragments but not with LMM from rabbit skeletal muscle (Table 2). It was therefore concluded that this antibody was a broad-reacting antibody to the head end of the myosin molecule.

Three polyclonal SMA sera which stained only smooth muscle (SMA-V) were tested for absorption with human uterine smooth muscle proteins. One serum (OB) was absorbed by smooth muscle myosin. No absorption was demonstrated with actin, tropomyosin or with skeletal muscle myosin.

One of four monoclonal sera (Bern) with SMA fluorescence (Fig. 3) was absorbed out by the muscle proteins available: complete absorption was demonstrated using uterine smooth muscle myosin (Table 3). Details of this case, an elderly man with Waldenström's macroglobulinaemia and recurrent chest infections, are given in the paper of Wager *et al.* (1971).

A summary of the immunofluorescent patterns and absorption results is given in Table 4. In comparison to the human autoantibodies, rabbit anti-chicken gizzard myosin antiserum gave no reaction with cardiac or striated muscle. It gave a type of SMA-T pattern on rat kidney staining the glomeruli and tubules, and polygonal staining on rat liver. There was thus some cross reactivity between this heterologous antiserum to smooth muscle myosin and the cytoplasmic myosins in liver and kidney.

TABLE 4. Comparison of immunofluorescence and absorption results between human myosin autoantibodies and rabbit anti-smooth muscle myosin serum

	Immunofluorescence on:					Absorbed with:				
Sera tested	Skeletal muscle (type I fibres)	Cardiac muscle myofibrils	Purkinje tissue myofibrils	Smooth muscle	Cytoplasmic myosin staining	Skeletal muscle myosin	Smooth muscle myosin	Actin	Tropomyosin	Antibody specificity
DRA	+	+	+	−	−	+	−	−	−	Type I skeletal muscle and cardiac myosin
WEN	+	+	+	+	+	+	+	−	−	Broad-reacting anti-myosin
BERN	−	−	−	+	−	−	+	−	−	Smooth muscle myosin
Rabbit chicken gizzard myosin	−	−	−	+	+	−	+	−	−	Smooth muscle and cytoplasmic myosin

DISCUSSION

Myosins in mammalian tissues exist as a diverse class of filamentous proteins which have in common both enzyme activity and the ability to bind reversibly with actin filaments. The myosin molecule consists of an alpha helical rod attached to two globular heads which contain the ATPase and the actin binding sites. The molecule can be split transversely across the rod body by tryptic digestion to form heavy and light meromyosin fragments (Lowey *et al.*, 1969), in an analogous manner to the peptic digestion of immunoglobulin. The myosin molecule consists of two symmetrical halves along its long axis (Margossian & Lowey, 1973). In contrast to actin, muscle and cytoplasmic myosins vary considerably in their physical, chemical and enzymatic properties (Pollard & Weihing, 1974). It is therefore not surprising that a variety of autoantibodies to myosin can occur.

Antisera raised in animals by immunization with purified contractile proteins have been used to investigate the ultrastructure of the myofibril. 'A'-band staining of the sarcomere by antimyosin antibodies was demonstrated by fluorescence and the accessibility of the myosin antigenic sites was shown to be partly dependent on the sarcomere length and state of contraction (Pepe, 1966). Antibodies have been raised to whole myosin, and to its sub-units meromyosin, S-1, rod and light chains (Lowey & Steiner, 1972).

Cardiac, slow skeletal smooth muscle and some cytoplasmic myosins are similar in that they consist of two heavy chains (mol. wt around 200,000 daltons) and two light chains. Limited amino acid sequencing has indicated that cardiac and skeletal myosin heavy chains are similar but not identical (Huszar & Elzinga, 1972). Major differences occur in the light chains of myosins from different tissues. Myosin from fast skeletal muscle contains three different light chains (Weeds & Lowey, 1971) and these are antigenically distinguishable when separated. Some of the antigenic sites exposed on the free chains, however, are hidden in the undissociated molecule (Horváth & Gaetjens, 1972).

Earlier studies by immunofluorescence using rabbit antisera to myosin from human pectoral muscle (Gröschel-Stewart & Doniach, 1969) showed that this antibody reacted with type I fibres of skeletal muscle and with heart muscle but not with type II fibres. Thus a 'zebra' type of staining pattern was produced on sections of skeletal muscle cut longitudinally which contained both fibre types. The antibody specificity of this myosin antiserum is thought to reside in the heavy chains of myosin (Arndt & Pepe, 1975). Zebra staining of skeletal muscle by immunofluorescence has been reported with some sera

from patients with rheumatoid arthritis, myasthenia gravis and various endocrine disorders, but the antigens had not previously been identified (Feltkamp & Feltkamp-Vroom, 1965).

Heterologous antisera to skeletal and smooth muscle myosins do not cross-react by immunofluorescence. The rabbit anti-chicken gizzard myosin studied in this paper stained renal glomeruli and tubules, and hepatocytes in a polygonal pattern but not skeletal muscle. Antisera to smooth muscle myosin are known to cross react weakly with the myosin filaments present in fibroblasts (Gröschel-Stewart *et al.*, 1975).

The identification of myosin autoantibodies in this study is based on the immunofluorescence patterns and on absorption with pure myosin preparations. Absorption experiments may be criticized on the basis that minor contaminants in excess antigen may be responsible. Relatively large amounts of myosin were needed for the polyclonal sera (equivalent to 3–40 mg myosin/ml of neat serum) to completely quench the IFL. Large quantities of actin are known to be needed to absorb out polyclonal anti-actin sera (Lidman *et al.*, 1976). No absorption of the anti-myosin sera occurred with actin or tropomyosin. The 'A'-band of skeletal muscle contains small amounts of 'C' protein (Offer, Moos & Starr, 1973) but absorption with an excess of pure 'C' protein did not alter the titre of the antimyosin antibodies. Absorption is necessary to confirm the antigen responsible for the IFL pattern since it has been shown that not all SMA-V sera are anti-myosin and other muscle antibodies can produce a zebra pattern.

Myosin autoantibodies are rare and so far no correlation is possible with human disease. Anti-myosin (serum DRA) occurring during a Coxsackie pericarditis presumably reflected cardiac damage by the virus. Muscle damage alone, however, which frequently occurs in everyday life, e.g. intramuscular injections including adjuvants such as pertussis apparently does not lead to the formation of detectable autoantibodies to myosin. In laboratory animals it is relatively easy to raise high-titre antisera to myosin but very difficult to produce satisfactory antibodies to actin despite multiple injections and the use of adjuvants. In man, chronic active hepatitis is frequently accompanied by autoantibodies to actin but not to myosin although both actin and myosin filaments lie just beneath the hepatocyte membrane. Clearly, cell breakdown and release of antigens is insufficient to explain these phenomena. Damaged cardiac muscle has been shown to take up antimyosin antibody when the sarcolemma is disrupted (Khaw *et al.*, 1976), and this may be one of the reasons why myosin antibodies are so rarely found in human sera.

We wish to thank Dr G. Offer for the gift of rabbit myosin and C protein samples; Professors M. Turner-Warwick, O. Wager and T. E. W. Feltkamp for patients' sera; Drs G. F. Bottazzo and C. D. Lambert for materials; Professor D. Doniach for advice and encouragement and Miss H. Fischler for help with the manuscript. A. J. F. was supported by a grant from the Wellcome Foundation and U.G.S. by the Deutsche Forschungsgemeinschaft.

REFERENCES

ARNDT, I. & PEPE, F.A. (1975) Antigenic specificity of red and white muscle myosin. *J. Histochem. Cytochem.* **23**, 159.

BOTTAZZO, G.F., CHRISTENSEN, A.F., FAIRFAX, A.J., SWANA, G., DONIACH, D. & GRÖSCHEL-STEWART, U. (1976) Classification of smooth muscle autoantibodies detected by immunofluorescence. *J. clin. Path.* **29**, 403.

FAIRFAX, A.J. & DONIACH, D. (1976) Autoantibodies to cardiac conducting tissue and their characterization by immunofluorescence. *Clin. exp. Immunol.* **23**, 1.

FELTKAMP, T.E.W. & FELTKAMP-VROOM, T.M. (1965) Antibodies against the various types of skeletal muscle fibres. *Immunology*, **9**, 275.

GABBIANI, G., RYAN, G.B., LAMELIN, J.P., VASSALLI, P., MAJNO, G., BOUVIER, C.A., CRUCHAUD, A. & LÜSCHER, E.F. (1973) Human smooth muscle antibody. Its identification as antiactin antibody and a study of its binding to nonmuscle cells. *Amer. J. Pathol.* **72**, 473.

GRÖSCHEL-STEWART, U. & DONIACH, D. (1969) Immunological evidence for human myosin isoenzymes. *Immunology*, **17**, 991.

GRÖSCHEL-STEWART, U., CHAMLEY, J.H., MCCONNELL, J.D. & BURNSTOCK, G. (1975) Comparison of the reaction of cultured smooth and cardiac muscle cells and fibroblasts to specific antibodies to myosin. *Histochemistry*, **43**, 215.

GRÖSCHEL-STEWART, U., SCHREIBER, J., MAHLMEISTER, C. & WEBER, K. (1976) Production of specific antibodies to contractile proteins, and their use in immunofluorescence microscopy. I. Antibodies to smooth and striated chicken muscle myosins. *Histochemistry*, **46**, 229.

HORVÁTH, B.Z. & GAETJENS, E. (1972) Immunochemical studies on the light chains from skeletal muscle myosin. *Biochim. biophys. Acta (Amst.)*, **263**, 779.

HUSZAR, G. & ELZINGA, M. (1972) Homologous methylated and nonmethylated histidine peptides in skeletal and cardiac myosins. *J. biol. Chem.* **247**, 745.

JOHNSON, G.D., HOLBOROW, E.J. & GLYNN, L.E. (1965) Antibody to smooth muscle in patients with liver disease. *Lancet*, ii, 878.

KHAW, B.A., BELLER, G.A., HABER, E. & SMITH, T.W. (1976) Localization of cardiac myosin-specific antibody in myocardial infarction. *J. clin. Invest.* **58**, 439.

LIDMAN, K., BIBERFELD, G., FAGRAEUS, A., NORBERG, R.,

TORSTENSSON, R., UTTER, G., CARLSSON, L., LUCA, J. & LINDBERG, U. (1976) Anti-actin specificity of human smooth muscle antibodies in chronic active hepatitis. *Clin. exp. Immunol.* **24**, 266.

LOWEY, S., SLAYTER, H.S., WEEDS, A.G. & BAKER, H. (1969) Substructure of the myosin molecule. I. Subfragments of myosin by enzymic degradation. *J. mol. Biol.* **42**, 1.

LOWEY, S. & STEINER, L.A. (1972) An immunochemical approach to the structure of myosin and the thick filament. *J. mol. Biol.* **65**, 111.

MARGOSSIAN, S.S. & LOWEY, S. (1973) Substructure of the myosin molecule. III. Preparation of single-headed derivatives of myosin. *J. mol. Biol.* **74**, 301.

OFFER, G., MOOS, C. & STARR, R. (1973) A new protein of the thick filaments of vertebrate skeletal myofibrils. Extraction, purification and characterisation. *J. mol. Biol.* **74**, 653.

PADYKULA, H.A. & HERMAN, E. (1955) The specificity of the histochemical method for adenosine triphosphatase. *J. histochem. Cytochem.* **3**, 170.

PEPE, F.A. (1966). Some aspects of the structural organization of the myofibril as revealed by antibody-staining methods. *J. cell. Biol.* **28**, 505.

POLLARD, T.D. & WEIHING, R.R. (1974) Actin and myosin and cell movement. *CRC Critical Reviews in Biochemistry*, **2**, 1.

RICHARDS, E.G., CHUNG, C.S., MENZEL, D.B., & OLCOTT, H.S. (1967) Chromatography of myosin on DEAE-Sephadex A-50. *Biochem. (Wash.)*, **6**, 528.

ROME, E. (1967) Light and X-ray diffraction studies of the filament lattice of glycerol-extracted rabbit psoas muscle. *J. mol. Biol.* **27**, 591.

SPUDICH, J.A. & WATT, S. (1971) The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. biol. Chem.* **246**, 4866.

WAGER, O., RÄSÄNEN, J.A., HALTIA, K. & WASASTJERNA, C. (1971) 'M' components with antibody activity: anti-smooth muscle, antithyroglobulin and anti-streptolysin-O activity in five M component sera. *Ann. clin. Res.* **3**, 86.

WEEDS, A.G. & LOWEY, S. (1971) Substructure of the myosin molecule. II. The light chains of myosin. *J. mol. Biol.* **61**, 701.