1. Robinson, L. B., Wichelhausen, R. H., and Brown, T. McP., *J. Lab. and Clin. Med.*, 1952, v39, 290.

2. Melen, B., *Acta Path. et Microbiol. Scandinav.*, 1952, v30, 98.

3. Sharp, J. T., Hijmans, W., Dienes, L., *J. Exp. Med.*, 1957, v105, 153.

4. Dienes, L., Weinberger, H. J., *Bact. Rev.*, 1951, v15, 245.

5. Park, J. T., Strominger, J. L., *Science*, 1957, v125, 99.

6. Lederberg, J., *J. Bact.*, 1957, v73, 144.

7. Dienes, L., Weinberger, H. J., Madoff, S., *ibid.*, 1950, v59, 755.

Received October 19, 1957. P.S.E.B.M., 1958, v97.

Demonstration of Myosin in Human Striated Muscle by Fluorescent Antibody. (23668)

I. KLATZO, B. HORVATH, AND E. W. EMMART

Nat. Inst. of Neurol. Diseases and Blindness and Nat. Inst. of Arthritis and Metabolic Diseases, N.I.H., U. S. Department of Health, Bethesda, Md.

The antigenic property of myosin provides an opportunity to study this protein using immunological technics. Kesztyüs, Nikodemusz and Szilagyi demonstrated that myosin prepared from rabbit skeletal muscle was antigenic in dogs(1). Ebert has shown that chicken cardiac myosin produced antibodies in rabbits and that by the precipitin reaction it was possible to correlate the time of appearance of cross striations of the heart muscle with the presence of myosin(2). More recently Finck, Holtzer and Marshall(3) applied Coons' fluorescent antibody technic for localization of myosin in the chick muscle. This technic depends on selective binding of specific, fluorescent antibody to antigen present in tissue sections. The site of localization of antigenic substances within the morphological structures can be then demonstrated using fluorescence microscopy.

Our investigation represents a study of distribution of myosin in striated muscle by fluorescent antibody technic. It is the purpose of these studies on the localization of myosin in normal muscle to establish a basis of comparison for further study on the behaviour of myosin in diseased fibers and thus bring some insight into the problem of neuromuscular disorders.

Materials and methods. Preparation of antigen: Myosin was prepared from muscle of rabbit and goat and from human muscle, obtained on autopsy performed less than 2 hours post mortem. The muscle (*M. psoas* in most cases) was kept in the deep freeze until needed, *i.e.* for a period from several days to several weeks. The preparation followed closely the procedure of Szent-Gyorgyi(4). According to this method, the muscle is extracted with 0.6 M potassium iodide (KI) containing sodium thiosulphate after preliminary washing with water and with .05 M KCl to remove the muscle's adenosine triphosphate (ATP). The myosin was precipitated by diluting the extract with water to a KI concentration of .025 M. The precipitate was washed with .025 M NaCl and dissolved by adding NaCl to .5 M. The myosin was purified by 3 precipitations, and was dissolved in .5 M NaCl. Heavy impurities were removed by ultracentrifugation for 1 hour at 39,460 rpm. The myosin solution was passed through a Seitz filter before using it in precipitin reactions. The purity of the myosin preparations was ascertained in the ultracentrifuge. All operations were carried out in the cold. In addition, actin content was estimated by the method of Szent-Gyorgyi(4) from the change of the logarithm of the relative viscosity on addition of ATP. Most preparations contained less than 5% proteins other than myosin. Some myosin preparations were stored at −20°C in presence of .5 M NaCl and 50% glycerol. For immunizing rabbits and for precipitin reaction the glycerol was removed and the myosin precipitated by dilution or

TABLE I. Method of Assay of Anti-Human Myosin Rabbit Sera.

Tube	1	2	3	4	5	6	7	8	9
(A) Anti-myosin rabbit serum									
Dilution of serum	0	0	0	1:2	1:4	1:8	1:16	1:32	1:64
ml of antiserum transferred		.5	.5	.5	.5	.5	.5	.5	.5
ml NaCl—0.5M	.5	.5		.5	.5	.5	.5	.5	.5
Myosin (1 ml 10 mg)	.5		.5	.5	.5	.5	.5	.5	.5
Precipitation after 24 hr—5°C (valued 0-4.0)			3.	2.	1.5	1.			
mg of N/ml (Kjeldahl) of myosin, serum & washed precipitates	.79*	5.69†	.99	.69	.29	.22	.16	.18	.16
(B) Control normal serum—no precipitation									
mg of N/ml	.84	4.38	5.66	3.26	2.21	1.51	1.19	1.03	1.03
Cal. nitrogen			5.22	3.03	1.93	1.38	1.11	.97	.90

* Myosin. † Serum.

dialysis. Such preparations proved also to be as satisfactory for immunizing rabbits and for precipitin reactions as fresh ones. *Assay of rabbit antibody to human myosin*: Antibody to human myosin was developed in 3 rabbits by intravenous injections of 8-10 mg of myosin in 0.5 M NaCl. Injections were given on alternate days for a period of 2 weeks. Five series of 6 injections each were administered allowing a period of rest between each series. The animals were bled from time to time, the serum removed, assayed for antimyosin antibody and the results compared with that of normal serum. The sera from 3 rabbits injected with myosin were assayed in 2-fold serial dilution from zero to 256 fold. Aliquots of 0.5 ml were transferred successively to tubes to which were added 2.5 mg of the antigen in 0.5 M NaCl solution. The degree of precipitation of the antigen-antibody reaction was evaluated from 0 to 4 and recorded immediately after preparation. The tubes were inverted several times and placed in the ice box at 5°C overnight. After 20 minutes at room temperature the amount of precipitate was again evaluated and recorded as the final reading. The procedure is shown in Table I for one lot of serum. In this assay dilution of antiserum was limited to 1:64 because differences in nitrogen below this dilution could not be determined. The reliability of this type of estimation of precipitation values was verified in some experiments by nitrogen determinations of washed precipitates and these values are compared with nitrogen of the control tubes containing no precipitates. Table I shows mg of nitrogen/ml (Kjeldahl) of twice washed precipitates and the estimated values obtained by visual observations of the density of the precipitates. For comparison a similar series of tubes using normal serum were prepared concurrently. These gave negative results. In addition, the nitrogen values of the aliquots of the serum sample and of the antigen aliquot were also obtained. In the assay of normal control serum no precipitation occurred and the values of the nitrogen per ml would be expected to represent the value of antigen plus that of normal serum aliquot. The actual nitrogen values obtained from this series conform closely to the calculated values (Table I). These data lend support to the method of evaluating the titre of the sera by the observed density of the precipitates. A tube containing serum and saline but no antigen, and another tube containing myosin and diluent were added to each series as controls. The slight cloudiness of these tubes could readily be distinguished from the antigen-antibody precipitates. The highest concentration of myosin which would readily produce precipitation without inhibition of the antibody was adopted for the assay. Fig. 1 shows this to be between 1.25 and .625 mg for the sera of all 3 immunized rabbits. The rise in the titre of antibody to human myosin following intravenous administration (approximately 10 mg per dose) in the rabbit was determined by assay of the antisera in serial dilution for a period of over 6 months. Fig. 2 shows the relationship of this rise to the periods of successive inocula-

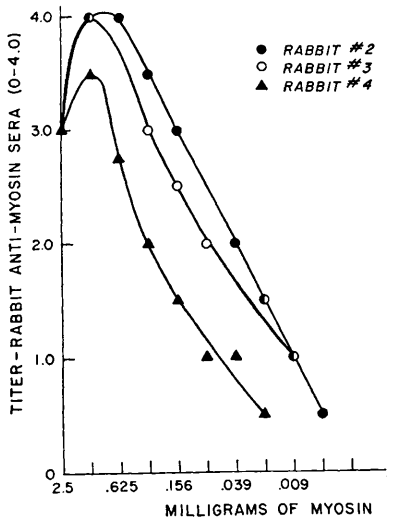

FIG. 1. Determination of equivalent zone between antigen and antibody showing the concentration of antigen (1.25 to 0.625 mg) required to give maximum precipitation with anti-human myosin sera from 3 rabbits.

tions, the rapid fall in titre with an extensive rest period of 2 months and an anamnestic rise in antibody titre with resumption of inoculations. A sample of anti-human myosin rabbit serum was mixed in serial 2-fold dilutions with serial dilutions of 3 myosin preparations derived from human, rabbit and goat muscle respectively. It was found that myosin of all 3 species reacted equally with antiserum of human myosin, as judged by the amount of precipitate in comparable tubes. The end points were also the same for all 3 myosin preparations tested. It will be noted that the species studied belong to 3 different orders of the mammals.

Preparation of fluorescent globulin conjugates: Following the determination of the titre of the rabbit antihuman myosin sera the globulin fractions were removed, pooled and conjugated to fluorescein isocyanate as described by Coons and Kaplan(5). The conjugated fluorescent globulin solution was dialyzed at 5°C against 0.15 M NaCl buffer, pH 7.4 to remove excess fluorescein. It was further purified by 2 ammonium sulfate fractionations at 5°C. After dialysis the fluorescent conjugate was further purified by ethanolic fractionation at −20°C. The precipitate was centrifuged down, the supernatant removed and the precipitate taken up in sufficient buffer to bring the volume to one-half the original globulin volume. *Preparation of muscle tissue*: The muscle tissue studied was derived mainly from human biopsy material. In addition, muscle from cat, rabbit, rat and mouse was used. The majority of muscle preparations were made by gentle teasing of the fibers after washing in 50% glycerol for a period of time, from several hours to several days. Other preparations were frozen in isopentane chilled with liquid nitrogen to −150°C, and sectioned at 8-10 mμ in a cryostat at −18°C. Human biopsy material in which pathological changes were suspected was in some instances fixed in chilled 10% formalin and embedded in diglycol stearate for sectioning. All preparations were treated with fluorescent anti-human myosin rabbit globulin solution for 20 minutes. The excess of fluorescent antibody solution was washed away with 5-6 changes of cold buffered saline solution. The preparations were mounted in 25% glycerol and examined and photographed under the fluorescence microscope. Study of the preparations in ultraviolet light was supplemented by observations in phase contrast and polarized light. In addition, some sections, particularly the pathological material, were stained by routine histological methods. As controls for the specificity of the fluorescent antibody staining, duplicate preparations were treated with fluorescent normal globulin solution. Also as an additional control, muscle sections were treated

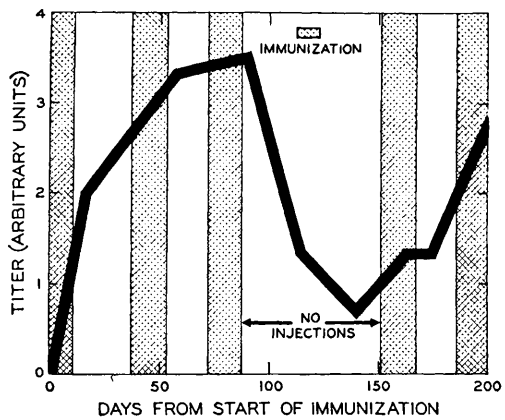

FIG. 2. Titer of antisera in relation to administration of myosin. Shaded areas—series of injections.

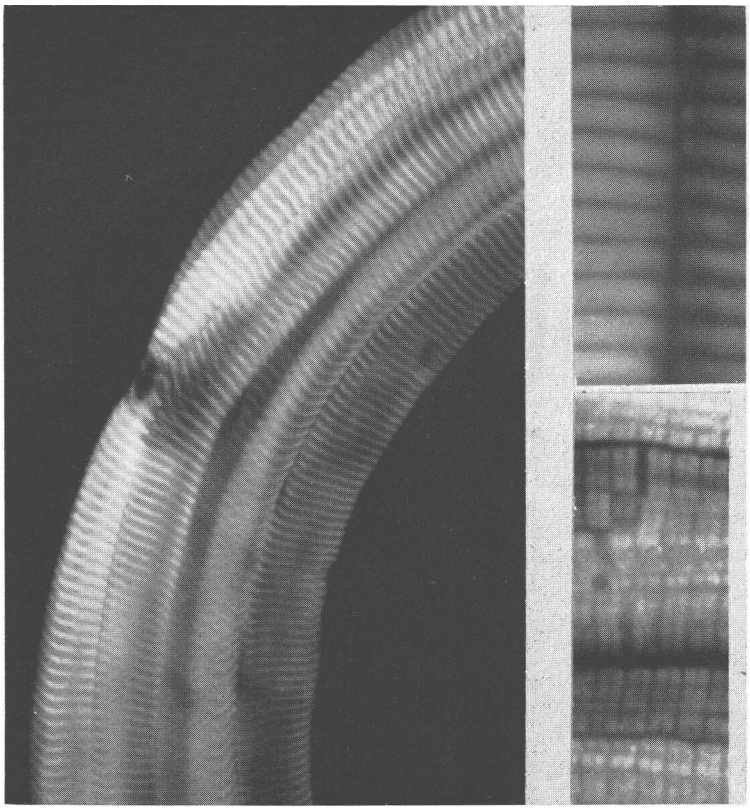

FIG. 3 (left). Human skeletal muscle. Teased preparation showing fluorescence of A bands; nuclei remain unstained. × 240.
FIG. 4 (upper right). Human skeletal muscle. A band is clearly visible. × 1250.
FIG. 5 (lower right). Human heart muscle. Intercalated discs remain unstained. × 1250.

with a preparation of fluorescent antistreptococcal hyaluronidase globulin which was available from other studies(6).

Results. In the preparations stained by incubation with fluorescent normal globulin and anti-hyaluronidase globulin the muscle fibers showed very faint, diffuse, non-specific fluorescence.

Normal human skeletal muscle and muscle of cat, rabbit, mouse and rat stained with labelled anti-human myosin antibody revealed brightly fluorescent cross bands, which were identified in phase contrast and in polarized light to be A bands (Fig. 3). The sarcolemmal nuclei always remained unstained; on the other hand, the sheaths occasionally showed some fluorescence, presumably due to non-specific absorption or from failure to wash away the excess of the conjugate. The intensity of the fluorescence was strongest in the teased preparations (Fig. 4). In the heart muscle, obtained from an autopsy case, the intercalated discs remained unstained (Fig. 5).

In muscle prepared by freezing and cutting in the cryostat most of the areas failed to show the characteristic striated pattern due both to the angle of cutting as well as distortion and fragmentation of myofibrils. In some areas, however, which were cut transversely, the myofibrils were sharply outlined as brightly fluorescent dots separated by non-staining interfibrillary spaces (Fig. 6).

Preparations fixed in formalin and embedded in diglycol stearate exhibited less intense fluorescence. Nevertheless, these preparations were valuable in the observations on pathological material because of the preservation of structural relationship between the fibers and interstitial elements.

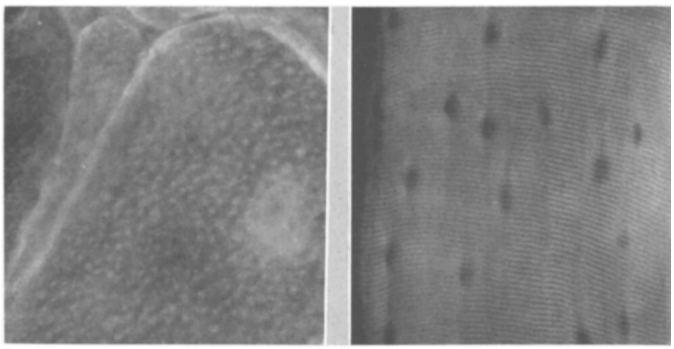

FIG. 6 (left). Cross-section of the rat muscle cut in the cryostat. Some of the myofibrils are brightly fluorescent. Fluorescence of the sacrolemmal sheath probably due to the failure of removing excess of the conjugate. × 400.

FIG. 7 (right). Human muscle obtained by biopsy in a case with myotonic dystrophy. Muscle fibers show slight decrease in size with a relative increase in number of nuclei. Pattern of cross-striation is well preserved. × 240.

Muscle tissue obtained from several patients afflicted with neuromuscular disorders presented histologically a variety of pathological changes. The muscle fibers in which pathological change was confined to an increase in the number of nuclei showed in ultraviolet light the preservation of the striated pattern with numerous oval or spherical unstained spaces, corresponding to the nuclei (Fig. 7). It was also noted that in some instances fibers undergoing severe degeneration were brightly fluorescent, and at the same time showed a complete loss of striated pattern, while the numerous condensed nuclei remained unstained (Fig. 8). Occasionally, single, small fluorescent bodies containing unstained spherical or oval spaces corresponding in size to the nuclei, were observed adjacent to the degenerating fibers (Fig. 9). Whether these structures were macrophages or remnants of the muscle fibers was not determined.

Discussion. The observations reported above demonstrated the specific staining of A bands in the striated muscle by antihuman myosin fluorescent antibody. The implication from this, however, that localization of myosin is confined to A bands must be considered with some reservations. It is well to keep in mind the difficulties associated with the estimation of the purity of myosin preparations. The methods employed for the estimation of purity (ultra-centrifugation, viscosimetry) are sensitive enough to exclude the presence of more than a few per cent actin, but are relatively insensitive to other impurities which may be associated with myosin. It is possible that even relatively small amounts of impurities in the preparation used for immunization may affect antibody re-

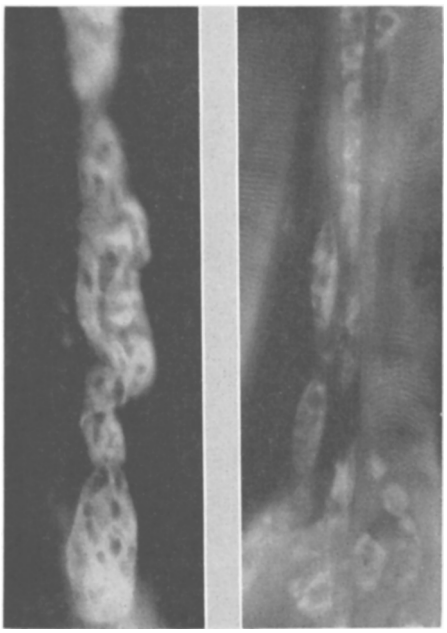

FIG. 8 (left). A severely atrophic fiber from the same case. It is brightly fluorescent with loss of striated pattern and with numerous unstained nuclei. × 400.

FIG. 9 (right). Human muscle from another case of myotonic dystrophy. Between 2 well-preserved fibers lies a long thin atrophic muscle fiber with fluorescent material. In addition there are several scattered smaller oval bodies which may be macrophages containing fluorescent material. × 400.

sponse to these substances. The occasional staining of sarcolemmal sheaths or Z band thus may be due to the presence of these additional antibodies other than myosin. Also, a theoretical possibility must be kept in mind that antigenic groups of myosin in certain sectors of the myofibril may be bound chemically to other substances and remain unreactive with antibody. This possibility prevents the absolute proof that myosin is absent in unstained segments such as the I band.

The presence of antibodies to myosin proper is demonstrated by the specific staining of myofibrils on cross sections, as these structures are known to be mainly composed of myosin. The finding that the nitrogen content of the washed precipitates agrees with theoretical figures indicates that the fluorescent globulin preparations mostly contained antibodies to myosin proper. It may be mentioned here that the fluorescence of the A band was already observed by Finck *et al.*(3).

The observation that anti-human myosin antibody reacted with muscle of other species raises the problem of antigenicity of myosin. Kesztyüs *et al.*(1) have found that of the 2 muscle proteins they studied actin was iso-antigenic and not species specific, myosin was not iso-antigenic. The observations reported here indicate that myosin is not species specific, since antibody to human myosin reacted with myosin prepared from the muscle of other animals.

The few observations on human muscle tissue undergoing degenerative changes should be regarded as preliminary ones. More experience in interpretation of fluorescent preparations must be obtained from the study of a larger amount of material. The quality of preparations which would preserve structural relationships should be improved and it also seems desirable to extend these studies to include other muscle proteins. It is interesting, however, at this stage to note that material staining specifically with anti-myosin fluorescent antibody may remain condensed in markedly atrophic fibers or may eventually be transferred to macrophages.

Summary. 1. Fluorescent antibody technic has been used for study of localization of myosin in normal and diseased skeletal muscle. 2. A method for assay of antibody to human myosin has been described. 3. Specific staining with fluorescent antibody was obtained in A bands of the striated muscle. 4. According to observations on staining with fluorescent antibody and to precipitin reactions myosin has to be considered as not species specific. 5. Preliminary observations on diseased muscle stained with fluorescent antibody revealed the preservation of striated pattern at the time of early pathology changes, and the presence of material reacting with the antibody to human myosin in severely degenerated fibers and in structures resembling macrophages.

1. Kesztyüs, L., Nikodemusz, S., Szilagyi, T., *Nature,* 1949, v163, 136.

2. Ebert, J. D., *J. Zool.,* 1953, v39, 333.

3. Finck, H., Hoztler, H., Marshall, J. M., *J. Biophys. and Biochem. Cytol.,* 1956, Suppl. 2, 175.

4. Szent-Gyorgyi, A. G., *J. Biol. Chem.,* 1951, v192, 361.

5. Coons, A. H., Kaplan, M. H., *J. Exp. Med.,* 1950, v91, No. 1, 1.

6. Emmart, E. W., Cole, R. M., May, E. L., Longley, J. B., *Resume. VII Congres Internat. de Pathol. Comparee—Lausanne,* 171.

Received October 19, 1957. P.S.E.B.M., 1958, v97.