POLYMORPHISM OF MYOSIN AMONG SKELETAL MUSCLE FIBER TYPES

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ABSTRACT

An immunocytochemical approach was used to localize myosin with respect to individual fibers in rat skeletal muscle. Transverse cryostat sections of rat diaphragm, a fast-twitch muscle, were exposed to fluorescein-labeled immunoglobulin against purified chicken pectoralis myosin. Fluorescence microscopy revealed a differential response among fiber types, identified on the basis of mitochondrial content. All white and intermediate fibers but only about half of the red fibers reacted with this antimyosin. In addition, an alkali-stable ATPase had the same pattern of distribution among fibers, which is consistent with the existence of two categories of red fibers.

The positive response of certain red fibers indicates either that their myosin has antigenic determinants in common with "white" myosin, or that the immunogen contained a "red" myosin. Myosin, extracted from a small region of the pectoralis which consists entirely of white fibers, was used to prepare an immunoadsorbent column to isolate antibodies specific for white myosin. This purified anti-white myosin reacted with the same fibers of the rat diaphragm that had reacted with antibodies against whole pectoralis myosin. This indicates that the myosins of white, intermediate, and some red fibers are sufficiently homologous to share antigenic determinants.

In a slow-twitch muscle, the soleus, only a minority of the fibers reacted with antitectoralis myosin. The majority failed to respond; hence, they are not equivalent to intermediate fibers of the diaphragm, despite their intermediate mitochondrial content.

Immunocytochemical analysis of two different muscles of the rat has demonstrated that more than one isoenzyme of myosin can exist in a single muscle, and that individual fiber types can be recognized by immunological differences in their myosins. We conclude that, in the rat diaphragm, there are at least two immunologically distinct types of myosin and four types of muscle fibers: white, intermediate, and two red. We suggest that these fibers correspond to the four types of motor units described by Burke et al. (Burke, R. E., D. N. Levine, P. Tsairis, and F. E. Zajac, III. 1973. J. Physiol. (Lond.) 234:723–748.) in the cat gastrocnemius.
Mammalian skeletal muscles consist of mixtures of fiber types which can be readily distinguished by ultrastructural and cytochemical criteria (22). Cytochemistry, in particular, reveals underlying functional differences. For example, localization of myosin ATPase in cryostat sections indicates that the pH-lability of this enzyme varies among fibers (29). Muscles composed primarily of fibers with an alkali-stable ATPase have a higher level of actomyosin ATPase activity than those muscles in which fibers with an alkali-labile ATPase predominate. The actomyosin ATPase of whole muscles, moreover, is directly proportional to the speed of contraction (3).

By stimulating single motoneurons in a mixed muscle, it has been possible to identify the muscle fibers which comprise an individual motor unit, and to correlate cytochemical features with actual physiological responses of specific muscle fibers (8, 16). With this direct approach, Burke et al. (8) have demonstrated that muscle fibers with high alkali-stable ATPase activity correspond to motor units having a fast contraction time, whereas fibers with low ATPase reflect a slow rate of contraction. Experimental alteration of the nerve supply has provided additional information concerning the role of the nervous system in maintaining both the microscopic composition and the biochemical and physiological characteristics of the muscle. By switching the nerve from a slow to a fast muscle, the speed of contraction is decreased, myosin ATPase activity is reduced, and the pattern of fiber types is converted, in part, to one which resembles that of a slow muscle (4, 6, 56). The pattern of muscle fibers is therefore significantly influenced by the type of innervation.

There is now increasing evidence for biochemical heterogeneity of myofibrillar proteins within whole muscles. These include tropomyosin (12, 13), troponin (33), and myosin (14, 31, 35, 36). The heterogeneity of myosin includes not only the heavy chains but also the low molecular-weight subunits or “light chains” (42, 58, 67). The different forms of a myofibrillar protein within an individual muscle may each be associated with a particular type of muscle fiber, or they may coexist within a single fiber (66). Some of these isoenzymes are directly influenced by the type of nerve supply. After cross-innervation, troponin as well as the light and heavy chains of myosin from a fast or slow muscle acquire characteristics which resemble those of the isoenzyme obtained from the reciprocal muscle (1, 6, 35, 60, 65, 68).

We have utilized an immunocytochemical approach to determine whether the chemical heterogeneity observed in the protein composition of whole muscles is related to the pattern of distribution of fiber types. This procedure has been used in other laboratories to localize actomyosin (28) and myosin (2) within a population of fibers in fresh frozen sections of skeletal muscle. We have demonstrated that not only does more than one immunologically distinct type of myosin exist within individual mammalian skeletal muscles, but that there is a preferential distribution of these isoenzymes with respect to the pattern of fiber types (24, 25).

MATERIALS AND METHODS

Preparation of Muscle

Adult male albino rats (90-120 days old) were killed with chloroform, and the soleus was exposed by blunt dissection of the dorsal hind limb. A thin strip of muscle was isolated from the abdominal surface of the left hemidiaphragm midway between origin and insertion. It was tied to a wooden splint, severed beyond the ligatures, and then placed against the soleus with the muscle surfaces apposed. The two muscles were tied together, cut free, and frozen in isopentane cooled to -160°C with liquid nitrogen. The frozen specimens were transferred, after about 20 s, to a cryostat at -18 to -22°C. The soleus and diaphragm were then sectioned simultaneously as a single block. This procedure insures that sections of the two muscles are equal in thickness and are exposed to identical experimental conditions.

Mature chickens (approx. 1 yr old) were used for the preparation of myosin and for cytochemical studies. Myosin extracted from the entire pectoralis (including the supracoracoideus) was used as the immunogen. Selected regions of the pectoralis were used to analyze the microscopic composition of the muscle, and also for the preparation of small quantities of myosin to be used as an immunoadsorbent.

A conspicuous “red” region is present at the anterior deep surface of the pectoralis. It forms a circumscribed band of fibers which extends from the ventral toward the dorsal border of the muscle. The “white” region is defined here as a small triangular portion of the pectoralis formed by cutting parallel to the tendon of insertion in a posterior direction toward the tip of the sternum, then parallel to the sternum in an anterior direction, and finally along the posterior border of the anterior red band. Apprx. 1 cm of the anterior border of this segment was discarded, and the entire deep surface through half the thickness of the muscle was removed, leaving

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only the superficial posterior-ventral region. The remain-
der of the pectoralis comprises the "mixed" region. Seg-
ments from each region were isolated by cutting parallel
to the fibers, tied, excised, and frozen in isopentane.
Cryostat sections were examined by the cytochemical
procedure described below.

For the preparation of white myosin to be used as an
immunoabsorbent, the entire white region of the pector-
alis was isolated by systematic dissection of several chick-
en. Representative regions from all specimens were
examined by cytochemistry. In all cases, the population
consisted entirely of white fibers.

Preparation of Antibodies

Specific antiserum to chicken pectoralis myosin was
elicted in rabbits by immunization with 1–3 mg of pro-
tein in 1 ml of 0.5 M KCl, 0.05 M potassium phosphate
(pH 7.0) emulsified with an equal volume of complete
Freund's adjuvant (Difco Co., Detroit, Mich.). The im-
munogen was myosin, prepared as described by Holtzer
and Lowey (38) with the addition of 3 mM ATP to the
extracting buffer. The myosin was further purified by
ion-exchange chromatography (54) in the buffer system
used by Offer et al. (47).

Antimyosin (IgG): For the preparation of im-
munoglobulin, two rabbits were immunized, and antisera
were pooled from many bleeds over a minimum period
of 1 yr. Serum from rabbit (14-1) was the same as that
characterized in the studies of Lowey and Steiner (43). Pooled sera (9-2 and 9-3) represent five to six bleeds
from rabbits (924) and (965). One preparation represented a single bleeding from each of the two
rabbits (924 and 965).

Immunoglobulin was isolated from serum by precipi-
tation with 40% saturated ammonium sulphate, fol-
lowed by chromatography on O-(diethylamino-
ethyl)cellulose (DEAE-cellulose). Fluorescein isothio-
cyanate (FITC) was coupled to immunoglobulin and sepa-
rated from unreacted dye either by ion-exchange chro-
matography (69) or by gel filtration (55). The former
procedure has been described in detail by Lowey and
Steiner (43). Gel filtration was used exclusively in the
latter stages of this study to conserve material and to
obtain better recovery of conjugated antibody. IgG or
purified antibody (10 mg/ml) in 0.1 M KC1, 0.01 M
potassium phosphate (pH 7.0) was brought to pH 9.5
with 0.1 vol of 0.4 M sodium bicarbonate, and added to
8–10 mg of 10% fluoresceinisothiocyanate on Celite
(Sigma Chemical Co., St. Louis, Mo.). After shaking for
a few minutes at room temperature, the suspension was
centrifuged, and the supernate layered on a 1.5 x 20-cm
column of Sephadex G-25 (coarse) equilibrated with
0.02 M potassium phosphate (pH 7.0). The yellow pro-
tein band was readily separated from the unreacted dye
by elution with the same buffer, and collected in about
two times the volume of applied protein. The ratio of
absorbance of the conjugated protein at 280 nm to that
at 495 nm was about 1.8. This material was dialyzed
against 0.1 M KCl, 0.01 M potassium phosphate (pH
7.0), clarified by centrifugation, and stored in the frozen
or freeze-dried state at -20°C. A comparison of the
staining pattern obtained with fluorescent antibodies
fractionated by ion-exchange chromatography with that
observed for fluorescent antibodies isolated by gel fil-
tration showed no appreciable difference.

Affinity-purified antimyosin (Ab): Specific antibody was isolated from immune serum by ad-
sorption on an immunoadsorbent column. The activation
of Sepharose 4B (Pharmacia Fine Chemicals, Inc.,
Uppsala, Sweden) by cyanogen bromide (about 200 mg/
ml of packed Sepharose) was carried out essentially as
described by Omenn et al. (48). Myosin, isolated from
whole pectoralis or from the white region, was added at a
concentration of 5–10 mg of protein per milliliter packed
gel in a coupling buffer consisting of 0.5 M KCl, 0.05 M
sodium pyrophosphate, pH 6.2. The reaction mixture
was stirred in the cold overnight and any nonbound
myosin removed by washing repeatedly with coupling
buffer. Clarified serum in 0.15 M KCl-0.02 M potas-
sium phosphate (pH 7.0) was applied to a 2 x 8-cm
column of immunoadsorbent at a flow rate of 100 ml/h;
the bound antibody was eluted either at pH 2, or with 4
M guanidine hydrochloride. The low pH solution was
neutralized immediately, and the guanidine was dialyzed
out as rapidly as possible to minimize denaturation.
Immunoadsorbent columns were stored in 0.02% azide
at 4°C, and could be used several times. The yields
of specific antibody were about 50%. The antibodies pre-
pared by the procedures described above gave a single,
sharp precipitin line when reacted with unchromato-
graphed myosin in double diffusion; the absence of addi-
tional bands indicates that antibodies against contami-
nants such as C-protein (46) are present in negligible
amounts.

Enzyme Cytochemistry

Transverse cryostat sections, approx. 10 μm, were
mounted on glass slides, and succinic dehydrogenase
activity was demonstrated using nitro blue tetrazolium
according to the method of Nachlas et al. (44). They
were incubated for 25 min at 37°C, dehydrated, and
mounted in Permount (Fisher Scientific Co., Pittsburgh,
Pa.).

Myofibrillar ATPase activity was localized in sections
serial to those described above. The procedure is based
on the method of Padykula and Herman (49), but modi-
fied to demonstrate selectively an alkali-stable ATPase
(30). Sections were fixed for 5 min at 4°C in 2% formalin
containing 0.068 M CaCl₂, 0.33 M sucrose buffered with
0.2 M sodium cacodylate at pH 7.6. This was followed
by preincubation for 15 min at room temperature in
0.018 M CaCl₂, 0.1 M 2-amino-2 methyl-1-propanol
buffer (Sigma, no. 221) at pH 10.4. The sections were
then incubated for 45 min at 37°C in a medium contain-
ing 0.0027 M ATP, 0.018 M CaCl₂, 0.05 M KCl at pH
9.4 (Sigma, no. 221).
**Immunocytochemistry**

The procedure for localizing myosin was, in general, similar to the method developed in the laboratory of Coons et al. (11), and used recently to localize actomyosin (27, 28; Gröschel-Stewart, U., personal communication). Transverse sections serial to those used for enzyme cytochemistry were cut at 4 μm and mounted on slides that had been coated by dipping into a 1% solution of ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetate (EGTA), after a procedure in which EDTA was introduced to prevent contraction of fresh-frozen sections of muscle (51). Longitudinal sections were used to determine localization with respect to the sarcomere. The slides were placed on moistened filter paper in covered petri dishes, and the sections were exposed to fluorescein-conjugated antibody at protein concentrations of about 0.5-3.0 mg/ml. Control immunoglobulin from non-immunized rabbits contained 2.0-20 mg/ml protein. They were incubated for 30 min to several hours at room temperature, or overnight at 4°C, washed for 20 min with 0.01 M phosphate buffer at pH 7.2, and mounted in equal volumes of phosphate buffer and glycerol. Each antiserum was used in many experiments, and the pattern of response for a particular preparation was highly reproducible. Moreover, the same staining pattern was obtained for several different antisera (14-1, 9-2, 9-3).

The sections were examined with a Zeiss fluorescence microscope with a no. 53 barrier filter and FITC excitation filter, an achromatic-aplanatic dark-field condenser, and a Zeiss Neofluar 16/0.4 or Planapochromat 100/1.3 objective. Phase-contrast optics were used to evaluate the longitudinal distribution of labeled antibody within the sarcomere. Fluorescent images were recorded on Kodak type 103 a-G spectroscopic plates.

**RESULTS**

**Differential Response to Antimyosin in the Rat Diaphragm**

Ultrastructural and cytochemical features of the rat diaphragm have been analyzed extensively in this laboratory, and criteria have been derived for the identification of three types of muscle fibers: red, white, and intermediate. The same criteria can be applied to other muscles of the rat and to muscles of other mammalian species (22). The form and distribution of mitochondria and dimensions of the Z lines have been particularly useful in recognizing these fiber types. Mitochondrial content, demonstrated by localizing succinic dehydrogenase activity, is inversely related to cross-sectional dimensions of the fibers (Figs. 1a and 2a).

It is highest in small red fibers, especially along the periphery, lowest in large white fibers, and moderate in intermediate fibers. In the red fiber, large mitochondria with closely packed cristae form conspicuous subsarcolemmal aggregations and interfibrillar rows. These mitochondria are smaller, and cristae are less abundant in the intermediate fiber, and they are sparse in the white fiber. The width of the Z-line is also inversely related to fiber diameter, being widest in red fibers and narrowest in white fibers (21).

**Antipeptoralis myosin:** A differential response to antipeptoralis myosin (IgG) is evident among fiber types in the rat diaphragm (Fig. 1b). Fibers which have reacted with labeled antibody appear bright when examined with the fluorescence microscope. Certain other fibers exhibit a very low level of fluorescence, which is negligible by comparison. For purposes of discussion, the latter fibers are designated unreactive and their response to antibody is said to be negative. Fiber types were identified by comparison with serial sections in which the mitochondrial enzyme, succinic dehydrogenase, is localized (Fig. 1a). Immunoglobulin against chicken pectoralis myosin reacts with all white and intermediate fibers and with about 40% of the red fibers as well (Fig. 2a and b). This correlates well with the localization of an alkali-stable ATPase, which has the same pattern of distribution among fibers (Fig. 2b and c). Certain red fibers (about 60% of this category) do not react with this antmyosin, and they have little or no ATPase activity. Red fibers can therefore be subdivided into two categories, of which one has characteristics in common with white fibers.

Alkali-stable ATPase activity, when present, is localized entirely within the myofibrils, and in longitudinal sections is confined to the A-band regions. This ATPase is sulfhydryl-dependent (i.e., inhibited by p-hydroxymercuribenzoate), and activity is not demonstrated at neutral pH or when the substrate (ATP) is replaced by either ADP or AMP. This indicates that the ATPase is associated with myosin and not with some interfibrillar structure.

**Affinity-purified antipeptoralis myosin:** An immunoabsorbent column was prepared by coupling chicken pectoralis myosin to Sepharose. Antibodies against pectoralis myosin would be adsorbed by this column, whereas any antibodies against proteins other than myosin or against myosin from sources other than skeletal muscle, e.g., vascular smooth muscle, would be largely excluded. Specific antibody isolated by this procedure reacts with all white, intermediate, and some red fibers (Fig. 3a and b). The same fibers
FIGURE 1 Rat diaphragm, serial transverse sections. (a) Succinic dehydrogenase. (b) Fluorescein-labeled antipectoralis myosin (14-1). The same fibers are given the same designation in each section. Fiber types are identified on the basis of mitochondrial content, demonstrated by localizing succinic dehydrogenase activity (a). It is highest in small red fibers (R) especially along the periphery, lowest in large white fibers (W), and moderate in intermediate fibers (I). There is a differential response among fibers to antipectoralis myosin (b). All white (W), intermediate (I), and about half of the red fibers react with the antibody and therefore appear bright (black R) with the fluorescence microscope. About half of the red fibers (white R) fail to react; these unreactive fibers have a very low level of fluorescence which is barely perceptible in the micrograph. × 270.

have high ATPase activity (Fig. 3c). Affinity-purified antimyosin therefore reacts with the same fibers that had reacted previously with the whole immunoglobulin fraction (cf. Figs. 2b and 3b). It is, thus, unlikely that the staining pattern arises from a nonmyosin contaminant. In longitudinal sections, furthermore, the positive reaction is confined to the A-band regions, and control sections treated with fluorescein-labeled non-immune globulin exhibit no reaction.

The negative response of certain red fibers persists over a wide range of protein concentration (0.5–3.0 mg/ml) and time of exposure to the antibody (30 min to 4 h). The invariance of the staining pattern and the correlation with ATPase activity provides strong evidence for the existence of two types of red fibers.

Source of the Immunogen

Inasmuch as skeletal muscles are not homogeneous, it is difficult to prepare ample quantities of immunogen which are "pure" with respect to fiber type. Even the chicken pectoralis, which is commonly viewed as a white muscle, is heterogeneous, and the nonwhite fibers present could have contributed a red component to the immunogen. With succinic dehydrogenase activity as a criterion for identification, the fiber composition of three different regions of the pectoralis was examined. Only about 10% of the total weight of the muscle consists entirely of fibers which resemble white fibers in skeletal muscles of the rat, that is, fibers with a low mitochondrial content (Fig. 4a). A circumscribed region which accounts for about 1%
Figure 2  Rat diaphragm, serial transverse sections. (a) succinic dehydrogenase. (b) antipectoralis myosin (14-1). (c) alkali-stable ATPase. All white (W), intermediate (I), and about half the red fibers (R) react with antimyosin (b). The same fibers have a high level of ATPase activity; unreactive fibers have a low level of activity (c). $\times 270$. 

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FIGURE 3 Rat diaphragm, serial transverse sections. (a) Succinic dehydrogenase. (b) Affinity-purified antipectoralis myosin (9-3). (c) Alkali-stable ATPase. All white (W), intermediate (I), and some of the red fibers react with the affinity-purified antibody (b), and the positive response corresponds to a high level of ATPase activity in the same fibers (c). The same staining pattern was evident with other antisera (14-1 and 9-2). × 270.
FIGURE 4 Chicken pectoralis, succinic dehydrogenase. Figs. 4a-c are representative of the white, red, and mixed regions of the pectoralis, respectively. The white region (a) consists entirely of large fibers with a low mitochondrial content (similar to white fibers in muscles of the rat). In the red region (b), all fibers are rich in mitochondria. In the mixed region (c), fibers with a low mitochondrial content predominate, but some are rich in mitochondria. × 270.
is red to the eye and consists entirely of fibers which are rich in mitochondria (Fig. 4b). The remainder consists of a mixed population; fibers with a low mitochondrial content predominate, but about 10–20% have a high mitochondrial content (Fig. 4c). Fibers of the red region can be subdivided on the basis of their response to antipectoralis myosin. Certain fibers (Fig. 5a) react with antipectoralis myosin (Fig. 5b), and also have a high level of alkali-stable ATPase activity. All other fibers have a low ATPase activity and do not react with antimyosin. They therefore resemble the unreactive red fibers of the rat diaphragm. All fibers in the white and mixed regions of the pectoralis have a high ATPase activity and a positive response to antimyosin.

The fibers of the chicken pectoralis are in many respects comparable to red, white, and intermediate fibers of other skeletal muscles. Identification, however, is based primarily on mitochondrial content of the fibers. It should be emphasized that, while they resemble the fibers of certain skeletal muscles of the rat, we do not imply that all of their properties are identical.

**Positive Response to Antimyosin**

The positive response of red fibers of the rat diaphragm to antibodies against pectoralis myosin indicates either that the myosin of these fibers has antigenic determinants in common with white myosin or that the immunogen contained a red myosin. The latter is a distinct possibility, since the chicken pectoralis, which is the source of the immunogen, is heterogeneous, as described above.

Systematic dissection of the small white region of the pectoralis (Fig. 4a) consistently yields a pure population of fibers which are white based on low mitochondrial content, high ATPase activity, and a positive response to antimyosin. This region was isolated from the pectoralis of several chickens and used to prepare a white myosin. By using this myosin as an immunoadsorbent, antibodies

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**Figure 5** Red region of chicken pectoralis, serial transverse sections. (a) Succinic dehydrogenase. (b) Affinity-purified antipectoralis myosin (9-3). All fibers are rich in mitochondria (a). Certain fibers react with antimyosin, but many are unstained (b). The same staining pattern was obtained with antipectoralis myosin (IgG). × 270.
specific for white myosin could be selectively adsorbed from a population directed against whole pectoralis myosin. If these affinity-purified antibodies were to react only with white fibers of the rat diaphragm, this would indicate that the previous reaction with red fibers did reflect a red component in the immunogen. However, anti-white myosin (Fig. 6c) reacts with the same three fiber types that had reacted previously with antipectoralis myosin (Fig. 6b). Therefore, red fibers (Fig. 6a) continue to be subdivided on the basis of their response to anti-white myosin (Fig. 6c), and the pattern correlates with the localization of ATPase activity as in previous experiments. This suggests that the myosins of white, intermediate, and some red fibers are sufficiently homologous to share antigenic determinants.

The possibility exists, nevertheless, that the myosin of this class of red fibers contains some unique determinants which might be revealed if antibodies against shared determinants were selectively absorbed by white myosin. Unabsorbed antibodies would be expected to stain red but not white fibers. To test this hypothesis, sections were exposed to antipectoralis myosin absorbed with varying amounts of white myosin. At low concentrations of white myosin, where the antibody was only partially absorbed, a weak but otherwise unaltered staining pattern was observed. The distribution of reactive fibers was the same as in control preparations; namely, white, intermediate, and some red fibers reacted as in previous experiments. When white myosin was present in antigen excess, none of the fibers showed any fluorescence. No single category of fibers, therefore, has a response which can be preferentially inhibited by white myosin. This is consistent with the uniform reaction of the same fibers with anti-white myosin, and with the conclusion that the myosin of certain red fibers has antigenic determinants in common with white myosin.

**Negative Response to Antimyosin**

The negative response of certain red fibers of the rat diaphragm could indicate that a particular type of red myosin was absent from the chicken pectoralis, or that it was not present in the immunogen in quantities adequate to elicit a response in the rabbits used to prepare the antibody. If the explanation is the latter, it would be expected that the chicken pectoralis would contain a population of fibers unaltered by the antibody. Survey of different regions of the chicken pectoralis shows that it does indeed contain a population of fibers which fails to react with any antipectoralis myosin (Fig. 5b). The negative response of certain red fibers in the rat muscle, therefore, does not reflect the absence of a particular red myosin from the pectoralis. It suggests, rather, that this red myosin is sufficiently distinctive to have been selectively removed in the preparation of the immunogen, or that its concentration in the immunogen was too low to raise an appreciable antibody titer. The possibility that an individual rabbit was unresponsive to a particular red myosin is unlikely, since antisera prepared from several different rabbits all failed to react with the same group of red fibers. We therefore favor the interpretation that the negative response indicates the absence of a particular red myosin in the immunogen rather than an immunological tolerance of the rabbit.

**Extent of Homology between Myosins of Red and White Fibers**

The above results do not imply that the myosin of those red fibers which react with antimyosin is necessarily identical to that of white fibers. In fact, one preparation of immunoglobulin directed against chicken pectoralis reacts only with white fibers of the rat diaphragm. Red and intermediate fibers (Fig. 7a), which respond strongly to all other antimyosins (Fig. 7b), are nearly unresponsive to this preparation (Fig. 7c). This immunoglobulin represents a single bleeding from each of two rabbits and therefore is likely to contain a more “restricted population” of antibodies. All other antisera used in this study represent many bleedings pooled from two rabbits over a longer period of time after the initial injection of the immunogen.

This preparation of immunoglobulin was used to advantage in characterizing the fibers of the diaphragm and also the soleus muscle of the rat. The absence of a response in the red fibers which had reacted with all other antimyosins indicates that their myosin content may not be identical to that present in the white fibers.

**Differential Response to Antimyosin in Other Skeletal Muscles**

The immunocytochemical approach offers a means of distinguishing fiber types within a population where differences may not otherwise be readily apparent.

**Rat Soleus:** The soleus muscle is used
Figure 6  Rat diaphragm, serial transverse sections. (a) Succinic dehydrogenase. (b) Affinity-purified antipectoralis myosin (9-3). (c) Affinity-purified anti-"white" myosin (9-3). Anti-white myosin (c) reacts with the same fibers that react with antipectoralis myosin (b), namely all white (W), intermediate (I), and certain red fibers (R). × 270.
Figure 7  Rat diaphragm, serial transverse sections. (a) Succinic dehydrogenase. (b) Antipectoralis myosin (14-1). (c) Antipectoralis myosin (rabbits 924 and 965, single bleeds). This preparation reacts only with white fibers (W); all red fibers (R) and intermediate fibers (I) are unreactive. Compare with Fig. 7b in which all white (W), intermediate (I), and some red fibers (R) are reactive. × 270.
widely in biochemical and physiological investigations as an example of a homogeneous muscle which consists of typical red or intermediate fibers. In the rat soleus, there are two types of fibers: the majority (about 75%) resemble "intermediate" fibers on the basis of mitochondrial content (Fig. 8a), but the width of the Z-line is significantly greater than in typical intermediate fibers (23). Their failure to respond to any of the antimyosins described above (Fig. 8b), and their lack of alkali-stable ATPase activity indicate that they are not equivalent to intermediate fibers of the rat diaphragm. The minority of the fibers (25%), often classified as red on the basis of their high mitochondrial content (Fig. 8a), react with all antimyosins (Fig. 8b), including the preparation of immunoglobulin which had reacted only with white fibers in the diaphragm. All reactive fibers have a high level of alkali-stable ATPase activity, which, as in the diaphragm, correlates with their immunological cross-reactivity.

**Chicken anterior and posterior latissimus dorsi:** The anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) of the chicken are representative of slow red and fast white muscles, respectively. Although these muscles consist "almost exclusively" of either red or white fibers, there are, nevertheless, immunocytochemical differences within the fiber population (2). A majority of the fibers in the ALD and a few in the PLD react with fluorescein-labeled antibody against ALD myosin. Antibody against PLD myosin, on the other hand, stains almost all fibers in the PLD but only a minority in the ALD (2). We have found that antibody directed against white myosin from chicken pectoralis reacts with a minority of the fibers of the ALD (Fig. 9a) and with most of the fibers of the PLD.

![Figure 8 Rat soleus, serial transverse sections. (a) Succinic dehydrogenase. (b) Antipectoralis myosin (14-1). A minority of the fibers, which have the highest mitochondrial content (a), react with the antibody (b). The majority do not react. All antisera used in this study (including 9-2 and 9-3) reacted with the same population of fibers. A blood vessel (b.v.) is indicated to facilitate comparison of fibers in serial sections. × 270.](image-url)
FIGURE 9 Chicken ALD (a) and PLD (b), affinity-purified antipectoralis myosin (9-3). Some of the fibers of the ALD (a) react with antipectoralis myosin, but most are unreactive. Most of the fibers of the PLD (b) react with the antibody; two unreactive fibers are included in the illustration. Serial sections of the same fibers also reacted with anti-white myosin. × 270.

(Fig. 9b). The staining pattern in the PLD with antipectoralis myosin is therefore comparable to that observed with the homologous antibody. In addition, the fibers which are reactive also have a high level of alkali-stable ATPase activity. In the studies of Arndt and Pepe (2), the antibody against PLD had been first absorbed with ALD myosin, and thus was presumably specific for the majority of the PLD fibers. The fact that our anti-white myosin showed the same selective response without any prior absorption emphasizes the high degree of specificity of this antibody.

DISCUSSION

Chemical Heterogeneity of Myosin and the Pattern of Fiber Types

With the exception of ATPase activity, none of the criteria for distinguishing fiber types has provided significant insight into the nature of the contractile proteins. The use of antibodies to detect immunological differences among myosins and actomyosins within individual skeletal muscles has provided a promising approach to the problem of analyzing the protein composition of individual fibers (2, 28). We have prepared antibodies against myosin from a population of muscle fibers which is uniformly white according to cytochemical criteria. With these antibodies specific for white myosin as a marker, we have demonstrated that the myosins of three types of fibers in the rat diaphragm are sufficiently homologous to share antigenic determinants. All white, intermediate, and about half the red fibers react with this antibody, and the positive response correlates well with a high level of alkali-stable ATPase activity in the same fibers. Moreover, this homology in sequence is not confined to a limited region of myosin, since antibodies against the proteolytic subfragments as well as the light chains of myosin show the same staining pattern among fibers.1 The

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myosin of those red fibers which fail to react is distinguished not only by a lack of immunological cross-reactivity, but also by a low ATPase activity. On the basis of both immunological and enzymic criteria, therefore, there are two categories of red fibers in the muscle of the rat diaphragm.

These results should not be interpreted to mean that the myosin of the reactive red fiber is necessarily identical to that of the white fiber. We have described one situation in which only white fibers are stained by a particular antiserum. Therefore, the population of myosin molecules in the white fiber is distinctly different from that in the reactive red fiber. This observation may be explained by hypothesizing a minimum of three isoenzymes in the rat diaphragm: a so-called white myosin characteristic of the white fiber, and two types of red myosin, one of which is sufficiently homologous in sequence with white myosin to share antigenic determinants. When these shared determinants are not represented by cross-reacting antibodies in the sera, the red fibers will not show any fluorescence. An alternative explanation would be the existence of only two basic kinds of myosin: a white myosin in the white fiber and a red myosin in the unreactive red fiber. Varying proportions of these two myosins could account for the staining of the reactive red fiber and also the intermediate fiber. When the antibody titer of a serum is high with respect to antibodies against rat white myosin, the typical pattern in which all the white, intermediate, and half the red fibers are stained would be observed. However, if the titer is low, only those fibers with the highest concentration of white myosin, presumably the white fibers, would be expected to show a positive reaction.\(^2\)

It should be recognized that, in these sera, only a fraction of the antibody population, which was raised against chicken myosin, will cross-react with rat myosin. The concentration of cross-reacting antibody will vary among individual sera, and would be expected to be lowest in a single bleed taken soon after the initial immunization. Moreover, the affinity of antibodies increases markedly with time after antigen administration (17), and an early bleed might, therefore, be expected to contain antibodies with poor binding ability.

Although only a small portion of the myosin molecule has been sequenced to date, some generalizations concerning the number of isoenzymes in a particular muscle can be made. The most extensive sequence data available are those pertaining to the light chains of myosin. Frank and Weeds (20) have shown that two chemically homologous light chains of mol wt 20,700 (Alkali 1) and 16,500 (Alkali 2) are present in myosin isolated from fast-twitch muscles of the rabbit. The occurrence of these two chains in unequal molar concentrations (37, 66) suggests that they are probably not equally distributed between the two "heads" of the myosin molecule. Instead, it is more likely that there is a mixed population of homogeneous myosin molecules. The recent fractionation of myosin into two populations (Alkali 1 myosin and Alkali 2 myosin) by an antibody affinity column specific for Alkali 1 has provided a direct verification of the isoenzyme hypothesis (37). These isoenzymes could coexist within a single fiber or each could be associated with a particular type of muscle fiber. Electrophoretic analysis of isolated fibers from a white region of the rabbit psoas muscle has shown that the two species do in fact exist within a single fiber (66).

Heterogeneity in myosin has also been demonstrated by electrophoretic mobility (36) and by chemical analysis of the heavy chains (61, 63). Starr and Offer demonstrated the presence of two homologous N-terminal sequences in the heavy chains of rabbit myosin from mixed fast muscles. The similarity between the ratio of these sequences and the ratio obtained for the relative amounts of the two alkali light chains suggested that a specific interaction might occur between the two different heavy and light chains. However, chemical analysis of the heavy chain from a myosin subfragment containing either Alkali 1 or Alkali 2 showed that each light chain can be associated with either kind of heavy chain (62). Therefore, one must conclude that there are at least four isoenzymes of myosin in a fast-twitch muscle.

Far less chemical information is available for slow-twitch muscles, but an electrophoretic analysis of myosin from rabbit and cat soleus has shown two light chains of the "Alkali" type described above. These light chains are more similar to each other in molecular weight than are Alkali 1 and 2, but their chemical composition suggests extensive amino acid differences between the homologous chains (64). Although the stoichiometry of the light chains of slow-twitch myosin has not been determined, it is likely that at least two isoenzymes exist in this muscle.

Chemical studies on the myosins from slow-
twitch and fast-twitch muscles have, therefore, shown the existence of as many as six isoenzymes. This is probably a conservative estimate, since sequence data on the heavy chains, in particular those of slow-twitch myosin, will no doubt reveal further heterogeneity. Any attempt to correlate these isoenzymes with the pattern of fiber types must be viewed as tentative at present. By using antibodies specific for white myosin, we have shown that closely related myosins are present in the white, intermediate and half of the red fibers of a mixed muscle. Preliminary studies with antibodies against the N-terminal 41 residues of the Alkali 1 light chains suggest that the same fibers contain Alkali 1 type myosin. As antibodies to other unique sequences become available, such as an antibody to slow-twitch myosin, we should be able to determine whether the myosin of the soleus is related to that of the unreactive red fiber in the rat diaphragm. Thus, immunochemistry offers one approach for relating individual myosin isoenzymes to the individual fibers comprising a muscle.

Functional Implications of Heterogeneity

Although the relationship of functional activity to the pattern of fiber types is not well understood, it seems reasonable that the presence, within a muscle, of a mixture of different types of fibers would serve to increase the versatility of that muscle. Specific characteristics, such as speed of contraction and resistance to fatigue, could be distributed in various combinations to elicit optimal performance. The exact relationship between the structural features of a particular muscle fiber and its physiological properties has long been a matter of conjecture. Certain assumptions have been made, largely by extrapolation of data obtained from whole muscles. For example, it has been argued that the soleus, which is widely used as an example of a slow-twitch red muscle, is composed entirely of red fibers and that the red fiber is therefore a slow fiber. However, the fibers of the soleus are neither typically red as defined in this paper, nor are they necessarily all alike. Furthermore, certain fast-twitch muscles are composed of fibers which have ultrastructural and cytochemical features which resemble those of red fibers (26, 32, 53).

Functional characteristics have, of necessity, been attributed to individual fibers on the basis of indirect evidence. Typically, physiological measurements are made on selected whole muscles, and cytochemical characteristics of the constituent fibers are examined simultaneously. The individual fibers of a mixed muscle have, in this way, been classified according to both sets of criteria, and the fibers have been designated “fast-twitch glycolytic” and “fast-twitch-oxidative-glycolytic” to indicate the properties of white and red fibers, respectively (5, 52). Both types of fibers have a high level of myosin ATPase activity. In the above studies, a third type of fiber with low ATPase activity was equated with fibers of the slowly contracting soleus, which have intermediate oxidative enzymic activity. It was therefore designated “intermediate” or “slow-twitch oxidative”. This fiber is equivalent to what we have called an unreactive red fiber, referring to its low ATPase activity and its lack of immunologic cross-reactivity. There is general agreement concerning the existence of this fiber, but confusion has arisen because of the variety of designations applied to it. This fiber has been called either type I (19), intermediate (15), β (31) or type C (59). More important than the particular nomenclature, however, is the careful definition of the criteria used to identify a particular type of muscle fiber. These criteria, which include oxidative activity, ATPase activity, and contraction time of whole muscles, have led to the recognition, by most investigators, of three basic fiber types.

In our studies, we have consistently identified an additional type of fiber. We have defined this fiber as intermediate on the basis of cross-sectional dimensions, mitochondrial content, and width of the Z-line (21, 50). This designation, moreover, is based on direct observation of microscopic features relative to other fibers in a heterogeneous population. We have shown here that this fiber also has a high level of alkali-stable ATPase activity and a positive response to anti-white myosin. Thus, four types of fibers can be distinguished; the designations red, white, and intermediate are based on mitochondrial content; and two categories of red fibers are described as reactive or unreactive based on both ATPase activity and response to anti-white myosin.

There has been evidence to suggest that the cytochemical characteristics of individual muscle fibers are related to the physiological properties of specific types of motor units. The proportion of motor units with a fast or slow speed of contraction reflects the percentage of white or red fibers, respectively, as identified by mitochondrial content (34, 70). More recently, a new experimental
approach has permitted direct correlation of physiological data and the microscopic appearance of individual skeletal muscle fibers. When a single motoneuron is stimulated, glycogen is depleted from those muscle fibers which are innervated by it. The muscle fibers which comprise a motor unit are thus “marked” by the loss of glycogen in response to stimulation (41). By comparing “depleted,” i.e., stimulated, fibers with serial sections of the same fibers exposed to a variety of histochemical reactions, it became apparent that the muscle fibers which comprise a given motor unit are of one type (16). The histochemical features of these muscle fibers can thus be correlated with actual recordings of electrical and mechanical properties. Accordingly, the motor units in the gastrocnemius muscle of the cat have been designated FF (fast-contracting, fast-fatigue), FR (fast-contracting, fatigue-resistant) and S (slowly-contracting, fatigue-resistant) to describe their rate of contraction and resistance to fatigue (8, 10). A fourth motor unit designated as “unclassified” has a fast contraction rate, but fatigue-resistance is “intermediate.” Alkali-stable ATPase activity localized in the muscle fibers was shown to be high in fast and low in slowly contracting motor units. Mitochondrial enzymic activity was high in motor units with high fatigue-resistance, low in motor units with low fatigue-resistance, and intermediate in those with intermediate fatigue-resistance. These experiments support earlier conclusions that ATPase activity is directly proportional to speed of contraction, whereas mitochondrial content is related directly to resistance to fatigue.

Our observations of four types of muscle fibers in the rat diaphragm correlate well with these four types of motor units in the cat gastrocnemius. Succinic dehydrogenase and ATPase were localized in both studies, and therefore these enzymic activities can be used as a basis for comparison. The white fiber has a low mitochondrial content and high level of alkali-stable ATPase activity in both the rat and cat muscles, and therefore corresponds to the FF motor unit. Red fibers are rich in mitochondria, and ATPase activity is either high or low; these probably reflect the FR or S motor unit, respectively. The intermediate fiber is intermediate in mitochondrial content, but ATPase activity is high. It therefore may correspond to the unclassified motor unit, more recently referred to as F (int) or fast-contracting with intermediate fatigue-resistance (9). In addition, preliminary observations on the cat gastrocnemius indicate that, as in the rat diaphragm, all white and intermediate fibers and those red fibers with high ATPase activity, i.e., FF, F(int), and FR motor units, react with antibody specific for white myosin. Red fibers with low ATPase (type S motor units) are unreactive. That is, the muscle fibers in all fast-contracting motor units have myosins which share antigenic determinants and which possess similar ATPase activity. Slowly contracting motor units differ in that their muscle fibers lack immunological cross-reactivity and have low ATPase activity.

In the soleus, the predominant fiber type, which is slow-contracting and fatigue-resistant in both the rat (40) and the cat (7), has a negative response to antopectoralis myosin, a low level of alkali-stable ATPase activity, and a moderately high mitochondrial content. However, these fibers are not necessarily equivalent to fibers with similar characteristics in a heterogeneous muscle, as discussed above. Also, in the cat soleus, the distribution of mitochondria is not comparable to that of any of the fibers of a mixed muscle (45), and physiological properties of the motor units are not identical to those of a mixed muscle (7). A minority of the fibers of the rat soleus, which most likely correspond to fast-contracting motor units described by Kugelberg (40) in the same muscle, react with antopectoralis myosin, and have a high level of alkali-stable ATPase activity as well as a high mitochondrial content.

On the basis of our cytochemical studies, together with physiological measurements of individual motor units, four types of muscle fibers can be defined in fast-twitch (mixed) muscles. The fibers of the slow-twitch soleus may be related to these fibers, or they may represent yet another category. Further characterization at the ultrastructural level as well as more extensive immunological and chemical analysis of the proteins in these fibers may serve to clarify some of the ambiguities. Meanwhile the immunocytochemical approach offers a fundamental criterion for recognizing fiber types, and it can be used when other characteristics may not be readily apparent, for example, during development or after physiological or pathological alteration.

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