DISTRIBUTION OF MYOSIN ISOENZYMES AMONG SKELETAL MUSCLE FIBER TYPES

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ABSTRACT

Using an immunocytochemical approach, we have demonstrated a preferential distribution of myosin isoenzymes with respect to the pattern of fiber types in skeletal muscles of the rat. In an earlier study, we had shown that fluorescein-labeled antibody against "white" myosin from the chicken pectoralis stained all the white, intermediate and about half the red fibers of the rat diaphragm, a fast-twitch muscle (Gauthier and Lowey, 1977). We have now extended this study to include antibodies prepared against the "head" (S1) and "rod" portions of myosin, as well as the alkali- and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB)-light chains. Antibodies capable of distinguishing between alkali 1 and alkali 2 type myosin were also used to localize these isoenzymes in the same fast muscle. We observed, by both direct and indirect immunofluorescence, that the same fibers which had reacted previously with antibodies against white myosin reacted with antibodies to the proteolytic subfragments and to the low molecular-weight subunits of myosin. These results confirm our earlier conclusion that the myosins of the reactive fibers in rat skeletal muscle are sufficiently similar to share antigenic determinants. The homology, furthermore, is not confined to a limited region of the myosin molecule, but includes the head and rod portions and all classes of light chains. Despite the similarities, some differences exist in the protein compositions of these fibers: antibodies to S1 did not stain the reactive (fast) red fiber as strongly as they did the white and intermediate fibers. Non-uniform staining was also observed with antibodies specific for A2 myosin; the fast red fiber again showed weaker fluorescence than did the other reactive fibers. These results could indicate a variable distribution of myosin isoenzymes according to their alkali-light chain composition among fiber types. Alternatively, there may exist yet another myosin isoenzyme which is localized in the fast red fiber. Those red fibers which did not react with any of the antibodies to pectoralis myosin, did react strongly with an antibody against myosin isolated from the anterior latissimus dorsi (ALD), a slow red muscle of the chicken. The myosin in these fibers (slow red fibers) is, therefore, distinct from the other myosin isoenzymes. In the rat soleus, a slow-twitch muscle, the majority of the fibers reacted only with antibody against ALD myosin. A minority, however, reacted

with antibodies to pectoralis as well as ALD myosin, which indicates that both fast and slow myosin can coexist within the same fiber of a normal adult muscle. These immunocytochemical studies have emphasized that a wide range of isoenzymes may contribute to the characteristic physiological properties of individual fiber types in a mixed muscle.

KEY WORDS   muscle · fiber types · myosin · isoenzymes · immunocytochemistry

Most skeletal muscles are composed of mixtures of structurally different fibers, but the relationship to functional and chemical properties is not well understood. It is becoming increasingly apparent that there is significant chemical heterogeneity among many of the myofibrillar proteins, depending on the source of the muscle. Examples include myosin (27, 22), tropomyosin (7, 8), and troponin (21).

In the mammal, two general categories of muscle are described as fast-twitch or slow-twitch, depending on contraction time. In addition, white muscles tend to be faster than red muscles (38). Fast-twitch muscles, moreover, have a higher level of myosin ATPase activity than slow-twitch muscles (2). Accordingly, myosin extracted from these muscles is referred to as either "fast" (white) or "slow" (red) depending on whether its ATPase activity is high or low. In each of the two types of myosin there are two chemically different classes of light chains (42, 30, 51), but they have been most extensively characterized in fast-twitch muscles. One class is liberated from myosin by treatment with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and hence is designated DTNB light chains; the other class is removed by exposure to alkaline pH and has therefore been termed alkali light chains. Two types of alkali light chains can be distinguished on the basis of size. The larger alkali 1 (mol wt 20,700) has extensive sequence homology with the smaller alkali 2 (mol wt 16,500), but alkali 1 has an additional 41 N-terminal residues (11). This sequence, which accounts for the difference in molecular weight, is termed the "difference peptide." Otherwise, alkali 2 is largely identical to alkali 1 except for five residues in its N-terminus which substitute for those present in alkali 1. The two alkali light chains of myosin most likely reflect isoenzymes which are predominantly homodimers (25, 32), that is, myosin containing only alkali 1 or alkali 2 light chains. The light chains of slow-twitch muscles are less well-characterized, but two classes

have also been demonstrated. One class consisting of two chemically similar light chains is related to the alkali light chains of fast-twitch muscle, and a second class has some properties in common with the DTNB light chains (49).

Differences among myosins can be correlated directly with specific fibers within a population by localizing ATPase activity in transverse sections (9, 46). Fibers with a high level of alkali-stable (myosin) ATPase predominate in muscles which have a fast contraction rate (19). Hence, these fibers are often referred to as "fast" fibers (see Discussion). Individual fibers can also be identified by immunological differences in their myosins. We have shown, using an immunocytochemical approach, that the same fibers which react with antibodies specific for white (fast) myosin also have high alkali-stable ATPase activity (15), and are thus presumably correlated with fast contraction time. In the present study, we have shown that antibodies against slow myosin react with fibers having low ATPase activity, that is, "slow" fibers.

It is evident from a number of studies that there are immunological differences among myosins even within the same species. In fact, these differences may be more significant than differences among species, so that antibody directed against an antigen from one species may cross-react with the related protein from another species. It has been demonstrated, for example, that antibodies against chicken gizzard myosin react with smooth muscle not only from the chicken, but also from the rat, rabbit, and human (18). The same antibodies, however, failed to react with striated muscle, even in the chicken. Within a single striated muscle, furthermore, there are differences in the response to antibodies against skeletal muscle myosin among the component fibers, as demonstrated by the procedures of immunocytochemistry (17, 1). Tissue or cell specificity, therefore, appears to be greater than species specificity.

By taking advantage of the cross-reactivity of myosin in different species, it has been possible to localize isoenzymes of myosin within a heterogeneous skeletal muscle of the rat. Antibodies were

prepared against myosin from chicken skeletal muscles since they consist of relatively homogeneous populations of fibers. Using an antibody against white myosin from the pectoralis, we were able to demonstrate that the myosins in three of the four fiber types present in the rat diaphragm are sufficiently homologous to share antigenic determinants (15). To determine whether this homology has its origin in a limited region of myosin, or whether it extends to most of the molecule, we have examined the pattern of response to antibodies against the subfragments (S1 and rod), and both classes of light chains (31, 24). In addition, antibodies against the N-terminal sequences of alkali 1 and alkali 2 have been used, since these antibodies are capable of distinguishing between the isoenzymes of fast (white) myosin (25, 44, 32). The present study shows that the distribution among fiber types is the same as that observed using antimyosin; that is, all white (low oxidative), intermediate, and certain red (high oxidative) fibers react with these antibodies. The myosins of these fibers are, therefore, probably very similar. However, we present evidence which suggests that there could be variable mixtures of fast myosin isoenzymes in certain fibers, or that there may be yet another type of myosin which is different from either slow or fast myosin. In the rat soleus, a muscle with predominantly slow fibers, we show that both fast and slow myosin can coexist within a small population of fibers.

MATERIALS AND METHODS

The experimental procedures described here are an extension of those detailed in an earlier paper by Gauthier and Lowey (15).

Skeletal Muscles

Adult male albino rats and female chickens were killed with chloroform, and the muscles were exposed by blunt dissection. Thin strips were tied to wooden splints and frozen in isopentane cooled to −160°C with liquid nitrogen. Diaphragm and soleus from the rat, or anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) (or pectoralis) from the chicken, were sectioned simultaneously as a single block so that the fibers could be compared directly under identical experimental conditions (15).

For preparation of the immunogen, myosin was extracted from the entire chicken pectoralis. A selected region consisting only of white fibers was used to prepare small quantities of "white" myosin. By using this myosin as an immunoadsorbent, antibodies specific for white ("fast") myosin could be selectively adsorbed from a

population directed against whole pectoralis myosin (see reference 15). Myosin extracted from the chicken ALD was used as an immunogen to elicit antibodies against red ("slow") myosin. Either freshly dissected muscle or previously frozen muscle (obtained from Pel-Freez Biologicals, Inc., Rogers, Ark.) was used. Rat myosin was prepared exclusively from frozen diaphragm muscle (Pel-Freez Biologicals, Inc.).

Antigens

Chicken pectoralis myosin was prepared as described by Holtzer and Lowey (26), and further purified by ion-exchange chromatography (39) in the buffer system used by Offer et al. (34). A slightly modified procedure was followed for the isolation of myosin from frozen rat diaphragm and chicken ALD muscles. After precipitation at low ionic strength, the myosin was redissolved in 1 M KCl, 10 mM ATP, 15 mM MgCl$_2$, 1 mM EGTA, 0.05 M potassium phosphate (pH 7.0), and clarified by high-speed centrifugation before fractionation with solid ammonium sulphate (Schwartz/Mann [Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. J.], ultrapure grade). The material precipitating between 30 and 60% saturation (4°C) was collected, dialyzed free of ammonium sulphate, and applied to an ion-exchange column as previously described for chicken pectoralis myosin. For freshly dissected ALD muscles, an alternative procedure was to disperse the precipitated myosin in 0.5 M NaCl, 0.04 M sodium pyrophosphate (pH 7.5), against which it was dialyzed. The dialyzate was then changed to 0.02 M sodium pyrophosphate (pH 7.5), and the myosin was chromatographed on a diethylaminoethyl (DEAE)-cellulose column equilibrated in the same buffer (39, 30).

Antisera

The preparation of antisera was carried out as described by Holt and Lowey (24). Antisera were usually pooled from many bleeds over a period of ~1 yr; for example, anti-pectoralis myosin sera (9-2 and 9-3) represent pools of 5-6 bleeds each from two rabbits. Anti-S1 serum (15-2) and anti-rod serum (18-1) have been characterized in an earlier paper (31). Anti-ALD myosin serum represents a pool of four bleeds from a single rabbit taken 3 mo after the initial immunization. Goat serum specific for the Fc fragment of rabbit IgG was kindly contributed by Dr. A. Nisonoff.

Antibody Purification

Specific antibody was isolated from immune sera by adsorption to purified antigen coupled to Sepharose 4B (15, 25). Thus, anti-pectoralis serum was applied to an immunoadsorbent consisting of white (fast) myosin (see above) coupled to Sepharose. The bound antibody was eluted with 4 M guanidine hydrochloride and rapidly dialyzed against buffered saline to remove the denatur-

ant (15). Specific anti-S1 and anti-rod antibodies were isolated by passing their respective sera through the same white myosin immunoabsorbent column used to purify antimyosin. By a similar procedure, antibodies were prepared against the alkali 1 and 2 light chains and the DTNB light chain (43). The isolation of antibodies specific for the N-terminal sequence of alkali 1 (anti-Δ1) and alkali 2 (anti-Δ2) has been described by Holt and Lowey (25) and by Silberstein and Lowey (44). (We thank Laura Silberstein for her generous contribution of antibodies against the light chains.) Antiserum against ALD myosin was first passed through a column of Sepharose coupled to pectoralis myosin to adsorb any contaminating antibodies against white myosin. It is known that the chicken ALD has a small proportion of "white" (fast) fibers whose myosin could contribute to the immunogen (1, 15). The unretained serum, which is presumably specific for "red" (slow) myosin, was then applied to an immunoadsorbent of ALD myosin; the bound antibody was eluted with guanidine hydrochloride as described above.

The specificity of these antibodies was examined by double diffusion in agar. Antibodies to chicken pectoralis myosin, S1 and rod showed a single precipitin line when diffused against pectoralis myosin (Fig. 1). Antibody to ALD myosin also gave a single, sharp line when reacted with the immunogen, but showed no reaction with pectoralis myosin. The lack of a precipitin line does not necessarily exclude the presence of soluble antibody-antigen complexes; a more rigorous test for the absence of any cross-reaction between anti-ALD and pectoralis myosin was the failure of anti-ALD to stain cryostat sections of PLD or pectoralis muscle (see Fig. 6).

The specificity of the antibodies against the different classes of light chains, and against the N-terminal sequences of alkali light chains, has been described elsewhere (24, 25, 32). Anti-Δ1 and anti-Δ2, in particular, do not form precipitating complexes with immunogen, and thus it was necessary to characterize these antibodies by radioimmunoassay (Lowey and Silberstein, unpublished data).

For direct immunofluorescence, fluorescein isothiocyanate (FITC) was coupled to the rabbit antibodies as described by Gauthier and Lowey (15). For indirect immunofluorescence, FITC was conjugated to the immunoglobulin fraction of goat anti-rabbit Fc. In addition, a preparation of purified goat antibody labeled with fluorescein (donated by Dr. A. Nisonoff) was used in some experiments; no appreciable difference in staining pattern was observed.

Enzyme Cytochemistry

Transverse sections, ~10 μm, were cut in a cryostat at −18° to −22°C and mounted on glass slides; succinic dehydrogenase activity was demonstrated according to the method of Nachlas et al. (33). In serial sections, ATPase activity was localized by a procedure which
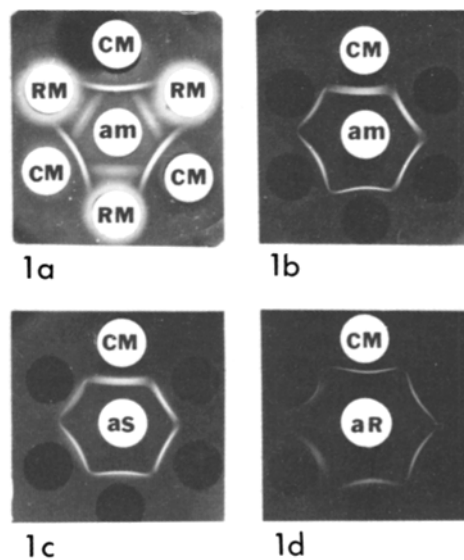

1a 1b

1c 1d

FIGURE 1 Immunodiffusion of myosin with affinity-purified antibodies against chicken pectoralis myosin and its subfragments. (a) antimyosin, 9-3 (am) in central well against chicken pectoralis myosin (CM), 6.5 mg/ml, and rat diaphragm myosin (RM), 10 mg/ml, in the peripheral wells. (b-d) two-fold serial dilution (7 mg/ml) of chicken pectoralis myosin (CM) against (b) anti-myosin, 9-2 (am); (c) anti-S1, 15-2 (aS); and (d) anti-rod, 18-1 (aR) in the central wells. Concentration of antibodies was ~3-4 mg/ml, except in a, which was 15 mg/ml.

distinguishes two types of activity, depending on stability after acid or alkali preincubation (20).

For the demonstration of alkali-stable ATPase activity, 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 (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 Chemical Co., St. Louis, Mo., No. 221) at pH 10.4. The sections were then incubated for 45 min at 37°C in a medium containing 0.0027 M ATP, 0.018 M CaCl₂, 0.05 M KCl at pH 9.4 (Sigma, No. 221).

Acid-stable ATPase activity was demonstrated using unfixed sections. They were preincubated for 15 min in 0.018 M CaCl₂, 0.05 M potassium acetate (pH 4.35). They were then incubated at pH 9.4 as described for alkali-stable ATPase activity.

Immunocytochemistry

Transverse cryostat sections serial to those used for enzyme cytochemistry were cut at 4 μm, mounted on slides treated with EGTA, and incubated as described previously (15). The sections were exposed to fluorescein-labeled antibody at protein concentrations of ~0.75

mg/ml for the direct method. For demonstrating immunofluorescence by the indirect method, sections were exposed to unlabeled antibodies at protein concentrations of 0.01–0.27 mg/ml and then subsequently reacted with fluorescein-labeled goat antibodies against rabbit immunoglobulin (protein concentration, 0.4–0.7 mg/ml). Control preparations using immunoglobulin from non-immunized rabbits showed no immunofluorescence after either the direct or indirect procedures.

Sections were examined with a Zeiss fluorescence microscope with a No. 53 barrier filter and FITC excitation filter, with an achromatic-aplanatic dark-field condenser and a Zeiss Neofluar 16/0.4 objective. Fluorescence was recorded on Kodak type 103 a-G spectroscopic plates.

RESULTS

Immunological Characterization of Antibodies Against Pectoralis Myosin

The antibodies used in these immunocytochemical studies were prepared against myosin from chicken muscles. Although we have demonstrated that antibodies to chicken pectoralis myosin can cross-react with myosin from rat diaphragm muscle (15), the extent of the reaction would be expected to be far weaker than that between the antibody and its immunogen. A heterologous ligand usually reacts with only a portion of the total antibody population; and, in addition, the antibody will almost always have a greater affinity for the homologous antigen than for the cross-reacting antigen. This is illustrated by the double diffusion pattern in Fig. 1a; antibodies to purified chicken pectoralis myosin showed a single strong precipitin band against chicken myosin, but a relatively weak line of partial identity against rat diaphragm myosin. The extent of cross-reaction was quantitated by the precipitin reaction which showed that only ~30% of the antibodies prepared against chicken myosin were precipitated by rat diaphragm myosin.

Despite the weaker immunological reactions in a heterologous system, we have chosen to use labeled antibodies against chicken myosin to stain rat diaphragm muscle for the following reasons: Large quantities of relatively homogeneous muscle are present in the chicken; for example, a specific region isolated from the pectoralis contains a homogeneous population of white fibers (15). In addition, the large amounts of protein available in chicken muscle, especially the pectoralis, are essential for the preparation of the subfragments and subunits needed in this study.

Comparable muscles in the rabbit would also provide large amounts of protein, but the rabbit is the most convenient animal in which to raise antibodies against a large number of different antigens. Moreover, we had available to us antisera against subfragments and light chains from chicken myosin, which have been well-characterized in earlier and current studies by Lowey and co-workers (31, 24, 25, 44). Finally, because of the ease with which rats can be bred, they are a convenient animal for any systematic study of development (16) or of physiological alteration.

Differential Response to Antibody Against Pectoralis (White) Myosin in a Fast-Twitch Muscle

The rat diaphragm consists of a mixed population of muscle fibers. Three types can be distinguished on the basis of the form and distribution of mitochondria using either the light or electron microscope (13). Mitochondrial content is easily demonstrated by localizing succinic dehydrogenase activity, which is highest in small red fibers, especially at their periphery, lowest in large white fibers, and moderate in intermediate fibers. Red fibers can be further subdivided by their alkali-stable ATPase activity and their response to antimyosin (15). About 40% of the red fibers have a high level of ATPase activity and react strongly with antibodies against white ("fast") myosin from the chicken pectoralis. In contrast, ~60% of the red fibers have a low level of ATPase and do not react with the antibodies. Fibers which have reacted with fluorescein-labeled antimyosin appear bright when examined with the fluorescence microscope (Fig. 2b). These fiber types can be identified by comparison with serial sections in which succinic dehydrogenase is localized (Fig. 2a). Thus, it is evident that antibody reacted with all white, intermediate, and certain of the red fibers. Other red fibers were unreactive. An alkali-stable ATPase was present in the same fibers which had reacted with antibody (Fig. 2c). In addition, the same staining pattern was evident when immunofluorescence was demonstrated by an indirect method (Fig. 3a). When sections were exposed to unlabeled antibody and subsequently reacted with fluorescein-labeled goat antibodies against rabbit immunoglobulin, all white, intermediate, and some red fibers responded, as with the direct procedure. Specificity of the indirect method is therefore sufficiently high. Inasmuch as
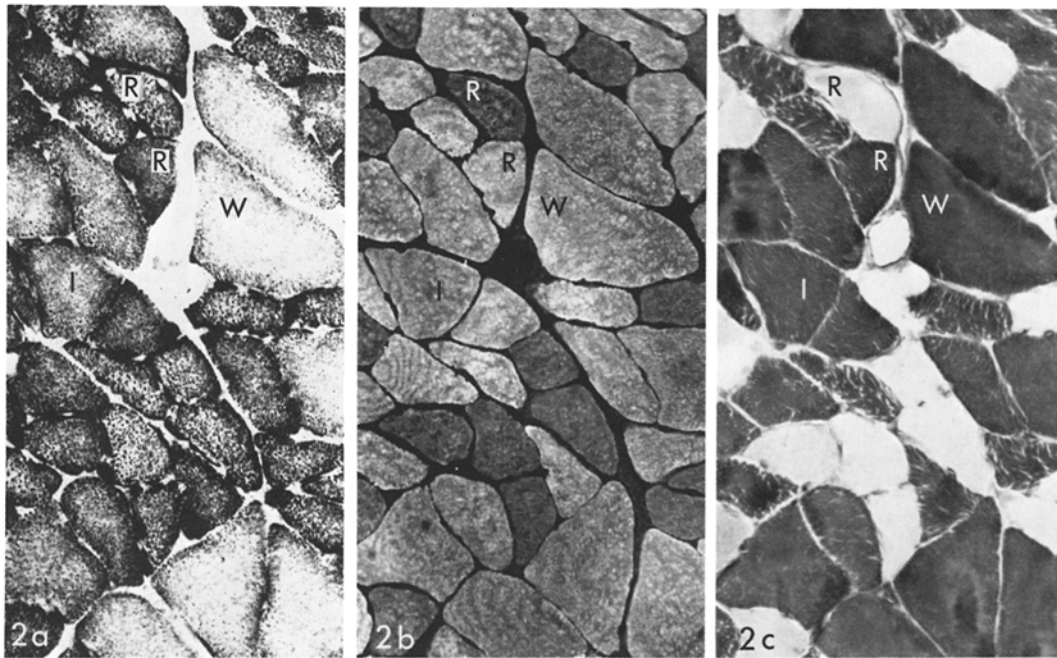
FIGURE 2 Rat diaphragm, serial transverse sections. (a) succinic dehydrogenase. (b) anti-pectoralis (fast) myosin (direct immunofluorescence). (c) alkali-stable ATPase. Fibers are identified by their mitochondrial content (a), which is highest in small red fibers (R), lowest in large white fibers (W), and moderate in intermediate fibers (I). All white (W) and intermediate (I), and ~40% of the red fibers (fast red fibers, black R) react with anti-fast myosin (b) and therefore appear bright with the fluorescence microscope. The same fibers have a high level of ATPase activity (c). × 226.

this procedure greatly amplifies the amount of fluorescein bound for a given antigenic site, it was used routinely in experiments where the level of fluorescence was otherwise low (few antigenic determinants or low-affinity antibody) or where the supply of antibody was limited.

The antimyosin used in these experiments was purified using an immunoadsorbent column to isolate antibodies specific for white ("fast") myosin. The immunoadsorbent was prepared from myosin which had been extracted from a region of the pectoralis consisting entirely of white fibers (15). Therefore, the positive response to this affinity-purified antibody indicates that the myosins of the three reactive fibers are sufficiently homologous to share antigenic determinants. The extent of the homology will become evident from the results described below.

Response to Antibodies Against the Proteolytic Subfragments

Antibody against the "head" (S1) and the "rod" portion of white myosin reacted with the

same fibers that had reacted with antibody against whole myosin (Fig. 3). Hence, the homology of the myosins in the three types of reactive fibers includes both the S1 and rod portions of the molecule. There is, however, a difference in the level of the response to antibodies against S1 within the population of reactive fibers. Those red fibers which reacted were less intensely stained than the white or intermediate fibers (Fig. 3b). This differential staining pattern, furthermore, was apparent using either direct or indirect immunofluorescence. In contrast to the pattern observed with S1, the same fibers were stained at an equally high level by antibodies against the rod (Fig. 3c). These experiments indicate that the antigenic determinants located in the head region of myosin are not distributed equally among fiber types.

Distribution of Low Molecular-Weight Subunits

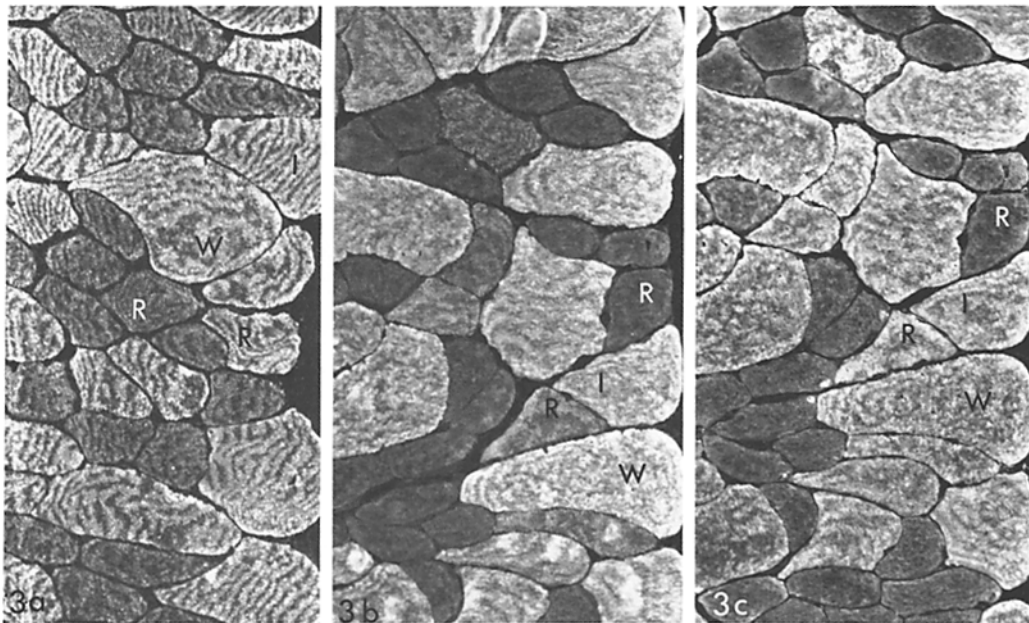Antibodies against the DTNB- and the alkali-light chains also reacted with the white, interme-

FIGURE 3 Rat diaphragm, transverse sections. (a) anti-pectoralis (fast) myosin (indirect immunofluorescence). (b) anti-S1 (with the exception of Fig. 2b, illustrations represent immunofluorescence demonstrated by the indirect method). (c) anti-rod. The pattern of staining with antimyosin (a) is the same as that observed when immunofluorescence was demonstrated by a direct method (Fig. 2b). Antibodies against the proteolytic subfragments of myosin, S1 (b) and rod (c), react with the same fibers that reacted with antibody against whole pectoralis myosin (a). However, the level of response to anti-S1 (b) is lower in fast red fibers (black R) than in other fast fibers (W, I). Compare Fig. 3b with 3c, which is serial to it. × 226.

diate, and some red fibers (Fig. 4a, b, and c). In this case, fluorescence could best be observed by the indirect method, since presumably far fewer antigenic sites are available for staining than with myosin or its subfragments. As a consequence of the sequence homology between alkali 1 and 2, antibodies to the individual light chains show considerable cross-reactivity with the heterologous antigens (24). For this reason, antibodies had to be prepared which are specific for unique regions in the alkali 1 and 2 light chains. Thus, an antibody has been isolated which is specific for the "difference peptide" (Δ1) in alkali 1 (25); this antibody reacted with the same three fibers observed previously (Fig. 4c). Another antibody has been isolated which recognizes a small region (Δ2), probably the N-terminal sequence, of alkali 2 (44, 32); again, this antibody reacted with the same fibers (Fig. 5c). These results demonstrate that the sequence homology of the myosins in white, intermediate and some red fibers is not confined to a limited region of the molecule, but

includes the head, the rod, and all the classes of light chains.

There is, however, a variation in the level of response to antibodies against Δ2, which is comparable to that observed with antibodies against S1. Almost all reactive red fibers stained less intensely with antibodies to Δ2 than did the white and intermediate fibers (Fig. 5c). These same reactive fibers were stained with equal intensity by antibodies against Δ1 (Fig. 5b). Although there is some variation in the level of fluorescence in some of the fibers that have reacted with anti-Δ1 (Fig. 5b), this is the result of fading of fluorescence when exposed to ultraviolet light, and it does not reflect the response to the antibody. The lower intensity is regional and is not related to fiber type. In another example (Fig. 4c), fluorescence is uniform throughout the illustration. In contrast, the lower level of fluorescence in the fast red fiber in response to anti-Δ2 (Fig. 5c) is related to fiber type, and it is observed consistently in all preparations. These results suggest that the alkali 1 and
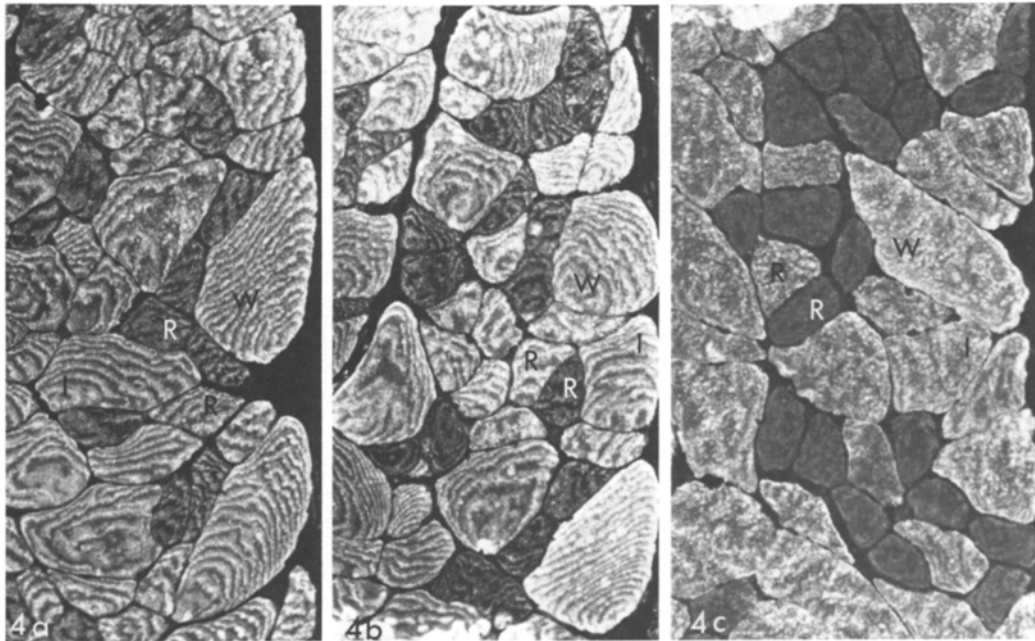
FIGURE 4   Rat diaphragm, transverse sections. Antibodies against myosin light chains. (a) anti-DTNB. (b) anti-alkali 2. (c) anti-alkali 1 (Δ1). All three antibodies react with the same fibers that reacted with antibodies against whole pectoralis myosin (Figs. 2b and 3a) and the proteolytic subfragments of myosin (Figs. 3b and c). × 226.

2 light chains are not distributed uniformly among fiber types.

Differential Response to Antibody Against ALD (Red) Myosin in a Fast-Twitch Muscle

In an effort to characterize the negative red fiber of the rat diaphragm, we have prepared antibodies against myosin from a comparable type of fiber in the chicken. The anterior latissimus dorsi (ALD) has been widely used as an example of a slow red muscle. Most of the fibers resemble negative red fibers in the rat muscle based on their high mitochondrial content, low myosin ATPase activity, and a negative response to antibodies against white myosin (1, 15). Myosin extracted from this muscle was used as an immunogen to elicit antibodies against red myosin. However, a small population of fibers in the ALD have a high ATPase activity and a positive response to anti-white myosin (15), and thus could contribute a white myosin to the immunogen. Therefore, the antiserum was adsorbed on an immunoadsorbent column prepared from white myosin from the pectoralis. Antibodies specific for white myosin would be bound to this column, whereas the

unretained fraction would presumably represent antibodies specific for red ("slow") myosin. The specificity of the immunoglobulin fraction of this adsorbed serum was shown by its ability to react with cryostat sections of chicken muscles. As expected, it reacted very strongly with the slow ALD, but failed to react with the fast pectoralis (Fig. 6), or with the posterior latissimus dorsi (PLD), another fast muscle. Since the muscles, in this case, are from the homologous species, staining (Fig. 6) is far more intense than in all other illustrations. Consequently, the contrast between reactive and unreactive fibers is greater. The negative response of the fast muscles is therefore even more meaningful in establishing specificity of the antiserum. As an additional purification step, the adsorbed anti-ALD was passed through an immunoadsorbent column prepared from ALD myosin. Both the purified antibody and the adsorbed immunoglobulin reacted strongly with the negative red fibers of the rat diaphragm, but not with the positive red fibers or the white or intermediate fibers (Fig. 7b). The two red fibers will henceforth be referred to as "slow" and "fast," respectively (see Discussion). Although a few fast
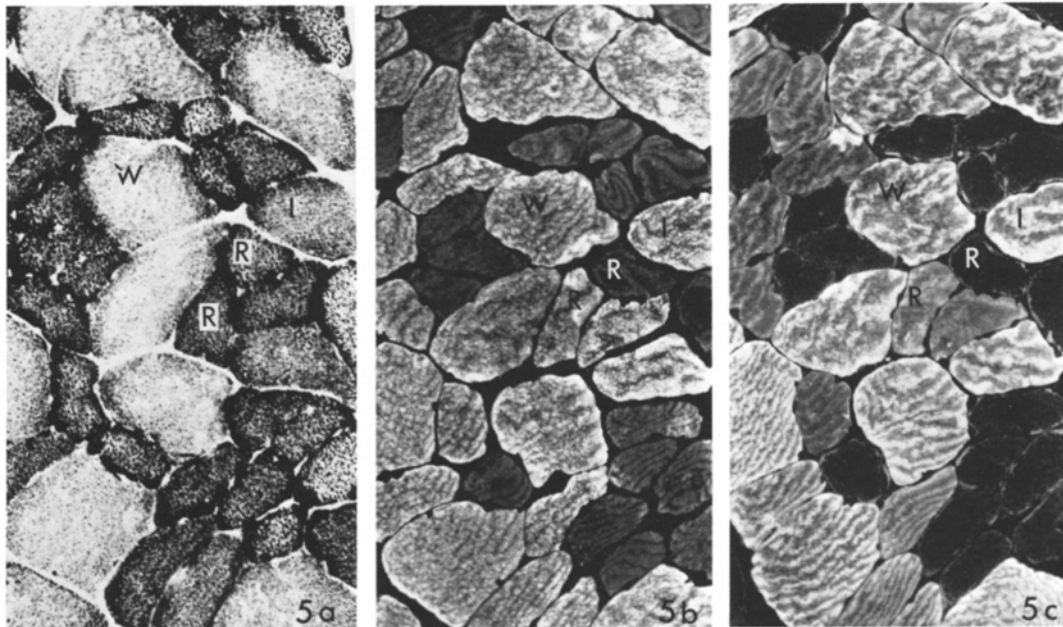
FIGURE 5 Rat diaphragm, serial transverse sections. (a) succinic dehydrogenase. (b) anti-Δ1. (c) anti-Δ2. The pattern of response is the same as that observed with all other antibodies against fast myosin, but the level of response to anti-Δ2 (c) is lower in fast red fibers (black R) than in other fast fibers (W, I). The lower level of fluorescence in some fibers that have reacted with anti-Δ1 (b) is a result of fading of fluorescence when exposed to ultraviolet light. It is regional and is not related to fiber type. Compare with Fig. 4c, which illustrates response to the same antibody as in Fig. 5b, but which does not exhibit regional loss of fluorescence. × 226.

red fibers reacted with this antibody (Fig. 7b), the staining pattern was largely reciprocal to that obtained with antibodies to isoenzymes of white myosin (Fig. 7a). These immunological studies emphasize the difference between the two types of red fibers in the rat diaphragm (compare Fig. 7a with Fig. 7b). This difference, moreover, correlates well with the localization of ATPase activity in the same fibers. The ATPase activity of a particular adult skeletal muscle fiber is stable after either alkali or acid preincubation, but usually not both (19). In the rat diaphragm, fibers which reacted with antibodies specific for isoenzymes of white myosin had a high level of alkali-stable ATPase activity, whereas fibers which reacted with antibodies against ALD myosin had a high level of acid-stable ATPase activity.

Pattern of Distribution of Myosins in a Slow-Twitch Muscle

The soleus muscle of the rat consists of two types of fibers, both of which are rich in mitochondria (14). The majority of the fibers failed to react

with antibodies against the isoenzymes of fast myosins (Figs. 8, 9, 10a, and 10b), but did react with antibodies against ALD myosin (Fig. 10c); they also had high acid-stable ATPase activity. A minority of the fiber population (~25%) reacted with antibodies to fast myosin (Fig. 8a) and with antibodies to S1 (Fig. 8b) and rod (Fig. 8c). These fibers also reacted with antibodies against the DTNB light chain (Fig. 9a), the alkali 2 light chain (Fig. 9b), and the peptides Δ1 (Figs. 9c and 10a) and Δ2 (Fig. 10b). As in the rat diaphragm, the overall pattern of response to these different antibodies is the same as that which had been observed with antibodies against whole white myosin. The minority of the fibers of the soleus, therefore, resemble the fast fibers of a fast-twitch muscle. The intensity of the response to antibodies against S1 and Δ2 is reminiscent of the weak reaction shown by the fast red fiber in the rat diaphragm. Although the level of fluorescence in soleus fibers reacting with antibody against Δ2 is somewhat variable, it is, for the most part, less intense than in white or intermediate fibers of the diaphragm (Fig. 10b). As in the diaphragm, these
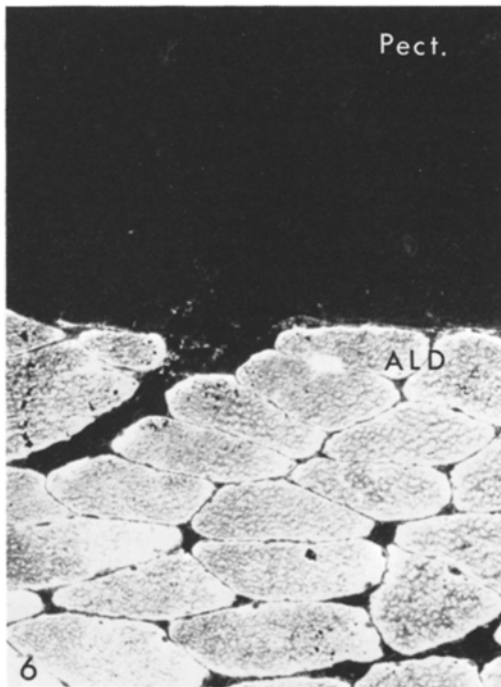
FIGURE 6 Specificity of anti-ALD (slow) myosin. Chicken ALD sectioned simultaneously with chicken pectoralis. This antibody reacts strongly with the slow ALD (lower), but does not react with the fast pectoralis (upper). × 226.

fibers had high alkali-stable, but low acid-stable ATPase activity. Unexpectedly, this same fiber population also reacted strongly with antibody against ALD myosin (Fig. 10c). This indicates that both fast and slow isoenzymes of myosin can coexist within individual muscle fibers.

DISCUSSION

We had shown in a previous study that the rat diaphragm, a fast-twitch muscle, is composed of four types of fibers. Classification was based on several criteria: the localization of an alkali-stable ATPase, mitochondrial content (oxidative activity), and the response of the individual fibers to an antibody prepared against white myosin from the chicken pectoralis muscle (15). Of these criteria, the reaction with antibody is the most definitive characterization of a particular fiber. The level of oxidative activity is a convenient way to describe a muscle fiber in the resting condition, but it has the disadvantage that it can be altered by physical activity, such as strenuous exercise (23, 3, 10). The structural proteins in a fiber are

less responsive to change under normal conditions, although prolonged electrical stimulation can lead to an increased synthesis of slow (or red) myosin at the expense of fast (or white) myosin (45, 35, 41). The distribution of ATPase activity among fibers is a good indication of the type of myosin, insofar as it can discriminate between fast and slow myosin, but it cannot distinguish between isoenzymes within the fast or slow category. For example, the A1 type of myosin (that with alkali 1 light chains) has the same Ca^{2+}-activated ATPase as the A2 myosin (that with alkali 2 light chains), and the two isoenzymes differ only in their actin-activated ATPase (48, 47). The histochemical determination of ATPase measures only the Ca^{2+}-activated ATPase activity and, therefore, would be insensitive to subtle differences among isoenzymes. For the above reasons, it was decided to use the highly specific interaction between an antibody and its antigen to study the distribution of the myosin isoenzymes with respect to fiber type. This approach has the additional advantage that more than one isoenzyme can be localized within a single fiber.

Distribution of Red and White Myosin In a Fast-Twitch Muscle

By staining the rat diaphragm with an antibody specific for myosin isolated from a homogeneous population of white fibers in the chicken pectoralis, we were able to show that all the white and intermediate fibers and about half the red fibers in the diaphragm contained a cross-reacting myosin (15). These results implied not only that there was homology between the myosins from the rat and the chicken, but, more important, that the myosins in the white, intermediate, and some red fibers of a single muscle can be quite similar. Since myosin is a large molecule with hundreds of potential determinants, it was not clear from these experiments alone just how extensive the sequence homology might be; it was entirely possible that only a very limited region of sequence was shared by the myosins of the different fibers, and that these molecules were really quite different in the bulk of their chemical composition. It appears from the results described in the present paper that this is not the case; the same fibers which had reacted with antibody to whole myosin reacted with antibodies prepared against the head, the rod, and all the light chains of white myosin. Even antibodies directed against small unique peptide
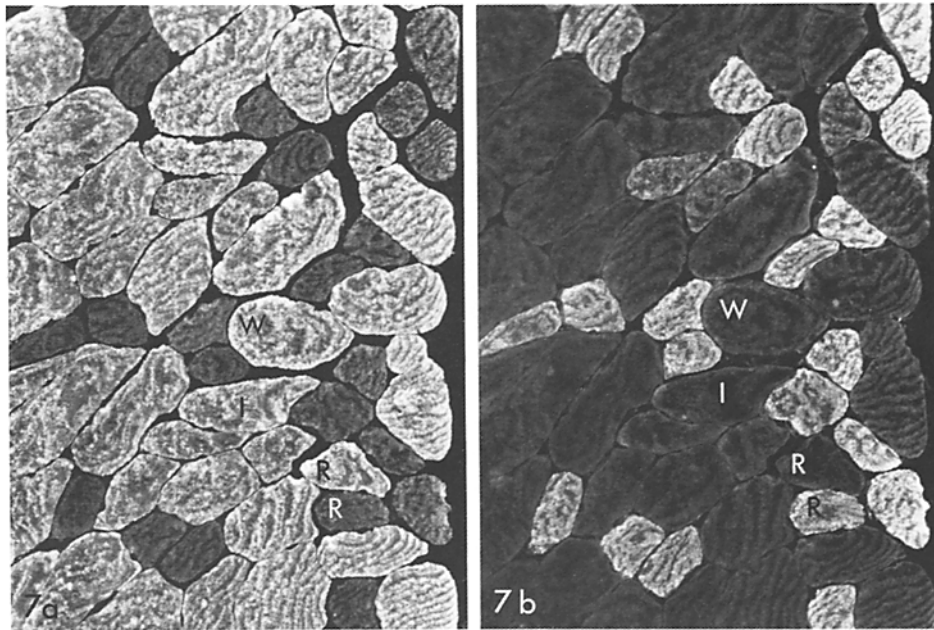
FIGURE 7 Rat diaphragm, serial transverse sections. (*a*) anti-pectoralis myosin. (*b*) anti-ALD myosin. The pattern of response to anti-ALD myosin (*b*) is reciprocal to that seen with anti-pectoralis myosin (*a*). It reacts strongly with slow red fibers (black *R*), but not with fast red fibers (white *R*) or white (*W*) or intermediate fibers (*I*). An occasional fast red fiber reacts with anti-ALD myosin (upper center, Fig. 7*b*). × 226.
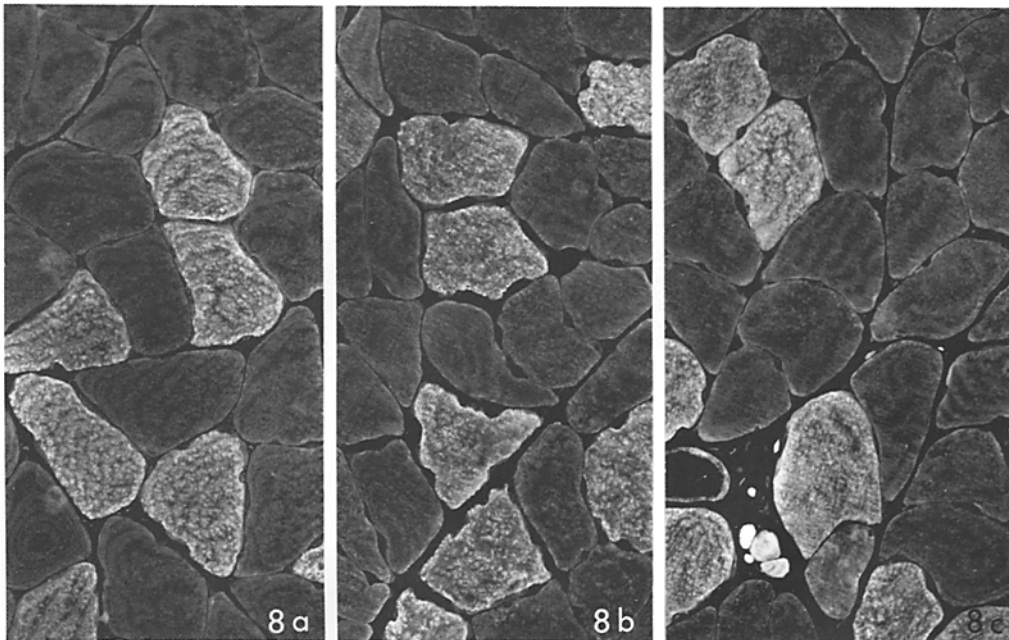

FIGURE 8 Rat soleus, transverse sections. (*a*) anti-pectoralis (fast) myosin. (*b*) anti-S1. (*c*) anti-rod. A minority of the fibers (~25%) react with all three antibodies. The majority are unreactive. × 226.
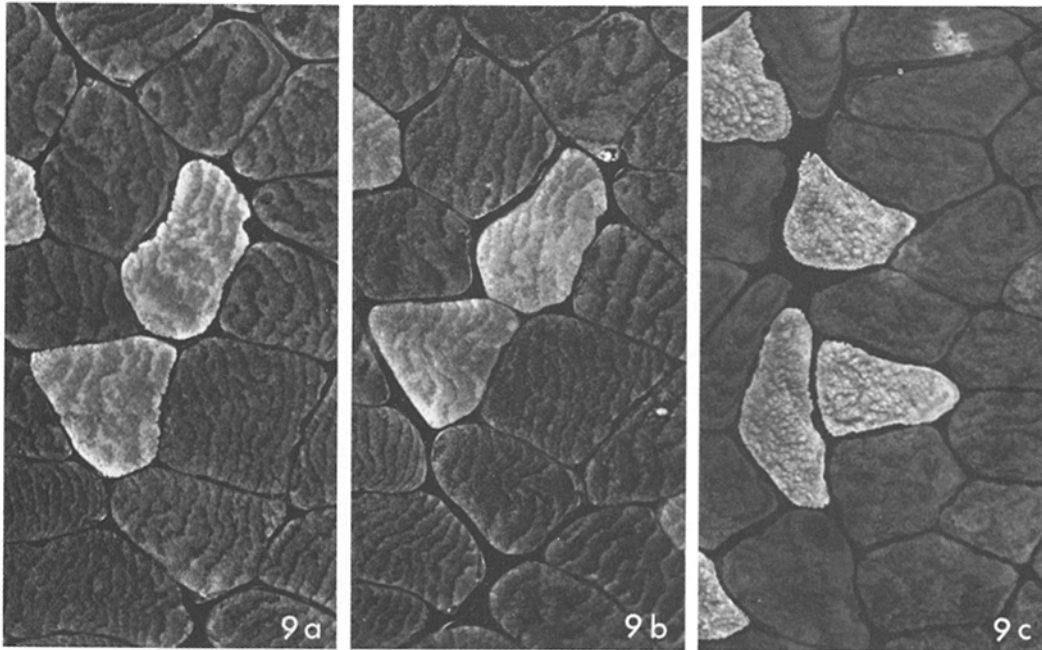
FIGURE 9 Rat soleus, transverse sections. Antibodies against myosin light chains. (*a*) anti-DTNB. (*b*) anti-alkali 2. (*c*) anti-alkali 1 (Δ1). The pattern of response is the same as that observed with antibodies against whole pectoralis myosin (Fig. 8*a*) and the proteolytic subfragments of myosin (Figs. 8*b* and *c*). Only a minority of the fibers react. × 226.
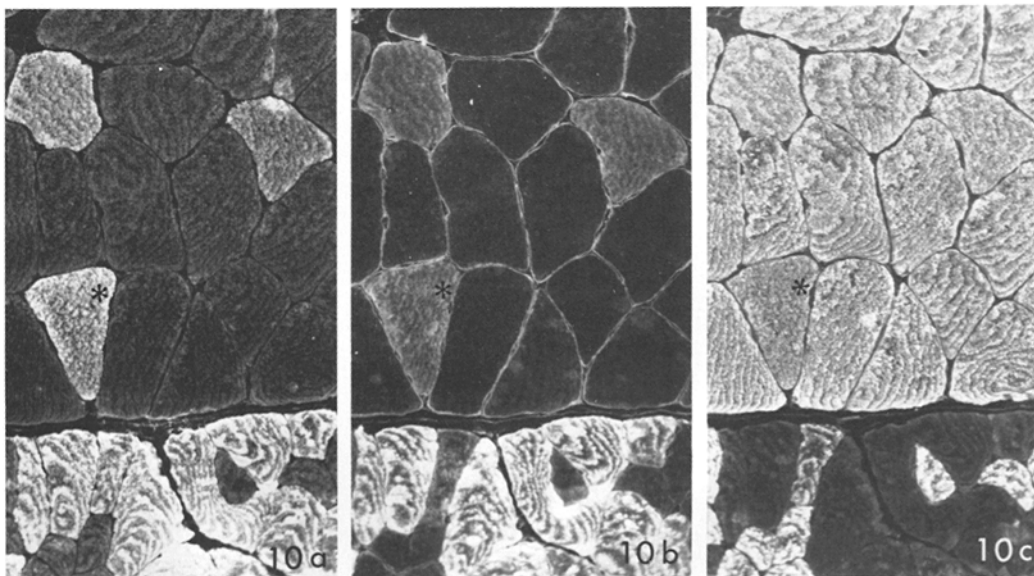

FIGURE 10 Rat soleus, serial transverse sections. Soleus is sectioned simultaneously with rat diaphragm so that the two muscles can be compared directly under identical conditions. (*a*) anti-Δ1. (*b*) anti-Δ2. (*c*) anti-ALD. A minority of the fibers react with antibodies against the two fast light chains (*a* and *b*). The level of fluorescence in soleus fibers reacting with anti-Δ2 (*b*) is less intense than in white or intermediate fibers of the diaphragm; it is comparable to that of the fast red fibers of the diaphragm. Antibody against slow myosin (*c*) reacts with the same fibers that reacted with antibodies against fast myosin (*a* and *b*) as well as with the majority of the fibers, which did not react with anti-fast myosin. × 196.

regions of the alkali 1 and 2 light chains stained the same reactive fibers in the diaphragm. These antibody markers have confirmed the work of Weeds and colleagues (50), who first showed by gel electrophoresis that the alkali 1 and 2 light chains can coexist within a single fiber. These experiments are consistent also with the observation of Pette and Schnez (36) that single isolated fast fibers have a light chain pattern characteristic of fast white myosin. The conclusion from these studies is that the myosins in the white, intermediate, and certain red fibers are remarkably similar if not identical. Moreover, one would expect these myosins to be quite different from the myosin in those red fibers which did not show any staining with the antibodies discussed above. This prediction was supported by the staining patterns obtained with an antibody prepared against myosin from the chicken ALD, a predominantly slow red muscle. This antibody reacted with that population of red fibers which had previously failed to stain with antibody and which had a low alkali-stable ATPase activity. It has been shown that the light chain pattern of this type of fiber is characteristic of that from a slow-twitch muscle (36).

Distribution of Isoenzymes Within a Population of Fast Fibers

Since we have now introduced an antibody specific for those red fibers formerly called "unreactive" (15), it was necessary to modify our nomenclature to distinguish between the two types of red fibers. We have been reluctant to assign a designation which reflects speed, since the speed of contraction of individual fibers of the rat diaphragm has not actually been measured. However, these fibers have many cytochemical features which resemble those observed in fibers of the cat gastrocnemius (see below), where physiological measurements have been made (5). We shall therefore refer to the two types of red fibers in the rat muscle as "slow" (unreactive with anti-white myosin) or "fast" (reactive with anti-white myosin) (Table I).

So far, we have emphasized the similarity of the myosins in the fast fibers. Of equal importance is the finding that the myosins in the fast red fibers and in the white fibers are not identical. Antibodies specific for S1 and for the alkali 2 light chain stain the fast red fibers less intensely than the white or intermediate fibers. A differential antibody staining pattern among reactive fibers had also been reported in an earlier paper (15), and two possible explanations were proposed: one involved a new isoenzyme in the fast red fiber (by "new" we mean different from fast white or slow red myosin), and the other suggested a mixture of fast and slow myosin in the fast red fiber. The second hypothesis now seems less likely, since there was no significant response by these fibers to antibody against ALD myosin. This suggests the existence of three types of myosin, one in the white fiber, one in the slow red fiber, and a third type in the fast red fiber. However, an additional explanation must now be considered; since antibody against $\Delta 2$ stained the fast red fiber less intensely than did antibody to $\Delta 1$, it is possible to account for the staining pattern by postulating a variable distribution of the A1 and A2 myosin isoenzymes, with the highest concentration of A2 myosin in the white fiber and the lowest amount in the fast red fiber. To account for the weak staining with anti-S1 one would need to postulate that these antibodies recognize conformations in the myosin head associated with the presence of A2 light chain. Unfortunately, no definitive solu-

TABLE I

Ultrastructural and Cytochemical Characteristics of Fiber Types in Relation to Physiological Properties of Motor Units

Fiber Type*	Mitochondria (SDH)	Z-line	ATPase (alkali-stable)	Anti-white myosin (Pectoralis)	Anti-red myosin (ALD)	Motor unit‡	Speed of contraction	Fatigue resistance
White	Low	Narrow	+	+	−	FF	Fast	Low
Intermediate	Int.	Narrow	+	+	−	F (int)	Fast	Int.
Red (fast)	High	Wide	+	+	−	FR	Fast	Int. to high
Red (slow)	High	Wide	−	−	+	S	Slow	Very high

* Based on ultrastructural and cytochemical characteristics of muscle fibers in the rat diaphragm and semitendinosis (12, 13, 15, and footnote 1).
‡ Data represent individual muscle units from the cat gastrocnemius (5, 6).

tion to the question of which isoenzymes are present in the different muscle fibers is available at present. However, these results do demonstrate, for the first time, that the myosins of all fast fibers are not necessarily identical. Chemical analysis of single fibers could eventually give an answer to this question.

Physiological Implications of Myosin Isoenzyme Distribution

We have described the pattern of response to antibodies against fast and slow isoenzymes of myosin among the muscle fibers of the rat diaphragm, a fast-twitch muscle. Four basic types of fibers can be identified, namely: white, intermediate, fast red, and slow red. The greater response to antibody specific for the alkali 2 light chain and for S1 serves, moreover, to distinguish the intermediate fiber from the less responsive fast red fiber, which it otherwise resembles closely. Additional evidence for these two types of fibers has been obtained by examining, with the electron microscope, fibers which have been exposed to antibody against white myosin.[1] Based on ultrastructural characterization alone, three fiber types can be recognized: the white fiber has a narrow Z-line and low mitochondrial content; the intermediate fiber has a narrow Z-line, but mitochondrial content is moderately high; the red fiber has both a wide Z-line and a high mitochondrial content. The red fiber can now be subdivided on the basis of a positive or negative response to anti-white myosin visualized directly with the electron microscope. Both categories of red fiber have wide Z-lines. The fast red and intermediate fibers, both of which react with the antibody, can therefore be distinguished on the basis of Z-line width, since it is wide in the two red fibers and narrow in the intermediate fiber. This confirms the identity of the intermediate fiber as one of four intrinsically different fiber types (Table I).

We suggested (15) that these four fibers correspond to four types of motor units described by Burke and his associates (5) in the cat gastrocnemius, also a fast-twitch muscle. The white fiber has a low mitochondrial content and high alkali-stable ATPase activity in both the rat and cat muscles, and therefore corresponds to the FF (fast-contracting, fast-fatigue) motor unit (Table

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[1] Gauthier, G. F. Ultrastructural identification of muscle fiber types by immunocytochemistry. Manuscript submitted for publication.

I). The intermediate fiber is intermediate in mitochondrial content, but ATPase activity is high, and therefore it presumably corresponds to the so-called F (int) motor unit (fast-contracting, intermediate fatigue resistance). Red fibers are rich in mitochondria, but ATPase activity is either high or low; these most likely represent the FR (fast-contracting, fatigue-resistant) and S (slow-contracting, fatigue-resistant) motor units, respectively. In both the cat and rat muscles, furthermore, all white and intermediate and those red fibers with high ATPase activity (i.e., FF, F (int), and FR motor units) react with antibodies specific for white myosin. Red fibers with low ATPase activity (type S motor unit) are unreactive, but they react with antibodies against ALD myosin (Table I). Muscle fibers corresponding to fast-contracting motor units also react with antibodies against various portions of white myosin, from which it is concluded that, as a group, the fast fibers have myosins which are remarkably similar.

We have discussed the possibility that the myosin of the fast red fiber is not identical to that of the other fast fibers in the rat diaphragm. Preliminary observations indicate that the myosins within the population of fast fibers of the cat gastrocnemius are also not identical. The staining of one type of red fiber (comparable to the fast red fiber in the rat muscle) is far more intense with anti-$\Delta 1$ than with anti-$\Delta 2$. The presence of a different myosin in this fiber type would be consistent with the physiological characteristics of the corresponding motor unit. The FR motor unit has a somewhat longer contraction time than other fast units (5). The difference in time to peak tension is small but statistically significant, and it suggests that the FR unit represents a separate category on the basis of its slower speed of contraction as well as its greater fatigue-resistance (R. E. Burke, personal communication).

The majority of the fibers in the rat soleus, a slow-twitch muscle fail to react with antibodies against any of the subfragments or light chains of fast myosin, but do react with antibody against ALD myosin. Therefore they resemble the slow red fibers of a mixed muscle, but they are not necessarily identical (4, 15). There is also, in the rat soleus, a small population (~25%) of fibers which react with antibodies against white myosin as well as its subfragments and light chains. This group of fibers most likely corresponds to a group of fast-contracting, fatigue-resistant motor units observed in the same muscle (29). They have a

high mitochondrial content and high alkali-stable ATPase activity. In addition, they have lesser amounts of the alkali 2 light chain than do white fibers of the diaphragm, and in this respect, they resemble the fast red fiber of the diaphragm. However, these results do not necessarily indicate that either population of soleus fibers is identical to any of the fibers of a fast-twitch muscle. We have shown that the fast-contracting fibers contain slow as well as fast myosin. This unusual property is not shared by any of the fibers of the rat diaphragm, with the possible exception of an occasional fast red fiber. This is the first demonstration of the presence of both slow and fast myosin within a single fiber in a normal adult muscle. Before this, we had shown that slow and fast myosin coexist in developing muscle fibers (16), and this has also been observed in chronically stimulated muscles (37, 40).

In conclusion, muscle fibers with fast myosin are present in a slow-twitch muscle (soleus) as well as in a fast-twitch muscle (diaphragm) of the rat, but the proportion of fast fibers is clearly greater in the faster muscle. This correlates well with the relative proportion of fast motor units demonstrated by Kugelberg (28) in the fast tibialis and slow soleus muscles of the same species. The presence within an individual muscle of different types of fibers and of different isoenzymes of myosin could provide an unlimited potential for modulation of physiological properties. The tendency is for a particular fiber to have either a fast contraction time or high resistance to fatigue, which suggests that speed is acquired at the expense of fatigue-resistance. However, some fatigue-resistant fibers have the capacity to contract rapidly. The fast red fiber in the diaphragm is an example of such a fiber. It appears, furthermore, to have a myosin composition which is distinct from that present in fast fibers with low fatigue resistance. The FR motor unit, which most likely corresponds to this muscle fiber, contracts somewhat less rapidly than other fast units but, nevertheless, has the combined advantages of speed and resistance to fatigue.

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