ULTRASTRUCTURAL IDENTIFICATION OF MUSCLE FIBER TYPES BY IMMUNOCYTOCHEMISTRY

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ABSTRACT

In a fast-twitch muscle, three types of fibers (red, intermediate, and white) can be distinguished on the basis of mitochondrial content. Red fibers, identified by abundant mitochondria, can be further differentiated on the basis of a positive or negative response to antibodies specific for white ("fast") myosin. Because there is also a difference in Z-line width among fibers of the same muscle, the possibility existed that the two red fibers, which differ in type of myosin, might also differ in dimensions of the Z line. We therefore examined, with the electron microscope, fibers which had been exposed to antibody against white myosin. In those fibers which react with the antibody, an electron-opaque band is evident in the H-band region, thereby distinguishing reactive from unreactive fibers. 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 fibers ("fast" and "slow") have wide Z lines, and thus are distinguished from white and intermediate fibers, which react with the antibody but which have narrow Z lines. On the basis of combined immunocytochemical and ultrastructural characteristics, four types of fibers can be recognized in a single muscle. Moreover, it is demonstrated here that a wide Z line does not necessarily imply a slow speed of contraction.

KEY WORDS skeletal muscle · fiber types · myosin · immunocytochemistry · muscle ultrastructure · thick filament

Based on ultrastructural as well as light microscope characteristics, three types of fibers can be recognized in individual fast-twitch mammalian muscles (6, 7, 11). The "white" fiber has a narrow Z line and low mitochondrial content; the "intermediate" fiber also 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. Using an immunocytochemical approach, it has been possible, with the light microscope, to distinguish two categories of red fibers in the diaphragm of the rat (8). One category reacts with antibodies specific for white ("fast") myosin, and the other fails to react. Fibers which react (all white and intermediate and ~40% of the red fibers) also have a high level of myosin ATPase activity and are therefore equivalent to type II fibers (5). They predominate in muscles which have a fast contraction rate (10), and hence are often designated "fast" fibers. These fibers have many of the cytochemical features which are characteristic of fast-contracting muscle units in the cat gastrocnemius, where physiological measurements have been obtained (1). We have, therefore, referred to those red fibers of the rat diaphragm which have a positive or negative response to anti-white

myosin as "fast" or "slow" red fibers, respectively (9). However, since there are differences in width of the Z line among the fibers of this muscle, it was possible that the two red fibers might differ with respect to Z-line width as well as the type of myosin present. Therefore, we have compared reactive and unreactive fibers, using the electron microscope directly. We have shown that both fast and slow red fibers have wide Z lines, and thus, together with white and intermediate fibers, they comprise four readily distinguishable categories of fibers.

MATERIALS AND METHODS

Preparation of Antibodies

Rabbit antiserum against chicken pectoralis myosin represents a pool (9–3) of several bleeds (8). The serum was purified using an immunoadsorbent column prepared from white ("fast") myosin coupled to Sepharose. Antibodies specific for white myosin were thus selectively adsorbed from a population directed against whole pectoralis myosin. This antibody preparation was used in an earlier study and is described more fully elsewhere (8, 9). Evidence for cross-reactivity is also presented below (see Results). Goat immunoglobulin (IgG) directed against the Fc fragment of rabbit IgG was prepared from serum contributed by Dr. A. Nisonoff.

Immunocytochemistry

Adult male albino rats were killed with chloroform, and the abdominal surface of the left hemidiaphragm was exposed by means of a mid-ventral incision. Strips of muscle, ~3 mm wide, were glycerinated during a 24-h period according to a procedure described by Rome (15). The tied strips of muscle were placed alternately, for 30 min, in standard salt solution (100 mM KCl, 1 mM $MgCl_2$, 6.67 mM phosphate, pH 7.0) and in glycerol solution (50% glycerol, 6.67 mM phosphate, pH 7.0) at 0°C. After 3 h they were placed in fresh glycerol solution and left for 24 h at 0°C. They were finally stored in fresh glycerol solution at −20°C. For the immunocytochemical procedure, the glycerinated muscle was washed three times in cold (0°–5°C) phosphate buffer (100 mM KCl, 10 mM phosphate, pH 7.2) for 20 min each. The tissue was blotted with filter paper, removed from the splints, and then supported with 7% agar on the stage of a Sorvall TC-2 "Smith-Farquhar" tissue sectioner (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). Transverse "sections," 50 μm thick, were cut and transferred to cold phosphate buffer. Six to 10 sections were incubated in ~0.3 ml of anti-white myosin solution at a protein concentration of ~2 mg/ml in phosphate buffer for ~16 h at 0°–5°C. Immunoglobulin from nonimmunized rabbits had a protein concentration of 1–2 mg/ml. The reacted sections were washed four times in phosphate buffer for a total of 2 h and then incubated in goat IgG (~2 mg/ml protein) for an additional 16 h at 0°–5°C. They were then washed four times in cold phosphate buffer for 2 h total.

Electron Microscopy

After incubation, the washed sections were fixed in 6.25% glutaraldehyde buffered with 0.067 M sodium cacodylate (pH 7.2) for 1 h at 0°–5°C. After washing three times in 0.1 M cacodylate, 0.25 M sucrose for 30 min total, they were postfixed in 1.0% osmium tetroxide buffered with 0.14 M veronal acetate (pH 7.4) for 1 h at 0°–5°C, and washed twice in cacodylate-sucrose solution, 3 min each. The sections were then dehydrated and embedded in Epon. Each entire section was oriented in a flat-embedding mould (Ladd Research Industries, No. 23650) with the fibers perpendicular to either the flat or lateral surface of the block (i.e., parallel to the cutting face) to facilitate preparation of longitudinal sections. For the examination of untreated muscle, 1-mm strips were excised from the animal and fixed immediately in 6.25% glutaraldehyde; they were processed for electron microscopy as described above, except that the strips were cut into 1-mm cubes and stained with 1% uranyl acetate before dehydration and embedding. Ultrathin sections parallel to the longitudinal axis of the fibers were cut on a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate and lead hydroxide, and then examined with a Siemens Elmiskop IA.

RESULTS

Ultrastructure of Three Fiber Types

Initial identification of skeletal muscle fiber types was based on the form and distribution of mitochondria as well as width of the Z line. Because glycerination causes disruption of mitochondrial ultrastructure, it was necessary to evaluate each fiber over an extensive area, only part of which is evident in any one micrograph. Criteria for identification are therefore best illustrated in fibers that have not been altered by experimental treatment (Figs. 1, 2, and 3). In the red fiber (Fig. 1), large mitochondria with closely packed cristae form conspicuous peripheral aggregations and interfibrillar rows, and the Z line is characteristically wide. The intermediate fiber (Fig. 2) is similar except that mitochondria are somewhat less abundant and the Z line is only about half as wide. In the white fiber (Fig. 3), peripheral mitochondria and interfibrillar rows are rare, and the Z line is narrow as in the intermediate fiber. The same ultrastructural features were observed in fibers exposed to antibody, but they are not necessarily included in the micrographs used to illustrate the

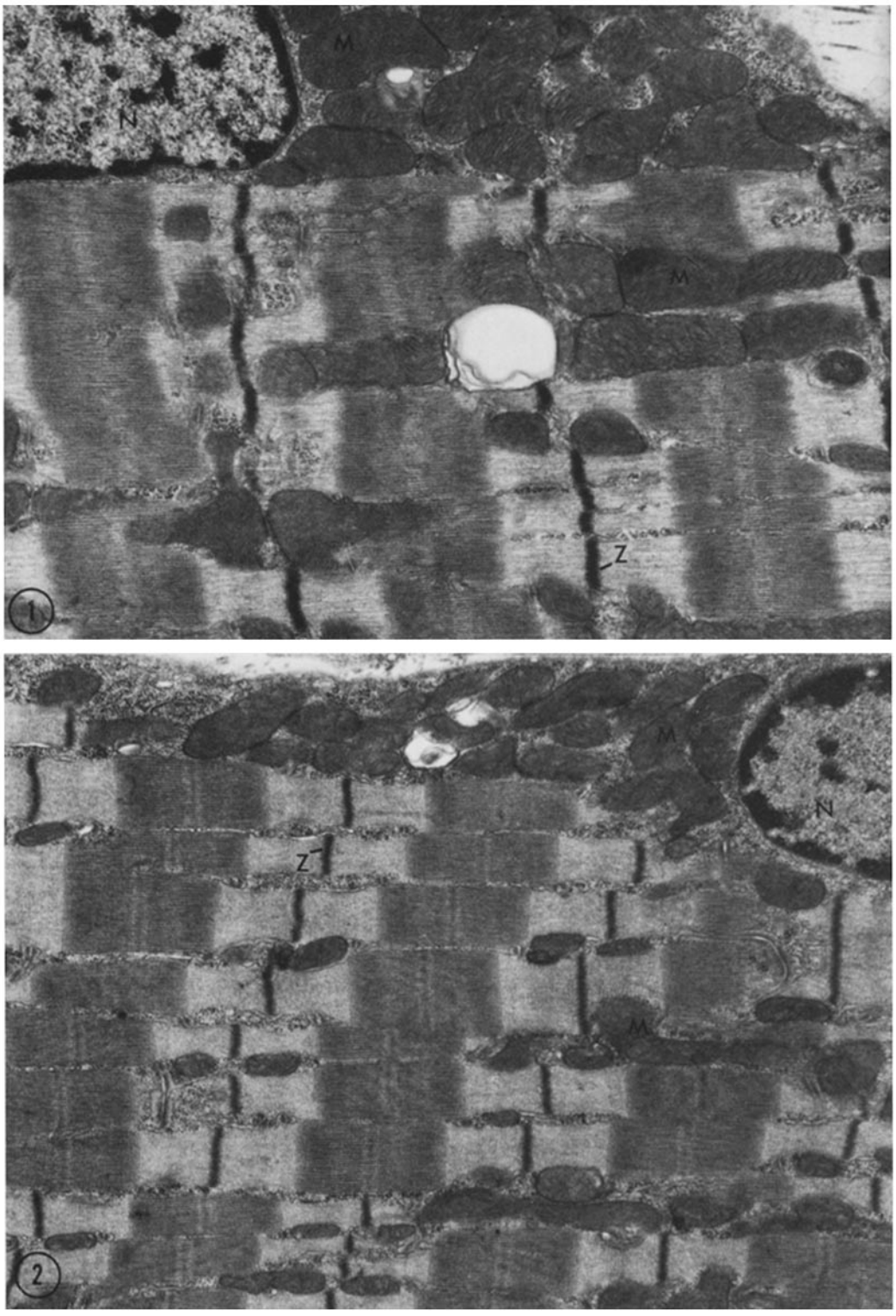


FIGURE 1 Red fiber. Large mitochondria (*M*) with closely packed cristae are located at the periphery of the fiber in the region of the nucleus (*N*) and also in rows between myofibrils. The Z line (*Z*) is characteristically wide. × 15,000.

FIGURE 2 Intermediate fiber. Mitochondria (*M*) are moderately abundant, but somewhat less than in the red fiber (Fig. 1); the Z line (*Z*) is only about half as wide as in the red fiber. × 15,000.

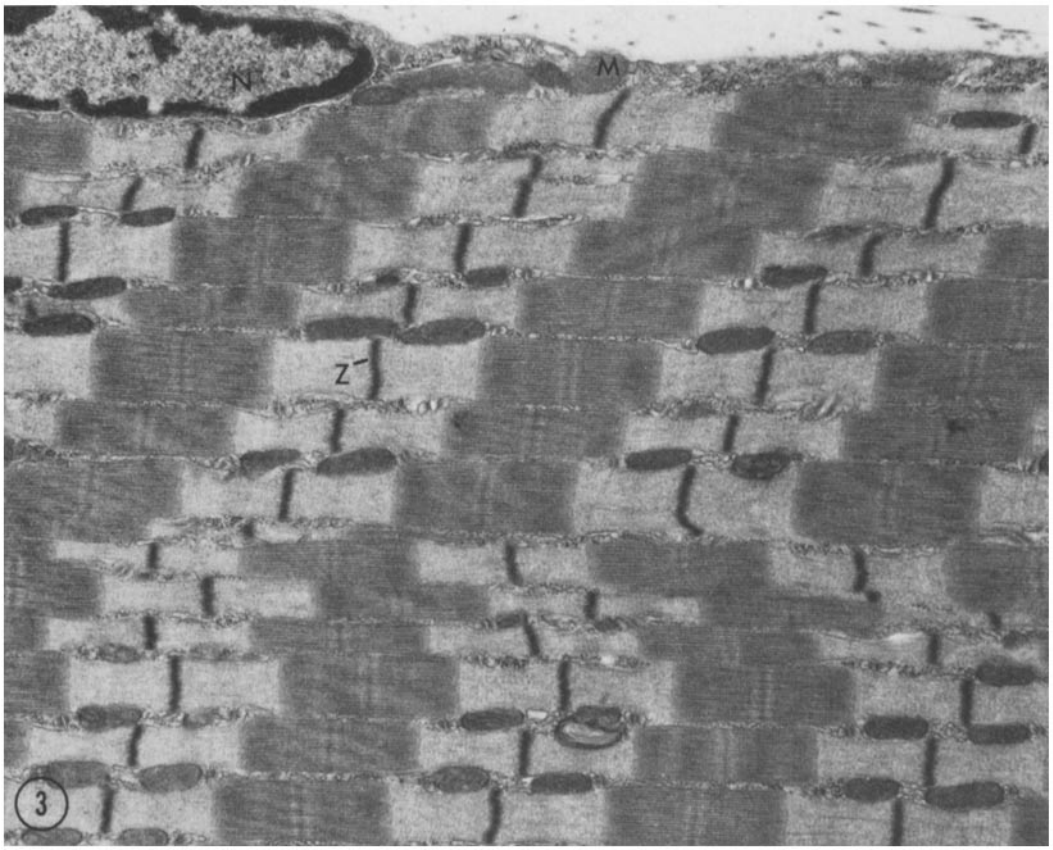


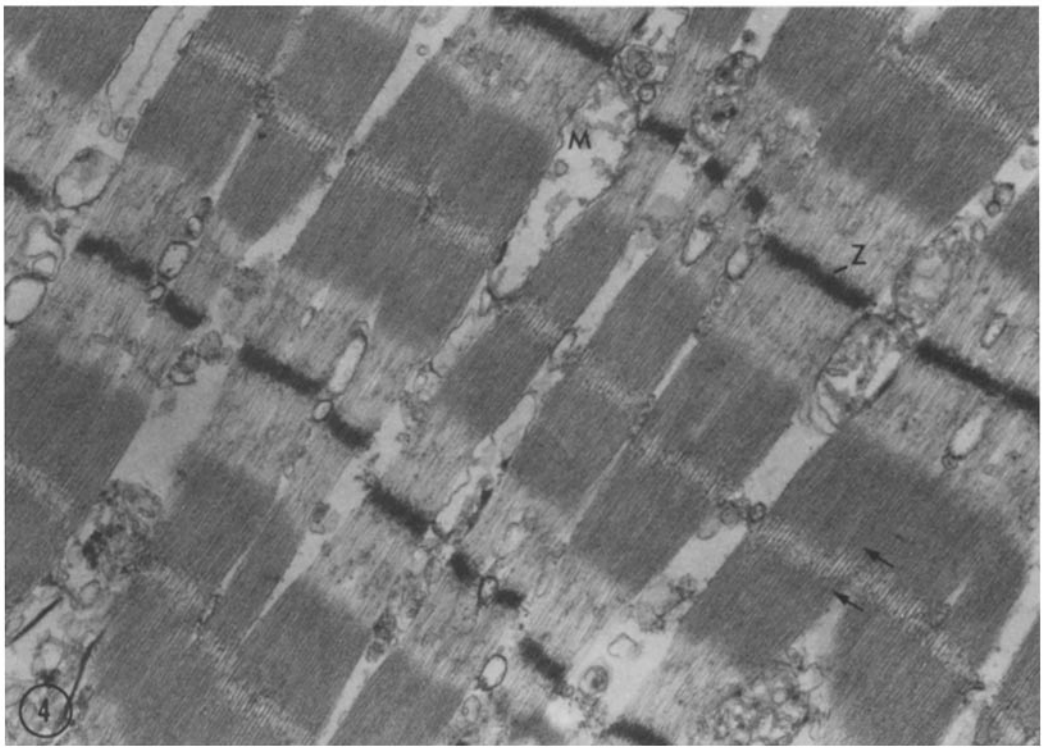
FIGURE 3 White fiber. Peripheral mitochondria (*M*) are few in number, and interfibrillar rows are absent. The Z line (*Z*) is about half as wide as that of the red fiber (Fig. 1). × 15,000.

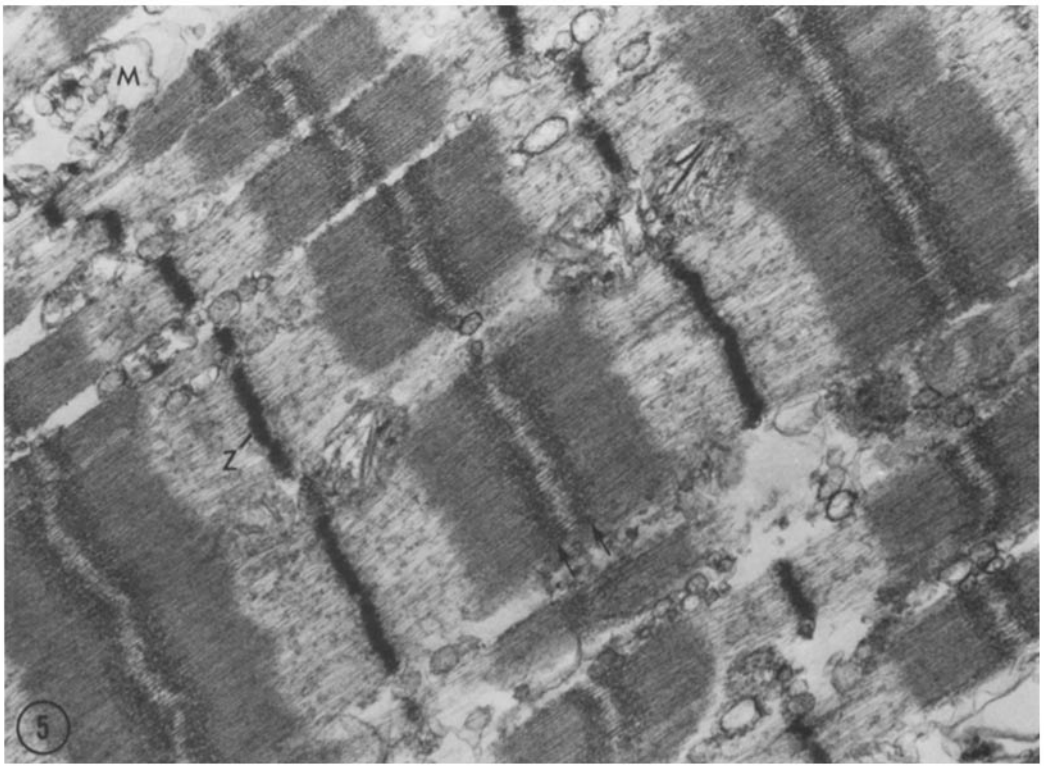
response to the antibody. Mitochondria, for example, are often represented only by membranous configurations (see Figs. 4, 5, and 6), which remain after treatment with glycerol.

Response to Anti-White Myosin

By taking advantage of the reaction of antibodies with proteins of the thick filament, it has been possible to "stain" the A-band region of a myofibril without additional labeling (3, 4, 12). In these earlier studies, the antibodies were reacted with isolated bundles of glycerinated muscle fibers, and the resulting "staining" exhibited a gradient, with the greatest intensity occurring at the periphery of the fibers (13). This method is satisfactory for localizing proteins within individual sarcomeres, but it is not ideal for the comparison of one fibril with another. For example, a positive vs. a negative response in adjacent fibrils could be attributable to a difference in the level to which the antibodies have diffused into the fibers. In the present study, antibodies were reacted with transverse sections of glycerinated, but not teased, muscle. A large population of fibers is thus included in the sample. Because the cells comprising skeletal muscles are long and more or less cylindrical, transverse sections automatically expose the cytoplasm without the need for disruption of surface membranes.[1] By this procedure the proteins in even a relatively thick section would be expected to be available to the antibody; use of a thick section ensures that the muscle fibers be of adequate length to facilitate orientation in blocks processed for electron microscopy. The procedure

[1] The purpose of glycerol in the present method was to prevent contraction of the fibers. Glycerination was chosen over fixation to minimize inactivation of antigenic sites in a system where the concentration of cross-reacting antibodies is already low (9).





FIGURES 4 and 5 Red fibers exposed to anti-white myosin. Z lines (*Z*) are wide, and membranous remnants of large mitochondria (*M*) are present between myofibrils of both fibers. There is no evidence of binding of antibody with myofibrils of the "slow" red fiber (Fig. 4), but in the "fast" red fiber (Fig. 5), a distinct electron-opaque band (arrows) is apparent on either side of the unstained bridge-free zone. This band occupies only the proximal part of the H band, which is visible because the fibers are highly stretched (compare the width of the A and I bands). × 21,500.

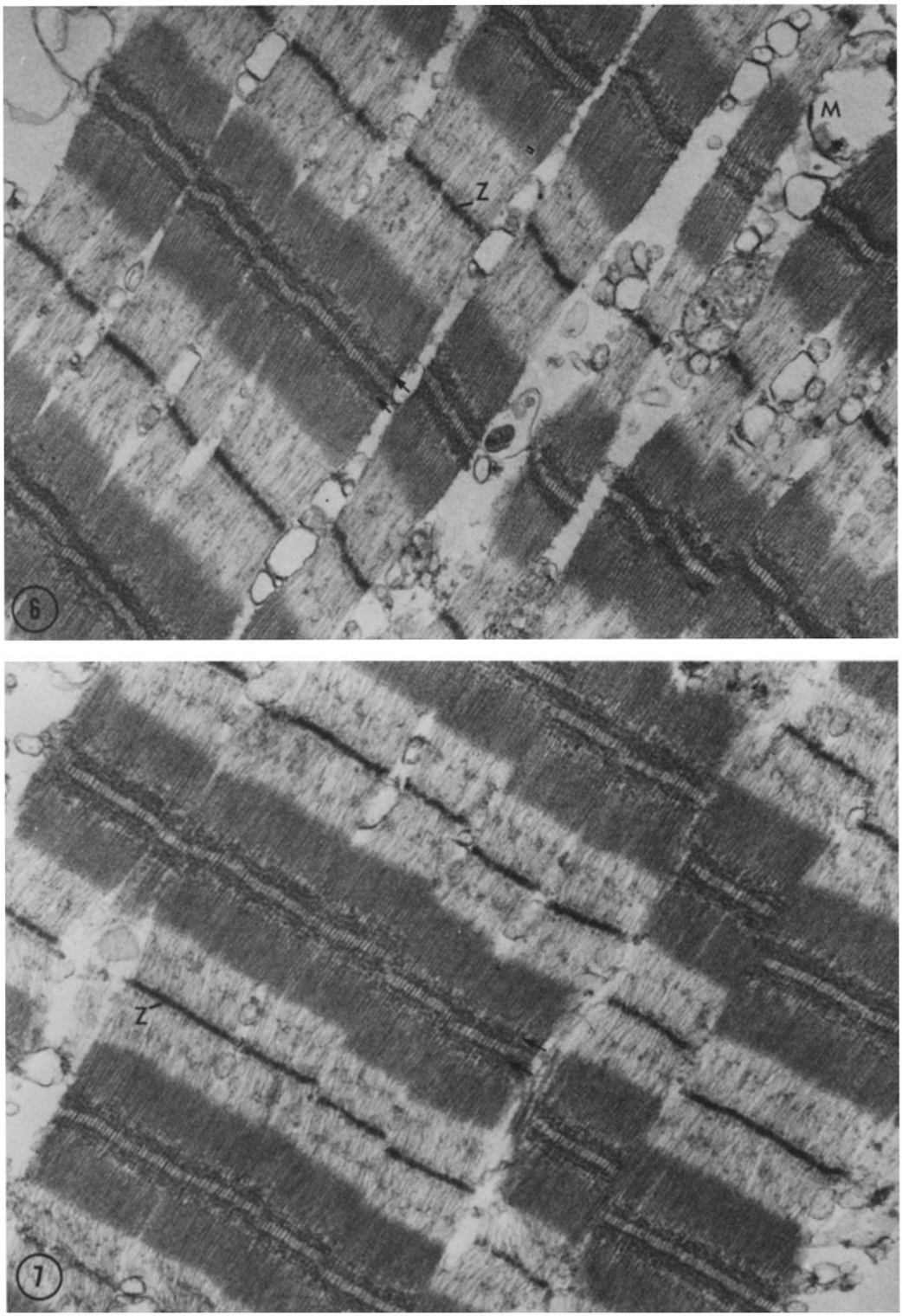


FIGURE 6 and 7 Intermediate and white fibers, respectively. Both fibers have narrow Z lines (*Z*) and both react with anti-white myosin. A circumscribed electron-opaque band (arrows) is present on either side of the bridge-free zone. The lateral limits of the H band are less evident than in Fig. 5 because the sarcomeres are less stretched. × 21,500.

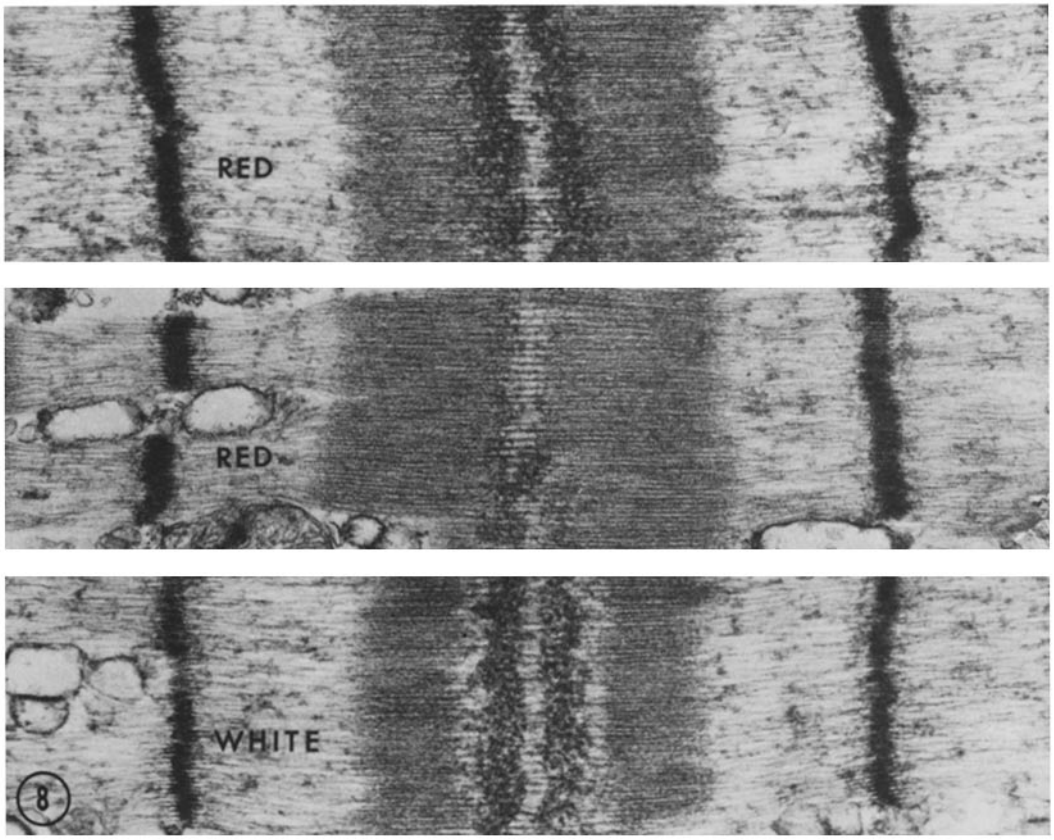


FIGURE 8 Single sarcomeres of fast red (upper), slow red (middle), and white fibers. Both fast and slow red fibers have wide Z lines when compared with the white fiber. Anti-white myosin is bound at a limited (proximal) region in each half of the A band of the fast red and the white fiber, but not the slow red fiber. The somewhat narrower A band in the white fiber is not related to magnification, which is the same as that of the red fibers. It is a consistent observation which may reflect an alteration in the thick filament in response to binding of antibody. × 36,000.

also has the advantage that all of the muscle fibers and myofibrils are exposed to the same experimental conditions, since diffusion can take place in a longitudinal direction. In this type of preparation, many fibers can be compared in a single ultrathin section.

Antibodies against chicken pectoralis myosin cross-react with myosin from the rat diaphragm. These antibodies showed a single precipitin line against rat myosin when examined by gel diffusion in agar (9). However, quantitative precipitin analysis showed that only ~30% of the antibodies precipitated rat myosin. Therefore the amount of antimyosin which reacts with the muscle is insufficient to be visualized directly, and an indirect method had to be used in which the fibers were subsequently treated with goat IgG which reacts with the primary antibody. No exogenous label was necessary, since the interaction of antibody with the thick filament provides sufficient increase in electron opacity to permit recognition of reactive and unreactive fibers wth the electron microscope. In fibers which react with the antibody, two discrete electron-opaque bands are evident on either side of the pseudo-H or bridge-free zone (Figs. 5, 6, and 7). Because the localization is circumscribed, and does not extend through the entire A band, reactive fibers can be readily distinguished from unreactive fibers without relying on a quantitative estimate of intensity of the antibody reaction. Fibers which do not react show no electron-opaque band in this region (Fig. 4). When nonimmune globulin is substituted for the antibody, there is also no visible reaction. There is no

gradient in staining among the myofibrils of any fiber or among the fibers. This indicates a uniform transverse distribution of antibody. It can be concluded that the presence of both reactive and unreactive fibers is the result of a preferential interaction with antibody and not a result of incomplete diffusion.

The results show that all fibers with narrow Z lines, namely white (Fig. 7) and intermediate fibers (Fig. 6), react with antibody to white myosin. Fibers with abundant mitochondria and wide Z lines can be either reactive (Fig. 5) or unreactive (Fig. 4). That is, both fast and slow red fibers have wide Z lines when compared with either the intermediate or the white fiber (Fig. 8). There is no apparent difference in staining pattern among fiber types. The electron-opaque band occupies a greater proportion of the H-band in some fibers (Figs. 6 and 7) than in others (Fig. 5). However, this is evident only because the sarcomeres in Fig. 5 are highly stretched, and the lateral limits of the H band are consequently located at a greater distance from the stained region.

DISCUSSION

Polymorphism Among Skeletal Muscle Fibers

It is becoming increasingly apparent that morphological differences among skeletal muscle fibers reflect significant differences in functional activity. However, the exact relationship between structural and physiological properties is not well understood. Interpretation of data from various laboratories is complicated by the numerous systems of nomenclature currently in use. The combination of ultrastructural and immunocytochemical criteria have led us to define four categories of fibers. However, it is evident that other categories may exist as well.

The differential response of individual fibers to antibody for a particular type of myosin is highly specific and provides a definitive characterization of the contractile apparatus of the fiber (9). Two criteria that have been particularly convenient for the identification of individual fibers are the form and distribution of mitochondria and the width of the Z line. On the basis of these two characteristics alone, it is possible to distinguish three types of skeletal muscle fibers within a fast-twitch (mixed) muscle (7, 11). Two have narrow Z lines and few or moderate numbers of mitochondria (white and intermediate fibers), and one has a wide Z line and abundant mitochondria (red fiber). The latter can be subdivided according to their reaction with antibody specific for fast or slow myosin (8, 9), and this is evident when examined with the light microscope. However, no direct ultrastructural evidence was available to support this conclusion. Because of the differences in Z-line width which are evident in the fibers of the same muscle, the possibility existed that the two red fibers might also differ in dimensions of the Z line. In that case, the fast red fiber might, in fact, have been equivalent to the intermediate fiber. It was therefore necessary to examine, with the electron microscope, fibers which had been exposed to antibody against one type of myosin. It has been shown, in the present study, that red fibers, identified by abundant mitochondria, all have wide Z lines. Because one of these fibers reacts with antibody specific for fast (white) myosin, while the other is unreactive, they can be equated with the two red fibers (fast and slow) identified using the light microscope. The intermediate fiber represents a separate category. By combining immunocytochemical with ultrastructural characteristics, therefore, four categories of fibers can be recognized in a single fast-twitch muscle: the white fiber, with narrow Z line and few mitochondria, has a positive response to anti-white myosin; the intermediate fiber, which has a narrow Z line but moderately abundant mitochondria, also has a positive response to anti-white myosin; there are two red fibers, both having wide Z lines and abundant mitochondria, but differing in their response to the antibody.

Apart from providing the identification for distinct categories of skeletal muscle fibers, these observations demonstrate that the myosin in fibers with a wide Z line can be either fast or slow. A wide Z line, therefore, does not necessarily correspond to the presence of slow myosin. The fast red fiber, moreover, reacts not only with antibody against fast (white) myosin, but also with antibodies specific for all the light chains of fast myosin (9). It can be concluded that the myosin of this fiber is closely related to that of the fast white fiber.

Finally, it should be emphasized that these distinguishing features serve merely as markers which permit important differences among skeletal muscle fibers to be recognized. It is not intended that they represent a restricted classification. Our recent observations indicate that, within a population of fast fibers, there are unequal distributions of myosin isoenzymes (9). It is likely that further

subdivisions among fibers will become evident as additional polymorphism of the myofibrillar proteins is revealed. Any system of classification, if it is to be useful, must remain flexible.

Preferential Localization of Antigenic Sites of Myosin Within the Sarcomere

Binding of antibody specific for white myosin is evident only along a circumscribed region of the A band (within the H band) at either side of the bridge-free zone. Its absence from the remainder of the A band may be the result of insufficient electron opacity provided by an unlabeled antibody. Nevertheless, the pattern of localization indicates that there is a difference in the distribution of antigenic sites along the length of the thick filament. It is possible that restriction of antibody binding to a position within the H band reflects the greater accessibility of this region because of an absence of overlap of thick and thin filaments. However, exclusion from the overlap region would explain the absence of binding only in the distal part of the A band. Staining is limited even within the non-overlap region, where it occupies only the most proximal part and does not appear to vary with sarcomere length. (Compare the width of the H band [e.g., Fig. 5] with that of the electron-opaque band.) The staining pattern most likely reflects an alteration in the configuration of the thick filament near the bridge-free zone, but it could also indicate a preferential binding of a nonmyosin contaminant at this site. However, the methods used to prepare the antigens and antibodies were chosen to minimize the possibility of impurities, and the procedures used to analyze the antisera did not detect any contaminating antibodies (8, 9). It is therefore unlikely that a contaminant would be present in such quantities that it would be more readily visualized with the electron microscope than antimyosin. In addition, the same staining pattern was evident when fibers were reacted with antibody specific for the "head" (S1) portion of white myosin (9). Since S1 is prepared by proteolytic digestion of myosin and is subjected to chromatographic procedures different from those used for whole myosin, it is unlikely that the same contaminant would be present in these two very different preparations, although the possibility cannot be excluded altogether. Furthermore, any contaminant in either antibody preparation would have to be specific for fast fibers, since only fast fibers exhibit the characteristic electron-opaque band.

The pattern of antibody binding observed in the present study is consistent with a structural differentiation along the thick filament such as that which has been described by Sjöström and Squire (16). It correlates particularly well with the location and dimensions of the proximal or "P"-zone observed by these investigators in sections prepared by cryo-ultramicrotomy. The P-zone consists of eight transverse lines, spaced ~14–15 nm apart, immediately adjacent to the bridge-free zone. It is believed that these lines represent rows of crossbridges which have become altered, either because of a change in myosin packing within the thick filament or because of the presence of additional proteins (2, 16). The electron-opaque band observed in the present study is also located in a limited region of the A-band near the bridge-free zone, and each band exhibits a periodicity which could also reflect the positions of altered crossbridges. Although this band was not analyzed quantitatively, it was possible to relate its dimensions to the known length of the A-band. It is estimated that the unstained central region surrounding the M-line is equivalent to the bridge-free zone, and that the stained band occupies a width of ~0.15 μm immediately adjacent to the bridge-free zone. Proteins other than myosin are known to be associated with the thick filament, and therefore the staining pattern observed here may not entirely reflect myosin heads. The predominant nonmyosin protein, namely C-protein, is located in the middle of each half of the A-band (3,14), but most of the stained band described here lies proximal to this position, and thus does not coincide with the location of C-protein. However, small amounts of additional nonmyosin protein within the proximal region have been reported (2, 3). Nevertheless, the demonstration here of a response, within a limited region of the A-band, to an antibody specific for myosin (see above) provides additional support for the suggestion that there may be intrinsic differences in the arrangement of myosin molecules along the length of the thick filament.

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