Development of Muscle Fiber Specialization in the Rat Hindlimb

NEAL A. RUBINSTEIN and ALAN M. KELLY
Department of Anatomy, School of Medicine, and Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT The appearance of fast and slow fiber types in the distal hindlimb of the rat was investigated using affinity-purified antibodies specific to adult fast and slow myosins, two-dimensional electrophoresis of myosin light chains, and electron microscope examination of developing muscle cells. As others have noted, muscle histogenesis is not synchronous; rather, a series of muscle fiber generations occurs, each generation forming along the walls of the previous generation. At the onset of myotube formation on the 15th d of gestation, the antimyosin antibodies do not distinguish among fibers. All fibers react strongly with antibody to fast myosin but not with antibody to slow myosin. The initiation of fiber type differentiation can be detected in the 17-d fetus by a gradual increase in the binding of antibody to slow myosin in the primary, but not the secondary, generation myotubes. Moreover, neuromuscular contacts at this crucial time are infrequent, primitive, and restricted predominantly, but not exclusively, to the primary generation cells, the same cells which begin to bind large amounts of antislow myosin at this time. With maturation, the primary generation cells decrease their binding of antifast myosin and become type I fibers. Secondary generation cells are initially all primitive type II fibers. In future fast muscles the secondary generation cells remain type II, while in future slow muscles most of the secondary generation cells eventually change to type I over a prolonged postnatal period. We conclude that the temporal sequence of muscle development is fundamentally important in determining the genetic expression of individual muscle cells.

Adult skeletal muscles are composed of large numbers of fibers with different physiological and biochemical properties as well as different contractile protein isozymes. For example, a series of papers have defined the myosin isozyme(s) in each of the three commonly accepted mammalian skeletal muscle fiber types (2, 16, 17, 38). Using a protein-A peroxidase immuno-cytochemical technique, Lutz et al. (28) have demonstrated that slow, type I fibers contain exclusively slow myosin. Fast, type II fibers can be subdivided into type IIb fibers, containing exclusively fast myosin, and type IIa fibers, having predominantly fast myosin, but also a small amount of slow myosin. These results apply to rabbit (28) and rat (see below) muscle, but apparently not to human muscle (8) in which both IIa and IIb fibers have only fast myosin.

Although muscle fibers synthesize contractile proteins in the absence of motoneurons, after innervation the neuron can control the particular isozymes subsequently synthesized. For example, when the nerves to a fast-twitch and a slow-twitch muscle are severed and forced to reinnervate the opposite muscle type, the former fast muscle switches to slow myosin synthesis, while the former slow muscle changes to fast myosin synthesis (49). Moreover, the fast-to-slow transition in these cross innervated fast muscles can be mimicked by chronic stimulation of the fast muscle's own intact motoneuron at a low, continuous frequency (43). These changes occur within individual fibers (32, 38).

Despite the occurrence of distinct fiber types in the adult animal, few differences have been detected among fibers during the early stages of embryogenesis. Published evidence suggests that all fibers originally have the same myosin type, although exactly which myosin type they have is a matter of controversy (18, 30, 36, 37, 39, 48, 50–52). In addition, the contractile and membrane properties and the concentrations of enzymes for energy metabolism also are uniform early in development (10, 12, 13), although they differ among fibers in the adult. The only apparent difference among early fibers relates to the lack

of synchrony of fiber formation during muscle histogenesis: during development, a series of fiber generations occurs (11, 23). Each succeeding (secondary) generation forms along the walls of the preceding (primary) generation. At most stages of early development, then, the fibers show morphologic heterogeneity related to their maturity.

The generation of distinct fiber types from an apparently homogeneous population of embryonic muscle fibers is the subject of this paper. From the onset of myotube formation in the distal hindlimb of the rat at 15 d gestation, all fibers react predominantly with antibody specific to adult fast myosin. We can detect the intiation of fiber type differentiation in the 17-d fetus by a gradual increase in binding of antibody specific to adult slow myosin in the primary generation, but not the secondary generation, myotubes. Moreover, neuromuscular contacts at this crucial time are infrequent, primitive, and restricted predominantly, but not exclusively, to the primary generation cells, the same cells that begin to bind large amounts of antislow myosin at this time. With maturation, most primary generation cells decrease their reaction with antifast myosin antibody and become type I fibers. Secondary generation cells are initially all primitive type II fibers. In future fast muscles, most secondary generation cells remain type II, while in future slow muscles most of the secondary generation cells eventually change to type I over a prolonged postnatal period. Whether type IIa fibers result from primary or secondary cells has not yet been determined.

MATERIALS AND METHODS
Antibody Preparation

Antibodies to fast and slow rabbit myosins were prepared previously (38). For the present studies, affinity-purified antibodies were used. IgG, obtained by 50% ammonium sulfate fractionation of antiserum, was affinity-purified by two rounds of absorption to appropriate myosins covalently bound to agarose. The procedure for purifying antifast myosin antibody follows: rabbit fast myosin, purified by column chromatography on DEAE Sephadex A50, was covalently bound to agarose with CNBr. Antifast IgG in 0.4 M KCl, 0.03 M phosphate, pH 7.3, was added to the agarose-myosin complex and stirred for 2 h. The agarose was washed with buffer and the bound antifast myosin antibody released from the myosin-agarose complex by lowering the pH to 2.7. To assure no cross reaction with slow myosin, the released antifast myosin antibody was absorbed with a slow myosin-agarose complex. Antislow myosin antibody was prepared similarly. Specificity of the antibodies to rat fast and slow myosins is identical to that shown previously for rabbit fast and slow myosins (Fig. 1 of reference 38). Immunoelectrophoresis suggests that both antibodies react only with heavy chains, not light chains (N. A. Rubinstein, unpublished observation). Antibody staining: Two staining methods were used. In most embryonic muscles, the indirect peroxidase-antiperoxidase (PAP) staining method of Sternberger (45) was used, as modified by Tapscott and Holtzer (personal communication). For some embryonic muscles and all postnatal muscles, the direct fluorescein-labeled antibody technique (16) was used. After sections were allowed to dry for 15–30 min, pre-immune IgG (5–10 mg/ml in standard salt) was applied for 30 min at 37°C. This decreased nonspecific binding considerably. After the preimmune IgG was removed, specific fluoresceinated antibody was applied to the section for 1 h at 37°C. Sections were washed briefly in standard salt, mounted in 25% glycerine, and examined with an Olympus Vanox Microscope equipped with epifluorescence. Although both the fluorescein and peroxidase methods gave qualitatively identical results, the PAP technique was more suitable for smaller muscles, since it allowed multiple, high power photographs without fading.

Myosin Preparations

Purified myosins could not be prepared from most embryonic muscles, since the amount of material available precluded extensive technical maneuvers. Actomyosin, however, could be prepared by the method of Adelstein et al. (1). Actomyosin preparations were analyzed on two-dimensional gels (29). The second dimension was 17.5% in acrylamide.

Animals

Embryonic rats were obtained by Caesarean section from date-mated Sprague-Dawley rats. The fetuses were killed and the skin was quickly dissected from the hindlimbs. The limbs were oriented in tragacanth and frozen in melting isopentane (−160°C). Serial 6-μm frozen sections were obtained and either stained directly with fluorescein-labeled antibodies or fixed and stained with the PAP technique. Usually, the entire distal hindlimb was serial-sectioned. Because many fibers in the developing limb do not extend from one end to the other, it was not possible to determine the exact number of fibers in any one muscle. As a convention, we report the fiber count obtained in the thickest part of the muscle and compare adjacent sections in this region. A number of animals at each day of development from day 14 in utero through 3 d postpartum were examined. In addition, muscles from animals 14 d postpartum and from adult animals were also examined. Only representative stages of development are reported below.

Electron Microscopy

Material was fixed in 2% paraformaldehyde, 2% glutaraldehyde, impregnated with osmium, and embedded in Spurr (Polysciences, Warrenton, Pa.).

RESULTS

Our fluorescein-labeled, affinity-purified antibodies stain the three adult fiber types differentially. Fig. 1 a and b are serial sections of an adult rat extensor digitorum longus (EDL) muscle. Approx. 80% of the fibers react only with antibody to fast myosin (type IIb), 3% with antibody to slow myosin (type I), and 17% react strongly with antifast myosin antibody (AF) and faintly with antislow myosin antibody (AS) (type IIa). Slow fibers are nonrandomly dispersed throughout the muscle. Antimyosin staining also defines three fiber types in the adult soleus (Fig. 1 c and d). While most fibers react exclusively with AS, a sizeable minority react both intensely with AF and lightly with AS. These are reminiscent of type IIa fibers in the EDL and have been described previously (17). Finally, a small number of fibers react only with AF, similar to the reaction of type IIb fibers in the EDL.

At most stages of development, we examined the EDL and tibialis anterior, predominantly fast muscles in the adult rat, and the soleus, a predominantly slow muscle. The EDL and tibialis anterior muscles border the tibia, and with this landmark the primordia of these muscles could be identified in the 15-d fetus. The soleus has no easily recognizable landmark and could not be confidently identified until 18 d of gestation.

While we are unable to detect muscle in the distal hindlimb at 13–14 d gestation, by 15 d we can identify a number of distinct muscle masses (Fig. 4). Although one can already see neurons invading the EDL and the tibialis anterior, neuromuscular contacts are infrequent and occur as focal, close membrane contacts between axons and myotubes (Fig. 2). Usually, one or two axon terminals participate in these junctions, but it is unusual to find synaptic vesicles within their axoplasm. Although this type of focal contact persists until 19 d gestation, its significance is unknown.

The primordium of the EDL consists of small myotubes aggregated into groups of two to six cells (Fig. 3). Within these groups, the plasma membranes of neighboring myotubes are interconnected by various forms of membrane specializations, principally close junctions (23). Mononucleated cells are interspersed through and between these groups. No basal lamina surrounds the cells.

Initially, the immature muscles do not show fiber type distinctions with antibody staining. These muscle fibers react moderately well with AF (Fig. 4 a), but only faintly with AS (Fig. 4 b). At higher magnification, the EDL shows moderate staining with AF (Fig. 4 c), equivocal staining with AS (Fig. 4
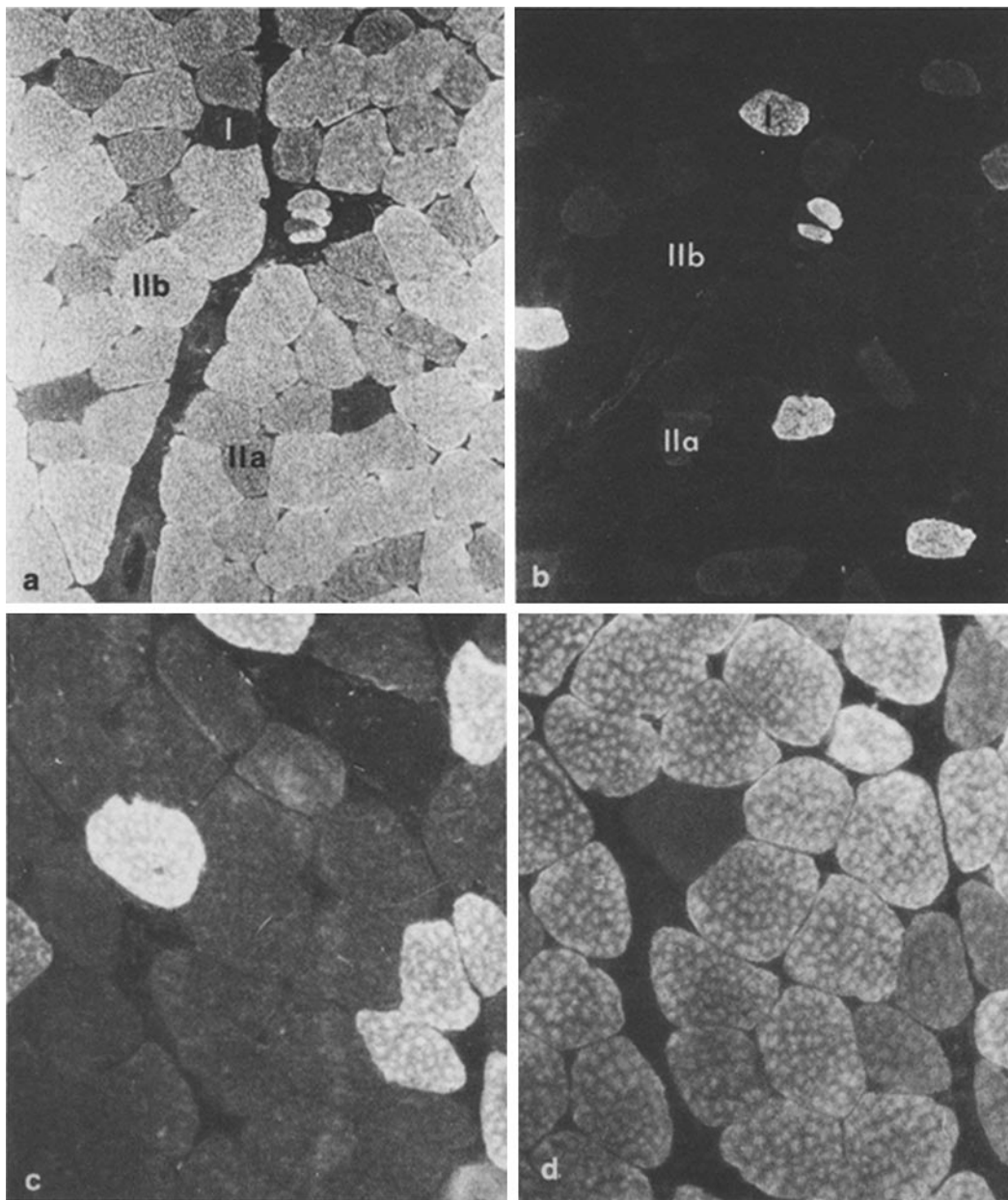
FIGURE 1 Serial sections of adult rat EDL and soleus muscles stained with AS and AF. Staining of the EDL with AF (a) or AS (b) shows three staining levels. Those fibers staining intensely with AF fail to react with AS (type IIb); those staining moderately with AF react lightly with AS (type IIa); and those failing to stain with AF react intensely with AS (type I). The soleus, likewise, shows three levels of staining with AF and AS: most fibers react only with AS. A sizeable minority react intensely with AF and lightly with AS, while a small number of cells react only with AF.
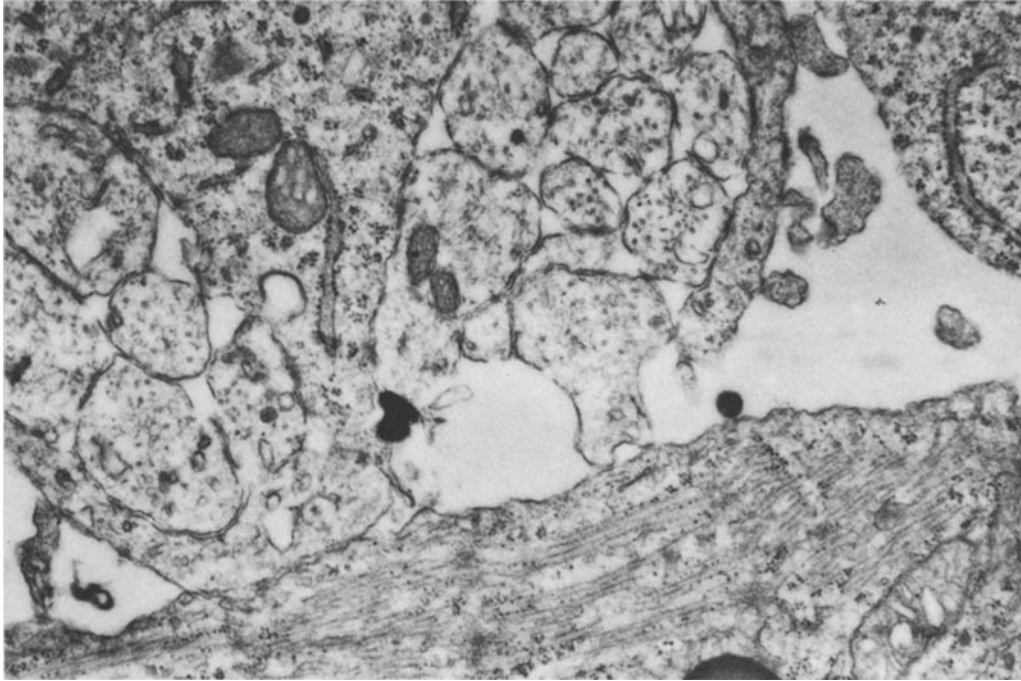
FIGURE 2 Neuromuscular contacts in the 15-d fetal hindlimb. A group of axons which are partially enwrapped by a primitive Schwann cell lie close to the surface of a myotube. One axon makes close membrane contact with the myotube plasma membrane. No basal lamina covers the myotube surface. × 24,000.
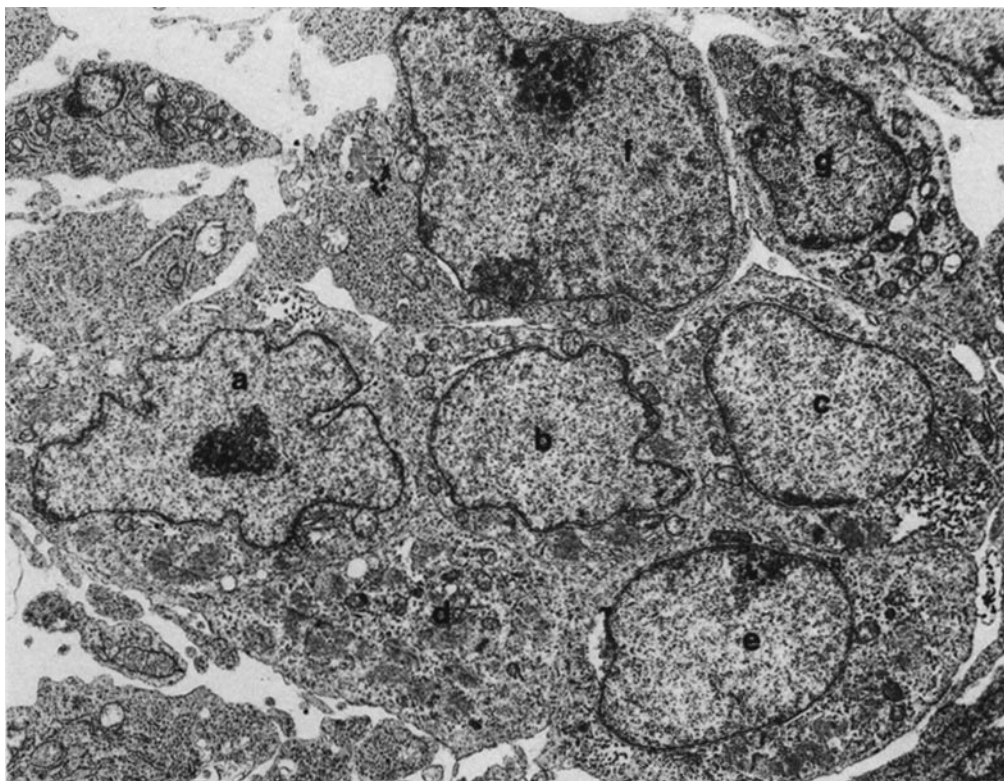

FIGURE 3 Cluster of cells from the EDL of a 15-d fetus. Five primitive muscle cells (a-e) and two undifferentiated mesenchymal cells (f and g) are clustered. Within this group, the plasma membranes of the various cells are intimately apposed and are frequently connected by membrane junctions. × 10,000.
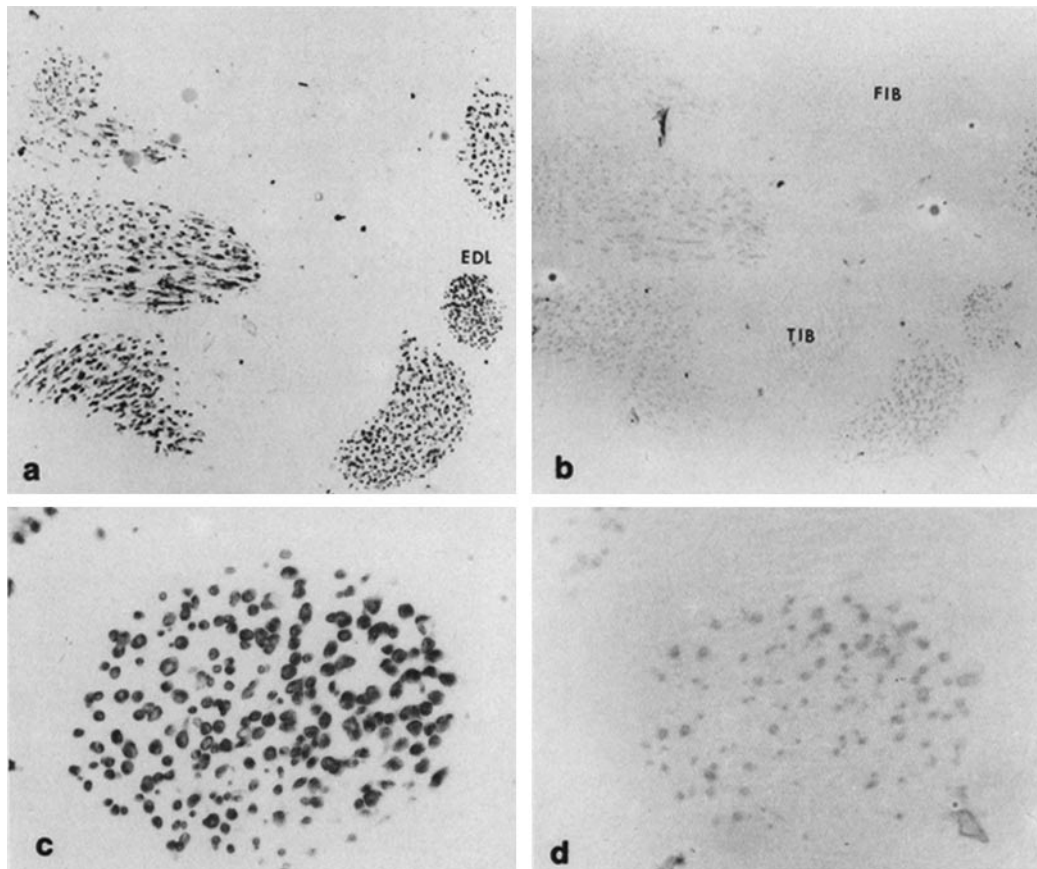
FIGURE 4 Distal hindlimb of a 15-d embryo. 6-μm serial cross sections of the entire distal hindlimb were stained with affinity-purified AF and AS, using the PAP staining method. Positive fibers appear black with this method. (a) AF. (b) AS. (c) The EDL, at higher magnification, showing all fibers reacting moderately with AF. (d) EDL showing faint, possibly nonspecific, staining with AS. The staining level with AS is similar to that of controls using pre-immune IgG. TIB, tibia. FIB, fibula.
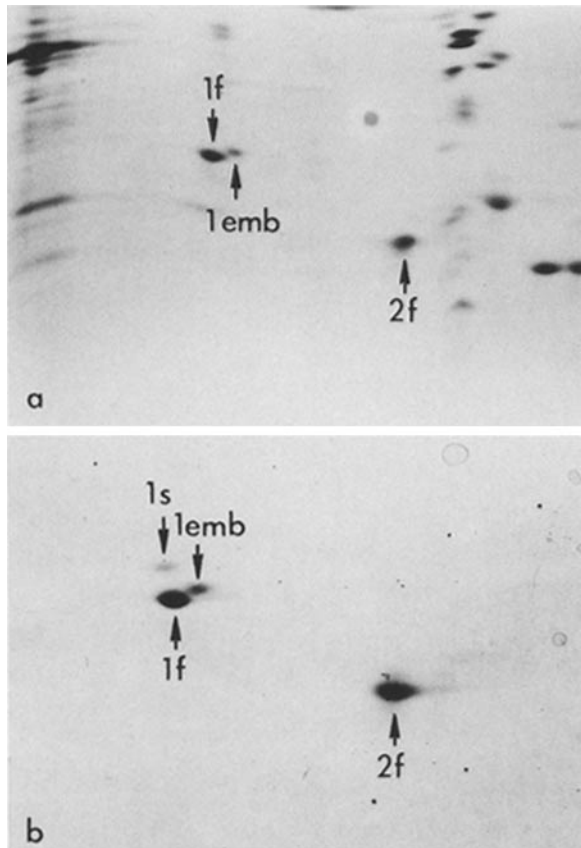
FIGURE 5 Light chains of myosins isolated from 15- and 17-d hindlimbs. Actomyosin was isolated from entire proximal and distal hindlimbs of 15-d fetuses and more purified myosin was prepared from 17-d fetuses. Proteins were electrophoresed as described by O'Farrell (29), using a 17.5% acrylamide concentration in the second dimension. (a) 15 d. (b) 17 d. Fast myosin light chains are 1f and 2f. Slow myosin light chains are 1s and 2s. The light chain unique to the embryo is 1emb.

d). Regardless of the presence or absence of a small amount of staining with AS at this stage, however, no distinctions among fibers can be identified by these two antibodies.

Two-dimensional electrophoresis of actomyosins from the whole 15-d limb reveals only fast myosin light chains, LC_{1f} and LC_{2f} (Fig. 5 a). LC_{3f} is decreased or absent in the embryo (30, 39, 44, 50). Another light chain, unique to the embryo, can also be seen (LC_{1emb}; 50). 2 d later, a small amount of a slow light chain, LC_{1s} (Fig. 5 b), has appeared. No LC_{2s} can yet be detected by this method.

At 17 d, large, primary generation myotubes are surrounded by numerous, smaller, secondary generation myotubes (Fig. 6), which are attached by close membrane junctions to the walls of the primary cells. When present, a single basal lamina circumscribes each of these primary-secondary cell complexes. The immature muscle fibers are in the myotube stage with central nuclei, a large central accumulation of glycogen, and peripheral myofibrils. This arrangement imparts a donut-shaped appearance after antibody staining (see below).

Two forms of neuromuscular contacts are seen. One consists of close membrane apposition between axon and myotube plasma membranes, as seen on day 15 (Fig. 2). The other form of neuromuscular junction can be recognized as a primitive motor endplate (Fig. 7 a and b). This consists of clusters of small axon terminals, many containing electron-opaque vesi-

cles, which abut the plasmalemma of large, primary generation myotubes. There are focal thickenings of the postsynaptic membrane at these synapses, and a small amount of basement membrane material is interposed between some, but not all, the apposed plasma membranes. These forms of synapses are limited to large primary generation cells and have not been encountered on secondary generation myotubes at this stage.

Antibody staining of the entire limb differs from that seen on day 15. All fibers in all muscles react strongly with AF (Fig. 8 a). Many fibers additionally stain very lightly, but definitively, with AS (Fig. 8 b). This staining is more intense than the staining seen with AS on day 15 or the staining with pre-immune IgG. Higher magnification pictures of the EDL reveal that not all cells react equally with AS (Fig. 8 c and d). In fact, only the large, primary generation fibers react clearly with AS, while the smaller secondary generation cells fail to bind this antibody appreciably. This pattern is more clearly defined by 18 d and will be discussed in detail below.

At 18 d, the morphology is comparable to that at 17 d, but primary generation cells have become significantly larger. While the two types of neuromuscular junctions persist, those identified as primitive endplates have further matured. The axon terminals, which have increased in size and have more synaptic vesicles, are consistently separated from the postsynaptic membrane of primary cells by 400- to 600-Å synaptic gaps uniformly filled with basal lamina (Fig. 9). The electron density of postsynaptic membranes has also increased. Although nerves are now seen approaching secondary generation fibers, there is no comparable evidence of endplate differentiation on secondary fibers. In addition, primordia of muscle spindles are also present at this stage.

With antibody staining, two types of fibers, reminiscent of type I and type II adult fibers, can clearly be distinguished throughout the entire limb. In Fig. 10, which is through the mid-belly of the EDL, ~300 primary and secondary generation cells can be seen, all of which react strongly with AF. 90% of the primary generation cells additionally stain strongly with AS (Fig. 10 b), whereas reactivity of small, secondary generation cells is weak and contrasts with their affinity for AF. Thus, the two fiber types present now are (a) those fibers reacting intensely only with AF, including most of the secondary generation cells plus a few primary generation cells, and (b) those fibers reacting strongly with both AS and AF, including most of the primary generation cells and a few secondary generation cells.

Fig. 11 shows a cluster of primary and secondary fibers, at higher magnification, stained with fluorescein-labeled AS. Even though the secondary generation cells are tightly apposed to the primary cell, only the primary generation cells react well with AS.

At 18 d, the soleus can first be identified and consists predominantly of uniform-sized fibers, probably equivalent to primary generation cells of the EDL, with very few secondary fibers. As in the EDL, all of the primary and secondary generation fibers react intensely with AF (Fig. 12 a), while 85% of the primary generation cells and 10% of the secondary generation cells additionally react strongly with AS (Fig. 12 b).

At 19 d gestation, differentiated endplates are larger and more readily found. Close membrane contacts are seen only rarely and occur on small myotubes. An occasional primitive motor endplate is seen on a secondary generation fiber, usually on one that has separated from the primary myotube.
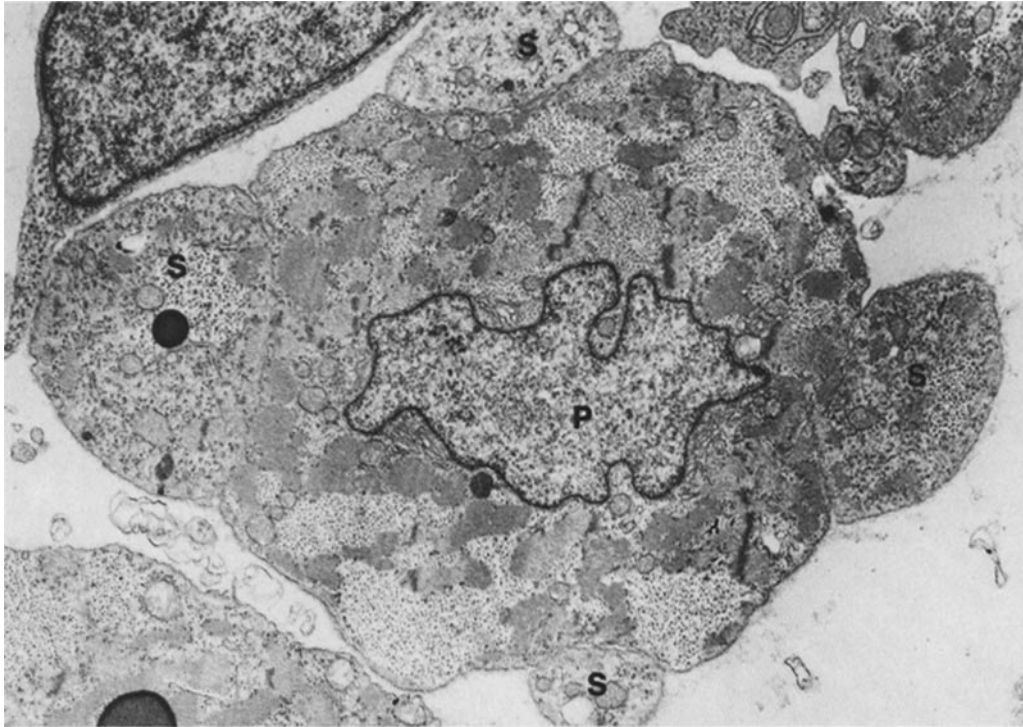
FIGURE 6 Cluster of cells in the EDL of a 17-d fetus. Characteristically, the muscle at this stage is organized into primary and secondary generation cells. In this illustration, a large primary myotube (P) containing a central nucleus and much glycogen is surrounded by four small secondary generation cells (S). × 14,000.

In the PAP-stained section of the entire leg, clear distinctions between the future fast EDL and the future slow soleus muscles are seen (Fig. 13 *a* and *b*) because of the quite different ratio of primary/secondary generation cells in the two muscles. In the EDL, all fibers continue to react strongly with AF (Fig. 13 *c*). The fibers reacting strongly with AS are not randomly scattered and usually occur one per group of muscle cells. These correspond to the primary generation cells seen in Fig. 10 *b*. Very rarely do any two adjacent fibers show an intense reaction with AS. Approx. 40% of the fibers, usually the larger cells, additionally react with AS (Fig. 13 *d*). Staining with AF, however, is no longer uniform in intensity from fiber to fiber. In the EDL, those fibers that stain less intensely with AF are the fibers reacting most strongly with AS. Those reacting most intensely with AF usually do not appear to stain with AS. By contrast, over 95% of the fibers in the soleus react not only with AF but also with AS (Fig. 13 *e* and *f*). In both muscles, the proportion of cells containing slow myosin at this time exceeds that found in the adult rat (3% in the adult EDL, 70% in the soleus), but the absolute number of cells containing slow myosin is close to that found in the adult. We believe that adult slow fibers in the EDL are formed predominantly from primary generation cells and are present in approximately their adult numbers by 19 d gestation. Secondary generation cells are practically all primitive fast fibers. Because the number of these secondary cells increases with development, the percentage of slow fibers declines as muscle histogenesis progresses. Thus, comparison of Figs. 10 *b* and 13 *d* shows a dilution, but not a decrease, of slow fibers.

2 d after birth, each fiber in the limb still reacts with AF, while some cells also react with AS. Staining of the EDL with AF still shows two levels of intensity; however, the fibers reacting with AS now stain more lightly with AF than they did

at 19 d gestation (Fig. 14 *a* and *b*). In the soleus, the larger fibers show less intense staining with AF and a greatly increased intensity with AS (Fig. 14 *c* and *d*). Now, however, there are a number of smaller cells applied to the walls of large myotubes in the soleus. Most of these stain exclusively with AF. They represent secondary generation fibers arising later in the development of the soleus than the EDL. This correlates with the more immature appearance of the soleus during development and the higher satellite cell mitotic activity at later stages in the soleus (21).

14 d after birth, fiber type differentiation has approached adult patterns. The EDL now contains a large proportion of AF-positive fibers; and, concomitantly, the percentage of AS-positive fibers has declined to approximately the adult level (Fig. 15 *a* and *b*). AS-positive fibers, moreover, now fail to react with AF. The soleus similarly shows two clear fiber types: small fibers staining intensely with AF but failing to bind AS, and large fibers staining lightly with AF and binding larger amounts of AS. Although the latter cells appear to occupy a larger proportion of the muscle, there are actually equal numbers of each type of cell at this stage. This high proportion of differentiating fast fibers may account for the slight speeding of contraction time in soleus muscles at this stage of development (10, 12). In the adult soleus, fewer cells contain only fast myosin. A transition from fast to slow cells in the rat between 5 and 35 wk after birth has been documented by Kugelberg (27).

In the EDL, the adult complement of predominantly fast light chains of LC_{1f} and LC_{2f} with a small amount of LC_{3f} can be seen on two-dimensional gels. LC_{1emb} is decreased (Fig. 16 *a* and *b*), and slow light chains are difficult to visualize. The soleus now shows moderate amounts of all fast and slow light chains (Fig. 16 *c*), complementing the staining pattern at this
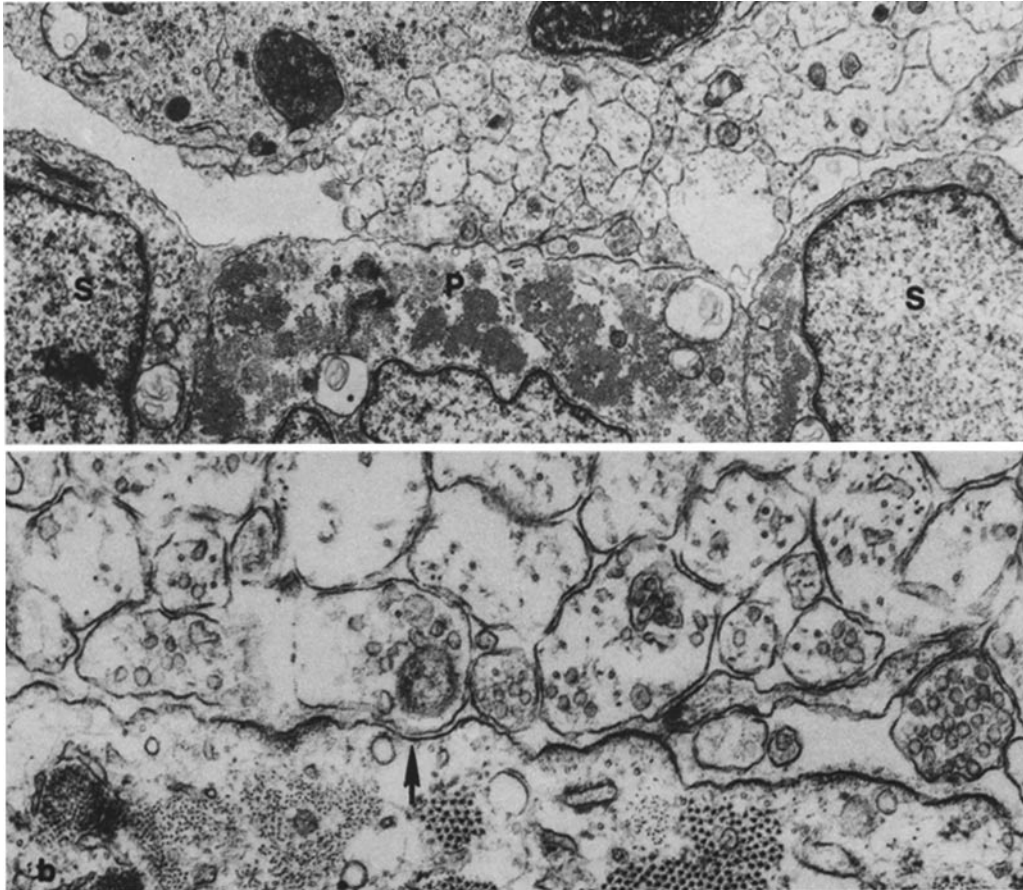
FIGURE 7 Neuromuscular contacts in the 17-d embryo. There are two types of neuromuscular contacts at this stage of development. One form consists of focal, close membrane junctions between nerve and muscle (see Fig. 2). In the second form of junction (a), endplate differentiation is occurring on a primary cell (P). Two secondary generation cells (S) border the area of neuromuscular contact, but there is no evidence of the nerve innervating either of these cells. × 10,000. At higher magnification (b), the primitive endplate can be seen to consist of clusters of axons that approximate the myotube plasma membrane. In most areas, the apposed membranes are separated by a 400- to 600-A synaptic gap that contains traces of basal lamina material. In one area (arrow), however, an axon comes within 150 Å of the myotube plasmalemma. There are a few synaptic vesicles within the terminal axons, and there is focally increased electron density of the primordial postsynaptic membrane. × 30,000.
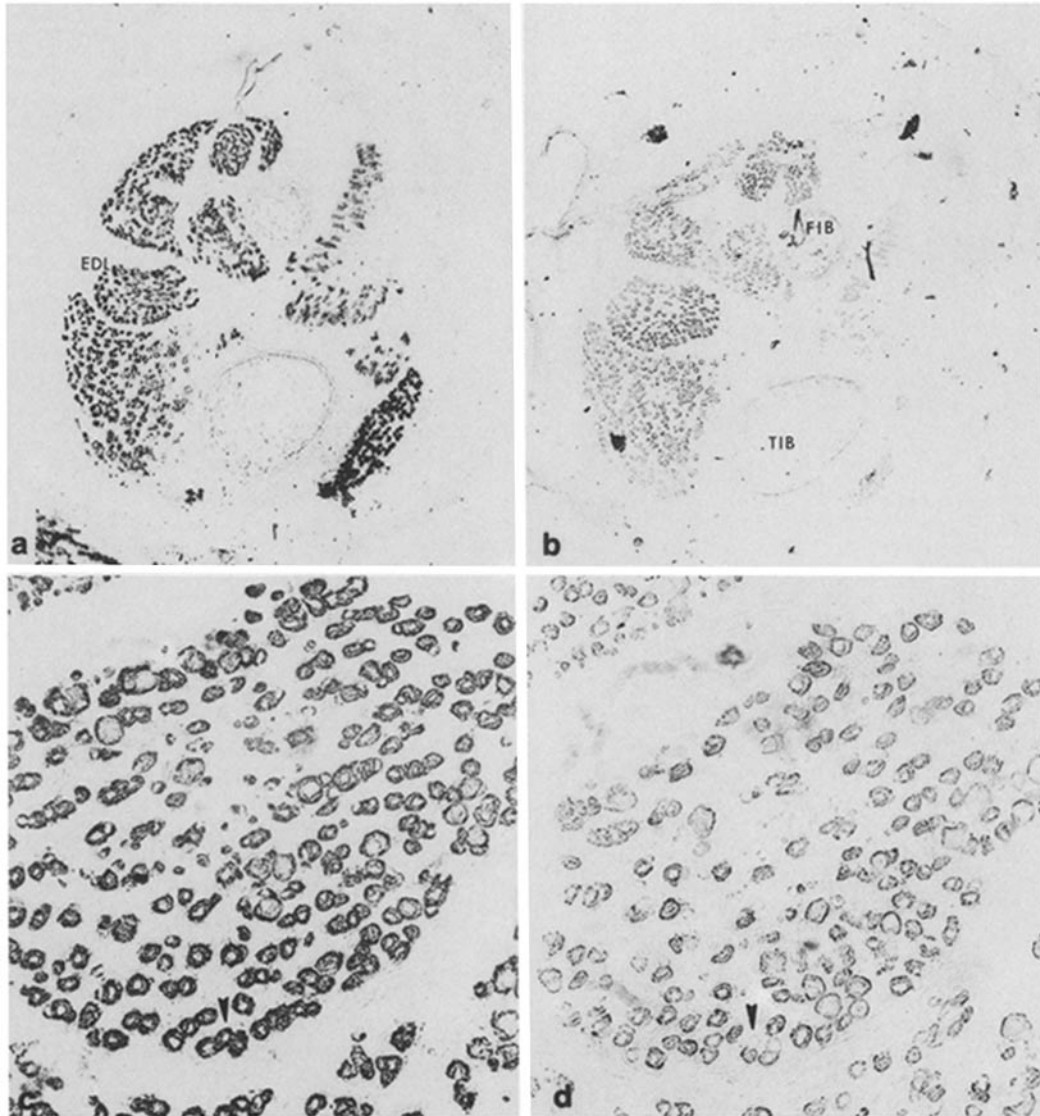
FIGURE 8 Distal hindlimb of a 17-d fetus. Serial sections of the distal hindlimb were stained with AF and AS by the PAP method. In the entire cross section of the limb, all fibers stain intensely and uniformly with AF (a), while many fibers additionally stain with AS (b). At this time, the reaction with AS, while lighter than that with AF, is more intense than at 15 d and more intense than staining with pre-immune IgG. The EDL at higher magnification shows the donut-shaped arrangement of myofibrils around the central core of nuclei and glycogen accumulations. While reaction with AF (c) is intense in all fibers, only the larger, primary generation cells react strongly with AS (d). Arrowhead points to a secondary generation cell which does not react with AS.
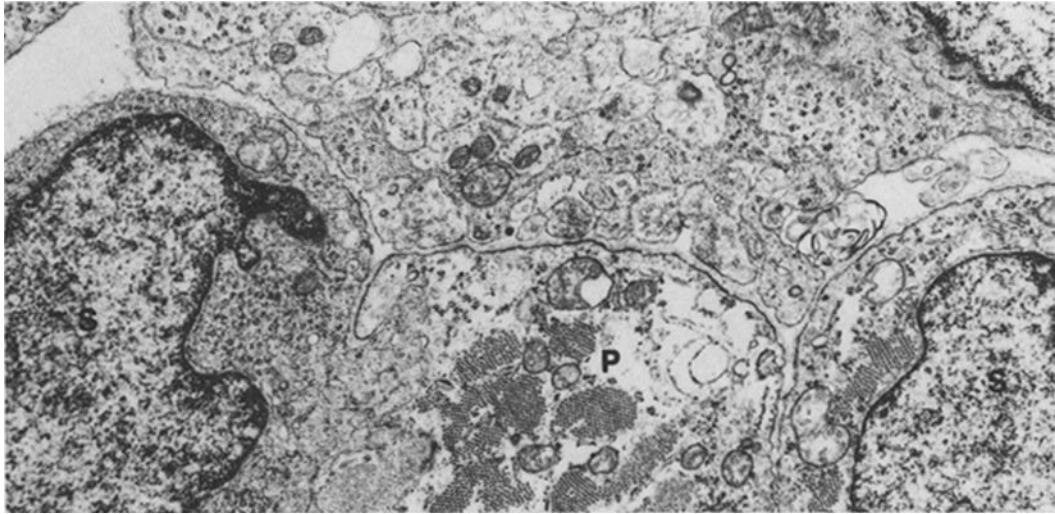
stage. In the adult soleus, however, the pattern is dominated by slow light chains LC_{1s} and LC_{2s} (Fig. 16 d).

Fig. 17 summarizes one possible interpretation of events leading to fiber type diversity.

DISCUSSION

Using antibodies specific to adult rabbit fast and slow myosins, we can first detect muscle in the distal hindlimb of the rat at 15 d gestation but do not see any differences among fibers until 2 d later. Differences in antimyosin staining among fibers arise because of a large increase in binding of AS in most primary generation fibers. These are the same fibers that appear to have the most mature neuromuscular junctions. Our discussion of these results is organized around several questions: What forms of myosin are present before and after the appearance of fiber type differentiation? What is the eventual fiber type destiny of primary vs. succeeding generations of muscle cells? And what role could the motoneuron play in initiating the diversity among fibers?

Myosin Types during Development

Although the earliest muscle cells do not seem to differ in their myosin isozymes, agreement has not been reached on the particular type of myosin initially present. Many investigators have shown predominantly, but not exclusively, fast myosin heavy and light chains in both fast and slow muscles of embryonic rats, rabbits, and chickens in vivo, although heretofore the earliest muscles formed had not been examined (30, 33, 37, 39, 48). The initial myosin synthesized has been observed, however, in tissue culture. Several groups have reported exclusively fast myosin light and heavy chains in cultured chicken muscles (36, 44), while Whalen et al. (50, 51) could not find any slow light chains in cultured rat muscle. Recently, two groups have noted very small quantities of slow light chains in cultured chicken and quail muscles (20, 46). The subject of the initial myosin complement requires further investigation.

In the present study, immunohistochemical staining with antislow myosin antibody plus two-dimensional electrophoresis

of light chains could not detect significant amounts of slow myosin during the initial stages of myogenesis on day 15. By 17-d gestation, however, light staining with antislow myosin antibody could be seen in most primary generation fibers and a miniscule quantity of slow LC_{1s} was seen on gels. Because the staining with AS on day 17 was barely detectable, we cannot rule out entirely the presence of small quantities of AS-reactive myosin in primary generation cells earlier, undetectable by the present methods.

Whalen and his colleagues (50–52) have presented evidence that the heavy chain found in the fetal rat is unique to the embryo and distinct from adult fast and slow heavy chains. Our data do not address this question directly. The heavy chain in all fibers of the embryo from day 15 through day 18 does react intensely with our AF. If a distinct embryonic heavy chain does exist, our AF, but not our AS, reacts strongly with it. However, with the appearance of intense staining of most primary generation myotubes with AS on days 17 to 18, a second type of heavy chain, possibly slow myosin heavy chain, has appeared. Thus, although the exact identity of the particular myosin isoenzymes which react with our antibodies may not be certain, these antibodies clearly distinguish two cell types at an earlier stage of development than has been reported previously.

Several other immunohistochemical studies of fiber type development in mammals have been published. Rowlerson (35) described fiber type differentiation in the rat limb using an antibody prepared against rat slow myosin, while Jenny et al. (19) studied newborn rabbit muscles using antibodies against rabbit fast and slow myosins. The results of both of these studies are similar to those presented in this paper. However, Gauthier et al. (18), studying the rat diaphragm with antibodies against chicken myosins, showed intense staining of all fibers with both antifast and antislow myosin antibodies at 19 d gestation. 1 d after birth, fiber type distinctions were evident. The discrepancy between the results of Gauthier et al. and those of Jenny et al., Rowlerson, and ours could be explained by invoking an embryonic heavy chain. Thus, while both the antifast and antislow myosin antibodies of Gauthier
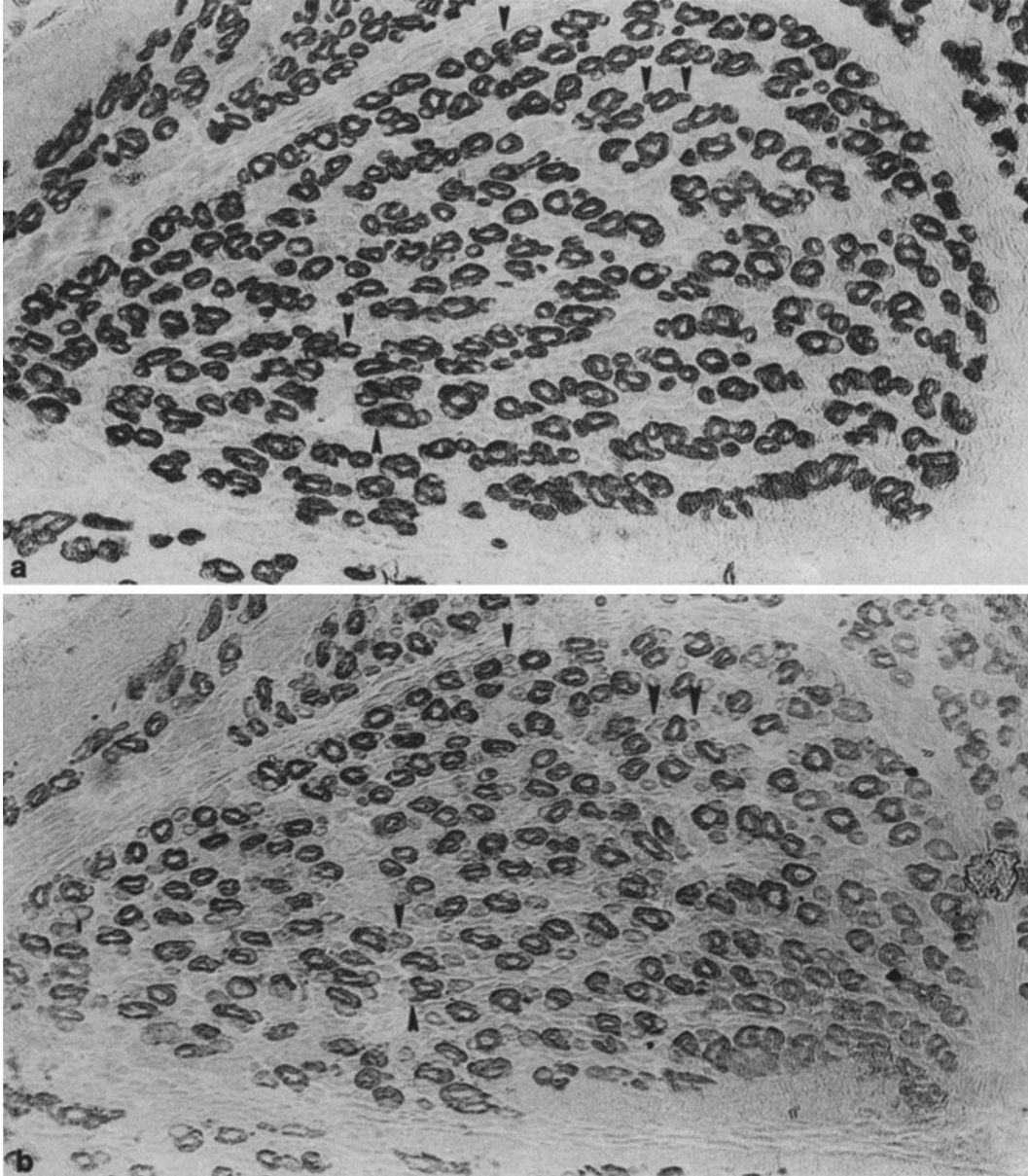
FIGURE 10   Serial sections of the EDL from the 18-d fetus stained with antifast and antislow myosin antibodies. All primary and secondary generation fibers of the EDL react strongly with AF (a); however, while most of the primary generation fibers also react with AS, few of the secondary generation fibers bind appreciable amounts of AS (b). Arrows point to secondary fibers staining strongly only with AF.
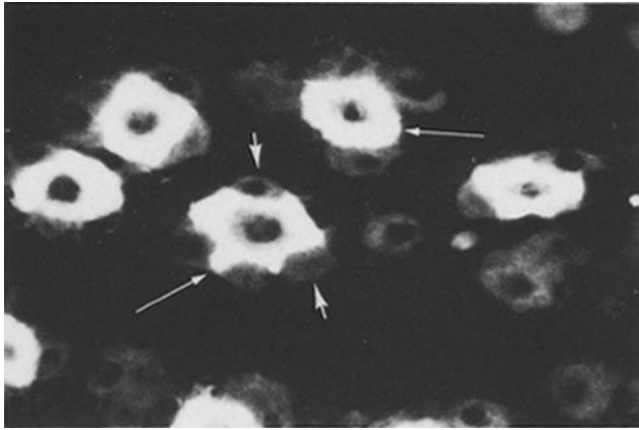
FIGURE 11 Staining of primary and secondary generation fibers with fluorescent AS. Sections of the EDL at 18 d were stained with fluorescein-labeled AS. Primary (thin arrows) and secondary (thick arrows) cells show differential staining. While the primary generation cells react quite intensely with AS, secondary generation cells fail to bind significant quantities of this antibody.

et al. might cross-react with the embryonic myosin, only the antifast myosin antibodies of the other investigators might show this cross reaction. The lack of cross reaction of our AS with the embryonic myosin has allowed us to detect the appearance of a distinct subset of fibers at an early stage.

Dhoot and Perry (14) have used a similar immunohistochemical technique to localize the fast and slow forms of troponin T, I, and C in developing mice. Although they began their examination after fiber type differentiation had begun, the scattered distribution of the slow forms of the troponins coincided with our described distribution of the slow form of myosin.

Destiny of Primary vs. Secondary Generation Fibers

Before birth most of the larger, primary generation myotubes start to become slow fibers in both future fast and future slow muscles. This is consistent with previous studies of Kelly and Schotland (22) and Ashmore et al. (3) using myofibrillar ATPase reactions. Since these fetal slow fibers have the same mosaic pattern of distribution throughout the muscle which characterizes the distribution of slow motor units in the adult, the appearance of slow fibers at 17–18 d gestation may indicate initial development of entire motor units. Their appearance approximately coincides with the onset of fetal movement in the rat hindlimb (15, 47, 53). Also, the movements could be reflex in nature, since differentiating spindles can be recognized by 18 d gestation (26; A. M. Kelly, unpublished observation).

Secondary generation cells may not be functionally innervated during the early stages of their development. Kikuchi and Ashmore (25) report that, in the chicken, secondary generation myotubes are not innervated when in contact with primary myotubes. In rat intercostal muscles, groups of primary and secondary generation myotubes appear to be innervated as a single unit, and secondary generation myotubes are not separately innervated until after birth (25). This correlates with the recent observation that during early postnatal development of the mouse lumbrical muscle, multiply-innervated primary fibers contribute to the formation of synapses on newly formed fibers (7).

While the destiny of primary cells to be slow fibers in the

adult is identical in both the soleus and the EDL, the time of appearance (21) and the destiny of the secondary fibers differ between fast and slow muscles. At 18 d gestation, the EDL contains numerous, small secondary fibers, most of which stain predominantly with AF; they remain this way through maturity. At the same time, the soleus is composed mostly of primary generation myotubes of uniform size, all staining moderately with both AS and AF, and a few small secondary fibers reacting predominantly with AF. It is not until after birth that secondary fibers appear in large numbers in this muscle. These, like their counterparts in the EDL, also stain selectively with AF. Unlike the EDL secondary fibers, however, after birth those in the soleus eventually transform into slow fibers (27, 37). We interpret this postnatal transformation of secondary generation fibers into type I fibers as an adaptation to changing patterns of muscle usage. The cue for the initial formation of type I fibers from primary generation cells may be different.

Speed of Contraction of Embryonic Muscles

Whether destined to become fast or slow in the adult animal, muscles of newborn animals have a low maximum velocity of contraction (V_{max}; 12). Within several weeks, fast muscles increase their V_{max} considerably. Slow muscles show a small, transient increase, followed by a return to the original low V_{max}. Barany (5) has noted that V_{max} is proportional to the myosin ATPase activity, which is lower with slow myosin than with fast myosin. Therefore, the pattern of slow fiber development coupled with the observations on the neuromuscular junctions suggests one contribution to the low V_{max} in developing fast and slow muscles despite the presence of large amounts of fast, or embryonic, myosin. In early development, only the large, primary generation fibers have well-developed neuromuscular junctions. Hence, any attempt to measure the V_{max} by stimulation of the muscle's motoneuron would predominantly reflect motor unit activation of these more differentiated primary generation fibers. These contain large amounts of slow myosin, unlike the great majority of fibers, which are secondary generation cells. We propose that stimulation of the motoneuron causes a contraction which is dominated by the large, slow myosin-containing primary generation muscle fibers and reflects the breakdown of ATP in those fibers alone. Thus, even though the great majority of fibers contain predominantly fast, or embryonic, myosin, there is reason to believe that these fibers do not yet fully contribute to the functional contraction of the fetal muscle after motoneuron stimulation. In the EDL, as secondary generation, future fast cells separate and become independently innervated, the motor unit composition of the muscle may be expected to change and will result in an increase in V_{max}.

Role of the Motoneuron in Determining Fiber Types

Although a single pioneering axon initially innervates an early muscle fiber, during most of embryogenesis and the first few weeks postpartum, rat muscle fibers are innervated by more than one motoneuron (4, 6, 34). In the adult animal, it is probable that slow myosin synthesis is determined by innervation by a motoneuron which is continuously active at low frequencies (31, 32, 38, 40–43). To account for fiber specificity during embryogenesis despite the presence of polyneuronal innervation, we hypothesize that specificity is imparted by the axon with the lowest firing threshold, despite functional inner-
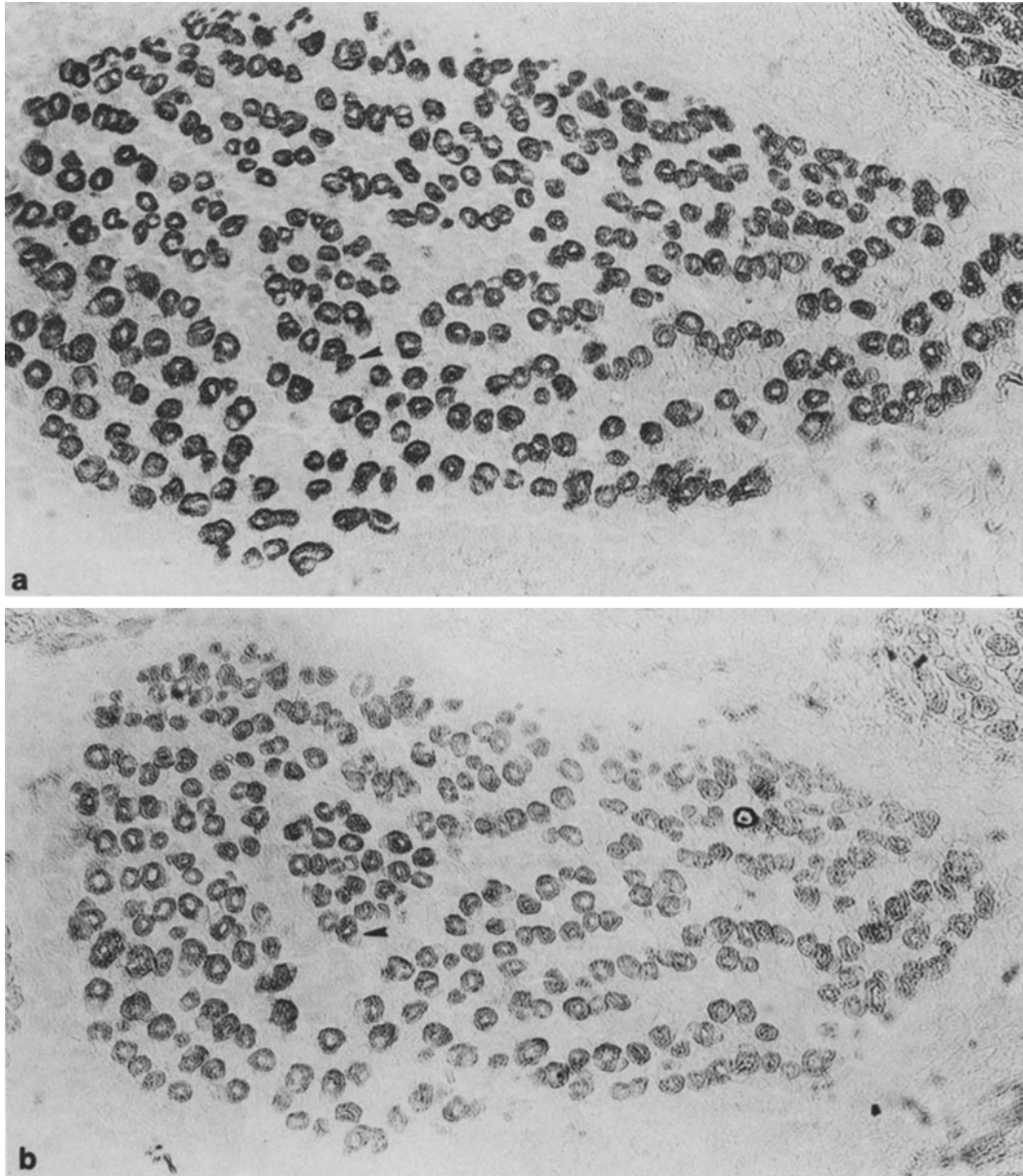
FIGURE 12 Serial sections of the soleus from the 18-d fetus stained with antifast and antislow myosin antibodies. All primary and secondary generation fibers react strongly with AF (a). Unlike the EDL, most of the fibers are primary generation cells, and very few secondary generation cells can be seen. Like the EDL, however, most of the primary generation fibers additionally react with AS (b), while few of the secondary fibers bind this antibody. Arrowhead marks a secondary generation cell reacting strongly with AF but not with AS.
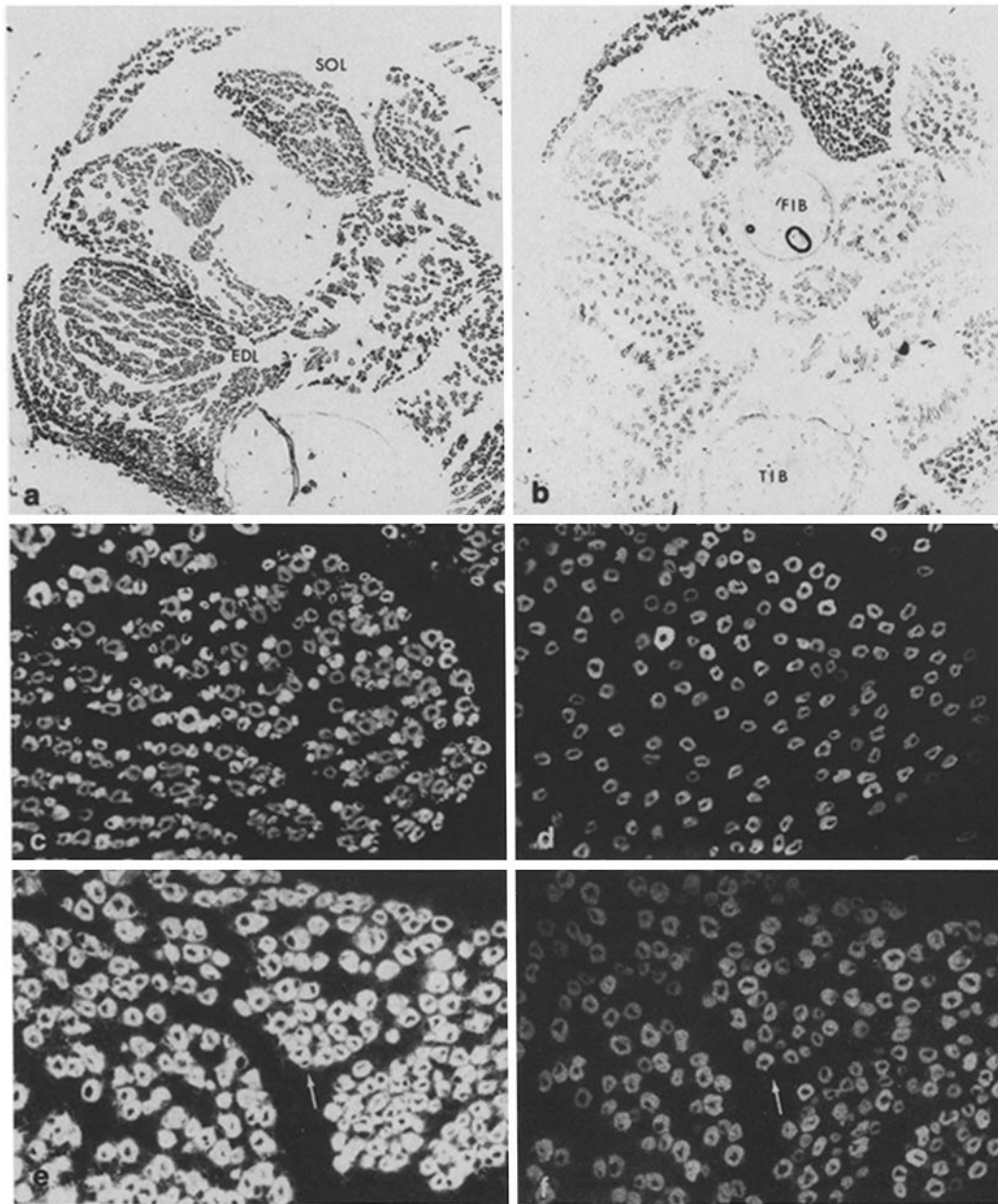
FIGURE 13   Antibody staining of the hindlimb of a 19-d embryo. Each pair (a,b; c,d; e,f) are serial sections. By 19 d gestation, the limb is well developed on both the extensor and flexor surfaces. Antibody staining by the PAP method reveals that all fibers in the limb still react strongly with AF (a), while only a select number of fibers reacts strongly with AS (b). The remaining sections were stained with fluorescein-labeled antibodies. With the fluorescein technique, positive fibers are light. The EDL shows two levels of staining with AF (c). Those fibers reacting strongly with AF but not with AS, while those fibers that react less intensely with AF, usually the larger, primary generation cells, bind AS avidly (d). In the soleus, almost all fibers now stain intensely with AF (e) and variably with AS (f). Occasional secondary fibers fail to stain with AS (arrows).
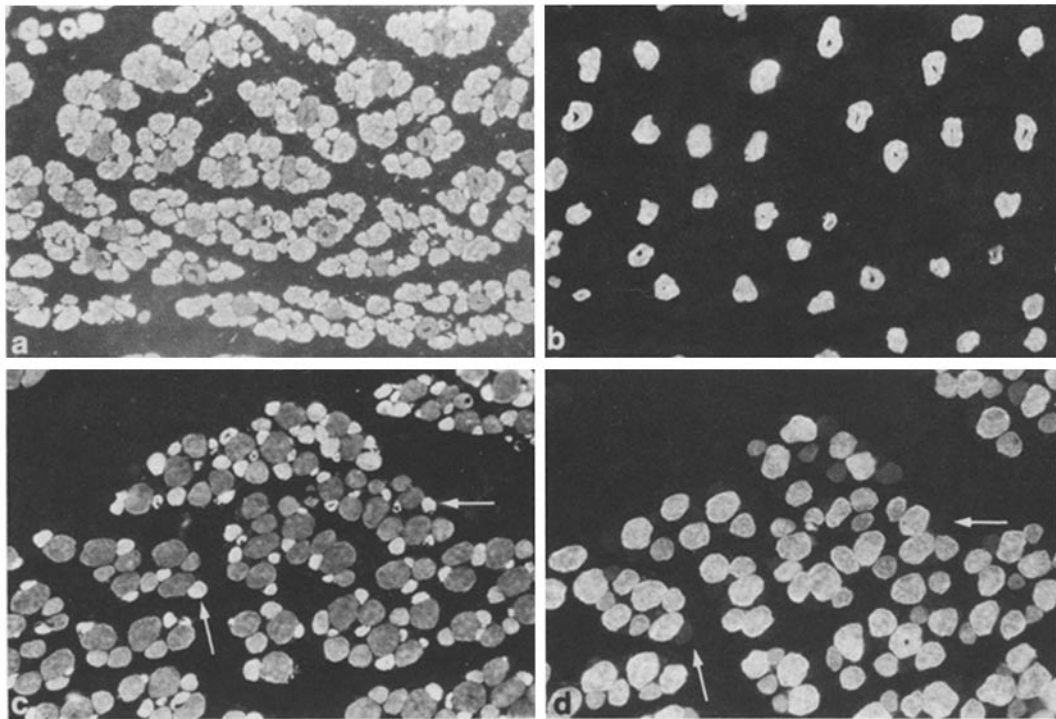
FIGURE 14   Serial sections of the EDL and soleus 2 d after birth. While all fibers of the EDL still react with AF (a), some differences in intensity among fibers can be discerned. A small percentage of cells additionally react intensely with AS (b). Most fibers, however, are negative with AS. Most fibers in the soleus react only lightly now with AF (c). However, a number of smaller, presumably secondary generation, fibers are seen closely apposed to larger fibers, and these smaller fibers react intensely with AF. Those fibers staining weakly with AF react strongly with AS (d), while those reacting strongly with AF react weakly, or not at all, with AS (arrows).
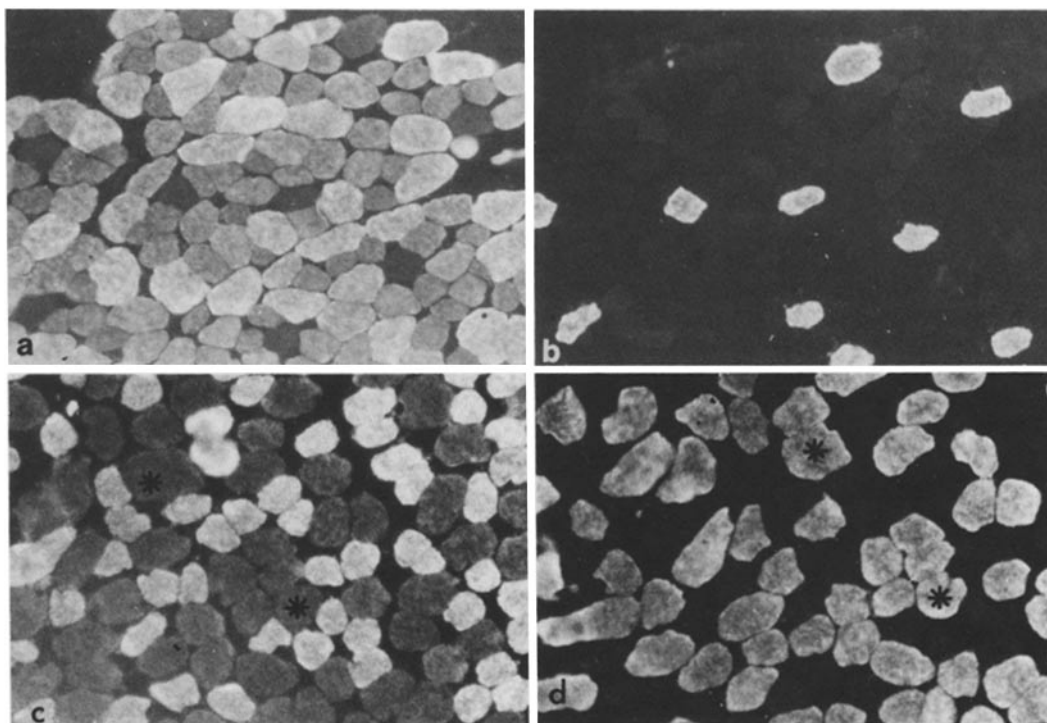

FIGURE 15   Serial sections of EDL and soleus muscles 2 wk after birth. In the EDL, the adult pattern of myosin staining has been approached. Most, but not all, fibers are positive with AF (a), while the fibers failing to react with AF now react with AS (b). In the soleus, 50% of the fibers react intensely with AF, while 50% are only lightly stained (c). Those fibers reacting strongly with AF fail to react with AS (d), while those reacting weakly with AF stain strongly with AS. Asterisks mark the same fibers in c and d.
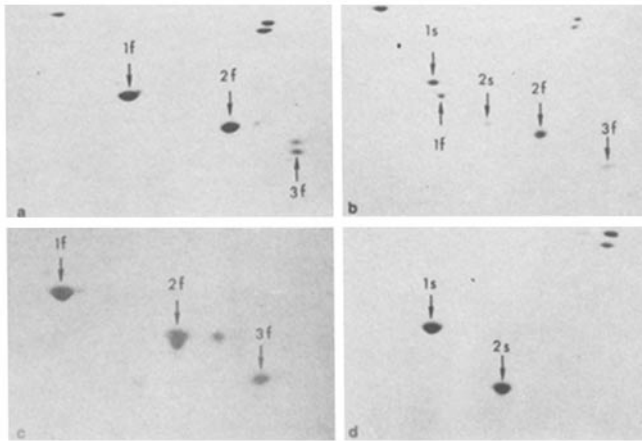
FIGURE 16   Light chains of the EDL and soleus muscles at 14 d after birth and in the adult animal. The EDL at all stages of development shows similar light chain complements. Predominantly fast light chains 1f, 2f, and 3f can be seen. The small amounts of slow light chains are usually not detectable. EDL light chains 2 wk after birth (a) and from an adult rat (b). The soleus 2 wk after birth shows all five fast and slow light chains, reflecting the fiber composition seen by antibody staining (c; see Fig. 15). By the adult stage, only slow myosin light chains can be seen, despite the presence of fast fibers in the muscle (d). Heavy loadings of myosin from this soleus did reveal slight quantities of fast light chains.

vation by other axons. This dominant axon could be the pioneering axon, suggesting that most pioneering axons are destined to become slow motoneurons, or it could be any other axon. The axon with the lowest firing threshold would probably be the most continuously active neuron in the group of neurons innervating a fiber and would, thus, be likely to specify slow myosin synthesis.

A pioneering axon induces a refractory state over the surface of the myotube so that no further synaptic points occur on the myotube (6); and, subsequently, secondary and tertiary cohorts of axons are constrained to multiply-innervate a myotube at the initial synaptic site. Is the refractoriness to diffuse innervation limited to the single, innervated myotube, or does it permeate an entire cluster of primary and secondary muscle cells and prevent the innervation of secondary fibers until separation from primary fibers (9)?

Although innervation of the primary generation fiber may inhibit innervation of the secondary cell, the signal for the initiation of slow fiber properties obviously does not traverse the junction between primary and secondary generation cells, for we rarely find secondary cells differentiating into slow fibers until after separation from the primary cell. In addition, it is still not clear whether the motoneurons impress specificity on the developing fibers or whether they allow the expression among fibers of inherent differences between primary and secondary generation cells.
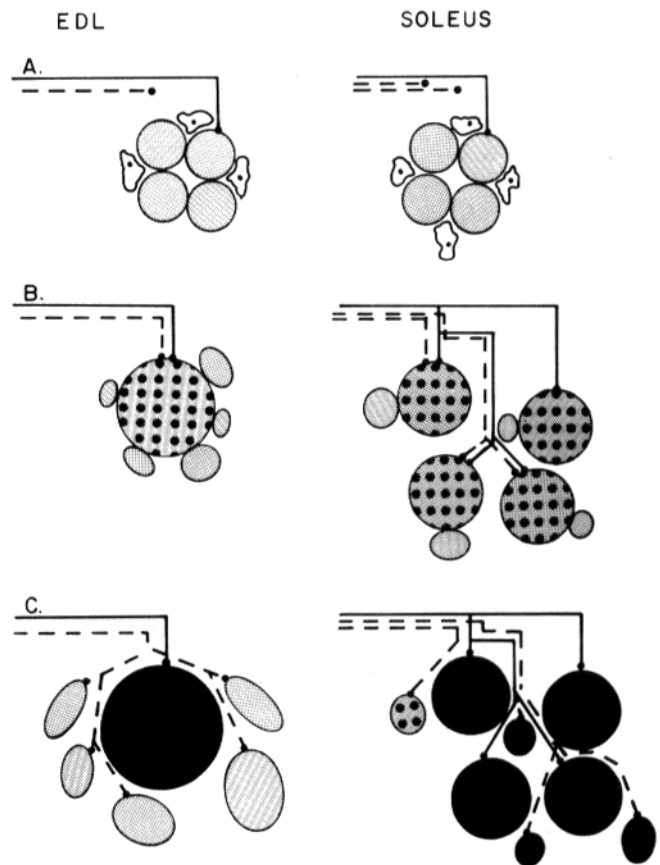
FIGURE 17   A possible sequence of events leading to muscle fiber diversity. In the following scheme, light shading indicates appreciable reaction of cells only with AF; shading with dots represents significant reaction with both AF and AS; dark shading signifies appreciable reaction only with AS. (A) A group of primary generation fibers surrounded by replicating mononucleated myoblasts are being innervated by a pathfinder motoneuron (solid line). Because the destiny of primary generation cells is to become type I fibers in the adult, one would expect the number of primary generation cells to be equal to the number of type I fibers in the adult animal. Similarly, because they are the first generation of fibers to arise, the number of type I fibers must be determined early in myogenesis. These hypotheses are supported by the data for the EDL muscle of the rat (see text). (B) Small, secondary generation cells have formed and are attached to the walls of the primary generation cells by close junctions. Successive cohorts of motoneurons (dashed lines) have been constrained to innervate the primary generation cell at the neuromuscular junction determined by the pathfinder motoneuron. Innervation of the secondary generation cells is not usually found at this time, possibly because of inhibition spreading from the innervated primary generation cell. The primary generation cell has begun to stain heavily with antibody to slow, as well as to fast, myosin, while secondary generation cells react heavily only with AF. (C) The secondary generation cells have relinquished their intimate connections with the primary generation cells and have become independently innervated. Their innervation apparently comes from the multiple innervation of primary generation cells (7). At this point, a major difference between developing fast and slow muscles can be seen. While practically all secondary generation cells in the fast muscle mature as type II fibers, most secondary generation cells in the soleus slowly convert to type I fibers during the first postnatal year (25). While the generation of type I fibers from secondary generation cells after birth may be due to the pattern of muscle usage, the initial formation of type I fibers from primary generation cells may not be due to the same cause.

REFERENCES

1. Adelstein, R., M. Conti, G. Johnson, I. Pastan, and T. Pollard. 1972. Isolation and characterization of myosin from cloned mouse fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 69:3693-3697.

2. Arndt, I., and F. Pepe. 1975. Antigenic specificity of red and white muscle myosin. *J. Histochem. Cytochem.* 23:159-168.

3. Ashmore, C., D. Robinson, P. Rattray, and L. Doerr. 1972. Biphasic development of muscle fibers in the fetal lamb. *Exp. Neurol.* 37:241-255.

4. Bagust, J., D. Lewis, and R. Westerman. 1973. Polyneuronal innervation of kitten skeletal muscle. *J. Physiol. (Lond.).* 229:241-255.

5. Barany, M. 1967. ATPase activity of myosin correlated with speed of muscle shortening. *J. Gen. Physiol.* 50:197-218.

6. Bennett, M., and A. Pettigrew. 1974. The formation of synapses in striated muscles during development. *J. Physiol. (Lond.).* 241:515-545.

7. Betz, W., J. Caldwell, and R. Ribchester. 1979. The size of motor units during post natal development of rat muscles. *J. Physiol.* (Lond.). 297:463-478.

8. Billeter, R., H. Weber, H. Lutz, H. Howald, H. Eppenberger, and E. Jenny. 1980. Myosin types in human skeletal muscle fibers. *Histochemistry.* 65:249-260.

9. Blackshaw, S., and A. Warner. 1976. Low resistance junctions between mesoderm cells during development of trunk muscle. *J. Physiol. (Lond.).* 255:209-230.

10. Buller, A., J. Eccles, and R. Eccles. 1960. Differentiation of fast and slow muscles in the cat hindlimb. *J. Physiol. (Lond.).* 150:399-416.

11. Church, J. 1969. Satellite cells and myogenesis: a study of the fruit bat. *J. Anat.* 105:419-438.

12. Close, R. 1964. Dynamic properties of fast and slow skeletal muscles of the rat during development. *J. Physiol. (Lond.).* 173:74-95.

13. Cosmos, E., J. Butler, and R. Scott. 1965. Phosphorylase activity in differentiating muscles of the domestic fowl. *J. Histochem. Cytochem.* 13:719.

14. Dhoot, G., and S. V. Perry. 1979. Distribution of polymorphic forms of troponin components and tropomyosin in skeletal muscle. *Nature (Lond.).* 278:714-718.

15. East, E. 1931. An anatomical study of the initiation of movement in rat embryos. *Anat. Rec.* 50:201-212.

16. Gauthier, G., and S. Lowey. 1977. Polymorphism of myosins among skeletal muscle fiber types. *J. Cell Biol.* 74:760-779.

17. Gauthier, G., and S. Lowey. 1979. Distribution of myosin isoenzymes among skeletal muscle fiber types. *J. Cell Biol.* 81:10-25.

18. Gauthier, G., S. Lowey, and A. Hobbs. 1978. Fast and slow myosin in developing muscle fibers. *Nature (Lond.).* 274:25-29.

19. Jenny, E., H. Weber, H. Lutz, and R. Billeter. 1980. Fibre populations in rabbit skeletal muscles from birth to old age. *In* Plasticity of Muscle. D. Pette, editor. Walter de Gruyter, Berlin. 97-109.

20. Keller, L., and C. Emerson. 1980. Synthesis of adult myosin light chains by cultured embryonic muscle. *Proc. Natl. Acad. Sci. U. S. A.* 77:1020-1024.

21. Kelly, A. 1978. Satellite cells and myofiber growth in the rat soleus and extensor digitorum longus muscles. *Dev. Biol.* 63:1-10.

22. Kelly, A., and D. Schotland. 1973. The evolution of the checkerboard in a rat muscle. *In* Muscle Development and the Muscle Spindle. B. Banker, R. Przybylski, J. van der Meulen, and M. Victor, editors. Excerpta Medica, Amsterdam. 32-48.

23. Kelly, A., and S. Zachs. 1969. The histogenesis of rat intercostal muscles. *J. Cell Biol.* 42:135-153.

24. Kelly, A., and S. Zachs. 1969. The fine structure of motor endplate morphogenesis. *J. Cell Biol.* 42:154-169.

25. Kikuchi, T., and C. Ashmore. 1977. Developmental aspects of the innervation of skeletal muscle fibers in the chick embryo. *Cell Tissue Res.* 171:233-251.

26. Kozeka, K., and M. Ontell. 1980. Prenatal development of muscle spindles in the mouse. *J. Cell Biol.* 87(2, Pt. 2):257 *a* (Abstr.).

27. Kugelberg, E. 1976. Adaptive transformation of rat soleus motor units during growth. *J. Neurol. Sci.* 27:269-289.

28. Lutz, H., H. Weber, R. Billeter, and E. Jenny. 1979. Fast and slow myosin coexist within single skeletal muscle fibers of adult rabbits. *Nature (Lond.).* 281:142-144.

29. O'Farrell, P. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007-4021.

30. Pelloni-Muller, G., M. Ermini, and E. Jenny. 1976. Myosin light chains of developing fast and slow rabbit skeletal muscle. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 67:68-74.

31. Pette, D., W. Muller, E. Leisner, and G. Vrbova. 1976. Time dependent effects on contractile properties, fiber population, myosin light chains, and enzymes of energy metabolism in intermittently and continuously stimulated fast twitch muscles of the rabbit. *Pfluegers Arch. Eur. J. Physiol.* 364:103-112.

32. Pette, D., and U. Schnez. 1977. Coexistence of fast and slow type myosin light chains in single muscle fibers during transformation as induced by long term stimulation. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 83:128-130.

33. Pette, D., G. Vrbova, and R. Whalen. 1979. Independent development of contractile properties and myosin light chains in embryonic chick fast and slow muscle. *Pfluegers Arch. Eur. J. Physiol.* 378:251-257.

34. Redfern, P. 1970. Neuromuscular transmission in new-born rats. *J. Physiol. (Lond.).* 209:701-709.

35. Rowlerson, A. 1979. Differentiation of muscle fiber types in fetal and young rats studied with a labelled antibody to slow myosin. *J. Physiol. (Lond.).* 297:27P.

36. Rubinstein, N., and H. Holtzer. 1979. Fast and slow muscles in tissue culture synthesize only fast myosin. *Nature (Lond.).* 280:323-325.

37. Rubinstein, N., and A. Kelly. 1978. Myogenic and neurogenic contributions to the development of fast and slow twitch muscles in the rat. *Dev. Biol.* 62:473-485.

38. Rubinstein, N., K. Mabuchi, F. Pepe, S. Salmons, J. Gergely, and F. Sreter. 1978. Use of type specific antimyosins to demonstrate the transformation of individual fibers in chronically stimulated rabbit fast muscles. *J. Cell Biol.* 79:252-261.

39. Rubinstein, N., F. Pepe, and H. Holtzer. 1977. Myosin types during the development of embryonic chicken fast and slow muscles. *Proc. Natl. Acad. Sci. U. S. A.* 74:4524-4527.

40. Salmons, S. 1980. The response of skeletal muscle to different patterns of use—some new developments and concepts. *In* Plasticity of Muscle. D. Pette, editor. Walter de Gruyter, Berlin. 387-400.

41. Salmons, S., and F. Sreter. 1976. Significance of impulse activity in the transformation of skeletal muscle type. *Nature (Lond.).* 263:30-34.

42. Salmons, S., and G. Vrbova. 1969. The influence of activity on some contractile characteristics of mammalian fast and slow muscles. *J. Physiol. (Lond.).* 210:535-549.

43. Sreter, F., J. Gergely, S. Salmons, anf F. Romanul. 1973. Synthesis by fast muscle of myosin light chains characteristic of slow muscle in response to long-term stimulation. *Nature (Lond.).* 241:17-19.

44. Sreter, F., S. Holtzer, J. Gergely, and H. Holtzer. 1972. Some properties of embryonic myosin. *J. Cell Biol.* 55:586-594.

45. Sternberger, L. Immunocytochemistry. John Wiley and Sons, New York. 2nd edition.

46. Stockdale, F., and H. Baden. 1979. On the origin of fast and slow muscle. *J. Cell Biol.* 83(2, Pt. 2):385 *a* (abstr.).

47. Strauss, S., and G. Weddell. 1940. Nature of the first visible contractions of the forelimb musculature in rat fetuses. *J. Neurophysiol. (Bethesda).* 3:358-369.

48. Syrovy, I., and Gutmann, E. 1977. Differentiation of myosin in soleus and extensor digitorum longus muscle in different animal species during development. *Pfluegers Arch. Eur. J. Physiol.* 369:85-89.

49. Weeds, A., D. Trentham, C. Kean, and A. Buller. 1974. Myosin in cross-reinnervated cat muscles. *Nature (Lond.).* 247:135-139.

50. Whalen, R., G. Butler-Browne, and F. Gros. 1978. Identification of a novel form of myosin light chain present in embryonic muscle tissue and cultured muscle cells. *J. Mol. Biol.* 126:415-431.

51. Whalen, R., G. Butler-Browne, S. Sell, and F. Gros. 1979. Transitions in contractile protein isozymes during muscle cell differentiation. *Biochimie.* 61:625-632.

52. Whalen, R., K. Schwartz, P. Bouveret, S. Sell, and F. Gros. 1979. Contractile protein isozymes in muscle development: identification of an embryonic form of myosin heavy chain. *Proc. Natl. Acad. Sci. U. S. A.* 76:5197-5201.

53. Windle, W., W. Minear, M. Austin, and D. Orr. 1935. The origin and early development of somatic behavior in the albino rat. *Physiol. Zoology* 8:156-185.