ABSTRACT Isozymes of myosin have been localized with respect to individual fibers in differentiating skeletal muscles of the rat and chicken using immunocytochemistry. The myosin light chain pattern has been analyzed in the same muscles by two-dimensional PAGE. In the muscles of both species, the response to antibodies against fast and slow adult myosin is consistent with the speed of contraction of the muscle. During early development, when speed of contraction is slow in future fast and slow muscles, all the fibers react strongly with anti-slow as well as with anti-fast myosin. As adult contractile properties are acquired, the fibers react with antibodies specific for either fast or slow myosin, but few fibers react with both antibodies. The myosin light chain pattern also shows a change with development: the initial light chains (LC) are principally of the fast type, LC1f and LC2t, independent of whether the embryonic muscle is destined to become a fast or a slow muscle in the adult. The LC3f light chain does not appear in significant amounts until after birth, in agreement with earlier reports. The predominance of fast light chains during early stages of development is especially evident in the rat soleus and chicken ALD, both slow muscles, in which LC1f is gradually replaced by the slow light chain, LC1s, as development proceeds. Other features of the light chain pattern include an "embryonic" light chain in fetal and neonatal muscles of the rat, as originally demonstrated by R. G. Whalen, G. S. Butler-Browne, and F. Gros. (1978. J. Mol. Biol. 126:415-431); and the presence of ~10% slow light chains in embryonic pectoralis, a fast white muscle in the adult chicken. The response of differentiating muscle fibers to anti-slow myosin antibody cannot, however, be ascribed solely to the presence of slow light chains, since antibody specific for the slow heavy chain continues to react with all the fibers. We conclude that during early development, the myosin consists of a population of molecules in which the heavy chain can be associated with a fast, slow, or embryonic light chain. Biochemical analysis has shown that this embryonic heavy chain (or chains) is distinct from adult fast or slow myosin (R. G. Whalen, K. Schwartz, P. Bouveret, S. M. Sell, and F. Gros. 1979. Proc. Natl. Acad. Sci. U. S. A. 76:5197-5201. J. I. Rushbrook, and A. Stracher. 1979. Proc. Natl. Acad. Sci. U. S. A. 76:4331-4334. P. A. Benfield, S. Lowey, and D. D. LeBlanc. 1981. Biophys. J. 33(2, Pt. 2):243a[Abstr.]). Embryonic myosin, therefore, constitutes a unique class of molecules, whose synthesis ceases before the muscle differentiates into an adult pattern of fiber types.
development. Using the procedures of immunocytochemistry, we have shown (18) that the fibers of the rat diaphragm, a fast-twitch muscle, react strongly with antibodies specific for both slow and fast myosin during early stages of development when the speed of contraction is still slow (22). We interpret this to mean that myosins with slow as well as fast characteristics coexist in the same fibers of this differentiating muscle. At later stages, when the speed of contraction has increased, myosin is segregated into different populations of fibers characteristic of the adult pattern of fiber types. The fibers of developing muscles also have a high level of ATPase activity which can be demonstrated after either acid or alkali preincubation (10, 18, 60). The presence of fibers with high acid-stable ATPase activity is correlated with a slow rate of contraction (21), and this is consistent with the observation of a "slow myosin" initially in a future fast muscle.

Although both physiological (9) and immunocytochemical data (18, 36) suggest a slow myosin in developing muscles, emphasis has been placed by some investigators on the presence of a light chain with characteristics of adult fast myosin (12, 40, 45, 57). Based on the light chain pattern, slow myosin is not readily evident in mammalian and avian muscles; trace amounts of slow light chains have been observed in the embryonic P.L.D., but these were ascribed to minor contamination by a slow muscle (42). More recent reports reaffirm the presence of small amounts of slow light chain in chicken embryos (6, 59), but the predominant light chain in early development is clearly more similar to a fast myosin light chain than a slow one (65).

The immunological and histochemical data on the question of the type of myosin present in developing muscles have also been conflicting: Rubinstein and his associates have reported that only fast myosin is synthesized in embryonic chicken and rat muscles (45, 46) and in cells cultured from chicken muscles (44). On the basis of their evidence, they proposed that all fetal muscles have an intrinsic genetic program which dictates the synthesis of fast myosin initially. However, several authors have suggested that developing muscles contain a distinct "embryonic" myosin (28, 53, 61, 65, 66) which is different from adult fast and slow myosins.

In an attempt to resolve some of these contradictions, we have extended our immunocytochemical studies to include additional selected muscles of the rat and chicken which have well-defined contractile properties. The same muscles have been analyzed by two-dimensional PAGE (38) for their light chain composition. In a separate study, to be reported elsewhere, we have analyzed the heavy chains of embryonic chicken myosin by peptide mapping of proteolytic digests of myosin (P. A. Benfield, D. LeBlanc, G. Waller, and S. Lowey, manuscript in preparation). We conclude from these combined studies that all the fibers of developing fast muscles of both the rat and chicken contain a distinctive embryonic myosin (or myosins). Although different from the adult slow and fast isoforms, this myosin is sufficiently homologous to show immunological cross-reactivity.

**MATERIALS AND METHODS**

**Skeletal Muscles**

Adult male albino rats and female white Leghorn chickens were killed with chloroform. In preparation for immunocytochemistry, the muscles were exposed by blunt dissection, and 3-mm strips were tied to wooden splints and then frozen in isopentane cooled to −160°C with liquid nitrogen. Embryonic muscles were excised whole, apposed to a tied adult muscle, and then frozen. Myosin was prepared from freshly dissected muscles or from frozen muscles by the standard procedures (17). Developing muscles were dissected and placed in equal volumes of glycerol and 13 mM phosphate (pH 7.0) at 4°C for ~1 h. They were then stored at −20°C. In the rat, a "19-d gestation" is defined as 3 d before term based on a 22-d gestation period. In the chicken, "days' incubation" refers to the number of days in ovo based on morphological criteria described by Hamburger and Hamilton (23), regardless of actual time of incubation. The 10-d specimens were from 9, 10, or 11-d chick embryos.

**Antibodies**

Antibody specific for the N-terminal sequence of alkali 1 (anti-A1) was prepared as described in Holt and Lowey (27). The purification of antibody specific for ALD myosin is described in Gauthier and Lowey (17). Antibody specific for the heavy chain of ALD myosin was prepared by absorbing the anti-ALD myosin with an excess of purified ALD light chains.

**Immunocytochemistry**

All specimens of developing muscle were mounted with an adult muscle and sectioned simultaneously so that differentiating fibers could be compared directly with adult fibers under identical conditions. Wherever possible, the adult muscle was the same as the developing counterpart. In some experiments (e.g., where the adult muscle was homogenous) a muscle with a mixed population of fibers was substituted. The selective positive response in this control ensured that a low or negative response to an antibody by the experimental muscle reflected a true absence of cross-reactivity and not a failure in the staining procedure. Conversely, the negative fibers present in the control ensured that a uniformly positive response by the experimental muscle was specific. In all the illustrations, sections are arranged so that the developing muscle is uppermost and the adult muscle below. In most instances, where two antibodies are compared, serial sections were used. Skeletal muscle fibers are sufficiently long that multiple transverse sections can be prepared, and therefore, portions of the same cell can be compared under the same conditions. Embryonic fibers (myotubes), however, are short, and serial sections do not necessarily contain sections of the same fiber.

Transverse cryostat sections (4 μm) were cut at −20°C and incubated as described in an earlier study (17). The sections were exposed either to antibody directed against slow myosin (anti-ALD) or to antibody specific for the A1 light chain of fast myosin (anti-A1). They were subsequently reacted with fluorescein-labeled goat antibody against rabbit immunoglobulin. Sections were examined with a Zeiss fluorescein microscope equipped with an epi-illumination system. A xenon XBO 75 W/DC lamp, a narrowband FITC excitation filter (485/20 nm), and a band-pass barrier filter (520–560 nm) were used with a Zeiss Neofluar 16/0.4 objective. The image was recorded on Kodak type 103A-G spectroscopic plates.

**Gel Electrophoresis**

Two-dimensional gel electrophoresis was performed according to O'Farrell (38). Isoelectric focusing gels were cast in glass tubing (90 × 2.5 mm internal diameter). Ampholines (LKB Instruments, Inc., Rockville, Md.) were used at a ratio of 4 (pH range 5–8): 1 (pH range 3.5–10). This gave a measured pH gradient (determined by slicing the gels and equilibrating in degassed, deionized water) of −4.5 to 6.8. Gels were run for 16 h at 30°C at a constant voltage of 400 V. Gels were removed from the tubes and equilibrated in 0.0625 M Tris HCl pH 6.8, 2.3% (wt/vol) SDS, 5% (vol/vol) mercaptoethanol, 10% (wt/vol) glycerol for 1 h, and stored at −30°C before running in the second dimension. In the second dimension, slab gels were run according to Laemmli (32) with a 12.5% running gel and a 5% stacking gel. Gels were run at 150 V constant voltage until an added bromophenol blue dye front reached the bottom of the gel (~3.5 h). They were then stained overnight in 0.025% Coomassie Brilliant Blue in 4% methanol, 9% acetic acid, and destained by diffusion in 5% methanol, 7.5% acetic acid.

Tissue samples (up to 50 mg wet weight, stored in 50% glycerol for 6 wk) were homogenized in 50–200 μl 9.5 M urea (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) 1.6% (wt/vol) ampholines pH range 5–8, 0.4% (wt/vol) 3.5–10 ampholines, 2% (vol/vol) Nonidet P-40, 5% (vol/vol) mercaptoethanol, 0.25% SDS (homogenization buffer). Loads of 10–40 μl were then applied to the isoelectric focusing gel. For muscle samples early in development (19-d gestation [rat], 10-d incubation [chicken]), muscles from 8–20 individuals stored in 50% glycerol for 6 wk were spun down in a microfuge tube. The pellet was then extracted in 50 μl homogenization buffer and the supernatant applied to an isoelectric focusing gel. Electrophoresis was then continued as described.
RESULTS

Electrophoretic Properties of Myosin Light Chains in Skeletal Muscles of the Rat

The three light chains characteristic of a fast-twitch muscle can be separated according to molecular weight by SDS PAGE (33); further separation on the basis of charge is achieved by using two-dimensional PAGE (65). The gel pattern is illustrated in the preparation from a predominantly fast muscle of the rat, the extensor digitorum longus (EDL) (Fig. 1d). These three light chains have been designated alkali 1, dithionitrobenzene, and alkali 2 light chains, in order of decreasing molecular weight, but they can also be referred to as fast light chains 1, 2, and 3, or LC1f, LC2f, and LC3f, respectively. We will use the latter nomenclature here. This terminology has the advantage that it denotes the speed of the muscle from which the myosin was extracted. The two principal light chains from a corresponding slow muscle, the rat soleus (Fig. 1g), have somewhat higher apparent molecular weights and slightly higher isoelectric points than LC1f and LC2f from the fast EDL. These are designated LC1s and LC2s. Small amounts of LC1f and LC2f are present as well, most likely originating from the fast fibers which comprise a minority of ~25% of the rat soleus (15). There is no equivalent to LC3 in this slow muscle. In the rat diaphragm, which has a mixed population of fibers, all five light chains are present, but LC1f, LC2f, and LC3f predominate (Fig. 1a).

At 19-d gestation, there is very little light chain typical of adult slow myosin in either the EDL or diaphragm, but two of the light chains characteristic of adult fast myosin, LC1f and LC2f are present (Fig. 1e and b). In addition, there is a small amount of light chain which has a molecular weight similar to that of LC3f, but which has a slightly lower isoelectric point (Fig. 1b and e). This light chain, which is unique to developing muscle, has been designated embryonic light chain 1, or LC1emb, by Whalen et al. (65). In the soleus at this stage of development, LC1f and LC2f predominate, along with substantial amounts of LC1s and also LC1emb (Fig. 1h).

At postnatal day 1, the light chain pattern in the two fast muscles has changed very little from that observed at 19-d gestation (Fig. 1c and f). The small amount of LC1f observed at the earlier stage in the diaphragm persists and increases slightly (Fig. 1e). LC1emb continues to be evident in both muscles. In the soleus, however, there is a marked change in the gel pattern at this time, as evidenced by a large increase in LC1s, relative to LC1f (Fig. 1i). LC1emb has decreased to only trace amounts.

Localization of Myosin Isozymes in Muscle Fibers of the Rat

Antibody was prepared against red myosin from the anterior latissimus dorsi (ALD), a slow muscle of the chicken. This antibody (anti-ALD) was used as an immunocytochemical marker to localize slow myosin within a population of muscle fibers of the rat. At 19-d gestation, there is very little light chain typical of adult slow myosin in either the EDL or diaphragm, but two of the light chains characteristic of adult fast myosin, LC1f and LC2f are present (Fig. 1e and b). In addition, there is a small amount of light chain which has a molecular weight similar to that of LC3f, but which has a slightly lower isoelectric point (Fig. 1b and e). This light chain, which is unique to developing muscle, has been designated embryonic light chain 1, or LC1emb, by Whalen et al. (65). In the soleus at this stage of development, LC1f and LC2f predominate, along with substantial amounts of LC1s and also LC1emb (Fig. 1h).

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fibers in transverse cryostat sections of skeletal muscles of the rat. Antibody against the difference peptide (Δ1) unique to the alkali 1 light chain of chicken pectoralis myosin (LC1Δ) (27) was used as a marker to identify fast myosin. The specificity of both antibody preparations has been described elsewhere (17), but is confirmed here by the ability to react selectively with only certain fibers within a small heterogeneous region of the adult chicken pectoralis (see below).

In the adult rat diaphragm, a fast-twitch muscle, there is a mixed population of fibers, which react with either anti-Δ1 or anti-ALD, but only occasionally with both antibodies (see Fig. 2 a and b, lower sections). At 19-d gestation, all fibers react strongly with antibodies against both fast and slow myosins (Fig. 2 a and b, upper sections). The EDL consists, in the adult, almost entirely of fibers which react with anti-fast myosin (Figs. 2 c and d, and 3 c and d, lower sections), but at 19-d gestation, all fibers react strongly with anti-fast as well as anti-slow myosin (Fig. 2 c and d, upper sections). In the soleus, a response to both types of antibodies is seen in all fibers at 19-d gestation (Fig. 2 e and f, upper sections), whereas in the adult muscle, fibers with only slow myosin predominate (Fig. 2 e and f, lower sections). The unstained central region of each fiber represents the position of the nucleus, which indicates that the muscle cells (fibers) are in the myotube stage of differentiation at this period of development. A difference in fiber diameter is evident, and smaller fibers probably represent later stages of differentiation (31), but there is no correlation with the level of immunofluorescence. Fibers with large or small diameters react with both antibodies equally.

At postnatal day 1, a differential response to the antibodies is apparent in the two fast muscles. Most fibers react strongly with anti-Δ1, but a few are less intensely stained (Fig. 3 a and c). The overall response to anti-ALD continues to be greater than in the adult muscles which have been sectioned simultaneously in the same block. The majority of the fibers of the diaphragm are moderately stained and some continue to be stained intensely (Fig. 3 b). An even larger proportion of fibers in the EDL react strongly with anti-ALD (Fig. 3 d). In the soleus, the response to anti-Δ1 has now diminished somewhat over that observed at 19-d gestation, but all fibers continue to react with this antibody, some quite strongly (Fig. 3 e). The level of response to anti-ALD is high in all fibers, but some fibers stain more intensely than others (Fig. 3 f). In the EDL and soleus, myotubes are still present, whereas in the diaphragm, all cells have differentiated to the myofiber stage, hence the unstained central nuclear region is no longer evident. Although a difference in fiber diameter is more apparent at this stage, there is, again, no relationship to staining pattern.

It is concluded that, during late prenatal and early postnatal development of these fast and slow muscles, there is abundant fast-type myosin as well as myosin with immunologic properties of slow myosin. The synthesis of the fast-type of myosin is then terminated in most of the fibers of the slow muscle, whereas the synthesis of the slow-type of myosin is terminated in the majority of the fibers of the fast muscles.

Myosin Light Chain Patterns in Skeletal Muscle Fibers of the Chicken

The two-dimensional polyacrylamide gel pattern of myosin light chains in the chicken pectoralis is similar to that of the EDL in the rat. There are three light chains which differ in molecular weight and isoelectric point (pl), LC1f, LC2f, and LC3f (Fig. 4 a). In the ALD, a slow muscle, there are two light chains comparable to those in the rat soleus, namely LC1f and LC2m and there are, in addition, detectable amounts of LC1l and LC2l (Fig. 4 d), as was observed in the rat soleus. It is possible that this reflects the small number of fast fibers normally present in this muscle. Separation of fast and slow light chains according to molecular weight is less pronounced than in the rat, e.g., in the mixed diaphragm muscle, but the pls are more widely separated.

At 10 d in ovo, LC1l and LC2l predominate in the pectoralis, along with small amounts of LC1f and LC2f (Fig. 4 b). There is also a small amount of light chain with the same molecular weight as LC3l, but with a lower pl, possibly corresponding to LC1em in the rat. In the ALD (Fig. 4 e), the predominant light chain is LC1l, but moderate amounts of LC2l and LC1a, and a trace amount of the putative LC1emb are present as well.

At 18 d in ovo, LC1l and LC2l again predominate in the pectoralis (Fig. 4 c). As in the slow muscle of the rat, there is a more pronounced change in the gel pattern of the slow ALD than in the pectoralis at the same stage of development. At 18 d, there is marked increase in LC1l, relative to LC1a (Fig. 4 f), and the pattern closely resembles that of the adult ALD.

Localization of Myosin Isozymes in Muscle Fibers of the Chicken

In the pectoralis muscle of the chicken, a fast white muscle, fibers which react with anti-fast myosin predominate, but a circumscribed red region (∼1%) of this muscle consists of two types of fibers (16) which react strongly with either anti-fast or anti-slow myosin (Fig. 5 a and b). This heterogeneous muscle is, therefore, a useful reference against which the relative intensity of staining can be compared at each stage of development. Furthermore, the positive response to the rabbit anti-
FIGURE 4 Two-dimensional gel patterns of myosin light chains from chicken skeletal muscles during development in ovo. Time of incubation is based on stages of development identified according to morphological criteria defined by Hamburger and Hamilton (23) (see Materials and Methods). Samples from adult ALD and pectoralis, and pectoralis at 10-d incubation, represent purified myosins. The remaining samples are obtained from tissue extracts as described in Materials and Methods. Adult pectoralis and ALD muscles contain predominantly fast and slow myosins, respectively. At 10-d incubation both muscles contain predominantly fast myosin light chains with small amounts of slow light chain (~10%). (The LC2f light chain is susceptible to proteolysis and this may explain the low amount of this light chain in the gel of ALD at 10-d incubation.) By 18-d incubation the amount of slow light chain is much reduced in the chicken pectoralis, but has increased in amount in the chicken ALD.

FIGURE 5 Adult chicken pectoralis. Serial transverse sections of the heterogeneous red region. (a) anti-Δ1. (b) anti-ALD. Response to the two antibodies is reciprocal; the fibers react strongly with either anti-Δ1 (a) or anti-ALD (b). The positive response is more intense than in adult rat muscles (Figs. 2 and 3), since the chicken is the homologous species. The high contrast between reactive and unreactive fibers illustrates the specificity of the antibodies. X 226.
chicken antibodies in this muscle is far more intense than in the rat muscles described above, since the chicken is the homologous species. Hence the contrast between reactive and unreactive fibers is high, and the negative response of certain fibers serves as a meaningful control for establishing the specificity of the antibodies.

The major part of the adult pectoralis muscle consists of fibers which react only with anti-fast myosin and not with anti-slow myosin (17). At 10 d in ovo, all fibers of the pectoralis react strongly with both fast and slow anti-myosins, but occasionally, some fibers have a lower level of response to anti-slow myosin than others (Fig. 6 a and b). The posterior latissimus dorsi (PLD) of the adult chicken also consists primarily of fibers which react with anti-fast, but not with anti-slow myosin (Fig. 7 c and d, lower sections). At 10 d in ovo, all fibers react strongly with both antibodies (Fig. 6 c and d). In the adult ALD muscle, most of the fibers react only with anti-slow myosin (Fig. 6 e and f, lower sections), but in the 10-d-old chick, all fibers of the ALD react with antibodies to fast and slow myosin (Fig. 6 e and f). At this stage of development, all three muscles, the pectoralis, PLD and ALD, are composed of myotubes. As in the developing rat muscles, there is no correlation between staining pattern and cross-sectional dimensions of the cells.

At 18 d in ovo, the response to anti-slow myosin in the pectoralis has diminished considerably, but all fibers continue to stain at least moderately, and some stain intensely (Fig. 7 b). All of the fibers react strongly with anti-fast myosin (Fig. 7 a). This staining pattern persists at least through 1-d posthatching (Fig. 8 a and b). Most of the fibers in the PLD no longer react with anti-slow myosin, but they continue to react strongly with anti-fast myosin (Fig. 7 c and d); a few fibers continue to stain intensely with both antibodies (Fig. 7 d). In the 18-d ALD, the level of response to anti-fast myosin varies, but it is, nevertheless, high in all fibers, and is comparable to or even greater than that of reactive fibers of the adult muscle included in the same section (Fig. 7 f, lower section).

The pronounced change in response to anti-slow myosin which occurs between 10 and 18 d in the fast PLD muscle but not the pectoralis illustrates the variation that can exist even within the same category of muscle. In the pectoralis, moreover, myotubes continue to be present at 18 d in ovo, and ~1-d posthatching, which further indicates that this muscle is less differentiated than the PLD at a particular stage of development.

The observation of a myosin with characteristics of slow myosin in developing muscle is apparently not related to species, since it is present in fast as well as slow muscles of the rat and also the chicken. The possibility exists that the isoencephal which reacts with anti-slow myosin has a light chain which is characteristic of adult LC1, (see Fig. 4 b) and that this light chain is associated, during development, with a heavy chain which resembles adult fast myosin. Alternatively, a distinctive "embryonic" myosin could exist which cross-reacts with antibodies specific for adult slow myosin. To determine whether the heavy chain is responsible for staining with anti-slow myosin, we absorbed anti-ALD myosin with total ALD light chains. The resulting antibody preparation would be expected to react only with the heavy chain of myosin. This absorbed antibody continues to react strongly with developing fibers in the fast EDL muscle of the rat as well as in the two fast muscles (PLD and pectoralis) of the chicken embryo (Fig. 9 a and b). It is concluded that the response reflects a property of the heavy chain, and that developing muscles contain a myosin which has a slow or embryonic type of heavy chain.

DISCUSSION

It has been suggested that, before innervation, skeletal muscle cells synthesize only adult fast myosin, and that synthesis of slow myosin is triggered by neuronal contact (45, 46). There is ample evidence for the influence of the nervous system on the type of myosin isoenzyme expressed by a muscle cell. This is well illustrated in muscles that have been cross-reinnervated. When the nerve supply is switched from a slow to a fast muscle, the speed of contraction is slowed, and the myosin of the newly innervated muscle has characteristics of slow myosin (4, 25, 54, 55, 64). Similarly, prolonged low-frequency stimulation of a fast muscle leads to a slower contraction time and to synthesis of slow myosin at the expense of fast myosin (41, 49, 54, 56).

Strong support for the concept of a "fast myosin" in the absence of the nervous system came from tissue culture experiments. Immunological cross-reactivity was observed between the myosin extracted from cultured cells and antibodies prepared against adult fast muscle myosin (44). No reaction was seen by immunofluorescence or gel diffusion when antibodies against slow muscle myosin were used. Likewise, myosin isolated from embryonic pectoralis muscle failed to react with antibodies specific for slow myosin, but did react strongly with antibodies against fast myosin (46). The embryonic anterior latissimus dorsi (ALD), a slow muscle in the adult, also contained fast myosin during early development. Moreover, many investigators have shown that the light chains of embryonic myosin comigrate with those of adult fast myosin, i.e., LC1, and LC2 (40, 45, 53, 65).

Despite the many similarities between embryonic myosin or tissue culture myosin and adult fast myosin, a large body of evidence has accumulated over the years in favor of the argument that they are, in fact, not identical. The most convincing

![Figure 6](https://example.com/figure6.jpg)

**Figure 6** Response of chicken skeletal muscle fibers to anti-\(\Delta 1\) (a, c, and e) and anti-ALD myosin (b, d, and f). 10-d incubation. All fibers in the developing pectoralis react with both antibodies (a and b), but some fibers have only a moderate level of response to anti-ALD (b); see also Fig. 9 a. The control muscle in the lower sections of a and b is from adult rat diaphragm; hence the level of fluorescence is low relative to that observed in adult chicken muscle (Figs. 5 and 9). The two sections in a and b are not serial, but observation with phase-contrast optics indicates that there are no muscle fibers present that fail to react with either antibody; that is, all fibers stain with anti-slow as well as anti-fast myosin. The apparently extensive space between fibers is occupied by connective tissue components. As in the rat muscles, there is no correlation between staining pattern and cross-sectional dimensions of the fibers. All fibers in the 10-d PLD react strongly both with anti-\(\Delta 1\) and anti-ALD myosin (c and d). The adult muscle was not sufficiently close to the developing muscle to be included in the micrograph, but the positive response to anti-\(\Delta 1\) and the absence of a response to anti-ALD is illustrated in other sections of adult PLD (see Fig. 7 c and d). In the adult ALD (e and f, lower sections), all fibers illustrated react with anti-ALD (f) but not with anti-\(\Delta 1\) (e). In the 10-d muscle, all fibers react with both antibodies. All three muscles are composed of myotubes at day 10. Sections in c and d and in e and f are serial. X226.
Response of chicken skeletal muscle fibers to anti-Δ1 (a, c, and e) and anti-ALD myosin (b, d, and f). 18-d incubation. Response to anti-Δ1 is high in both the pectoralis (a) and PLD (c) at 18 d. The response to anti-ALD is diminished in the pectoralis (b), but all fibers stain at least moderately, and some stain intensely. Only a few fibers of the PLD react with anti-slow myosin (d). All fibers of the ALD react with both antibodies. Adult muscles are rat diaphragm (a and b), chicken PLD (c and d), and chicken pectoralis, red region (e and f). All sections in which two antibodies are compared are serial. × 226.
FIGURE 8  Response of chicken pectoralis to anti-Δ1 (a) and anti-ALD (b). 1-d posthatching. Serial transverse sections with control adult rat diaphragm. The pattern of response is similar to that observed at 18-d in ovo (Fig. 7 a and b). All fibers stain intensely with anti-fast myosin. The level of response to anti-slow myosin is low in some fibers, but all fibers are nevertheless stained, some intensely. X 226.

FIGURE 9  Response of chicken pectoralis to anti-ALD myosin adsorbed with total ALD light chains. 10-d incubation. Serial transverse sections mounted with adult pectoralis, red region. In the developing muscle, all fibers react with anti-slow myosin before (a) and also after (b) adsorption, that is, with antibodies against the heavy chain. The contrast between reactive and unreactive fibers in the adult muscle is high, and therefore the latter are difficult to distinguish in the photograph. This obvious negative response verifies the specificity of the response of the developing fibers to anti-ALD myosin. X 226.

evidence came from the sequence of a peptide isolated by Huszar from the myosin of a 3-wk-old rabbit (28). This peptide was nonmethylated and had several amino acid substitutions relative to the homologous peptide from adult fast myosin. The
possibility remained that this peptide originated from slow muscle myosin, since the latter is not methylated and has not been sequenced. However incomplete, these data did prove that developing myosin was not identical to adult fast myosin.

**Localization of Isozymes by Immunocytochemistry**

Early immunocytochemical studies by Masaki and Yoshi- zaki (36) on avian muscles, both *in vivo* and in culture, demonstrated the presence of a myosin which cross-reacted with antibodies against slow (ALD) myosin. These findings were criticized by Rubinstein and Holtzer (44) because of the failure of the investigators to purify their antibodies by affinity chromatography; however, none of the earlier studies used antibodies purified beyond absorbing them with the heterologous antigen. The study by Gauthier et al. (18) was among the first to use antibodies that were purified by repeated adsorption onto matrices containing first the heterologous antigen (e.g., anti-ALD serum adsorbed with pectoralis myosin), followed by isolation of the antibody from a column matrix containing the homologous antigen (e.g., ALD-myosin). These highly purified antibodies persisted in their reaction with embryonic rat muscles. All the fibers in transverse, frozen sections of rat diaphragm at 19-d gestation and rat EDL at 1-d postnatal, reacted with antibodies specific for slow and fast myosin. Some of the fibers, which were inadvertently cut in longitudinal section, showed transverse striations reflecting the localization of the antibody in the A-band (see Fig. 5 in Gauthier et al. [18]). Thus, the previous immunocytochemical methods, along with the biochemical analyses, provided strong evidence that developing muscles contain more than simply adult fast myosin.

In the work described here, we have extended our immunocytochemical investigations to include a variety of slow and fast mammalian (rat) and avian (chicken) muscles. Consistent with our earlier, more limited observations (18), we find that all the fibers of a developing muscle, regardless of their future speed of contraction, react with antibodies specific for slow and fast myosin. In a recent paper, however, Kelly and Rubinstein (30) report that a limited population of fibers, "primary generation cells," react with anti-slow myosin as well as with anti-fast myosin, while the remaining "secondary generation cells" react exclusively with anti-fast myosin. This poses the problem of why partial reactivity is observed under experimental conditions (EDL from an 18-d rat fetus) in which we find that all the myotubes are stained (see Fig. 2). Even at 1 d postnatal, where differentiation of the EDL into fiber types is beginning to occur, all the fibers continue to react with both anti-slow and anti-fast myosin antibodies (Fig. 3). We believe the explanation can be found, in part, in the different immunological techniques: the indirect peroxidase-anti-peroxidase method used by Kelly and Rubinstein (30) can lead to a loss of reactive sites if the tissue is fixed; and the method of direct immunofluorescence is not as sensitive to low levels of reactivity. The technique of indirect immunofluorescence used in our study is both highly sensitive and, as shown by the differential staining pattern in adult control muscles, highly specific.

There is now general agreement that some myosin in embryonic and cultured muscle cells is of a slow-type (11, 36, 43). Whether developing muscles contain adult-type myosin iso- zymes, or whether a distinct myosin isozyme (or isoizymes) exists during the early stages of development cannot be resolved by immunological techniques alone, but requires biochemical analysis of the proteins.

**Characterization of Isozymes by Gel Electrophoresis**

We have shown, as have others previously, that the predominant myosin light chains of developing muscles have the same electrophoretic mobility as those of adult fast myosin. In agreement with the findings of Whalen et al. (65), we also observe an embryonic light chain in rat EDL, diaphragm and soleus muscles at 19-d gestation and 1-d postnatal. The absence of this minor band in all three adult muscles is convincing evidence that this spot is not an artifact. Whether such an embryonic light chain is present in chicken muscles is less clear. There is a spot on the acidic site of LC1, which is particularly pronounced in 10-d chicken pectoralis; however, a trace of this band persists in the adult. More data are needed to resolve this problem.

What is certain is the presence of ~10% slow light chains in 10-d chicken pectoralis (see Fig. 4b) (6). Since this work was completed, several other reports have appeared which confirm the presence of small amounts of slow light chain both in embryonic (37, 59) and in cultured chicken cells (29, 58).

This raises the question of whether the slow light chains alone might be responsible for the staining of 10-d chicken pectoralis by anti-ALD antibody. This is less likely for rat EDL muscle, since its gel pattern shows barely a trace of slow light chains at 19-d gestation (Fig. 1 e). Absorbing the antibody to ALD myosin with ALD light chains removed any doubt that at least some of the slow antigenic determinants must be located on the heavy chain of the myosin in embryonic rat and chicken muscles.

The presence of an excess of fast adult light chains does not necessarily imply that the associated heavy chains need be of the fast adult type. Wagner and Weeds (63) showed that the light chains had relatively little effect on the ATPase activity, and that the active sites were located primarily on the heavy chains. More recently, Wagner (62) prepared hybrids containing light chains from slow cardiac myosin and heavy chains from fast skeletal muscle myosin. Again, the principal conclusion was that the enzymatic activity of the molecule was determined largely by the heavy chain. "Embryonic myosin" can therefore be thought of as a population of molecules, which may consist of adult slow, fast, or embryonic light chains complexed to an adult fast, slow, or embryonic heavy chain.

The nature of the myosin heavy chain in developing muscles can be examined either by electrophoresis of native myosin in pyrophosphate gels (26) or by SDS-gel electrophoresis of proteolytic degradation products of myosin. The early studies of Sreter et al. (53) compared tryptic digestion patterns of adult rabbit skeletal myosin with those of embryonic myosin and found minor differences. With the introduction of two-dimen- sional gel electrophoresis, more definitive peptide maps became available. In particular, the work of Whalen et al. (66) showed that rat myosin, both in cultured and embryonic muscle cells, is an isozyme distinct from adult slow or fast myosin.

Recent peptide mapping studies of embryonic chicken pec- toralis myosin have shown that distinct isozymes also occur in developing avian muscles (6, 47). It could be argued that the 10% slow light chains observed in 10-d chick embryos were associated with 10% adult slow heavy chains, the contribution of the slow heavy chain would be too small to be detected in peptide maps of total embryonic myosin digest. To circumvent this problem, an immunoadsorbent specific for ALD myosin heavy chain was used to isolate a "slow-type" myosin from the pool of embryonic isozymes.
peptides from this small fraction, although slightly different from the characteristic pattern obtained for bulk embryonic myosin, nevertheless, showed no resemblance to adult slow myosin heavy chain (6; P. A. Benfield, D. LeBlanc, G. Waller, and S. Lowey, manuscript in preparation). We conclude that despite the immunological cross-reactivity, embryonic myosins are distinct in primary structure from adult slow or fast myosin, and constitute a unique class of isozymes. Independent evidence for the absence of adult fast myosin in embryonic chicken muscle has come from studies using monoclonal antibodies prepared against adult pectoralis myosin. Certain clones were found which did not react with myosin from embryonic tissue, thereby confirming the conclusions reached from electrophoretic studies (D. Winklmann and S. Lowey, unpublished observations).

**Relationship of Isozymes to Speed of Contraction**

The absence of adult slow myosin in embryonic muscles brings us to the question of why developing muscles are slow. According to Bárány's classic experiment with adult muscles (3), the type of myosin should reflect the speed of contraction of a muscle. In an attempt to answer this question, Kelly and Rubinstein (30) suggested that "primary generation cells," those cells which are innervated early in development, react with anti-slow myosin antibodies, and therefore are responsible for the slow speed of contraction. Incompatible with this hypothesis is our finding that all myotubes, both primary and secondary, react with antibodies against fast and slow adult myosin. Furthermore, as discussed previously, peptide analysis of the myosin from both 10-d embryonic chicken pectoralis (6) and 20-d embryonic rat muscles (66) indicates an absence of adult slow myosin. Also, the embryonic myosin which is present, at least in the chicken pectoralis, has an ATPase activity and 20-d embryonic rat muscles (66) indicates an absence of secondary, react with antibodies against fast and slow adult myosin. Differences in speed of contraction of a muscle may also be related to myofibrillar proteins other than myosin. For example, when the speed of contraction of the soleus is increased by cross-reinnervation, there is an increase in the amount of the fast isoform of troponin I (1). During postnatal development, both fast and slow forms of troponin I are present in individual muscle fibers, and only later do they become selectively distributed among fast or slow fibers (14).

There is increasing evidence for a role of the sarcoplasmic membrane systems in regulating the contractile properties of a muscle. Although the speed of shortening may reflect the type of myosin present in a muscle, the time to peak of an individual twitch response is more closely related to the sarcoplasmic membranes (24). Organization of the sarcoplasmic reticulum is more elaborate in fast than in slow muscle fibers, and it becomes fully developed only late in differentiation (39, 51, 52). The density of intramembrane particles and the level of calcium transport activity associated with them is greater in fast than in slow muscles, and both increase during the normal course of development, as speed of contraction is increased (5, 8, 34, 35, 48). In the absence of a fully differentiated membrane system, it might be expected that contraction would be slow, regardless of the type of contractile proteins present.

**Influence of the Nervous System**

It is clear that the nervous system has an influence on the type of myosin synthesized by a muscle fiber, but the extent of its influence is not certain. As discussed above, alteration of the nerve supply by cross-reinnervation or by chronic stimulation results in a transformation of myosin isozymes. We had suggested earlier that the presence of both fast and slow types of myosin within a fiber during development is likewise related to the pattern of innervation (18). At a time when speed of contraction is slow, individual motor endplates are contacted by several axonal endings (2, 7), and this is referred to as "polynuclear innervation." Both fast and slow types of myosin coexist within individual muscle fibers at this time. As adult contractile properties are attained, and polynuclear innervation is withdrawn, synthesis of one type of myosin appears to be "switched off," leading to the adult pattern in which each muscle fiber contains a single class of isozymes. The study described here presents additional evidence for such a hypothesis, but with modification. As shown by Whalen et al. (66) in rat muscle and by Benfield et al. (6) in the chick pectoralis, the myosin present in embryonic muscles is not equivalent to the fast and slow isozymes of adult muscles, and the adult isozymes do not appear until 2-3 wk after birth (or hatching). The adult form is preceded by yet another class of "neonatal" isozymes (P. A. Benfield, G. Waller, and S. Lowey, manuscript in preparation) (67). Hence the transformation during development involves more than a selective termination of synthesis within a mixed population of adult isozymes. Rather, an entirely new class of adult isozymes replaces a transient population of first embryonic and then neonatal isozymes. The sequence of events suggests that the adult pattern of distribution of myosin isozymes occurs only after the adult neuromuscular relationship has been established. Less certain is whether the distinctive isozymes observed during development can be synthesized regardless of the type of innervation pattern, or whether the motoneurons comprising the polynuclear configuration have a role in determining the type of myosin isozyme expressed by the muscle cell.

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