

SOME PROPERTIES OF EMBRYONIC MYOSIN

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ABSTRACT

Myosins from the following sources were purified by diethylaminoethyl-Sephadex chromatography: myotubes grown in vitro for 7–8 days, prepared from pectoralis muscles of 10-day old embryos, and breast and leg muscles from 16-day old embryos. The adenosine triphosphatase activities of these myosins were close to that of adult m. pectoralis myosin. The light chains of the embryonic myosins had the same mobilities in sodium dodecyl sulfate electrophoresis as those in adult pectoralis muscle myosin and were clearly distinguishable from those in myosin from tonic muscle m. latissimus dorsi anterior. The fastest light chain in embryonic muscle myosin—apparent mol wt 16,000—was present in smaller amounts than in adult myosin. The negative staining pattern of paracrystals of embryonic light meromyosin (LMM) was indistinguishable from that of adult fast muscle LMM. The significance of these results for differentiation of various muscle types has been discussed.

Myosin is synthesized and assembled into striated myofibrils very early in development, long before the skeletal muscles are physiologically active. For example, there are over 2×10^8 postmitotic, mononucleated, striated myoblasts in a single myotome of early 3-day chick embryos. By day 4 there are bi- and trinucleated myotubes in these *anlagen* of the trunk muscles (1). Aside from occasional twitches, however, these future mixed red and white trunk muscles are not physiologically active until hatching 18 days later. Similarly, scattered postmitotic, mononucleated, cross-striated myoblasts are present in 5 day breast muscle. By day 7 mononucleated cross-striated myoblasts are rare and, although goodly numbers of long, multinucleated myotubes are present, these future white muscles will not contract with any regularity until after hatching (2).

The following observations stress the similarities between the contractile proteins synthesized in early myogenic cells with those synthesized in

mature fibers:

(a) The earliest myofibrils bind fluorescein-labeled antibodies to myosin and tropomyosin in precisely the same fashion as do mature myofibrils (3, 4, 5).

(b) Thick and thin filaments of the earliest myofibrils have the same dimensions as the thick and thin filaments of mature muscle (6–9), and the thin filaments in the earliest myogenic cells can be decorated with heavy meromyosin to form typical arrowhead complexes (10).

(c) Glycerinated models of 3-day striated myoblasts contract when exposed to adenosine triphosphate (ATP) as do mature myofibrils but, as with mature myofibrils, contraction is blocked if the myofibrils are pretreated with antibody to either myosin or tropomyosin (11).

Perry and coworkers (12–15) have stressed the qualitative differences between “embryonic” myosin (e.g., myosin from newborn rabbits) and myosin extracted from mature muscles. They reported

that embryonic myosin, in addition to differing immunologically, had a lower ATPase activity and lacked the 3-methyl-histidine residues found in myosin from adult animals. They also suggested that in terms of the low molecular weight components (light chains) embryonic myosin was closer to myosin from adult red or cardiac than to white muscle myosin. These findings led these workers to conclude that:

(a) Myosin exists in two isozyme forms, embryonic or slow, and adult or fast.

(b) The switch which takes place at different stages of development in different species (from embryonic to the adult type) may occur in response to innervation and physiological activity.

(c) Myosin synthesized in postnatal animals would gradually replace the embryonic myosin of the myofibrils assembled earlier in development.

Recent work has more sharply defined the relation of various aspects of myosin to the type of muscle from which it was isolated. It seemed of interest, therefore, to re-examine the questions whether myosin of a given muscle differs from myosin of the same adult muscle and whether embryonic myosin corresponds to one of the types found in adult muscle.

The experiments to be reported in this paper suggest that the first myosin molecules to be synthesized in very young chick myotubes in fact possess essentially the same ATPase activity and the same three low molecular weight components that are found in mature fast muscle (16). Similarly, the negatively stained paracrystals of light meromyosin (LMM) obtained from 16 day breast muscle myosin are of the fast or white type (17) and are identical to those prepared from mature breast muscle.

MATERIALS AND METHODS

Myotubes grown *in vitro* were prepared from chick embryos as described (18). Mononucleated cells of breast muscles, carefully dissected under a dissecting microscope, of 10-day chick embryos were cultured for 8–10 days. DNA of the cultured cells was measured by the method of Burton (19). The cultured myotubes were transferred to stoppered 50-ml Erlenmeyer flasks, covered with fresh tissue culture medium, shipped in iced containers from Philadelphia to Boston, and washed on arrival with ice-cold 0.05 M phosphate buffer, pH 7.0.

To isolate myosin from tissue cultures at an optimal yield, actomyosin was first extracted at 0°C for 20 hr with a solution containing 0.6 M KCl, 10 mM Tris HCl, pH 8.5, 1 mM ethylenediaminetetraacetate

(EDTA), and 1 mM dithiothreitol (DTT), 5 ml g wet weight (approximately 3–4 g were used). After centrifugation (12,000 *g* for 20 min in a Sorvall SS-34 rotor), 5 mM MgSO₄ and 10 mM K₄P₂O₇ were added to the supernatant (crude actomyosin) under constant stirring to dissociate myosin. Actin was separated by centrifugation (150,000 × *g* for 1 hr, Beckman Model L centrifuge, rotor 50) and the myosin in the supernatant was purified by repeated precipitation at low ionic strength followed by dissociation in a solution containing 0.5 M KCl and 5 mM Tris HCl, pH 7.6. Further purification was achieved by column chromatography and (NH₄)₂SO₄ precipitation as described previously (16). Myosin was dialyzed against a solution containing 0.01 M phosphate (pH 7.0), 0.5 M NaCl, 0.1 mM EDTA, and 0.1 mM DTT before gel electrophoresis. Myosin was also prepared from the breast and leg muscles of 16-day old embryos according to our earlier procedure (20) except that a ribonuclease A treatment was included as described by Dow and Stracher (21). The preparation of LMM and the electron microscope examination of negatively stained paracrystals of LMM were carried out as described by Nakamura et al. (17).

Adult chicken myosin was prepared as before (21) from 3 month old fast twitch (m. pectoralis major and latissimus dorsi posterior), slow tonic muscle (m. latissimus dorsi anterior), and cardiac muscle of white leghorns.

For gel electrophoresis the myosin from 16-day embryos and that from adult muscles were further purified by column chromatography and (NH₄)₂SO₄ precipitation as described above. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described earlier (16). LMM paracrystals were prepared and examined by electron microscopy as described (17).

K-EDTA-activated ATPase was assayed in a medium containing 0.6 M KCl, 1 mM EDTA, 50 mM Tris HCl, pH 7.6, and 2 mM ATP. For assay of Ca-activated ATPase the medium contained 0.02 M KCl, 10 mM CaCl₂, 50 mM Tris HCl, pH 7.6, and 2 mM ATP, 25°C. The reaction was started by the addition of myosin (0.3 mg/ml). The liberation of inorganic phosphate was determined according to the method of Fiske and SubbaRow (22).

It is difficult to assign an "age" to the myosin extracted from the cultured myotubes. There is virtually no myosin in the mononucleated cells used to start the cultures and it is not until the third or fourth day of culture that the synthesis of myosin enters an exponential phase, which is then maintained for another 3 or 4 days. At the time of extraction of the cultures, between days 8 and 10, the bulk of the myosin synthesized *in vitro* was approximately 4 and 5 days old. By using a similarly adjusted age scale, the age of the bulk of the myosin from 16-day embryos would range from 6 to 10 days (i.e., signifi-

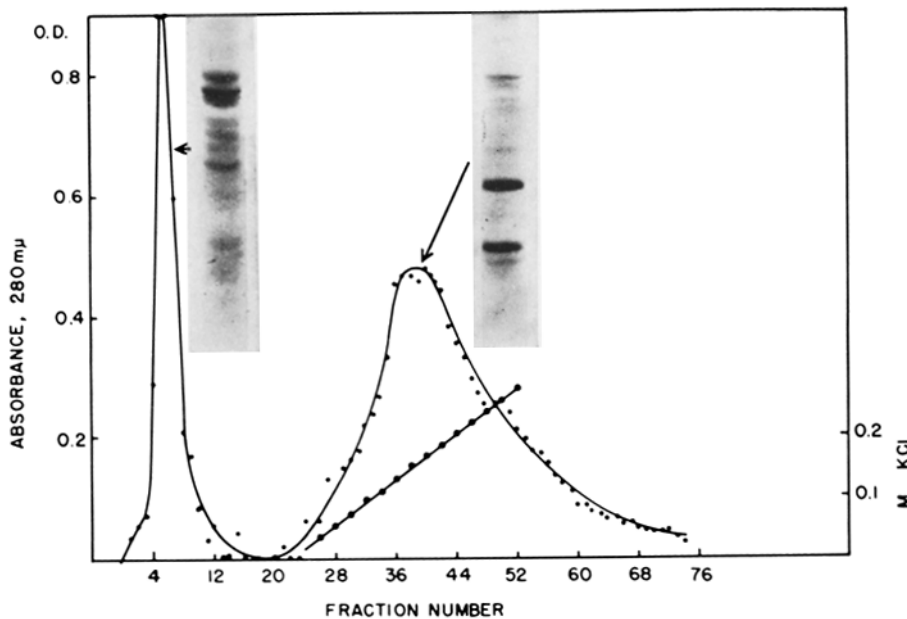


FIGURE 1 Chromatographic purification of myosin isolated from a culture of myotubes. About 50 mg protein was applied to a 30×1 cm DEAE-Sephadex A-50 column equilibrated with a 0.05 M K-pyrophosphate, pH 7.5. The protein was eluted with a linear gradient (0 – 0.5 M KCl in 0.5 M K-pyrophosphate) and 2-ml fractions were collected. Fractions 4–8 and 32–52 were combined, the protein was precipitated with 50% saturated $(\text{NH}_4)_2\text{SO}_4$, collected by centrifugation, and dialyzed exhaustively against 0.5 M KCl, 5 mM Tris HCl, pH 7.6. Samples of the pooled fractions were subjected to polyacrylamide gel electrophoresis in the presence of SDS. The patterns are shown as insets. (Amount of protein applied, 25 μg .)

cant quantities of myosin are not synthesized in breast muscle until approximately day 7). In brief, the myosins analyzed in these preparations are much younger than what is referred to in the literature as “embryonic” or “fetal” myosin.

RESULTS

ATPase Activity of Embryonic and Mature Myosin

The elution pattern of myosin extracted from cultured myotubes showed two peaks (Fig. 1). The first peak did not have ATPase activity and its gel pattern showed heavy contamination with unidentified proteins. The second peak contained ATPase activity and was used for enzyme studies and SDS gel electrophoresis. The ATPase activity of myosin from cultured myotubes is somewhat lower than that of myosin from 16 day embryonic breast muscle or leg muscle (Fig. 2). This is probably a lower limit for the activity of this myosin due to the ease of denaturing the embryonic myosin during extraction. The presence of trace

contaminants in the embryonic myosin which are difficult to remove would also lower its specific activity (1, 23). It should also be noted that there is no significant difference between the ATPase activity of myosin from breast white muscle and that from red muscles of the leg. The ATPase activity of myosin from adult tonic muscle latissimus dorsi is significantly lower than that of any of the other myosins. We assayed the myosin from cultured myotubes for the stability of its ATPase activity at alkaline pH. Mammalian slow muscle myosin shows considerable lability at pH 9 in contrast to the stability of fast (white) muscle myosin (21). Unfortunately chick myosin, whether from red leg muscle or from the latissimus dorsi anterior, does not exhibit alkali lability; myosin extracted from the cultured myotubes otherwise displays no alkaline lability. In summary, in the embryonic chick the earliest molecules of myosin synthesized display ATPase activity essentially similar to that found in molecules of myosin from mature muscle.

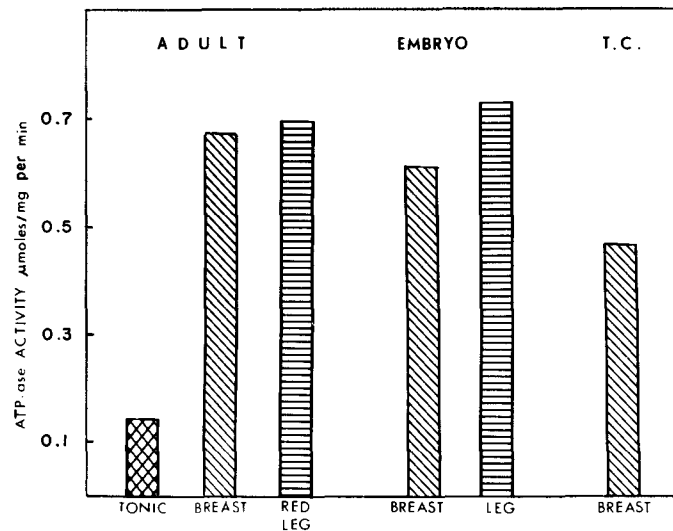


FIGURE 2 Ca^{2+} -activated ATPase activity of embryonic tissue culture (T.C.) and adult myosin. Conditions as described in Materials and Methods. The tonic adult muscle used was the latissimus dorsi anterior.

Light Chains from Embryonic Myosin

Studies based on SDS-polyacrylamide gel electrophoresis have demonstrated that the low molecular weight chains associated with adult rabbit myosins differ in fast, slow, and cardiac muscles (16, 24). The most prominent differences between fast, slow, and cardiac muscles are the presence of three chains in fast muscles and only two in slow and cardiac muscle. Differences in size and charge have also been demonstrated between the two slower components in the three types of muscle.

As shown in Fig. 3, myosin from the adult twitch muscle (*m. pectoralis*) of the chick differs from myosin from the adult tonic *m. latissimus dorsi anterior*. The former yields three light chains while the latter yields only two. Coelectrophoresis of the light chains from fast and slow myosins shows that even the two apparently corresponding chains differ in molecular weights.

If all embryonic muscles pass through a phase corresponding to slow or tonic, then myosin from cultured myotubes or from embryonic breast muscle should be expected to differ from the myosin from the corresponding mature muscle. As shown in Fig. 4, this did not happen. The embryonic myosins contain the same three low molecular weight components displayed by the homologous mature muscle. Myosin from cultured myotubes from very early embryos also shows the

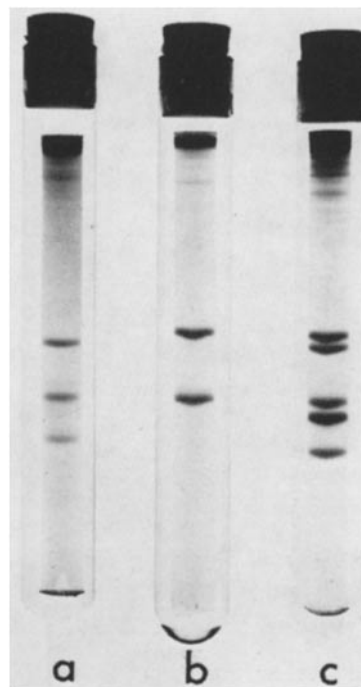


FIGURE 3 Electrophoresis of chicken myosin in 12.5% polyacrylamide gels. *a*, adult twitch (*m. pectoralis major*) muscle, 20 μg ; *b*, adult tonic (*m. latissimus dorsi anterior*) muscle, 30 μg ; *c*, coelectrophoresis of adult twitch (breast) and adult tonic muscle myosin (40–40 μg of each).

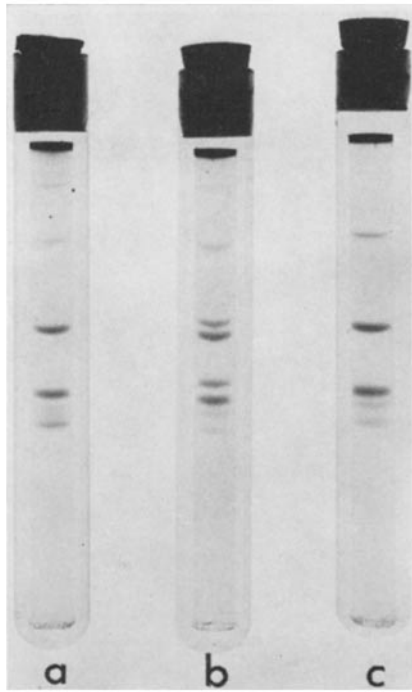


FIGURE 4 Coelectrophoresis of embryonic and adult myosin samples in 12.5% polyacrylamide gels. *a*, coelectrophoresis of myosins from embryonic chicken muscle (15 μg) and from adult twitch muscle (15 μg); *b*, coelectrophoresis of myosin from embryonic chicken muscle (20 μg) and from adult tonic muscle (30 μg); *c*, embryonic chicken twitch muscle, 30 μg .

characteristics of adult fast myosin both in terms of ATPase activity and the light chain pattern. Although the fastest light chain is always present in smaller amounts than in adult myosin, myosin from the *m. latissimus dorsi anterior*, a slow tonic muscle, contains a distinct set of subunits and displays low ATPase activity. On coelectrophoresis of the embryonic myosins with the two types of adult myosins, the light chains of embryonic myosins comigrate with those of the fast adult myosin and clearly do not correspond to those of tonic adult myosin.

Negatively Stained Paracrystals of LMM from Embryonic Myosins

Negatively stained light meromyosin aggregates from adult fast muscle are readily distinguishable from LMM paracrystals prepared from either adult rabbit, slow or, cardiac muscle. As shown in Fig. 5, the 439 Å period of adult white pectoralis

muscle LMM consists of a dark band and a 100 Å light band; the latter often contains one or two darker lines. The cardiac and slow LMM paracrystals are more complex. If embryonic myosin from presumptive fast white muscle is similar to slow, red muscle one would expect the paracrystals of LMM from 16 day embryonic breast muscle to be similar to those from adult slow muscle. As illustrated in Fig. 5, this was not the case. Negatively stained LMM paracrystals from 16 day embryonic breast or leg muscle do not differ from those prepared from white breast or adult leg muscles.

DISCUSSION

Efforts to correlate functional activity with aspects of myogenesis date back over a century. Harrison's classical experiment in 1904, however, (25) demonstrated unequivocally that the initiation of muscle development was totally independent of innervation. More recent work using cultures of myogenic cells only confirms this view (26). In the total absence of nerves or known hormones, replicating presumptive myoblasts yield postmitotic myoblasts which go on to fuse and form multinucleated myotubes. These myotubes, which synthesize creatine phosphokinase and myoglobin in addition to actin, myosin, and tropomyosin, persist *in vitro* for months without signs of trophic degeneration (1). If the synthesis, assembly, or maintenance of these molecules in mature muscle is dependent on some trophic influence supplied by nerves, then this dependence must be a secondary phenomenon which develops only in the postembryonic period.

That myosin isolated from fast or slow muscle displays different properties raises several fundamental issues regarding the genetic mechanisms and the physiological significance of this kind of molecular diversity. Differences in the size and charge of the light chains and the striking differences in the negatively stained LMM paracrystals in fast, slow, and cardiac muscles raise the possibility that several, possibly coupled, structural genes are required to produce a given functional thick filament. Obviously there must be genetic decisions during myogenesis to specify which heavy chain and which light chains are to be transcribed and translated in a particular muscle fiber. Currently it is not known at what time in development a myogenic cell is committed to synthesize the polypeptide chains of fast myosin or

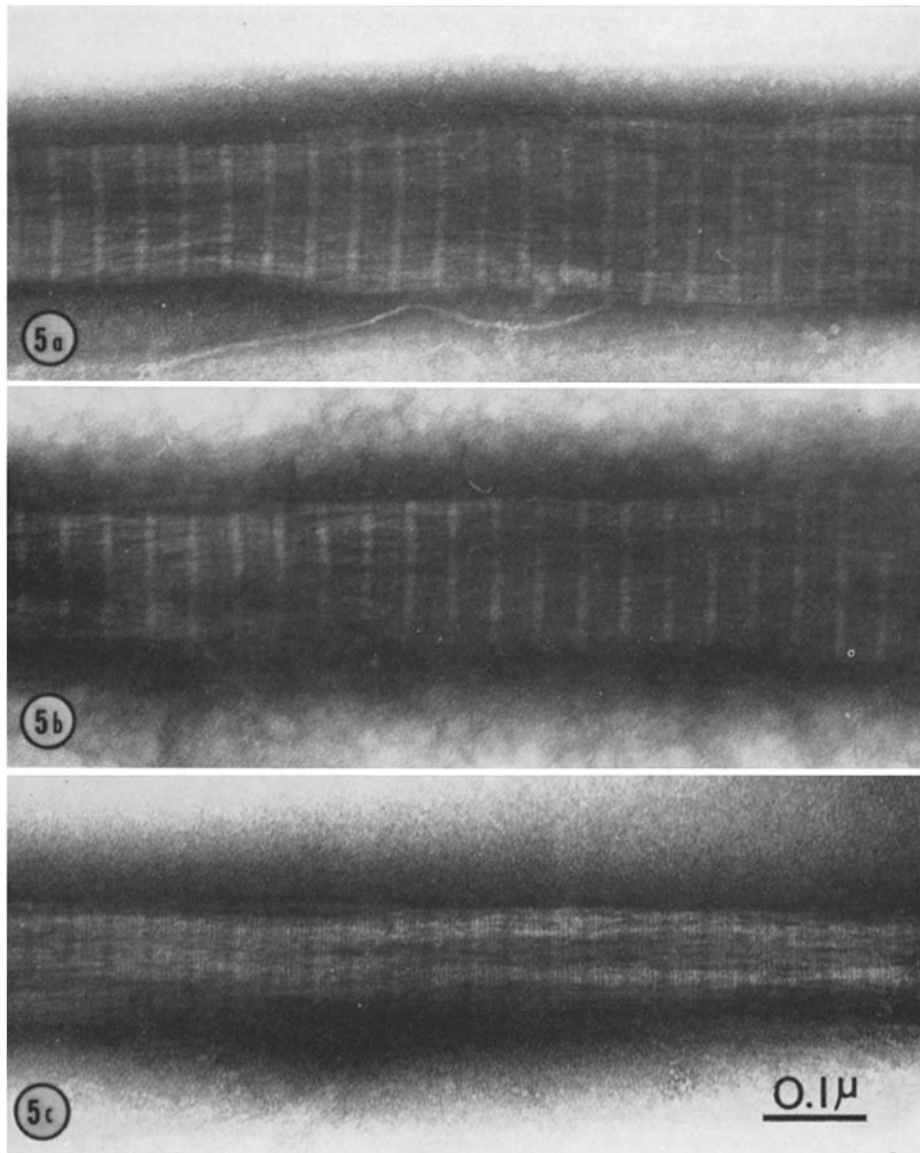


FIGURE 5 Comparison of the band pattern of embryonic and adult chicken muscle (breast) light meromyosin paracrystals (tryptic digest). Negative staining with 1% uranylacetate. *a*, adult chicken twitch muscle; *b*, embryonic chick muscle; *c*, adult chicken heart muscle. $\times 135,000$.

when myogenic cells are committed to synthesize those of slow myosin. Also unknown is the nature of such "inducing" factors as may be involved. In this connection the production of "hybrid" myotubes—myotubes formed by fusing presumptive fast and presumptive slow myoblasts—should be of some interest.

The data in this respect discourage the simple

notion that (*a*) before innervation and active contraction all muscles translate messages corresponding to the heavy and light chains of a less specialized myosin of the slow type, and (*b*) after innervation by "fast" nerves the functional muscles suppress the synthesis of "slow" myosin and begin to translate the messages for the heavy and light chains of fast myosin.

It is clear from our data that the light chains of myosin of cultured myotubes from early chick embryo pectoralis muscle and of myosin directly isolated from the same muscle of 16-day chick embryos are indistinguishable, by means of SDS electrophoresis, from the light chains of adult pectoralis myosin. The only change in the light chain pattern that may be associated with development is the increase in the relative amount of the faster moving (16,000 dalton) subunit. In this context it is important to stress the fact that, although the so-called red or slow muscles of the rabbit, or the slow tonic muscles of the chick, contain only two light chains which correspond roughly in their mobility to the two slower moving light chains of the fast or white muscle, the two slower moving chains in slow muscle are clearly distinguishable from their counterparts in fast muscle. Thus, the low content of the fastest chain in embryonic muscle, resulting in a fast band which apparently can be completely missed (27), may lead to the erroneous conclusion that embryonic myosin is equivalent to slow or red muscle myosin.

The essential similarity of embryonic breast muscle myosin to the adult type is also shown by comparison of the ATPase activities. Both myosin isolated from tissue cultures and that isolated directly from embryonic breast muscle have ATPase activities close to that of adult myosin and several times higher than that of myosin from the adult tonic latissimus dorsi anterior m. It should be noted that the apparently red leg muscles of the chick contain myosin whose ATPase activity is like that of white breast muscle. Furthermore, these muscles contain myosin which cannot be typed red or slow on the basis of its light chain pattern or on the basis of the appearance of negatively stained LMM paracrystals. In our hands the only type of muscle in the chick that shows the red, or slow, light chain pattern is the tonic anterior latissimus dorsi muscle. This illustrates the need for careful distinction among various muscle types. Red muscle may contain myosin that appears, with respect to ATPase activity and light chain pattern (see above), indistinguishable from white muscle myosin (see also reference 24). With respect to ATPase activity our data differ from those of Perry and his colleagues, who reported lower ATPase activity for embryonic myosin. The clue to the discrepancy may be found in the report of Dow and Stracher (27) which showed that in embryonic myosin

there is a time-dependent loss of ATPase activity in the absence of SH-protecting agents, the rate of loss decreasing with increasing maturity. We have not found it necessary to add DTT to stabilize embryonic myosin ATPase, but it may well be that under some, to us not clear, conditions it is necessary to do so in order to preserve the enzymatic activity.

The stability during the development of the pectoralis muscle of ATPase activity and of the pattern of synthesis of the light chains of myosin is consistent with the histochemical data (28). They show that the characteristics of the ATPase reaction in pectoralis muscle of the chick at hatching and during the period after it are those of the so-called α -fibers—alkali stable ATPase. This is in contrast to the dramatic changes in glycolytic enzymes during the posthatching period (29, 30). The changes have been attributed to differentiation in the pectoralis muscle. This process of differentiation, however, does not affect the ATPase activity, light chains, and LMM staining pattern of myosin. It would be of interest, as a counterpart of the present studies, to carry out a detailed investigation on embryonic tonic muscle although the technical difficulties, because of the small size of the m. latissimus dorsi, are considerable.

It has been reported that in the rabbit development is accompanied by a change in the 3-methylhistidine (13, 31) content of myosin. Huszar (32) has shown that the lack of methylation in the embryo is not due to the absence of the histidine residue—located in the subfragment-1 region of the molecule carrying the ATPase- and actin-combining sites—that would be methylated in the adult. Published data (31) as well as those obtained by us suggest that no such change in methylation takes place in the chicken. Whether differences between chicken and rabbit are related to differences in ATPase activity during development requires further work.

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