

Isozymes of myosin in growing and regenerating rat muscles

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Native myosin isozymes of rat muscles have been isolated by electrophoreses in non-dissociating conditions. Their mobilities were measured, using taenia coli myosin as an internal standard and their relative concentrations were determined by computer planimetry of the electrophoretograms. Three isozymes were observed in extensor digitorum longus (EDL), two in soleus (SOL), four in neonatal muscles and four in muscles three days before birth. Regenerates of minced EDL or SOL muscles in adult animals had no native myosin the third day after surgery; they were similar to neonatal muscles 15 days after surgery and to adult muscles 60 days after surgery.

Muscle myosin is a hexameric molecule in its native state. Various isozymes, differing by their heavy-chain primary structures or by the type of their alkaline light chains, can be separated in their native state by electrophoreses in non-dissociating conditions [1–4]. Slow muscle myosin can thus be separated from fast muscle myosin [1, 2]. Neonatal [5, 6] and embryonic [6] isomyosins have also been separated by this technique, whilst their chemical individuality has been supported by immunochemical methods or peptide mapping after enzymatic cleavage of the myosin heavy chains. Using taenia coli myosin as an internal standard, we are able to measure accurately R_F values for the various isoforms of myosin in adult, neonatal and embryonic muscles and, using an interactive computer-controlled scanning device, to measure the relative proportions of the various isoforms.

Adult muscles regenerating from a local cold injury express transiently fetal myosin heavy chains [7]. We have therefore analysed the isomyosins of regenerating muscles, using a model described by Carlson [8] in which the muscle is minced and orthotopically autografted. After an initial phase of degeneration and fragmentation of the graft, regeneration proceeds, recapitulating ontogeny. 15 days after surgery, the regenerate is contractile [9] and partially innervated [10]. 60 days later, the fibres are hypertrophied [8], their tension may be back to normal [11], their force-velocity is similar to that of the original muscle [12] and the functional recovery is satisfactory [11]. We show in this work that the isozymic transition characteristics of muscle growth from embryo to adult stages [6] also occur in adult rat during muscle regeneration.

MATERIALS AND METHODS

Striated muscles excised from rats (Wistar, outbred local stock, obtained from the *Katholieke Universiteit te Leuven*) were

Abbreviations. SOL, adult soleus muscle; EDL, adult extensor digitorum longus muscle; FM₁, FM₂ and FM₃, myosins of adult fast muscle 1, 2 or 3; SM₁, SM₂ and SM₃, myosins of adult slow muscle 1, 2 or 3; fM₁, fM₂, fM₃, fM₄ and fM₅, myosins of neonatal muscle 1, 2, 3, 4 or 5; eM₁, eM₂, eM₃ and eM₄, myosins of embryonic muscle, 1, 2, 3 or 4; R_F , ratio of the electrophoretic mobility of myosin to the mobility of an internal standard.

Enzyme. Myosin ATPase (EC 3.6.1.3).

Embryonic muscles (symbolized 'eM') were obtained by careful dissection of the thigh of 18-day-old conceptus, removing the skin and the bones. Neonatal muscles (fM) were excised from the thighs of new-born rats, within a few hours of the birth. Adult soleus (SOL) and extensor digitorum longus (EDL) were dissected from rats of various masses (50–200 g), removing surrounding blood vessels, nerves and tendons. Regenerating muscles were obtained using the method of Carlson [8]. Rats (50–70-days old) were anesthetized with 0.3 ml subcutaneously of thalamonal®. SOL or EDL muscles were dissected out, avoiding as much as possible any damage to the nerves and the blood vessels. The muscles were placed in sterile petri dishes and cut into small pieces with small scissors. The mince was grafted back into the animals. The regenerated muscles were excised for examination either 3 days or 15 days (SOL 15d; EDL 15d) or 60 days (SOL 60d; EDL 60d) after the surgery. All dissections and surgeries were performed under sterile conditions. Some taenia coli muscles (to be used as internal standards) were obtained from adult guinea pigs.

Immediately after dissection, the muscle was weighed, frozen in liquid nitrogen and ground in a mortar (cooled with liquid nitrogen). The muscle powder was transferred into a tube and mixed on ice for 20 min with 4 vol. Guba-Straub buffer (0.3 M NaCl, 0.1 M NaH₂PO₄, 0.05 M Na₂HPO₄, 1 mM MgCl₂, 10 mM Na₄P₂O₇, 0.1% Na₃N, pH 6.5; 2-mercaptoethanol at a final concentration of 0.1% was added just before use). The mixture was centrifuged (20000 rpm; 10 min). The supernatant was mixed with 1 vol. glycerol and stored at –20 °C.

This crude extract of myosin was used without further purification for polyacrylamide gel electrophoresis in non-dissociating conditions, using a technique similar to that described by Hoh et al. [2] and d'Albis et al. [1], with a few modifications. Gels at a concentration of 4% were prepared just before use by filling electrophoresis tubes (7.5 × 0.5 cm) up to 6 cm with a de-aerated polymerizing solution containing 5 ml of stock solution (9.7 g of acrylamide and 0.3 g of methylenebisacrylamide, Serva, in 25 ml of water), 10 ml of 0.1 M Na₄P₂O₇ (pH 8.5), 29.7 ml of water, 5 ml of glycerol, 0.1 ml of tetramethylenediamine. The pH was readjusted to 8.5 and 0.2 ml of 12.5% ammonium persulfate was added. The polymerizing solution was overlaid with 20 µl of water to ensure a flat horizontal gel surface and polymerization was allowed to proceed.

Electrophoreses were performed at 4°C in a Pharmacia apparatus (GE-2/4) which allows thermostating and recirculation of buffer between the anodic (lower) and the cathodic (upper) reservoirs. Running buffer was 20 mM $\text{Na}_4\text{P}_2\text{O}_7$ with 10% glycerol, 1 mM EDTA and 0.01% 2-mercaptoethanol, pH 8.6. The gel was then loaded with 20–80 μl of crude myosin extract diluted 1/30 to 1/300 (v/v) with a buffer containing 20 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50% glycerol 0.01% 2-mercaptoethanol and 0.1% bromophenol. All myosin samples contained in addition 20% of a crude extract of taenia coli myosin used as an internal standard. Electrophoreses were performed at a constant voltage gradient of 8 V/cm for 30 min (to eliminate persulfate ions [1]) followed by 14 V/cm for 19.5 h. Gels were stained 1 h at 70°C in a Serva Blue solution (25 mg of Serva R250, 20 ml of methanol, 70 ml of acetic acid in 1 l water) and destained in 7% acetic acid with 5% methanol.

Gels were scanned with a traveling microscope (Nikon measuroscope) modified in two ways. The X-displacement was controlled by a stepper motor (1 mm/s) and the light transmitted by the gel was measured with a silicon photodetector (OSD-50/0 Centronic) fitted to the eye-piece and electronically stabilized. The signals were digitized and transmitted to a desk computer (Tektronix 4051). We found that it was not realistic to construct a program that would automatically analyze the densitograms, because changes in transmitted light are not only due to stained protein bands but also to many artefacts (air bubbles, dust specks, scratches on the gel) that cannot be easily signaled to the computer. For this reason we designed an interactive program that worked as follows. The operator moved a little arrow over the densitogram shown on the screen and selected in turn the baseline, the starting point for the computation (value 0 for R_F), the endpoint of the computation (value 1 for R_F), the localization of each significant peak (controlled by visual inspection of the gel). The machine then computed the R_F values of all selected peaks. An example of this procedure is shown in Fig. 1.

In a second step of analysis, the densitograms were shown again in the computer screen. The operator selected, with the aid of the little arrow, the beginning and the end of each peak. The machine integrated the densitogram between the limits set by the operator using the 'dropping line' procedure for overlapping peaks. The area of each peak was then divided into the summated areas for all the skeletal muscle myosins. In this way areas of each myosin peak were expressed as fractions of the total area for skeletal myosin.

This method assumes that the molar absorption of the stained myosin is the same for all the isozymes. We have not checked this assumption, since purified samples are not available for all the myosin isoforms. On the other hand, we have checked that areas were proportional to myosin concentrations over the range of concentrations used in this research.

We found that the electrophoretic mobilities of the various myosin isozymes might vary noticeably not only in consecutive runs, but even in samples that were run together. Therefore we preferred to measure R_F values, defined as the ratio of the mobility of myosin to the mobility of an internal standard. Glutamate dehydrogenase is suitable as an internal standard because it migrates much more rapidly than the fast myosins [1]. However we preferred to use taenia colimyosin since it has only one form, which moves a little faster than the fastest myosins of neonatal muscles, and it offers the supplementary advantage of checking the myosin stability during the preparation and the electrophoresis: we observed that electrophoreses of 3-day regenerates show no native myosins, not even that of the internal standard (the taenia coli myosin). This fact shows

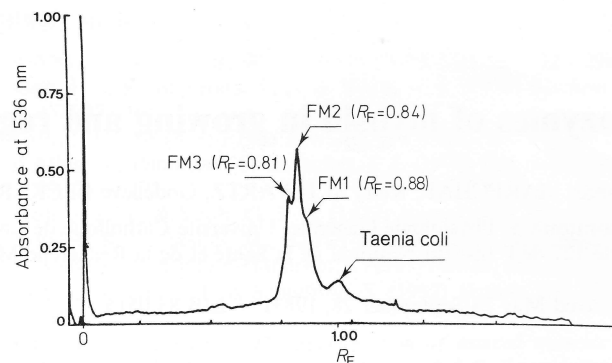


Fig. 1. Densitogram of an EDL muscle as shown on the screen of the desk computer. The little arrows are positioned by the operator who selects five points that signal in turn, the beginning of the gel (R_F value of 0), the three peaks of the three fast isozymes, and the peak of taenia coli isozyme (R_F value of 1.0). The computer then calculates the R_F values of the three fast myosin peaks. The coordinated axes are added by the computer

that native myosins have been degraded in the extracts by very active proteolytic enzymes.

Means, standard errors of the means (SEM) and t -tests were calculated in the usual way using, when applicable, paired difference t -tests.

RESULTS

Adult muscles

In a first series of experiments, we have analyzed the native myosins of two muscles of the adult rat: the extensor digitorum longus (EDL), a muscle of the fast-twitch type, and the soleus (SOL), a muscle of the slow-twitch variety.

We have observed three forms of native myosin in EDL (Fig. 2A). These forms are present in all of the 16 muscles that were analyzed although their relative proportions may vary widely. Their mobilities are shown in Table 1. They were computed in units of mobility of taenia coli myosin, used as an internal standard. The fastest myosin has an R_F value equal to 0.852 ± 0.007 ($n = 16$). We have called this myosin FM₁ (fast myosin of skeletal muscle 1). The next myosin, FM₂, migrates somewhat more slowly, its R_F value being 0.826 ± 0.006 ($n = 16$). The slowest migrating myosin, FM₃, has an R_F value of 0.797 ± 0.006 . The method of measuring the rate of migration of native myosin is not only accurate, but also reproducible. This latter property is measured by the standard errors of the means quoted above: they are well below 1%. A natural consequence of this reproductibility is that the three zones are well defined. The paired differences (FM₁–FM₂) and (FM₂–FM₃), equal to 0.026 ± 0.002 and 0.029 ± 0.003 , respectively, have a high statistical significance ($P < 0.001$). Integrations of the three peaks yield estimates of their areas in arbitrary units that can be used to compute the relative concentrations of the three isozymes. The muscle content of each myosin form has been computed as a percentage of the total content in myosin (obtained by adding the integration values of the three forms). The three myosins are present at similar concentrations (Table 2): $35.2 \pm 3.3\%$ for FM₁, $33.7 \pm 2.1\%$ for FM₂ and $30.5 \pm 3.0\%$ for FM₃. These data suggest that fast muscles contain one third of each fast form of myosin. We think, however, that such a conclusion is too simple, because the variations in the relative myosin content of

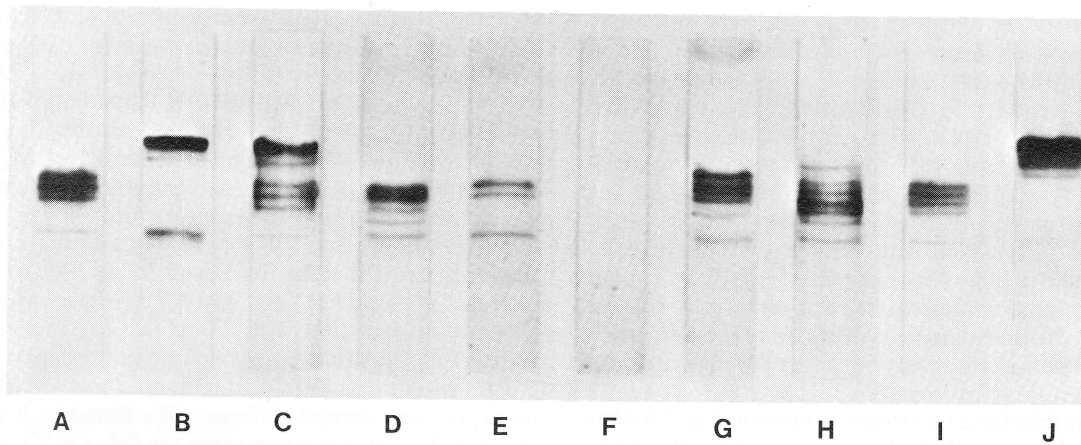


Fig. 2. Electrophoreses of myosin in non-dissociating conditions. (A) Rat EDL; (B) rat SOL; (C) coelectrophoresis of rat EDL with rat SOL; (D) hind limb of new-born rat; (E) hind limb of embryo, three days before birth; (F) regenerating EDL, 3 days after surgery; (G) regenerating EDL, 15 days after surgery; (H) regenerating SOL, 15 days after surgery; (I) regenerating EDL, 60 days after surgery; (J) regenerating SOL, 60 days after surgery. In every cases, the lowest zone is taenia coli myosin, used as an internal standard

Table 1. Mobilities of adult, neonatal and embryonic myosins

SM = myosin of adult slow muscle; FM = myosin of adult fast muscle; fM = myosin of neonatal muscle; eM = myosin of embryonic muscle

Muscle	Mobility of myosin form							
	SM ₂	SM ₁	SM' ₁	FM ₃ fM ₅ eM ₄	FM ₂ fM ₄ eM ₃	FM ₁ fM ₃ eM ₂	fM ₂ eM ₁	fM ₁
<i>Adult muscles</i>								
EDL (n = 16)				0.797 ± 0.006	0.826 ± 0.006	0.852 ± 0.007		
SOL (n = 15)	0.677 ± 0.009	0.733 ± 0.006	0.760 ^a ± 0.016					
<i>Growing muscles</i>								
Neonatal (n = 11)					0.824 ± 0.005	0.855 ± 0.003	0.900 ± 0.003	0.942 ± 0.004
Embryonic (n = 11)				0.81 ^b	0.838 ± 0.002	0.872 ± 0.003	0.904 ^c ± 0.004	

^a Form observed in four experiments.

^b Form observed in two experiments.

^c Form observed in five experiments.

Table 2. Proportions of native myosins in adult and in growing muscles

SM = myosin of adult slow muscle; FM = myosin of adult fast muscle; fM = myosin of neonatal muscle; eM = myosin of embryonic muscle. Myosins of similar R_F (cf. Table 1) are arranged in columns

Muscle	Proportion of myosin form							
	SM ₂	SM ₁	SM' ₁	FM ₃ fM ₅ eM ₄	FM ₂ fM ₄ eM ₃	FM ₁ fM ₃ eM ₂	fM ₂ eM ₁	fM ₁
% total myosin								
<i>Adult muscles</i>								
EDL (n = 16)				30.5 ± 3.0	33.7 ± 2.1	35.2 ± 3.3		
SOL (n = 15)	71.2 ± 3.9	26.2 ± 3.4	2.6 ^a ± 1.3					
<i>Growing muscles</i>								
Neonatal (n = 11)					31.5 ± 3.3	44.0 ± 2.4	17.5 ± 3.2	6.7 ± 3.2
Embryonic (n = 11)				8.1 ^b ± 5.5	58.5 ± 7.8	28.2 ± 5.2	5.2 ^c ± 2.5	

^a Form observed in four experiments.

^b Form observed in two experiments.

^c Form observed in five experiments.

the three forms between muscles exceed widely the variations expected from error measurements. For example, we found in one muscle 19% (FM₁), 51% (FM₂), 30% (FM₃); in another one we found 41% (FM₁), 19% (FM₂), 40% (FM₃); in a third one 39% (FM₁), 44% (FM₂), 17% (FM₃). Thus the relative content of FM₁ varies from 19% to 59%; that of FM₂ varies from 19% to 51%, and that of FM₃, from 17% to 47%. The error of the measurements, as estimated from densitometric records made on gels loaded with various amounts of EDL myosin of the same muscle, does not exceed $\pm 20\%$; it cannot account for the large variations in relative myosin content found between individual muscles. We are led to the conclusion that EDL muscles of adult rat may have very different contents in the various forms of fast myosins.

Rat soleus muscles have two forms of myosin (Fig. 2B) that do not coelectrophorese with the fast isozymes (Fig. 2C). They were found in the 15 soleus muscles that we have analyzed. The faster of these two forms was called SM₁ (for slow myosin skeletal muscle 1) and the slower one was called SM₂. SM₁ migrates with an R_F value of 0.733 ± 0.006 ($n = 15$); this is appreciably slower than the slowest migrating form of EDL (FM₃) but it is clearly faster than SM₂. The paired difference between R_F values of SM₁ and SM₂ is highly significant (0.056 ± 0.004 ; $n = 15$; $P < 0.001$). The two forms of slow myosin are present at different concentrations. SM₁ is the least concentrated: its mean concentration in 15 experiments is $26.2 \pm 3.4\%$ of the total slow myosin. Most of the slow myosin is of the SM₂ type: $71.2 \pm 3.9\%$ (mean of 15 experiments). A third component was observed in 4 of the 15 samples. Its R_F value is 0.760 ± 0.016 . It migrates at a faster rate than SM₁ but at a slower rate than FM₃. This component is thus different from either SM₁ or FM₃; we have called it SM₁'. When present, its concentration is low: $2.6 \pm 1.3\%$ of the total slow myosin. The simplest conclusion suggested by these data is as follows. Soleus muscle contains two main forms of myosin that are present in a 3:1 ratio ($[SM_2]/[SM_1] = 3$) and a third form is present in a quarter of the samples. However this conclusion is subjected to the same difficulty reported above for EDL. The between-muscle variations in the relative proportions of the two main slow myosins are very wide. The concentration of SM₁ varies between 10% and 64% of the total slow myosin, whilst that of SM₂ varies between 35% and 90%. Even the weak conclusion that SM₂ concentrations are always higher than SM₁ concentrations is not true, as there was one experiment in which SM₁ concentration (64%) was higher than SM₂ concentration (36%).

Neonatal and embryonic myosins

In a second series of experiments, we have analyzed the myosins of neonatal (1-day-old rat) muscles and embryonic (18-days post-conception, i.e. 3 days before birth) muscles.

Neonatal muscles contained four isozymes (Fig. 2D). The mobilities of these forms, measured relative to that of taenia coli myosin, are (Table 1): 0.942 ± 0.004 , 0.900 ± 0.003 , 0.855 ± 0.003 and 0.824 ± 0.005 ($n = 11$). We have called these forms fM₁ to fM₄ (for fetal myosin 1 to 4), in the order of decreasing mobilities. In one single case, we observed a fifth form, with R_F value of 0.81. In earlier experiments (in which it was not possible to evaluate R_F values) we observed five zones in neonatal muscles. We call fM₅ the fifth zone occasionally seen in neonatal muscles.

The mobilities of fM₁ and fM₂ are definitely higher than that of the fast adult myosin (FM₁). The mobilities of fM₃ and

fM₄ cannot be distinguished from those of FM₁ and FM₂. Coelectrophoreses of neonatal muscles with EDL yielded five zones with R_F values of 0.804 ± 0.005 , 0.831 ± 0.004 , 0.863 ± 0.004 , 0.901 ± 0.003 and 0.943 ± 0.004 ($n = 10$). Thus the two fastest forms (FM₂ and FM₁) of fast myosins comigrate with the two slowest forms of neonatal muscles (fM₄ and fM₃). The slow myosins of adult SOL comigrate far behind the four neonatal myosins (three experiments, not shown). The mean relative concentrations of the neonatal forms are given in Table 2. It shows that the two fastest forms (fM₁ and fM₂) are much less abundant (17.5% and 6.7% respectively) than the two slowest forms, fM₃ (44.0%) and fM₄ (31.5%). Eight of the samples had individual values that do not differ by more than 15% (of total myosin) from the means shown in Table 2. But one experiment seemed different. We found in it a very low value for fM₄ (10%), a low value for fM₃ (29%), a very high value for fM₂ (46%) and fM₁ (15%). This finding suggests that the fast neonatal forms (fM₁ and fM₂) might increase considerably just after birth, but for a very short period only.

Embryonic muscles have two main forms of native myosin that are present in all the experiments and two minor forms of myosin, present in a few experiments only. We have called them eM₁ (for embryonic myosin 1), eM₂, eM₃ and eM₄, starting from the fastest moving myosin. Their R_F values are shown in Table 1. The two main forms of embryonic myosin (eM₃ and eM₂) comigrated with the two slowest forms of neonatal myosins (fM₄ and fM₃) and with the two fastest forms of adult fast myosin (FM₂ and FM₁).

Most of the embryonic myosin is of the form eM₃ (58.5%) and some of it is of the form eM₂ (28.2%). It is interesting to notice that the proportion of eM₃ is higher than that of fM₄, whilst the reverse is true for eM₂, whose proportion is lower than that of fM₃. If embryonic and neonatal myosins are identical (or, at least, if eM₃ $\equiv$ fM₄ and eM₂ $\equiv$ fM₃) these facts suggest that the form eM₃/fM₄ is an embryonic form of myosin that is displaced around birth by the form fM₃/eM₂, typical of the perinatal period. The slow form eM₄ is present in low concentration (8.1%) and seems to have disappeared completely in neonatal animals. The fast form eM₁ is present at still lower concentrations (5.2%), but its neonatal counterpart, fM₂, is quite abundant (17.5%). These facts indicate that eM₄ and eM₃ (probably identical to fM₅ and fM₄) are embryonic forms that disappear around birth, whilst fM₃ and fM₂ are neonatal forms (probably identical to eM₂ and eM₁) that appear at the perinatal period.

Regenerating muscles

In a third series of experiments, we have analyzed regenerating muscles. EDL or SOL muscles of adult rats were dissected, minced and orthotopically autografted. The regenerates were examined 3, 15 or 60 days after surgery for their contents in the various isoforms of myosin (cf. Tables 3 and 4).

Electrophoretograms of three-day-old regenerates (EDL = 5; SOL = 5; gel F in Fig. 2) show no zone corresponding to skeletal muscle myosin. The gels were faintly and uniformly stained, as if some proteins were evenly distributed throughout them. In any case, the native myosins have been destroyed. They may have been denatured already in the graft, as histological sections of one-day-old graft demonstrate disorganization of the grafted muscle, but they may also have been denatured in the extracts or during the electrophoreses, as the taenia coli myosin added as an internal standard had also disappeared.

Table 3. *Mobilities of myosins from regenerating fast and slow muscles*SM = slow myosin, adult; FM = fast myosin, adult; fM = neonatal myosin; eM = embryonic myosin; *n* = number of experiments

Muscle	<i>n</i>	Time after surgery	Mobility of myosin form								
			SM ₂	SM ₁	SM' ₁	FM ₃ fM ₅ eM ₄	FM ₂ fM ₄ eM ₃	FM ₁ fM ₃ eM ₂	fM ₂ eM ₁	fM ₁	
d											
EDL	8	15				0.792 ^a ± 0.004	0.820 ± 0.003	0.853 ± 0.003	0.885 ± 0.003	0.933 ± 0.005	
SOL	5	15		0.749 ± 0.007		0.799 ± 0.005	0.830 ± 0.005	0.861 ± 0.003	0.886 ± 0.004	0.926 ± 0.002	
EDL	8	60				0.808 ± 0.005	0.834 ± 0.003	0.860 ± 0.005	0.903 ^b ± 0.005		
SOL	7	60	0.700 ± 0.006	0.730 ± 0.006	0.775 ± 0.010	0.810 ^c					

^a Form observed in five experiments.^b Form observed in four experiments.^c Form observed in one experiment.Table 4. *Proportions of native myosin in regenerating muscles*SM = slow myosin; FM = fast myosin; fM = neonatal myosin; eM = embryonic myosin; *n* = number of experiments

Muscle	<i>n</i>	Time after surgery	Proportion of myosin form							
			SM ₂	SM ₁	SM' ₁	FM ₃ fM ₅ eM ₄	FM ₂ fM ₄ eM ₃	FM ₁ fM ₃ eM ₂	fM ₂ eM ₁	fM ₁
d			% total myosin							
EDL	8	15				17.1 ^a ± 5.3	30.3 ± 4.4	24.2 ± 4.8	21.1 ± 2.0	7.0 ± 0.7
SOL	5	15		9.3 ± 1.6		14.8 ± 1.5	22.0 ± 2.8	28.7 ± 2.7	15.9 ± 3.1	8.6 ± 2.1
EDL	8	60				39.5 ^b ± 5.4	32.9 ± 1.9	21.0 ± 3.3	6.6 ^b ± 2.6	
SOL	7	60	42.5 ± 6.8	42.8 ± 6.3	13.4 ± 4.3	1.3 ^c				

^a Form observed in five experiments.^b Form observed in four experiments.^c Form observed in one experiment.

Eight regenerating EDL muscles were analyzed 15 days after surgery. Five of them had five bands with R_F values of 0.792 ± 0.004 , 0.820 ± 0.003 , 0.853 ± 0.003 , 0.885 ± 0.003 and 0.933 ± 0.005 (gel G in Fig. 2). The other three muscles had only four isoforms of myosin; the slowest form was absent. Table 4 shows the relative proportions of the myosins; it is seen that the fastest migrating form is the weakest (7.0% of total myosin). As was the case for adult and growing muscles, the variations between muscles were quite large. The 15-day-regenerating myosins do not comigrate with adult slow myosins (four experiments), but they do with adult fast myosins (five experiments), neonatal myosins (three experiments) and the embryonic myosins (two experiments). These comigration experiments indicate that the five isoforms of myosin that are found in 15-day-regenerating EDL are similar if not identical to the five neonatal myosins (fM₁ to fM₅).

Five regenerating SOL muscles were analyzed 15 days after surgery. All of them had six isoforms of myosin (gel H in Fig. 2). The R_F (Table 3) and distribution (Table 4) of the five faster forms are very similar to those found in 15-day-regenerating EDL. The sixth isoform is present in a small amount (9.6%) and migrates at a slow rate ($R_F = 0.749$), intermediate between

those of the slow myosins (SM₁ and SM₁'). In four coelectrophoreses of 15-day-regenerating SOL with adult slow myosin, we found that the sixth isoform of regenerating SOL comigrated with the adult slow myosins, but we could not decide whether the slow regenerating myosin comigrated with SM₁ or with SM₁'.

Eight regenerating EDL muscles were analyzed 60 days after surgery. All of them had three isoforms of myosin with R_F and distribution similar to those of fast adult myosin (gel I in Fig. 2; Table 3 and 4). Four muscles had one supplementary fast myosin ($R_F = 0.903$), but present in very small amount (6.6% of total myosin); this form seems most probably a remnant of the neonatal myosin (fM₂) observed in muscles regenerating for 15 days.

Seven regenerating SOL muscles were analyzed 60 days after surgery. All of them had three slow forms of myosin (gel J in Fig. 2; Tables 3 and 4). The two slowest forms were present in approximately equal amounts, about 43% (with large variations between muscles). The mobility of the slowest form was 0.700 ± 0.006 , somewhat faster than that of SM₂ (0.677 ± 0.009). The mobility of the second one was 0.730 ± 0.006 , just equal to that of SM₁. These isozymes comigrated with SM₁

and SM₂ in four experiments. Thus, regenerating SOL have slow adult myosins (SM₁ and SM₂) 60 days after surgery. They also have a third slow form whose mobility (0.775 ± 0.010) is equal to that of SM₁ (0.760 ± 0.016). Although this form was present in adult slow muscles, it is not constant (it was observed only 4 times in 15 experiments) and not abundant (2.6% of total slow myosin). By contrast, all SOL muscles regenerating for 60 days have that form in noticeable amount (13.4% of total myosin). In one experiment we found a small amount of a fast form of myosin ($R_F = 0.810$), presumably a remnant of the neonatal myosin FM₅.

DISCUSSION

Adult fast muscles

Existence of three fast muscle native isomyosins was first proposed by Hoh et al. [2] for chicken posterior latissimus dorsi. It has since been repeatedly confirmed: in chicken posterior latissimus dorsi [3, 4, 13], in fast muscles of rabbit [4, 5, 14, 15], in rabbit psoas [16], in fast rat muscle [6], in human fast fibers type 2 [17], in anterior tibialis of rabbit [18] and in rat EDL (this work).

Hoh [3] has called the three fast isomyosins of chicken posterior latissimus dorsi, FM₁, FM₂ and FM₃ in order of decreasing mobility and, later, he used the same names for the three fast isomyosins of rabbit fast muscles [5]. This seems justified because chicken and rabbit fast isomyosins comigrate together [4]. Srihari et al. [16] used the same names but Hoh et al. [19] used another nomenclature, simply numbering the isomyosins 1, 2 and 3. We have chosen to use the nomenclature proposed by Hoh [3] in this work.

Although heavy chains of fast myosins are heterogeneous [17, 20–22], the different forms of fast myosins separate in the same three zones during electrophoreses in non-dissociating conditions [17]. Furthermore, myosins of identical heavy chains separate according to their alkaline light chains [23]. Thus it appears that fast isomyosins separate on the basis of their alkaline light chains rather than on the basis of their heavy chains [3, 13].

Hoh [2, 3] proposed a most reasonable hypothesis to account for the function of the three fast isomyosins, namely that the distribution of the multiple forms of myosin modulates the speed and the power of muscle fibres.

There are indeed indications that isozyme distributions are not fixed in muscles. First, there are large differences between muscles of various types. Hoh et al. [2] reported a ratio FM₁:FM₂:FM₃ of 38:39:23 for chicken posterior latissimus dorsi, whilst d'Albis et al. [4] reported a ratio of 25:37:38 for the same muscle. Various other ratios have been reported; 39:35:26 for chicken pectoralis [4]; 37:34:29 for the same muscle [3]; 4:26:70 for chicken plantaris [3]; 20:38:42 for rabbit psoas [16]; 31:34:35 for rat EDL (this work). These variations might explain some of the variations in mechanical performances between these fast muscles. Secondly there are large individual variations between the isozyme proportions of the same muscle (EDL) obtained from animals matched for age and sex (see Results). Such 'between-muscle' variations might explain the large 'between-muscle' variations in shapes of the rat EDL force-velocity relation observed by us (unpublished experiments). Unfortunately, mechanical experiments were not performed on the muscles analyzed for their myosin isozymes, so that there is as yet no physiological evidence for this assertion. On the other hand, Hoh's hypothesis finds little biochemical support. The three fast isomyosins have virtually

the same ATPase activity, at least in chicken posterior latissimus dorsi [2]. The same holds true for mammalian myosin. Wagner and Weeds [24] have prepared homodimers of S₁ and tested their actin-activated ATPase activities. The turn over rate of ATPase activity of S-1 (LC₃) was twice that of S-1 (LC₁). Unfortunately, this is true only at low ionic strength (6 mM KCl): at higher ionic strength (45 or 75 mM KCl) no difference was found between S-1 (LC₃) and S-1 (LC₁) actin-activated ATPase activities [25]. Thus, the physiological significance of the existence of several forms of fast myosin in the same muscles is still unsettled.

Adult slow muscles

Slow muscle myosin migrates at an appreciably slower rate than fast myosin, as was first demonstrated by d'Albis and Gratzer [1], using chicken muscles (anterior and posterior latissimus dorsi) and confirmed several times since [2–4, 14, 15]. Soleus muscles usually show only one zone in electrophoreses in non-dissociating medium (rabbit [5, 26]; guinea pig [4]; human [15, 16]; rat [6]) like another slow muscle, chicken anterior latissimus dorsi [2, 13]. Some workers [4, 26] have reported the existence of two bands in electrophoreses of rat soleus, similar to those described in this work. Also, chicken anterior latissimus dorsi sometimes presents two bands [2, 3], or even three [4]. The reason of these variations is unknown.

In keeping with the nomenclature used for fast muscles isomyosins, Hoh [3] calls SM₁ and SM₂ the two components of slow muscle myosin (in the order of decreasing mobility). Srihari et al. [16] have called SM the slow myosin of rabbit soleus. We have adopted the same nomenclature because slow isozymes of rat muscles migrate at approximately the same rate as slow isozymes of chicken muscles. We have called SM₁ the third band that we have occasionally observed, as this band migrates faster than SM₁.

The two slow muscle isomyosins migrate more slowly than the fast muscle isomyosins because their heavy chains are different, as is well documented. But the basis on which the two main slow muscle isomyosins separate is not known. The regulatory light chain LC2S is partly phosphorylated, but this fact cannot be used to explain the separation of the two slow isozymes, since the proportion of phosphorylated LC2S to unphosphorylated LC2S is the same in the two zones [3, 4]. The two slow isozymes might separate on the basis of their alkaline light chain content, LC1Sa and LC1Sb, but there is no direct experimental evidence for this. On the other hand, the two slow muscle light chains accumulate at different rates during muscle growth; whilst the amount of LC1Sa is higher than that of LC1Sb in slow myosin of adult rabbit, the opposite appears to be true until 2 weeks of age [27]. This pattern would fit with that presented in Fig. 3, if SM₂ was an homodimer of LC1Sa and SM₁ an homodimer of LC1Sb. However, other interpretations are possible, such as hybrids of the type slow heavy chains/fast light chains, the existence of which is postulated in human single fibers [15, 17].

Embryonic and neonatal muscles

The interpretation of the isomyosin patterns of neonatal muscles is complicated because it seems probable that several forms of myosin appear and disappear rapidly in turn, whilst some of these isoforms have similar mobilities.

It is convenient to begin the discussion with the clearest facts. Neonatal muscles contain two isomyosins that are more mobile than the fast adult isomyosins (cf. Table 1). These forms

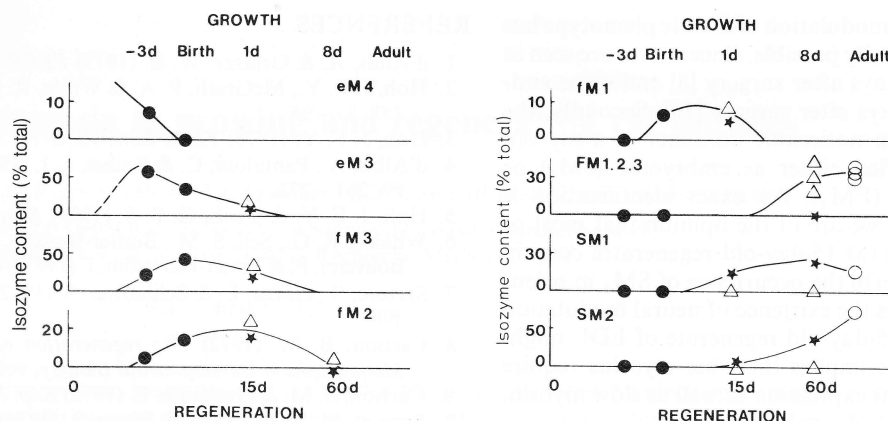


Fig. 3. Hypothetical reconstruction of the time course of myosin isozymes during growth and regeneration of adult muscle. eM₄ and eM₃: embryonic myosins; fM₁, fM₂ and fM₃: neonatal myosins; FM_{1,2,3} fast adult myosins 1, 2 and 3; SM₁ and SM₂: slow adult myosins 1 and 2. (○) Adult; (●) neonatal and embryonic muscles; (△) regenerating EDL; (★) regenerating SOL. The points are experimental values (Tables 2 and 4). The relative adjustment of the time scales of growth and regeneration is based on an hypothesis discussed in the text

have been observed first by Hoh and Yeoh [5] in neonatal rabbit muscles and, later, by Whalen et al. [6] in neonatal rat muscles. Following the suggestion of Hoh and Yeoh [5] we call fM₁ and fM₂ the two fastest forms of neonatal myosin (by order of decreasing mobilities).

Besides these two fast-migrating forms, neonatal muscles contain two (and sometimes three) other forms: we call them respectively fM₃, fM₄ (and fM₅). As their mobilities are indistinguishable from those of the fast adult myosins, i.e. FM₁, FM₂ (and FM₃), the three slowest forms of neonatal myosins might be identical with the fast adult myosins.

However, experiments by other authors strongly suggest that neonatal myosins are different from adult fast myosins by several criteria, such as: two-dimensional peptide maps of myosin heavy chains digested with cyanogen bromide [5] or chymotrypsin [6], complement fixation using guinea-pig antibodies to sodium-dodecyl-sulfate-denatured heavy meromyosin of 8-day-old rats [6], length of synthetic myosin filaments [6], indirect immunofluorescence assays on section of rat gastrocnemius [6], tibialis anterior or soleus [7] muscles. The discussion on the significance of these forms is postponed until after the discussion on the embryonic forms.

Three days before birth, the isozyme pattern is very different from that observed at birth. We shall accept the hypothesis that eM₁, eM₂, fM₄ and fM₅ are identical to the forms fM₂, fM₃, eM₃ and eM₄, respectively, for reasons already explained above (see Results). In this view, neonatal muscles contain two kinds of myosin heavy chains: an embryonic form, called MHC_{emb} by Whalen et al. [6], and a neonatal one, called MHC_{neo} by Whalen et al. [6]. Small amounts of adult slow or fast MHC cannot be excluded. Neonatal muscles contain also two (and sometimes three) forms of alkaline light chains LC_{1F} and LC_{3F} (and occasionally the embryonic form, LC_{1emb} described by Whalen et al. [28]). Five combinations are possible. As Hoh and Yeoh [5] have demonstrated in the longissimus dorsi of 2-day-old rabbit, fM₁, fM₂ and fM₃ contain MHC_{neo} combined (respectively) with two LC_{3F} or with one LC_{3F} and one LC_{1F} or with two LC_{1F}. We postulate that the forms eM₄ (≡ fM₅) and eM₃ (≡ fM₄) correspond to embryonic heavy chains (MHC_{emb}) combined respectively either with LC_{emb} or with LC_{1F}. This interpretation is also consistent with the long known fact that perinatal muscles have a lower content in LC_{3F} than adult muscles and also with the

fact that the synthesis of LC_{3F} begins around birth, in rabbit muscle [29] and in chicken breast muscles [30] at a time when LC_{1F} has already been present for several days.

We therefore agree with the conclusions of Whalen et al. [6], namely that three main classes of isomyosins succeed each other during ontogeny: two of them are transitory (embryonic and neonatal myosins) and the adult form is definitive. Fig. 3 shows a very schematic scheme of development that summarizes our current working hypotheses: it should be helpful in interpreting the isomyosin changes observed in regenerating muscles.

Regenerating muscles

The minced fragments of autografted muscles very rapidly show degenerative changes. Nuclear pycnoses appear within hours, together with disorganization of Z bands and sarcomere structures [8]. Electrophoreses in sodium dodecyl sulfate of 6-day-old regenerates demonstrate an increase of small proteins (in the region of 10–30 kDa), together with a decrease by one third of the heavy chain content. Actin also decreases by the same amount [31]. The high proteolytic activity of 3-day-old regenerates is also documented by a very important increase in the activity of lysosomal enzymes [32]. Therefore it is not surprising that native myosin has been degraded in 3-day-old regenerates (cf. gel F in Fig. 2).

Within three days after the surgery, a large increase in mononuclear cells occurs in the regenerates [8] and also in the activity of glucose-6-phosphate dehydrogenase [32], an enzyme indicative of cell proliferation. Seven days after surgery, numerous myotubes are seen and narrow fibres with central nuclei are common 15 days after surgery [8]. The regenerating muscles are reinnervated 15–30 days after surgery [10]. Since the morphological changes observed in regenerating muscles are so closely similar to those seen in growing muscles, it is reasonable to expect similar changes in the isomyosin patterns. This expectation is largely fulfilled.

Regenerates 15-days old have isomyosins that are quite similar to those observed in neonatal muscles, with two noticeable differences. First, the 15-day-regenerates of soleus muscles have a small amount of slow muscle myosin (Table 3). This cannot be a remnant of the grafted muscles, since 3-day-old regenerates have no native myosin whatsoever. This fact

indicates that some neural modulation of muscle phenotype has already occurred. This is easily possible, since nerves are seen in regenerates as early as 7 days after surgery [8] and some endplates are functional 15 days after surgery [10]. Secondly, the 15-day-regenerates contain noticeable amounts of a myosin form that can be identified either as embryonic (eM₄) or neonatal (fM₅) or adult (FM₃). An exact identification is impossible at present, but we are of the opinion that most of this form is FM₃ because (a) 15-day-old regenerates contain very little LC_{1emb} [12] and (b) the occurrence of SM₁ in soleus 15-day-regenerate indicates the existence of neural modulation at this early stage; thus 15-day-old regenerate of EDL might contain FM₃ as well (this implies that fast myosins require neural modulation for their expression as well as slow myosin, an hypothesis that is still to be proven).

Regenerates 60-days old have isomyosins that are similar to their parent muscles, but in different proportions. EDL also contains an early form of isomyosin (fM₂) and SOL contain a slow form (SM₁) whose significance is unknown (but which may be similar to isomyosin 4 of Hoh et al. [19]). Thus the regenerated muscles are fast-like or slow-like, in accordance with the original muscles. This conclusion agrees with earlier results on the mechanical force-velocity [12] and the distribution of alkaline light chains [12] or of lactate dehydrogenase isozymes [33].

The next question is whether the time course of isomyosins during regeneration is similar to that of isomyosins during ontogenesis. Any answer requires an adjustment of the respective time scales of growth and regeneration and obviously there are many ways of doing this. We propose to use the actin concentration as an indicator of equivalent degree of muscle development for growing or regenerating muscles. We have observed in earlier experiments [34] that 15-day-old regenerates have the same actin content as growing muscles of 1-day-old rats (about 0.07 µmol/g) and that 60-day-old regenerates have the same actin content (about 0.18 µmol/g) as growing muscles of 8-day-old rats. We have constructed Fig. 3 using this information. It summarizes the time courses of the various isomyosins during growth and regeneration, to the best of our present knowledge. It suggests that two main stages must be distinguished in muscle growth and regeneration. The first stage lasts for about one or two weeks. It is characterized by synthesis of isomyosins that are embryonic or neonatal in type. The second stage is characterized by large-scale syntheses of adult isomyosins, whose type (slow or fast) is modulated by neural influences. Regenerating muscles recapitulate ontogeny, but whilst the first stage matures at about the same rate as is the case in growing muscle, the second stage matures at a rate much lower than that observed in growing muscles. The first conclusion is in agreement with the findings of Jones [35] who reported that myoblasts extracted from regenerating muscles grow *in vitro* at the same rate as myoblasts extracted from newborn animals and of Sartore et al. [7] who reported that muscles regenerating from a cold injury express transiently heavy chain myosins that are fetal in type. The second conclusion is in agreement with the time course of creatine accumulation in regenerating muscle, which is 2–4-times slower than that observed in growing animals [36].

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