

“SLOW” MYOSINS IN VERTEBRATE SKELETAL MUSCLE

An Immunofluorescence Study

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ABSTRACT

Specific antisera were raised in rabbits against column-purified myosins from a slow avian muscle, the chicken anterior latissimus dorsi (ALD), and a slow-twitch mammalian muscle, the guinea pig soleus (SOL). The antisera were labeled with fluorescein and applied to sections of muscles from various vertebrate species. Two distinct categories of the slow fibers were identified on the basis of their differential reactivity with the two antisera. Fibers stained by anti-ALD appear to correspond in distribution and histochemical properties to physiologically slow-tonic fibers, i.e., fibers that display multiple innervation and respond to stimulation with prolonged contractures. In mammals, only a minority of fibers in extraocular muscles and the nuclear bag fibers of muscle spindles were brightly labeled by this antiserum. In contrast, fibers labeled by anti-SOL in mammalian muscle appear to correspond in distribution and histochemical properties to physiologically slow-twitch fibers. Anti-SOL was also found to stain a population of fibers in reptiles, amphibians, and fishes that did not react, or reacted poorly, with anti-ALD; in avian muscle, only a minor proportion of the slow fibers were labeled by anti-SOL. These findings point to the existence of two antigenically distinct, though partly cross-reacting, types of “slow” myosin in vertebrate muscle.

Multiply innervated skeletal muscle fibers that respond to stimulation with a long-lasting contraction rather than a twitch are present in various classes of vertebrates (see reference 17 for a review). However, it is not clear whether there is one homogeneous type of “slow” or “slow-tonic” fibers, nor is it clear what criterion should be used for their identification. Not only are there significant physiological differences between the slow fibers of amphibians, birds, and mammals, but also within a single species and a single muscle there seem to be several subtypes, or possibly even a continuous spectrum of fiber types (20, 31, 38). The slow fibers of frogs have often been regarded as the prototype of this category of muscle fibers.

The incapacity of propagating action potentials has been taken as a distinguishing feature of amphibian slow fibers, but conflicting reports have made this less certain. Uncertainty is also due to the probable existence of different types of slow fibers in amphibian muscle (see reference 31). The slow fibers of birds are known to be multiply innervated and to produce prolonged contractures, but, in contrast to amphibian slow fibers, they appear to be able to propagate action potentials and to twitch (15, 30). In mammals, multiply innervated slow fibers similar to those of the frog have been identified only in extraocular muscles (18), whereas singly innervated “slow-twitch” fibers are widely distributed in all body muscles.

The factors responsible for the contractile properties of vertebrate slow fibers have not yet been elucidated. Using skinned fiber preparations, Constantin et al. (8) found that the slow contraction of frog slow fibers is an inherent property of the contractile mechanism. Myosin could be the crucial component in this mechanism inasmuch as a close relationship between myosin ATPase activity and the speed of muscle shortening has been demonstrated in various vertebrate muscles (4). However, a precise characterization of myosin and other contractile proteins in the various slow muscles has been hampered by the fact that in amphibians and in mammals slow-tonic fibers are a minor component within predominantly fast muscles. This difficulty can be overcome by immunohistochemical procedures that permit the identification of antigenically different types of contractile proteins in single skeletal and cardiac muscle fibers (9, 13, 35). We have used this approach to analyze the types of myosin present in vertebrate slow fibers. Antisera against myosins from slow avian muscle and slow-twitch mammalian muscle were labeled with fluorescein and applied to sections of muscles from various species. Two distinct types of myosin were identified in slow fibers of vertebrate skeletal muscles.

MATERIALS AND METHODS

Preparation of Antigens

Myosins were isolated from the slow anterior latissimus dorsi (ALD) muscle of the chicken and the slow-twitch soleus (SOL) muscle of the guinea pig, essentially as described by Barany and Close (5), and purified by ion-exchange chromatography (34). Myosins from different fast muscles, the chicken pectoralis and the guinea pig tensor fasciae latae and masseter, were also prepared by the same procedure and used for the control of antiserum specificity. Myosin preparations were >95% pure when assayed by SDS gel slab electrophoresis (21). A comparative biochemical study of the two types of slow myosin will be reported elsewhere (U. Carraro, L. Dalla Libera, and S. Sartore, manuscript in preparation). Myosin solutions were stored at -30°C with 50% glycerol until used.

Production of Antisera

Five rabbits were immunized with ALD myosin and three with SOL myosin by intramuscular injection with 2 ml of antigen solution containing 1 mg of myosin in 0.4 M KCl and 50 mM sodium phosphate buffer, pH 7.4, emulsified with an equal volume of complete Freund's adjuvant. The rabbits received two boosts with the same amount of antigen in complete Freund's adjuvant after 2 and 4 wk. Blood was taken 8–10 d after the second boost, and the serum was tested for titer and specificity by double immunodiffusion (26) and immunoelectrophoresis (12). Additional boosts were given whenever necessary to obtain

high antibody titer. Antisera obtained from the bleedings were separately stored at -30°C .

Separation of Immune and Specific IgG

Immune IgG, i.e., the whole IgG fractions from immunized animals, were separated from each antiserum by ion-exchange chromatography (11, 24). Specific IgG, i.e., antimyosin IgG, were separated by affinity chromatography on the insolubilized immunogen. The immunoabsorbent was prepared using CNBr-activated Sepharose beads, essentially as described by Long et al. (25). Unreacted CNBr sites were saturated with 0.1 M glycine in 0.5 M KCl and 200 mM sodium phosphate buffer, pH 8.0. The crude IgG fraction obtained by ammonium sulphate precipitation was dissolved in 0.3 M KCl and 50 mM Tris-HCl, pH 7.5, mixed with insolubilized antigen for 20 h at 0° – 4°C with continuous agitation, then loaded into a chromatography column. The column was washed several times with buffer at pH 7.5 to eliminate nonspecifically bound IgG until the A_{280} of the effluent returned to zero. Bound IgG were eluted by lowering the pH with 0.3 M KCl and 0.2 M CH_3COOH , pH 2.8 (25). Specific IgG were rapidly brought to pH 7.0 with 0.2 N NaOH, dialyzed against PBS, and stored in small aliquots at -30°C or lyophilized until used.

Labeling of Specific IgG with Fluorochromes

For direct immunofluorescence study, immune or specific IgG were coupled to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TMRITC). IgG were dialyzed against 0.05 M sodium carbonate buffer, pH 9.5, and conjugated with FITC (Serva Feinbiochemica, Heidelberg, W. Germany) or TMRITC (Baltimore Biological Laboratories, Cockeysville, Md.) according to the dialysis bag technique (7) with 20 μg (FITC) or 40 μg (TMRITC) of fluorochrome per milligram of IgG. The reaction was carried out at 4°C for 12–18 h. Unbound fluorochrome was eliminated by dialysis against phosphate-buffered saline (PBS) or by gel filtration through a column of Sephadex G-25 equilibrated with the same buffered solution. The mean fluorochrome/protein ratio (F/P) was calculated by measuring the absorbance of TMRITC at 515 nm or FITC at 495 nm and IgG at 280 nm (32, 39, 40). Conjugates with a F/P ratio of ~ 2 generally were obtained.

In the beginning of this study, conjugates with different F/P ratios were separated by ion-exchange chromatography (39, 40), and only the fractions having a ratio of 1.5–2.5 were used in the immunofluorescence studies. This step was later omitted because identical results were obtained with the unfractionated conjugate.

Immunofluorescence Staining

Direct immunofluorescence procedures were mostly used. Transverse sections of frozen muscles were cut at $\sim 10\ \mu\text{m}$ in a cryostat, mounted on slides, and exposed to the appropriate dilution of FITC- or TMRITC-labeled antibodies. Incubation was carried out in a humidified chamber at 37°C for 30 min. The sections were then washed in phosphate-buffered saline (PBS) for 20 min, fixed in 1.5% paraformaldehyde in PBS for 10 min, and mounted in 1/1 (vol/vol) glycerol-PBS. A Leitz Dialux fluorescence microscope equipped with epiillumination and filter sets for revealing fluorescein or rhodamine was used. The specificity of the reactions was tested by the following controls: (a) staining with labeled IgG from preimmune serum; (b) staining with labeled antimyosin IgG absorbed with specific insolubilized antigen; (c) staining with labeled antimyosin IgG in the presence

of an excess amount of unlabeled specific antiserum. For indirect immunofluorescence, the sections were first treated with unlabeled antimyosin, then, after washing with PBS, with FITC- or TMRITC-labeled goat anti-rabbit IgG (purchased from Miles Laboratories, Inc. Elkhart, Ind. and from Cappel Laboratories, Inc. Downingtown, Pa., respectively). Both incubations were carried out at 37°C for 30 min with appropriate dilutions of antisera. The specificity of the reactions was tested with nonimmune or preimmune serum in the first step.

Enzyme Histochemistry

Cryostat sections serial to those used for immunofluorescence were processed for the histochemical demonstration of succinate dehydrogenase (27) and myosin ATPase after alkaline and acid preincubation (16, 29).

RESULTS

Specificity of Antibodies

IMMUNODIFFUSION AND IMMUNOELECTROPHORESIS: When tested by double immunodiffusion and immunoelectrophoresis against various myosin preparations, anti-ALD myosin antisera gave a distinct precipitin line with ALD myosin but never reacted with SOL myosin or with myosins from fast chicken or guinea pig muscles (Fig. 1 *a*). There was no significant variation in reactivity among antisera from various rabbits and from various bleedings, except for the presence in one of the five rabbits immunized of a second weak precipitin line closer to the antibody well.¹

Anti-SOL myosin antisera reacted only with SOL myosin when obtained from early bleedings (Fig. 1 *b*). In contrast, the response of anti-SOL antisera from late bleedings varied in different animals, and a reaction of nonidentity with ALD myosin was occasionally seen (Fig. 1 *c*).

IMMUNOFLUORESCENCE: Specificity of antibodies was also assessed in immunofluorescence reactions on cryostat sections of composite blocks of fast and slow chicken muscles for anti-ALD and fast and slow guinea pig muscles for anti-SOL. Identical results were obtained with direct and indirect immunofluorescence and with fluorescein- or rhodamine-labeled antisera. Most observations were made with direct immunofluores-

¹ Preliminary observations have shown that this weaker precipitin line is continuous with a line formed by this anti-ALD antiserum with myosin light chains prepared from ALD muscle. The other anti-ALD antisera did not react with light chain. The antigenicity of ALD myosin is therefore essentially due to heavy chains, which agrees with a previous study (1).

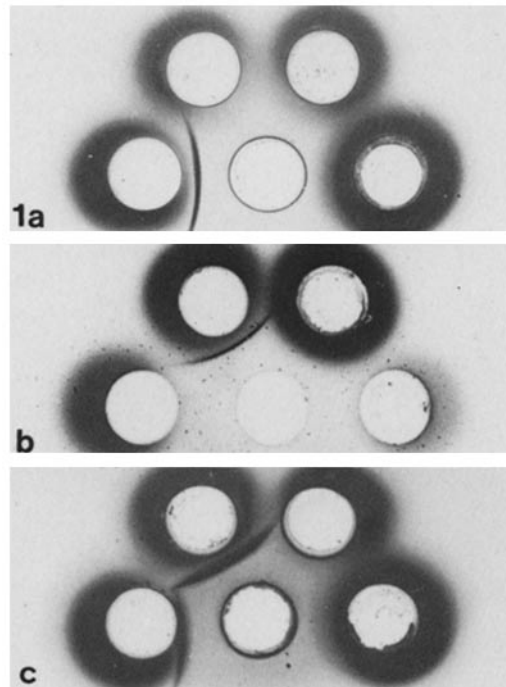


FIGURE 1 Ouchterlony double immunodiffusion assays. The central well contained: *a*, anti-ALD; *b*, anti-SOL from early bleeding (3 mo of immunization); *c*, anti-SOL from late bleeding (same animal, 9 mo of immunization). The outer wells beginning with the well on the left and proceeding clockwise contained: chicken ALD myosin, guinea pig SOL myosin, guinea pig tensor fasciae latae myosin, chicken pectoralis myosin. Each well contained 20 μ l of antigen or antiserum. Myosin concentration was about 1 mg/ml. Immunodiffusion was carried out in 1% agarose in 0.4 M KCl, 50 mM sodium phosphate buffer, pH 7.4. The plates were incubated for 3 d at room temperature, and stained in 1% amido black.

cence with fluorescein-labeled antimyosin antibodies.

Anti-ALD stained most fibers in the slow ALD muscle, whereas most fibers in the fast pectoralis were completely negative (see also reference 1). The reaction was specifically inhibited by pretreatment of the labeled antiserum with the corresponding myosin in the relative proportions determined by double immunodiffusion tests. No staining was observed with fluorescent preimmune serum. Anti-ALD myosin consistently gave a strong reaction without any background staining; an even stronger but qualitatively identical reaction was obtained with affinity-purified anti-ALD, which was prepared by labeling antibodies absorbed by a column of insolubilized ALD myosin. Both af-

finitly-purified and nonpurified anti-ALD were used throughout this study with similar results. There was no significant variability in the specificity of anti-ALD antibodies from different antisera, except for slight variation in the reaction with mammalian type I fibers (see below).

Anti-SOL stained all fibers in the guinea pig SOL, which is homogeneously composed of fibers showing alkali-labile and acid-stable myosin ATPase activity, whereas all fibers were negative in the tensor fasciae latae and in the masseter muscles, both of which are homogeneously composed of fibers showing alkali-stable and acid-labile myosin ATPase activity. Affinity-purified anti-SOL gave a brighter reaction without any background staining and was used throughout this study. Anti-SOL prepared from early and late bleedings gave qualitatively similar staining of the different fibers in mammalian muscles; on the other hand, anti-SOL from late bleedings gave a much stronger reaction with positive fibers in amphibian and avian muscle.

Distribution of Labeled Fibers

AMPHIBIANS AND REPTILES: When applied to sections of frog (*Rana temporaria*) or toad (*Xenopus laevis*), anti-ALD and anti-SOL were found to selectively stain two different populations of fibers in the tonus bundle of the ileofibularis muscle and in the deep portion of the rectus abdominis (Fig. 2*a*). A group of fibers was found to stain intensely with anti-ALD and weakly with anti-SOL, whereas another group of fibers showed a reciprocal pattern of staining (Fig. 2*a* and *b*). The two types of fibers also displayed a distinct reactivity with myosin ATPase: fibers staining intensely with anti-ALD were completely negative after preincubation in alkali and stained less intensely after preincubation at pH 4.6 (Fig. 2*c* and *d*). These fibers could also be recognized by their weaker reaction with succinate dehydrogenase. Two populations of slow fibers likewise could be distinguished in tortoise (*Testudo graeca*) and lizard (*Lacerta agilis*) muscle with antimyosin staining (not shown).

BIRDS: Anti-ALD was found to intensely stain >95% of the fibers in the chicken ALD myosin, the rare negative fibers being identified as fast fibers by their reactivity with anti-pectoralis myosin antiserum (Fig. 3*a* and *b*). Anti-SOL intensely stained only a portion of the fibers reacting with anti-ALD (Fig. 3*c*). Muscle fibers staining

intensely with anti-SOL comprised ~20% of the total fiber population in ALD muscle, the majority of the fibers being only weakly positive.

The subpopulation of slow fibers stained by anti-SOL corresponded in most cases to fibers that stained more intensely with myosin ATPase after preincubation at pH 4.6 (Fig. 3*d*), whereas they could not be distinguished after alkaline preincubation (Fig. 3*e*). In other chicken muscles investigated, anti-SOL was also found to react with a variable proportion of the slow fibers that were stained by anti-ALD. In addition, anti-SOL stained a number of fibers not reacting with anti-ALD and reacting with anti-pectoralis myosin (Fig. 3*c*). These fibers represent a particular category of fast fibers and will be dealt with in a separate report.

MAMMALS: In the mammalian muscles from the various species investigated (rat, guinea pig, rabbit, and man), anti-SOL stained all type I fibers, i.e., fibers showing alkali-labile and acid-stable myosin ATPase activity, whereas anti-ALD stained only a minor proportion of fibers in extraocular muscles and in muscle spindles. The typical type I fibers of mammalian body muscles were unstained by anti-ALD from early bleedings and very weakly stained by anti-ALD from late bleedings. A positive response of type I fibers to anti-ALD has recently been reported by Gauthier and Lowey (14). With our antisera, the reactivity of mammalian slow-twitch fibers with anti-ALD, even when present, was markedly weaker, in both direct and indirect immunofluorescence tests, than that shown by a number of extraocular and intrafusal fibers.

The response of human and guinea pig extraocular muscle fibers to anti-ALD and anti-SOL is illustrated in Fig. 4. Fibers reacting with anti-ALD were mostly localized in the peripheral orbital layer. These fibers were also stained by anti-SOL, though often less intensely; in addition, anti-SOL reacted strongly with a number of fibers distributed predominantly in the central and global layers, which failed to react with anti-ALD. Fibers with an intermediate degree of reaction with both anti-ALD and anti-SOL were also observed. Fibers staining with anti-ALD and/or anti-SOL all displayed acid-stable myosin ATPase activity, with a tendency for the fibers most reactive with anti-ALD to stain less intensely for myosin ATPase after acid preincubation. The latter fibers also showed a weaker reaction for succinate dehydrogenase.

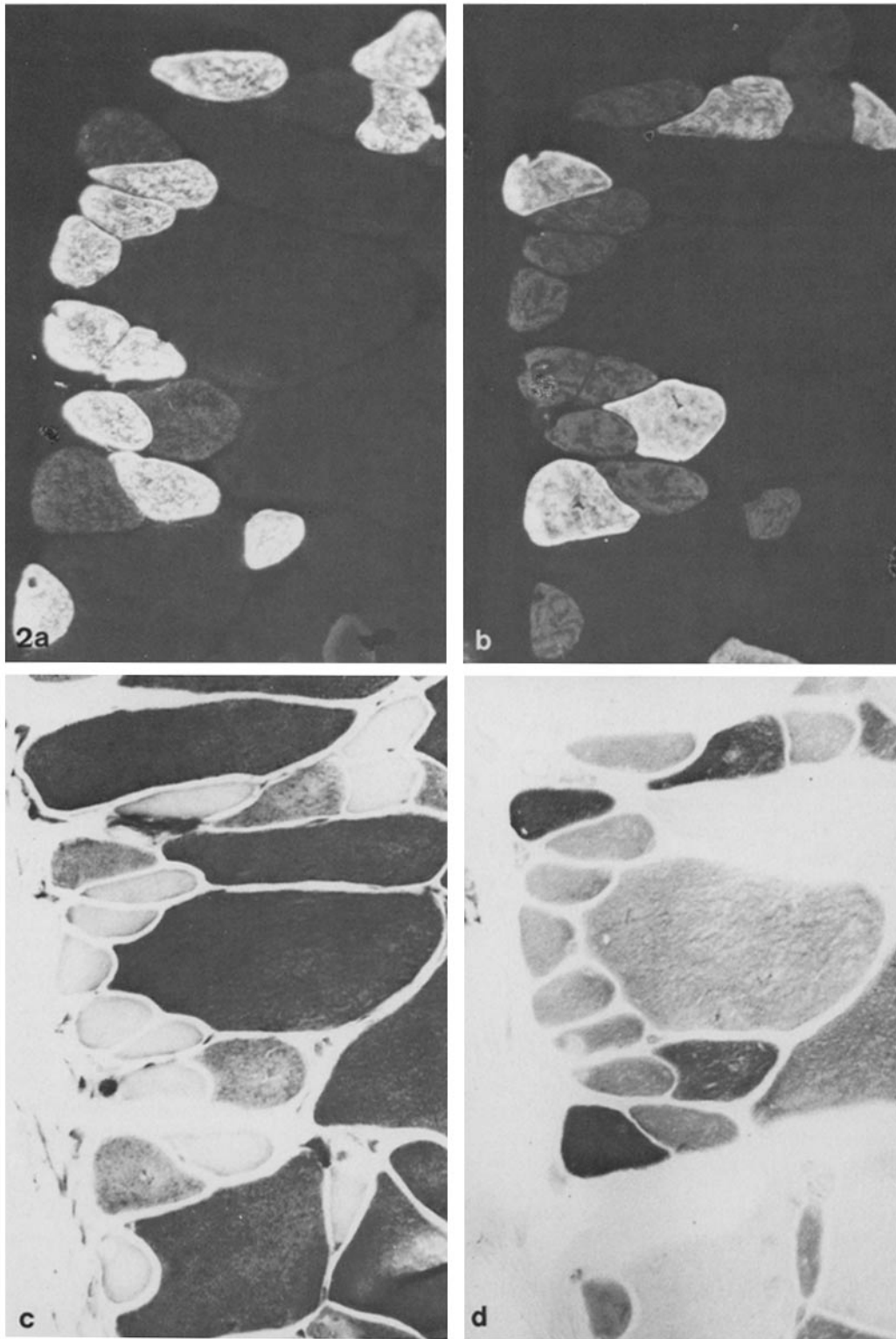


FIGURE 2 Frog muscle. Serial transverse sections through the deep medial portion of the rectus abdominis. Fibers stained strongly by anti-ALD (*a*) react weakly with anti-SOL (*b*) and show lower myosin ATPase activity after alkaline (*c*) and acid (*d*) preincubation. Fibers stained strongly by anti-SOL react weakly with anti-ALD and show higher myosin ATPase activity after alkaline and acid (pH 4.6) preincubation. $\times 400$.

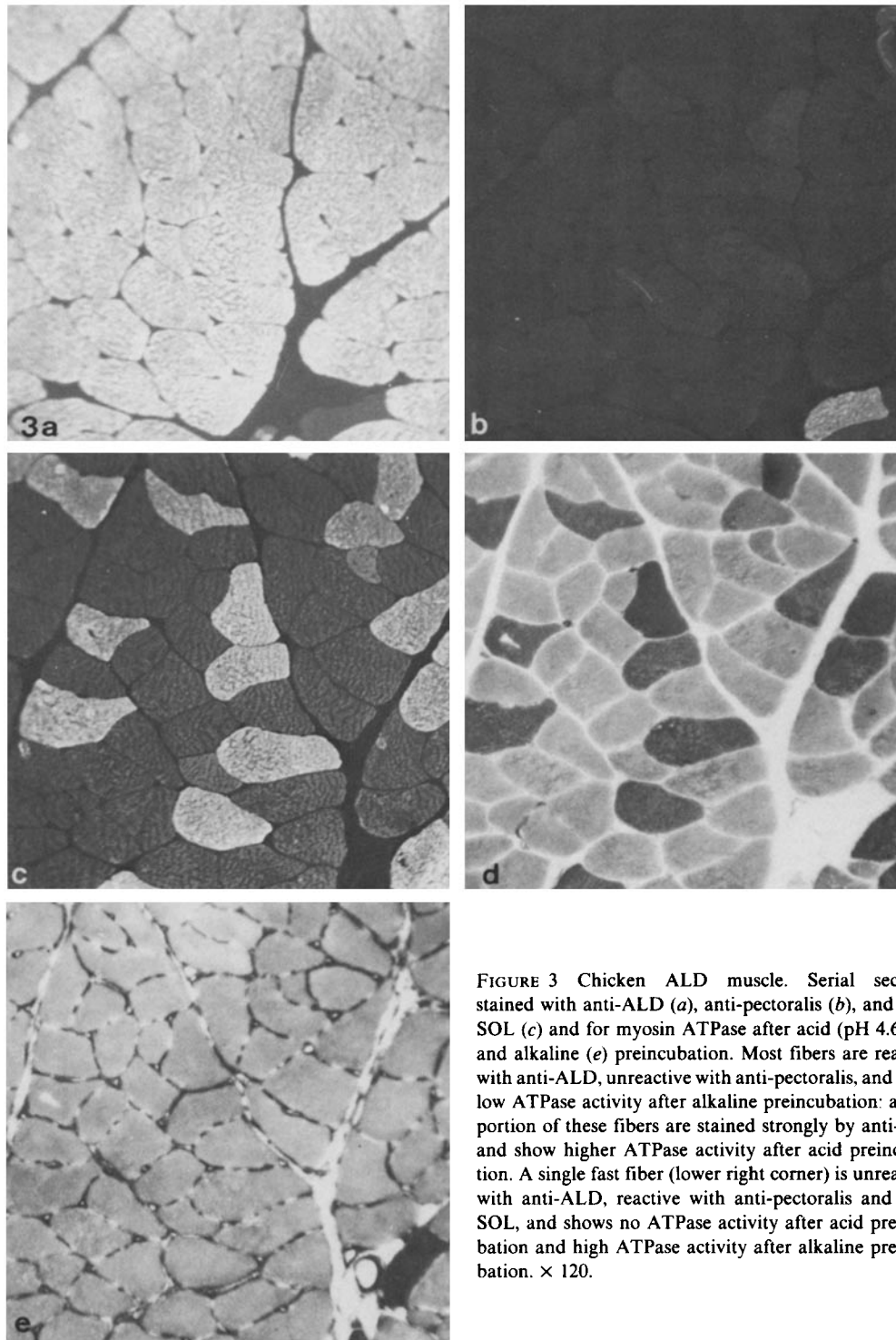


FIGURE 3 Chicken ALD muscle. Serial sections stained with anti-ALD (*a*), anti-pectoralis (*b*), and anti-SOL (*c*) and for myosin ATPase after acid (pH 4.6) (*d*) and alkaline (*e*) preincubation. Most fibers are reactive with anti-ALD, unreactive with anti-pectoralis, and show low ATPase activity after alkaline preincubation: a proportion of these fibers are stained strongly by anti-SOL and show higher ATPase activity after acid preincubation. A single fast fiber (lower right corner) is unreactive with anti-ALD, reactive with anti-pectoralis and anti-SOL, and shows no ATPase activity after acid preincubation and high ATPase activity after alkaline preincubation. $\times 120$.

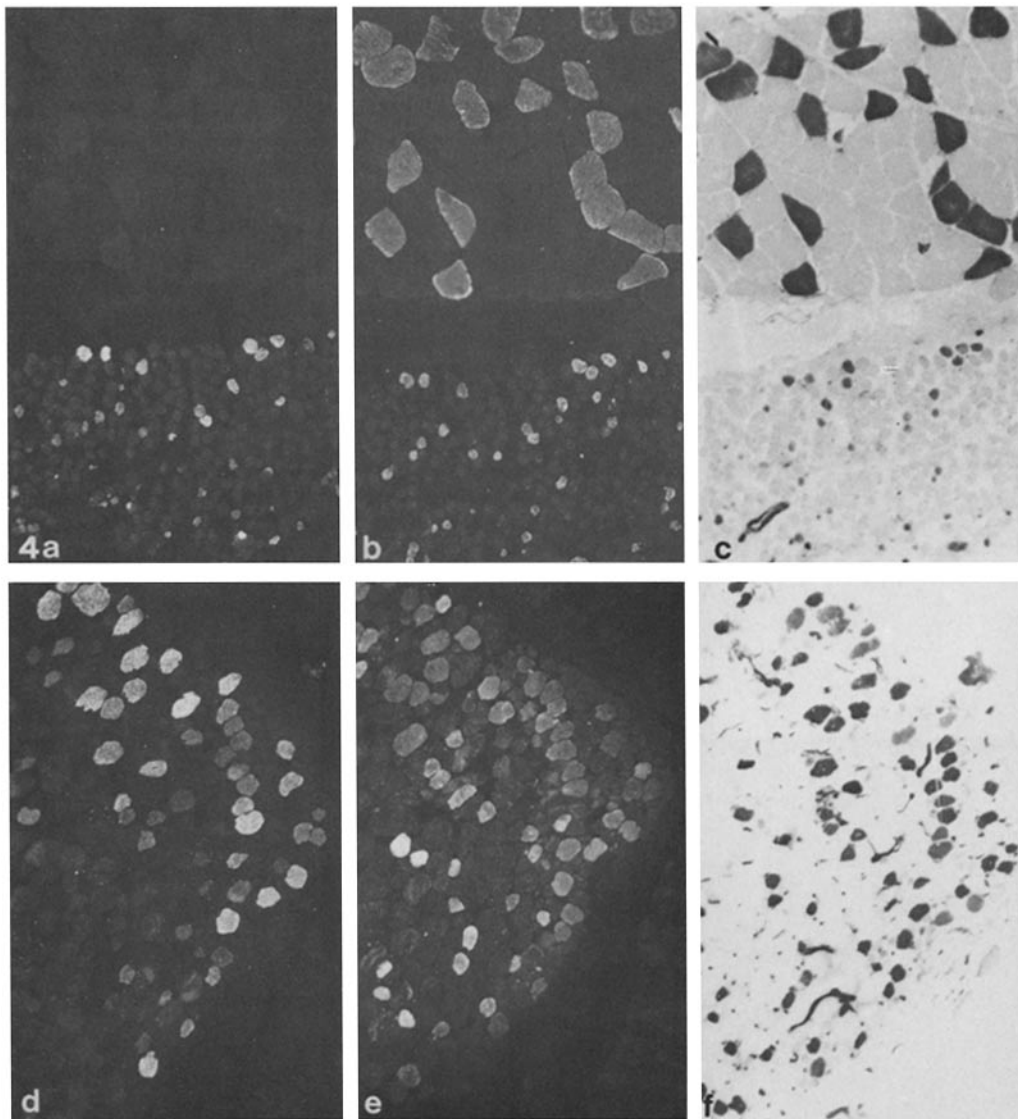


FIGURE 4 Mammalian extraocular muscles. (a-c) Serial sections through a block composed of human pectoralis (*top*) and oblique inferior (*bottom*) stained with anti-ALD (a) and anti-SOL (b) and for myosin ATPase after acid (pH 4.3) preincubation (c). Note the differential response of slow fibers in pectoralis and extraocular muscle to anti-ALD. (d-f) Serial sections through the peripheral portion of the rectus superior of the guinea pig stained with anti-ALD (d) and anti-SOL (e) and for myosin ATPase after acid (pH 4.3) preincubation (f). Note the presence of fibers strongly reactive with anti-ALD and weakly reactive with anti-SOL, fibers reactive with anti-SOL and unreactive with anti-ALD, and fibers weakly reactive with both antimyosins. Fibers strongly stained by anti-ALD often show less intense myosin ATPase activity. (a-c) $\times 90$. (d-f) $\times 150$.

The differential response of intrafusal and extrafusal fibers to the two antimyosins is illustrated in Fig. 5. Intrafusal fibers stained by anti-ALD were identified as nuclear bag fibers in serial sec-

tions processed for histology and enzyme histochemistry. The smaller of the two bag fibers, which corresponds to the bag, fiber according to the nomenclature proposed by Ovalle and Smith (28),

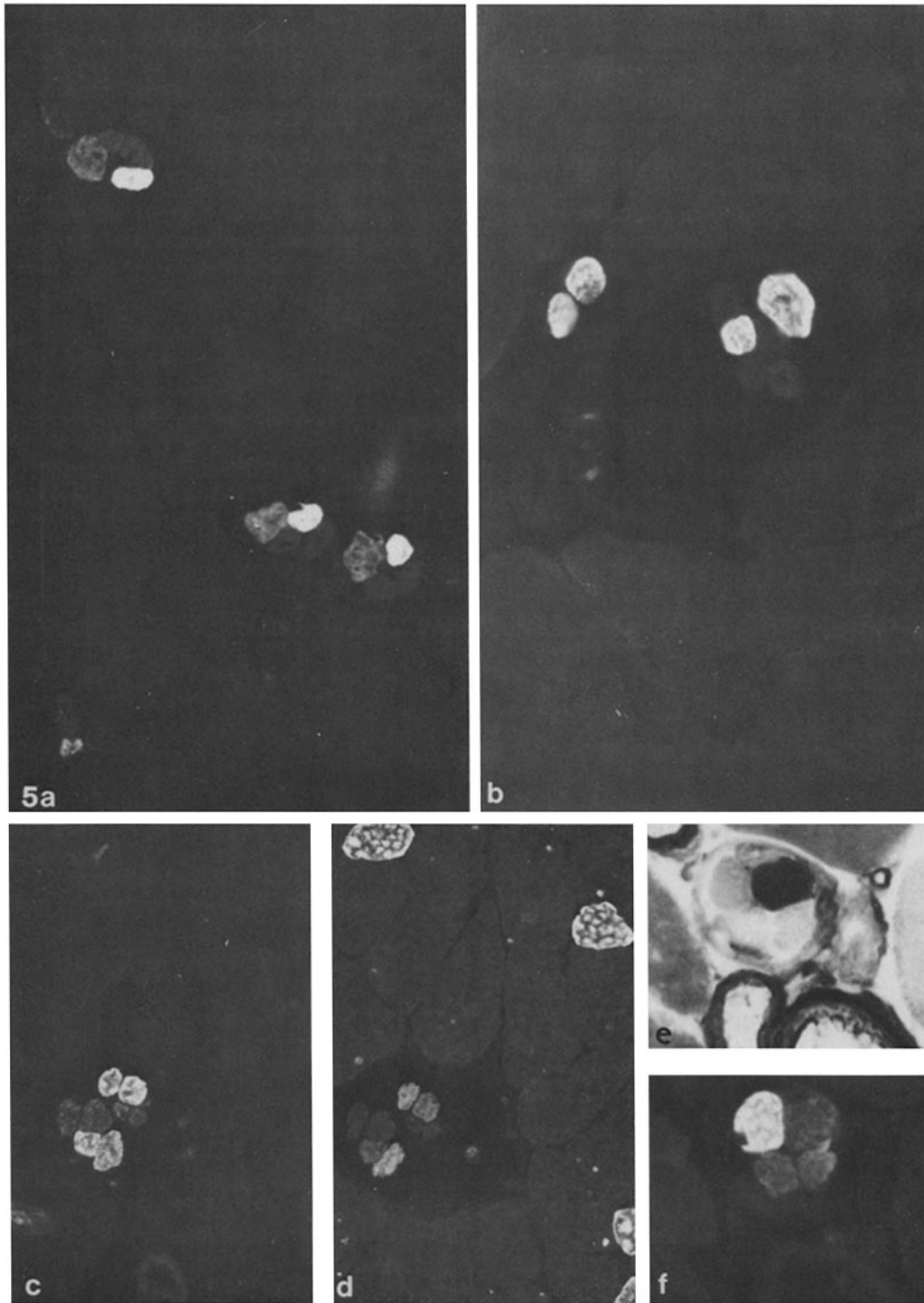


FIGURE 5 Mammalian muscle spindles. (a) Rat extensor digitorum longus muscle, anti-ALD staining. Several spindles with labeled nuclear bag fibers: note stronger reaction of the smaller bag₁ fibers. (b) Guinea pig soleus muscle, anti-ALD staining. Two muscle spindles are seen: the spindle on the right is sectioned through the juxtaequatorial region and shows two labeled bag fibers and four unlabeled chain fibers. The spindle on the left is sectioned through the extracapsular region and shows two labeled bag fibers. (c-d) Serial sections of the rat extensor digitorum longus muscle illustrating the differential response of intrafusal and extrafusal fibers to anti-ALD (c) and anti-SOL (d). (e-f) Serial sections of a muscle spindle in the rat soleus muscle processed for myosin ATPase after acid preincubation (e) and for immunofluorescence with anti-ALD (f). The bag₁ fiber, which is stained less intensely in the ATPase preparation, shows the strongest reaction with anti-ALD. (a and b) $\times 320$. (c and d) $\times 220$. (e and f) $\times 500$.

and which stains less intensely than bag₂ fiber for myosin ATPase after preincubation at pH 4.6, was always brightly stained by anti-ALD. In contrast, there was some variation in the degree of reactivity of the bag₂ fiber; occasionally, chain fibers also showed a very weak reaction with anti-ALD. Bag fibers also were stained by anti-SOL, the degree of reactivity usually being similar in bag₁ and bag₂ fibers; chain fibers were negative with this anti-myosin.

DISCUSSION

The findings reported here indicate that there are two distinct types of slow fibers in vertebrate muscle that can be identified on the basis of their differential reactivity with anti-chicken ALD and anti-guinea pig SOL myosin. Fibers stained by anti-ALD appear to correspond in distribution and histochemical properties to fibers known to display multiple innervation and to respond to stimulation with a contracture rather than a twitch. Such fibers, which could be called "slow-tonic" fibers to distinguish them from the "slow-twitch" fibers of mammalian muscles, are present in various vertebrate classes (see reference 17). Muscle fibers labeled by anti-ALD in the frog and the toad correspond to the "clear" fibers described by Lannergren and Smith (22) that show very low concentrations of fat droplets and mitochondria. These multiply innervated slow fibers were found to respond with only local contraction when stimulated electrically and to give a sustained contracture with acetylcholine. In mammals, fibers stained by anti-ALD were found in extraocular muscles, which are the only muscles in which fibers with physiological properties similar to those of amphibian slow fibers have been demonstrated (18). These slow-tonic fibers undergo contracture upon the administration of succinylcholine, a property which has recently been used to identify single slow-tonic fibers in extraocular muscles and to analyze their ultrastructural characteristics (2). In a correlated acetylcholinesterase and anti-myosin immunofluorescence study reported elsewhere (33), we show that the fibers labeled by anti-ALD in human extraocular muscles have multiple end-plates and therefore probably correspond to slow-tonic fibers.

Nuclear bag fibers in mammalian spindles were also stained by anti-ALD, in agreement with physiological studies indicating that bag fibers have properties similar to the slow-tonic fibers of amphibians: bag fibers give only nonpropagated po-

tentials in response to dynamic γ axon stimulation (23) and react with sustained contracture to succinylcholine (36). Functional and structural heterogeneity of bag fibers has recently been demonstrated (3, 6, 28). The finding that bag₁ or dynamic bag fiber has a slower contraction time-course and a greater sensitivity to acetylcholine than bag₂ or static bag fiber is consistent with our observation that bag₁ fibers often stain more intensely with anti-ALD than bag₂ fibers.

In conclusion, these findings indicate that there is marked immunological cross-reactivity, hence, close structural similarity, between myosins from slow-tonic fibers of different vertebrate classes. In other words, there seems to be a slow-tonic type of myosin, defined immunologically by an antiserum raised in the rabbit to chicken slow muscle myosin, which is present in slow-tonic fibers of vertebrates and may, in part, be responsible for their characteristic contractile properties. The fact that slow-tonic fibers from different species share a well-defined antigenic marker gives support to the concept of a basic similarity of these fibers (17) and can be useful for their histochemical identification. However, other properties of slow-tonic fibers may vary, as is apparently true in the case of the membrane differences between amphibian and avian slow-tonic fibers. Indeed, a variety of slow-tonic fibers appears to be present, even within the same species, as is shown by the variation in the degree of staining with anti-ALD.

The slow-tonic type of myosin is immunologically different from, though partially cross-reactive with, the type of myosin present in the slow-twitch SOL muscle of the guinea pig. This slow-twitch type of myosin, as defined by labeling with fluorescent anti-guinea pig SOL myosin, can be found in those fibers on the basis of histochemical myosin ATPase activity (10). These fibers are the predominant or exclusive component of slow-twitch muscles and are generally present as a minor component in fast-twitch muscles of mammals. The presence in chicken ALD of a number of slow fibers that display a stronger cross-reactivity with anti-SOL than the majority of slow ALD fibers is indicative of the presence of a slow-twitch type of myosin in avian muscle as well. The two types of slow myosin recently identified in chicken ALD (19) may derive from the two types of slow fibers described here. Physiological studies on chicken ALD should be reconsidered in the light of these findings, for the two types of slow fibers may differ in their contractile properties.

A slow-twitch type of myosin that reacts more strongly with anti-SOL than with anti-ALD can also be identified in specific fibers of reptiles and amphibians. These fibers are distinguishable from typical slow-tonic fibers for the different pH sensitivity of the myosin ATPase reaction. In fact, they stain more intensely than slow-tonic fibers after preincubation at pH 4.6, a feature they have in common with ALD fibers labeled by anti-SOL and with mammalian slow-twitch fibers. This finding provides independent evidence for the concept presented here of two distinct types of slow myosin in muscle fibers from different vertebrate classes. Two varieties of amphibian slow fibers, corresponding to those described in this study, were previously recognized by Smith and Ovalle (38) on the basis of histochemical and ultrastructural criteria. Two types of slow motor units with different contractile properties and supplied by motor axons of different sizes have also been described in *Xenopus* (37). Previous conflicting reports about electrophysiological properties of amphibian slow fibers (see reference 30) may be due to heterogeneity of slow fiber populations.

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