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The Leg Flexor Muscle of *Carcinus*. II. Distribution of Muscle Fiber Types

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ABSTRACT Three types of muscle fiber were recognized in the leg flexor muscle of *Carcinus maenas* on the basis of histochemical staining for the oxidative enzyme NADHD and analysis of fiber cross-sectional area. The distribution of these fiber types within the muscle is described. The oxidative capacity and cross-sectional area of the fiber was correlated with the fiber type determined physiologically. *Key words* crab leg muscle, NADHD histochemistry, fiber types, oxidative capacity

Investigation of the contraction time of crustacean muscle fibers has revealed a number of muscle fiber types. Two extremes can be easily recognized: "slow" and "fast." Intermediate types can also be identified (Atwood, '76), although they are better considered as presenting a continuum from one extreme to the other.

A variety of histological methods have also been used to identify muscle fiber types. These include histochemical staining for adenosine triphosphatase (ATPase) and oxidative enzymes, and measurements of sarcomere length.

Traditionally, sarcomere length has been used as an indicator of fiber type (Atwood, '72, '76). Recently sarcomere length and muscle fiber type have come to be regarded as synonomous, with short sarcomere fibers being equated to fast fibers and long sarcomere fibers being equated to slow fibers (Lang et al., '80; Ogonowski et al., '80). Such extrapolation may not be valid in all cases.

More recently, Lang et al. ('80) have used ATPase activity as well as sarcomere length to classify fibers. Some confusion seems to have arisen with attempts to correlate these morphological and histochemical measures with physiological results. This is well shown by the perplexing conclusion that "the oxidative capacity of the muscle fibers is not directly correlated with muscle fiber type (based on adenosine triphosphatase activity and sarcomere length)" (Lang et al., '80). Biologically, it would appear to be more meaningful to relate metabolic status to intrinsic physiological function. Close examination of the photomicrographs of Lang et al.'s ('80) histochemical sections (the reproduction being admittedly poor) suggests that they could readily support such a correlation between physiological fiber type, oxidative capacity, and ATPase activity. That is, physiologically slow fibers have a high oxidative capacity and a low ATPase activity, and physiologically fast fibers have a low oxidative capacity and higher ATPase activity. Such a conclusion would be in agreement with the well-established pattern in vertebrate muscle. Lang et al. ('80) appear to have arrived at their confusing conclusion by using the method of fiber typing based on sarcomere length and relating it to ATPase activity rather than relating these morphological characters to the fiber type determined physiologically.

The intrinsic muscle fiber type is best determined physiologically by intracellular depolarization of single muscle fibers since this avoids problems encountered in the interpretation of results obtained by axonal stimulation of motor units in polyneuronally innervated muscle fibers (e.g., Govind and Lang, '74). While the intracellular method has been used successfully to show a nondiscrete range of muscle fiber types from fast, through intermediate, to slow in the leg flexor muscle of *Carcinus* (Parsons, '82), it is difficult to obtain an accurate assessment of the spatial distribution of the fiber types within this muscle because reliable localization of the individual muscle fibers is generally only possible for the more superficial fibers.

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Histochemical methods of muscle fiber typing allow precise localization of individual fibers throughout the muscle. The enzyme NADHD has been used commonly as an indicator of the oxidative capacity of a muscle fiber (Nachlas et al., '58), and in crustaceans has been shown histochemically to be high in physiologically slow muscle fibers and low in physiologically fast muscle fibers (Ogonowski and Lang, '79). Physiologically intermediate fibers generally have intermediate histochemical levels of NADHD.

The work reported here describes the recognition of these fiber types on the basis of their staining response for the oxidative enzyme for NADHD and analysis of fiber cross-sectional area. The spatial distribution of the muscle fiber types within the flexor muscle is also described.

MATERIALS AND METHODS

Histochemistry

Walking legs from the crab *Carcinus maenas* were obtained by autotomy as described elsewhere (Parsons, '80). The cuticle covering the anterior and posterior faces of the merus was removed with a dental drill. The hypodermis was left intact to protect the underlying fibers. Two thin strips of cuticle were also left in place dorsally and ventrally, to help prevent splitting of the muscle during freezing. The merus was then rapidly frozen in liquid propane cooled with liquid nitrogen. During freezing the flexor muscle was held under tension by fixing the merus-carpus (M-C) joint open with Periphery Wax (Lactona Corp.). Frozen tissue samples were then allowed to equilibrate in a cryostat (Slee Medical Equipment Ltd., London) at −20°C for 1 h. The remaining cuticle was then removed by splitting with cold instruments. Muscle samples were mounted using a commercial mounting compound (Ames O.C.T.) and sectioned perpendicular to the apodeme at 12 μm. The flexor muscle was sampled at approximately 500-μm intervals along the merus. Sections were collected on coverslips, allowed to air dry for 1–2 hours, and then stained for NADHD using the method of Pearse ('72)[1] except that sections were mounted in Farrants medium. Control sections were incubated in the absence of the substrate NADH. In all cases control sections were unstained.

Analysis of muscle fibers

Sections taken from positions along the merus (see Fig. 1) were photographed and printed at 40 times magnification. Fiber ty... were marked-in on the photographs after ... croscopic examination of the sections at h... magnifications (≥ × 250).

Muscle fiber cross-sectional area was de... mined using a MOP-3 image analyzer (... Zeiss Inc.). No attempt was made to comp... sate for the change in muscle fiber area du... the different fiber insertion angles onto ... apodeme (see Results). Histograms of ... cross-sectional areas of the different mu... fiber types were constructed and compa... nonparametrically to determine if the dis... butions of area came from the same pop... tion, using a multiple comparison ... employing rank sums (Dunn, '64).

Electron microscopy

Flexor muscle tissue was prepared for e... tron microscopy as previously described (P... sons, '80). One-micron plastic sections w... cut from the same blocks and stained w... Paragon stain (Paragon Co., New York) ... differentiate mitochondria.

RESULTS

Examination of the NADHD-stained ... tions (Figs. 2, 3) of the flexor muscle allow... three types of muscle fiber, designated ty... S, F, and I, to be recognized on the follow... basis. Type S fibers had a heavily stained ... tinuous peripheral band of approximately ... form width. In some fibers deep invagina... of this peripheral band were present (Fig. ... In most cases a light background stain ... also present. Type F fibers had a li... stained, thin, discontinuous peripheral ba... and no background stain within the fiber (... 3a). Type I fibers showed intermediate ...

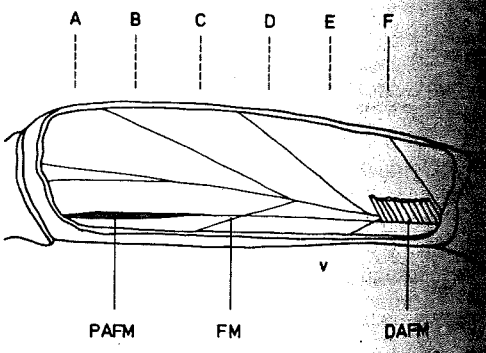

Fig. 1. Anterior view of the merus showing the loca... of sample sites (A-F) from which sections were analy... determine the distribution of muscle fiber types ... flexor muscle. Level A is the most proximal ... DAFM, distal accessory flexor muscle; FM, flexor ... PAFM, proximal accessory flexor muscle; v, ventral

[1]Note that the formula for the Tris buffer given by Pearse ('72) is incorrect; the correct formulation is given by Sober ('68).

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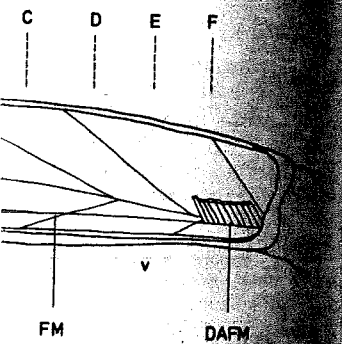

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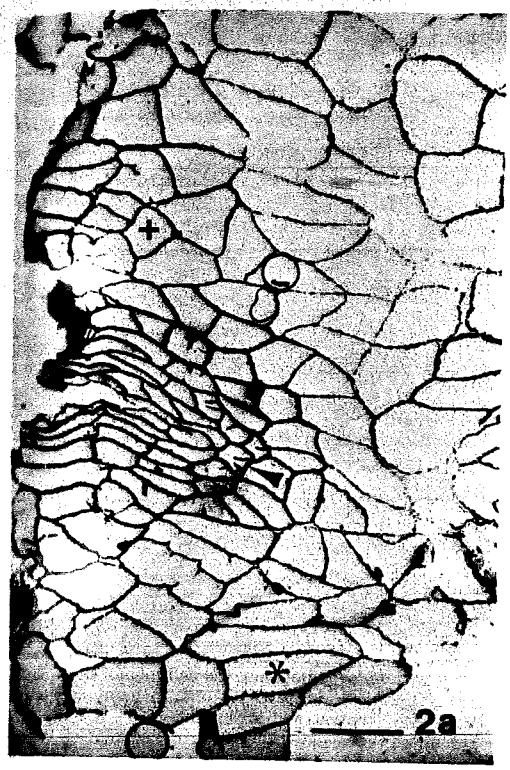

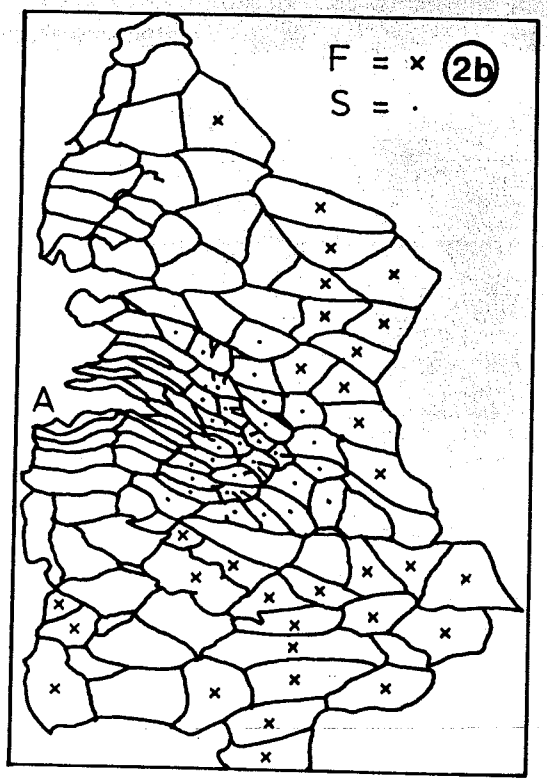

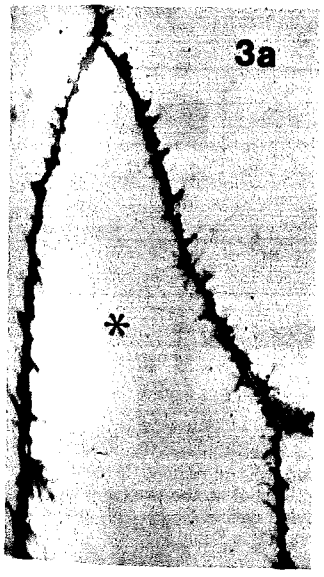

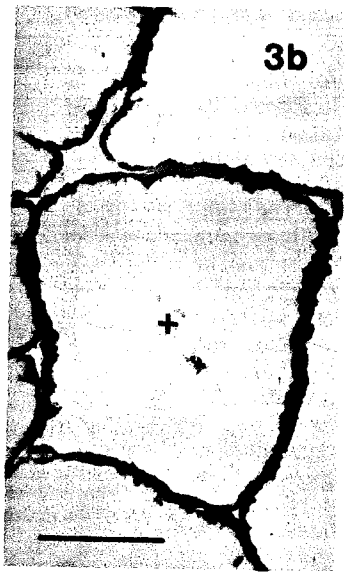

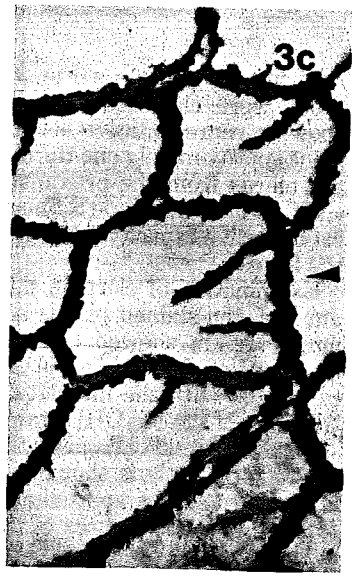

Fig. 2. a. Section of the leg flexor muscle (at level B) stained for NADHD. Individual fibers show different staining intensities and thicknesses at their periphery. High-power microscopic examination (see Fig. 3) is necessary for recognition of the three fiber types. The posterior surface of the flexor muscle is at the left. Some of the extensor muscle fibers are also shown. Three air bubbles are also present: one on lower margin and two just above center. The asterisk, "+" sign, and arrowhead indicate the fibers shown at higher magnification in Figure 3. Scale bar = 500 μm. b. Tracing of the flexor muscle fibers of Figure 2a to show location of fiber types determined by high-power examination as described in the test. The position of the flexor apodeme (A), which does not extend to the anterior surface of the flexor muscle at this level and was torn out during sectioning, is shown. Type I fibers are unmarked; type F and type S fibers are marked as shown. Scale bar = 100 μm.

Fig. 3. High-power photomicrographs of flexor muscle fibers from the section shown in Figure 2a. Note differences in stain intensity, thickness and continuity. See text for details. a, b, and c are at the same magnification. Note also the differences in fiber cross-sectional area. The asterisk, "+" sign, and arrowhead indicate the position of these fibers in Figure 2b. a. Type F fibers. b. Type I fibers. c. Type S fibers. Scale bar = 100 μm.

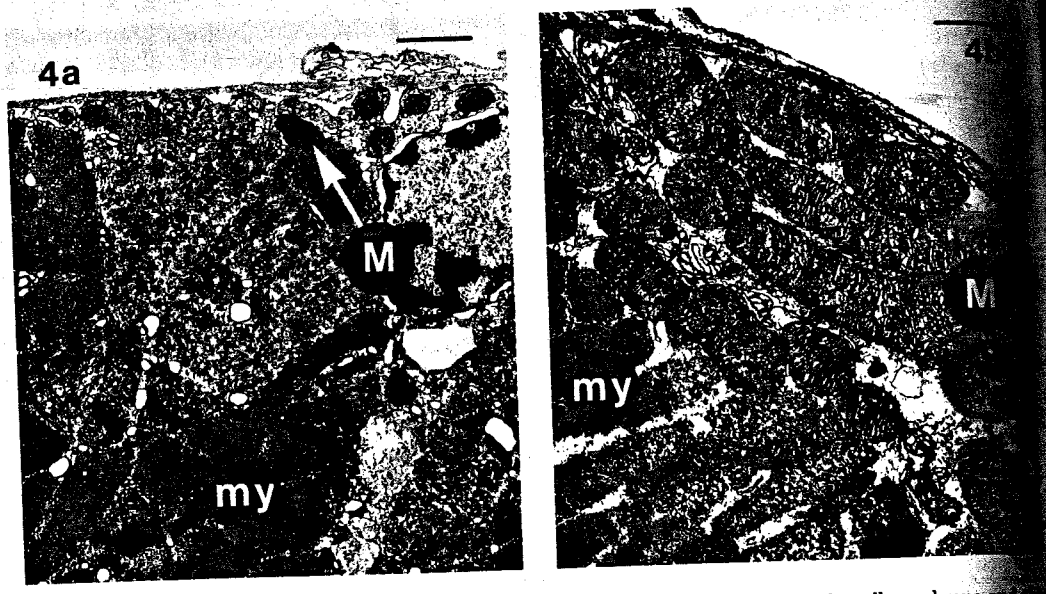

Fig. 4. Electron micrographs of flexor muscle fibers to show differences in mitochondrial content and distribution. a. Type F fiber. Myofibrils (my) are found close to the cell membrane, and a few small mitochondria (M) are scattered near the edge of the fiber. Scale bar = 1 μm. b. Type S fiber. The numerous large mitochondria (M) form a thick, densely packed layer between the cell membrane and myofibrils (my). Scale bar = 1 μm. In many fibers mitochondrial content and appearance were intermediate between the types shown in a and b. These fibers represent the range of type I fibers found histochemically.

intensity and thickness (Fig. 3b). Any doubtful types were classified as type I. Figure 2a shows a representative section taken from a point approximately one-third of the way along the merus from the proximal end. These results suggest that type S fibers have the highest oxidative capacity while type F fibers have the lowest.

Examination of electron microscopic and 1 μm Paragon-stained plastic sections of flexor muscle fibers showed that the number and distribution of mitochondria (Figs. 4a,b) is well correlated with the intensity and spatial distribution of the NADHD stain (Fig. 3).

The distribution of muscle fiber types within the flexor muscle is shown in Figure 5. Type S fibers make up approximately 14% of the total cross-sectional area of the flexor muscle at the proximal end. This decreases to zero in the central region and then increases again distally. Type I fibers are present at all levels, occupying between one-quarter to one-half of the total cross-sectional area. Type F fibers occupy over 70% of the muscle area near the center of the merus and more than 40% of the area at either end.

Histograms of fiber cross-sectional areas for the three fiber types at the six sample sites

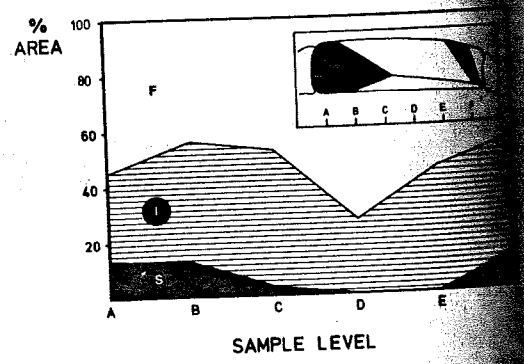

Fig. 5. The percentage of total flexor muscle cross-sectional area occupied by each fiber type. Type F fibers predominate in the more central regions (levels C, D, and E). Type S fibers are located at the proximal and distal ends of the muscle. Inset: Schematic plan of approximate location of type S fibers. Type F and I fibers overlie the S fibers.

along the merus are shown in Figure 6. Since the angle the fibers make to the apodeme varies along the length and the depth of the apodeme, these distributions of fiber cross-sectional areas were only tested for significant differences at each sample level, rather than overall, because the cross-sectional area of the

fibers is altered by the angles that the fibers make to the apodeme. In the case of the obscurred deeper fibers, these angles cannot be measured. At each level (A-F) the distributions of cross-sectional areas of the three fiber types were significantly different ($P < 0.05$). (Type S fibers at level C (Fig. 6) were not tested due to inadequate sample size.) The differences between the distributions of the three fiber types at each level are evident from Figure 6. Type F fibers have cross-sectional areas that generally span the whole range of areas encountered but have the largest mean area of the three fiber-type distributions. The distributions of type I fibers have lower mean areas, few larger fibers, and increased numbers of small fibers. Type S fibers have the smallest mean areas. Small area fibers predominate in the type S distributions and the areas show less variability than the type I and F fibers. These results indicate that the fibers defined histochemically on qualitative grounds also formed discrete populations as determined quantitatively according to fiber cross-sectional area.

Within the flexor muscle, type S fibers were always found in approximately the posterior (deeper) half of the muscle, close to the apodeme. Type I fibers were generally found surrounding the type S fibers, and in most cases

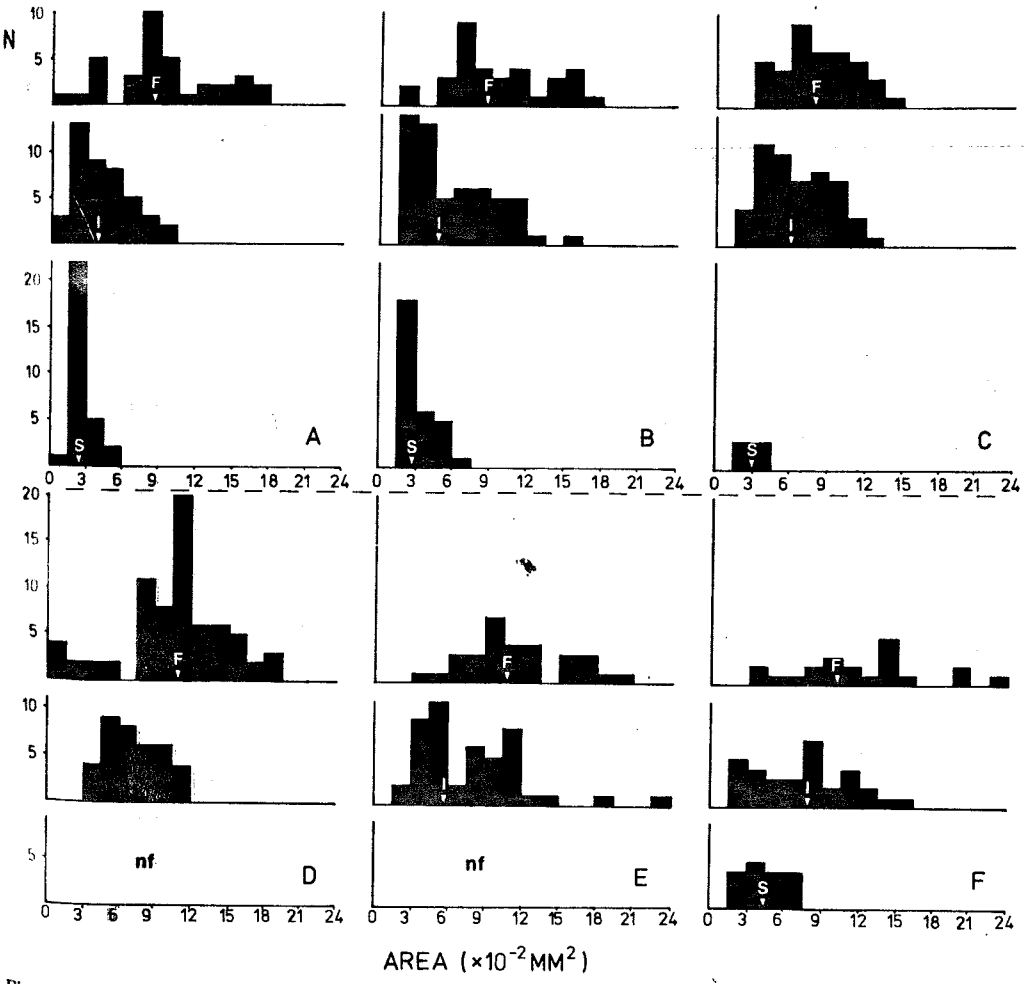

Fig. 6. Distribution of muscle fiber cross-sectional areas for the three fiber types (F, I, and S) at the six sample sites along the flexor muscle. Vertical axis = number of fibers; white arrow-head = mean value. nf = no fibers present (see Fig. 5).

in the more posterior regions of the muscle. Small numbers of type I fibers were also sometimes found on or near the most anterior muscle layer.

DISCUSSION

Three muscle fiber types have been recognized in the leg flexor muscle of *Carcinus maenas* on the basis of histochemical staining for the oxidative enzyme NADHD and the analysis of fiber cross-sectional area.

The distribution and intensity of staining for NADHD correlates well with the abundance and distribution of subsarcolemmal mitochondria (Figs. 3, 4). Electron micrographs and 1 μm plastic sections of type S fibers reveal large numbers of subsarcolemmal mitochondria. Where sarcolemmal invaginations are present, they are associated with abundant mitochondria which give some of the type S fibers their characteristic appearance in histochemical sections (Fig. 3c). Similarly the absence of large numbers of subsarcolemmal and inter-myofibrillar mitochondria in type F fibers correlates with the lighter NADHD staining seen histochemically (Fig. 3a). A similar correlation has been demonstrated in other invertebrates (Atwood, '72) and the vertebrates (Muller, '76; Mosse, '78, '79). Since it is known that physiologically slow muscles have a high oxidative capacity (high NADHD) it is reasonable to conclude that the type S fibers represent the slow fibers found physiologically (Parsons, '82). In addition, fatigue resistance, a property of slow muscle fibers, is also associated with high mitochondrial content (Hoyle and MacNeil, '68; Silverman and Charlton, '80). Type F fibers, with their lower oxidative capacity (low NADHD) and fewer mitochondria, therefore represent the physiologically fast fibers. Intermediate densities of mitochondria and intermediate staining for NADHD at the periphery of the fibers implies physiologically intermediate contraction profiles.

Histochemical staining of the flexor muscle shows that slow fibers are present in the proximal and distal regions but absent in the central region (Fig. 5). Intermediate muscle fibers are present throughout the muscle but predominate in the posterior half of the muscle. Fast fibers are generally found in the more accessible anterior layers of the flexor (Fig. 2b). Physiological examination of the flexor muscle fibers (Parsons, '82) shows a distribution that parallels that of the NADHD staining described above. Physiologically slow fibers are found in the posterior proximal and distal regions of the muscle but rarely in the central regions. Intermediate and fast fibers are found throughout the muscle, generally in the anterior layers. The functional implications of the localization of the different fiber types in the flexor muscle are considered (Parsons '82).

An exception to this general pattern described above was found in the most superficial anterior layer of the flexor muscle. Most of the fibers in this layer showed intermediate to slow contraction profiles, while the NADHD staining of these same fibers suggests fast contracting fibers. This apparent contradiction may be a consequence of the method of tension measurement. Tension was measured from the distal end of the carpus or from a disarticulated apodeme (Parsons, '82). The most anterior layer fibers insert onto the anterior, thin projection of the apodeme that runs for most of the length of the apodeme. Single fiber contractions acting on this edge would tend merely to bend it, thus dissipating some of the force so that it is not transferred to the transducer. Although only single fibers were stimulated, the tension produced by these fibers must act on the whole apodeme which is damped by the remaining quiescent muscle fibers. Further losses in measured tension would then be expected to occur in transmission of the contractile force through the muscle and apodeme to the distally placed transducer. To confirm that this is a sufficient explanation, isolation and measurement of single fiber contraction speeds and tensions in these superficial layers of the flexor must be undertaken (e.g., see Atwood et al., '65).

There was no indication that the slow fibers described here (Fig. 3c) could be further separated on the basis of NADHD staining as in the case in lobster claw propus muscles (Govind et al., '81; Kent and Govind, '81), although some type S fibers did show some light background staining (Figs. 2, 3). It may be that the extremely slow, very deeply and completely stained fibers that Kent and Govind ('81) found are unique to the highly specialized but simply innervated cheliped muscles.

A recurring deficiency in histochemical examinations of this type (see also Lang et al., '80) is the absence of a single standard method which can be used to compare indicators of muscle fiber type. The most direct and biologically relevant measure of fiber type is intracellular depolarization of single muscle fibers. Many studies of muscle fiber types use only histological parameters (e.g., Govind et al., '81) and make no attempt to relate fiber type

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based on these parameters back to a physiological contraction type. Without such comparison these studies must be considered incomplete.

The work presented here indicates that the correlation of oxidative capacity (as determined from NADHD distribution) and physiological fiber type allows accurate localization of fiber types within complex polyneuronally innervated crustacean muscles. We suggest that in future studies of crustacean muscles, muscle fiber types should be determined from both physiological contraction profiles of single muscle fibers and histological parameters—as, for example, was done in this study, where the oxidative capacity and the cross-sectional area of the fibers was determined. Ideally, all these measurements should be made on the same individual fibers.

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