Myosin Isozymes in Normal and Cross-reinnervated Cat Skeletal Muscle Fibers

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ABSTRACT Immunocytochemical characteristics of myosin have been demonstrated directly in normal and cross-reinnervated skeletal muscle fibers whose physiological properties have been defined. Fibers belonging to individual motor units were identified by the glycogen-depletion method, which permits correlation of cytochemical and physiological data on the same fibers. The normal flexor digitorum longus (FDL) of the cat is composed primarily of fast-twitch motor units having muscle fibers with high myosin ATPase activity. These fibers reacted with antibodies specific for the two light chains characteristic of fast myosin, but not with antibodies against slow myosin. Two categories of fast fibers, corresponding to two physiological motor unit types (FF and FR), differed in their immunochemical response, from which it can be concluded that their myosins are distinctive. The soleus (SOL) consists almost entirely of slow-twitch motor units having muscle fibers with low myosin ATPase activity. These fibers reacted with antibodies against slow myosin, but not with antibodies specific for fast myosin. When the FDL muscle was cross-reinnervated by the SOL nerve, twitch contraction times were slowed about twofold, and motor units resembled SOL units in a number of physiological properties. The corresponding muscle fibers had low ATPase activity, and they reacted with antibodies against slow myosin only. The myosin of individual cross-reinnervated FDL muscle units was therefore transformed, apparently completely, to a slow type. In contrast, cross-reinnervation of the SOL muscle by FDL motoneurons did not effect a complete converse transformation. Although cross-reinnervated SOL motor units had faster than normal twitch contraction times (about twofold), other physiological properties characteristic of type S motor units were unchanged. Despite the change in contraction times, cross-reinnervated SOL muscle fibers exhibited no change in ATPase activity. They also continued to react with antibodies against slow myosin, but in contrast to the normal SOL, they now showed a positive response to an antibody specific for one of the light chains of fast myosin. The myosins of both fast and slow muscles were thus converted by cross-reinnervation, but in the SOL, the newly synthesized myosin was not equivalent to that normally present in either the FDL or SOL. This suggests that, in the SOL, alteration of the nerve supply and the associated dynamic activity pattern are not sufficient to completely respecify the type of myosin expressed.

The influence of the nervous system on the myosin composition of skeletal muscle fibers has been well documented, but the extent of this influence remains unclear. Experiments with cross-reinnervation have provided striking evidence that chemical as well as physiological properties can be transformed by alteration of the nerve supply. When a fast muscle is reinnervated by a nerve that originally supplied a slow muscle, contraction is slowed and the myosin becomes similar to that present in a slow muscle (3, 24, 36, 40). Converse alterations occur in "slow" muscles reinnervated by "fast" nerves, but some observations suggest that the transformation is less complete (1, 7, 30, 37). Although contraction time of

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the cat soleus is faster when cross-reinnervated by a nerve from a fast muscle, this may not be accompanied by a change in the histochemical pattern of myosin ATPase activity (e.g., see reference 7). However, this does not necessarily imply that the myosin is unchanged after cross-reinnervation of the soleus.

To examine the characteristics of myosin isozymes in cross-reinnervated muscle fibers, we used the procedures of immunocytochemistry. This approach was used successfully in earlier studies of normal muscles, in which we described the localization of myosin isozymes with respect to individual muscle fibers in a mixed population (17, 18). By using antibodies specific for the N-terminal sequence of the alkali 1 and alkali 2 light chains of fast myosin,[1] we were able to show that differences in myosin composition exist even within a fast category of muscle fibers (15, 18). In the present work, contractile properties of single motor units were measured in the soleus (SOL) and flexor digitorum longus (FDL) muscles of the cat before and after cross-reinnervation, and the corresponding muscle fibers were "marked" by the glycogen depletion method. In this way, physiological data could be compared directly with immunochemical properties in sequential sections of the same marked fibers. By using the immunocytochemical approach, it was possible to identify changes in the contractile proteins that are not evident by other cytochemical procedures. We show that the myosin of identified muscle units can be transformed by cross-reinnervation. We conclude that the myosin synthesized by a muscle fiber is clearly influenced by the type of nerve supply, but that cross-reinnervation does not always effect a complete transformation of either the physiological properties or the myosin composition of the fiber, even in fibers which are known to be successfully cross-reinnervated.

MATERIALS AND METHODS

Surgical and Physiological Procedures: In experiments described in detail elsewhere (10, 14),[2] surgical cross-unions were made under aseptic conditions between the SOL nerve and FDL muscle, or between the FDL nerve and the SOL muscle. In addition, several FDL and SOL muscles were also subjected to self-reinnervation by cutting the muscle nerve and rejoining it to itself. After periods of 30 to 50 wk (mean 48.5 wk), the physiological and histochemical characteristics of cross-reinnervated whole muscles and of individual motor units were studied with the animals under pentobarbital anesthesia. Individual motor units were isolated by penetrating their motoneurons with micropipette electrodes (6), and motor units were characterized as to physiological type by the tests and criteria used for normal units in these muscles (8, 9).

At the end of each experiment, FDL and SOL muscles were removed, blotted dry, weighed, tied at approximately resting length to a metal rod or wooden splint, and frozen by immersion in isopentane cooled to −160°C with liquid nitrogen. Comparable FDL and SOL muscles from normal (unoperated) adult cats were used as additional control material. Frozen muscles were stored in a liquid nitrogen freezer.

Enzyme and Carbohydrate Cytochemistry: After prolonged stimulation of a single motoneuron, the muscle fibers innervated by it (a muscle unit) are depleted of their glycogen. These muscle fibers can thus be readily identified by the PAS procedure, since they appear as negative images among other stained fibers. Specimens of frozen FDL and SOL obtained from normal cats and from the same muscles that were used for the physiological measurements were sectioned transversely (~10 μm) in a cryostat at −20°C. Glycogen was localized in sections that had been fixed for 10 min in picric acid-ethanol-formol (Rossman's fixative) at 0–5°C. The sections were stained by the periodic acid-Schiff (PAS) reaction, and selected sections were first digested with amylase. This ensured that the staining pattern accurately reflected the distribution of glycogen, and also permitted evaluation of fibers that had been depleted of glycogen. Minimal residual (amylase-resistant) staining of stimulated muscle fibers represented carbohydrates other than glycogen, most likely associated with interfibrillar membranous structures. Alkali-stable (myosin) ATPase activity was localized in sequential sections according to the method of Guth and Samaha (21).

Immunocytochemistry: Antibodies specific for the N-terminal sequence of the alkali 1 light chain (anti-Δ1) and the alkali 2 light chain (anti-Δ2) of chicken pectoralis myosin were isolated as described by Holt and Lowey (26) and Silberstein and Lowey (35). Antibody against myosin extracted from the chicken anterior latissimus dorsi (anti-ALD) was purified as described earlier (18). Transverse cryostat sections (4 μm) serial to those used for enzyme and carbohydrate cytochemistry were used for the procedures of indirect immunofluorescence as described by Gauthier and Lowey (17, 18). Unfixed sections were incubated with the unlabeled antibodies and then reacted with fluorescein-labeled goat anti-rabbit immunoglobulin. The sections were examined with a Zeiss fluorescence microscope equipped with an epi-illumination system. A Xenon XB075 W/DC lamp, narrow-band FITC excitation filter (485/20 nm), and band-pass barrier filter (520–560 nm) were used with a Zeiss Neofluar 16/0.4 objective. The image was recorded on Kodak type 103 a-G spectroscopic plates.

RESULTS

Before describing the immunocytochemical observations on normal and cross-reinnervated FDL and SOL muscles, it is useful to summarize the physiological results obtained in the series of experiments from which the material was derived. Further details are available elsewhere (7, 14).[2]

Motor Units in FDL and SOL Muscles of Normal Cats

In the normal cat, the fast-twitch FDL muscle contains a mixed population of motor units and muscle fibers. The motor units in this heterogeneous population can be divided into clearly definable types on the basis of certain physiological properties (9). These include three types of fast-twitch units, denoted FF, FR, and F(int); and a single category of slow-twitch, type S units. Fast-twitch units make up almost 90% of the normal cat FDL, while type S units comprise only 11.5%.[2] Each unit type is associated with a cytochemically distinct muscle fiber type, which can be identified by glycogen depletion following prolonged stimulation of the motoneuron (9, 29).[2] Muscle fibers of types FF, F(int), and FR units all exhibit high alkali-stable ATPase activity, although the level of activity in the FR unit fibers is often slightly lower than in the other two units (Figs. 1 *a* and 2 *a*). In contrast, alkali-stable ATPase activity is much lower in the fibers of type S units. Oxidative enzymic activity is low in the fibers of type FF units but relatively high in those belonging to F(int), FR, and S units. For convenience in this paper, we will use the designations "FF," "FR," and "S" to denote muscle fibers with the cytochemical attributes of fibers known to be associated with these physiological motor unit types. Fibers inferred to belong to FF or FR units will also be referred to collectively as "fast" fibers.

The SOL muscle of the normal cat is a rare example of a

[1] *Abbreviations used in this paper:* anti-Δ1 and anti-Δ2, antibodies specific for the N-terminal sequence of the alkali 1 (LC1) and alkali 2 (LC3) light chains, respectively, of myosin from the chicken pectoralis; anti-ALD, antibodies against myosin from the anterior latissimus dorsi of the chicken; FDL, flexor digitorum longus muscle; FHL, flexor hallucis longus; SOL, soleus muscle; FF, fast-twitch, fatiguable motor unit; FR, fast-twitch, fatigue-resistant unit; F(int), fast-twitch unit with fatigue-resistance intermediate between FF and FR; S, slow-twitch unit, very resistant to fatigue; PAS, periodic acid–Schiff.

[2] Dum, R. P., M. J. O'Donovan, J. Toop, and R. E. Burke, manuscript in preparation.

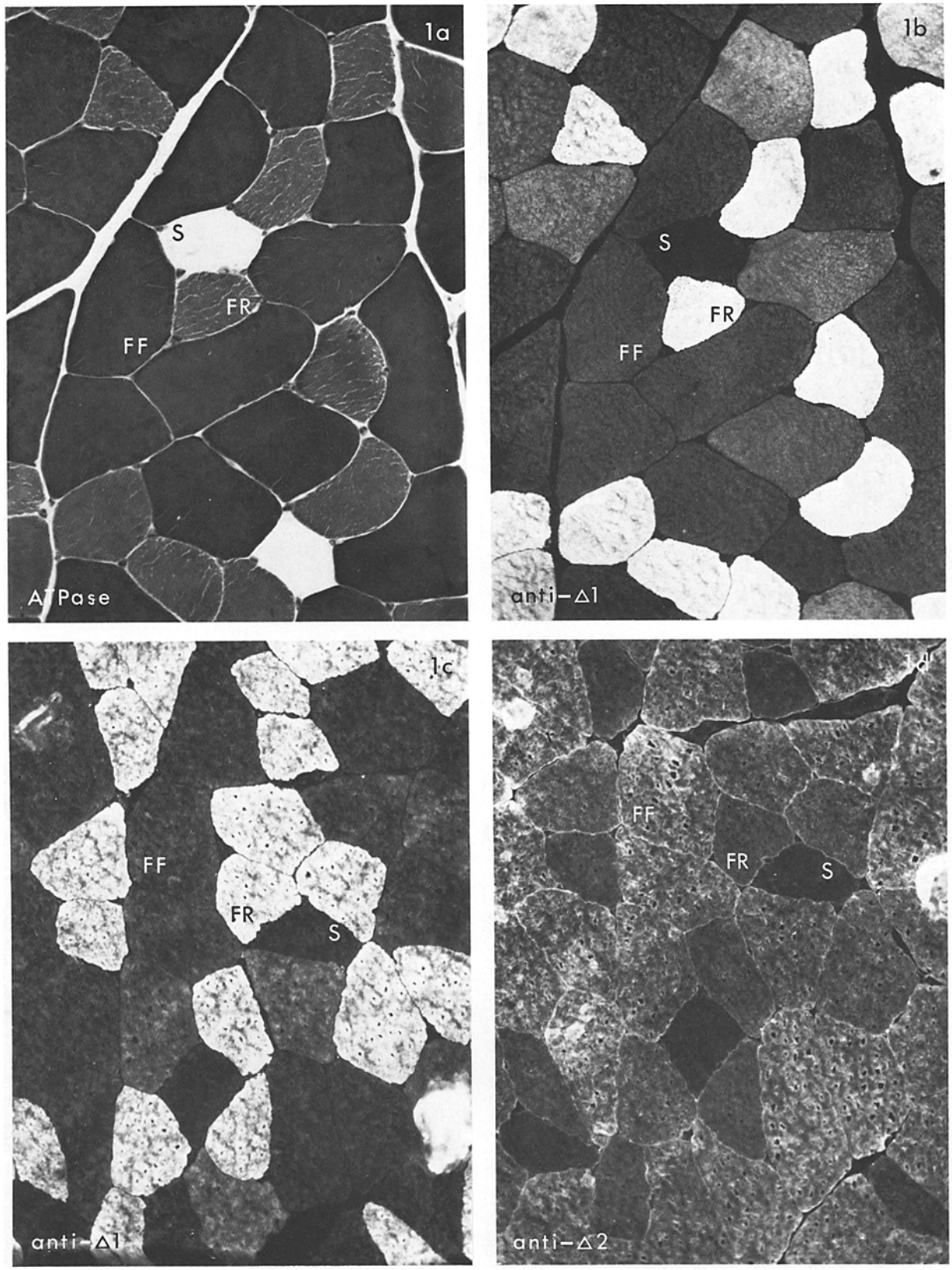

FIGURE 1 FDL, normal cat (#3217). Transverse sections. Fast fibers with high ATPase activity (*FF* in *a*) react moderately with anti-Δ1 (*b*); fast fibers with moderately high ATPase activity (*FR* in *a*) react strongly with anti-Δ1 (*b*). Fibers which stain most intensely with anti-Δ1 (*FR* in *c*) stain only weakly with anti-Δ2 (*FR* in *d*), though the level of staining varies. Fibers with very low ATPase activity (type *S* in *a*) do not react with anti-Δ1 (*b*) or with anti-Δ2 (see *c* and *d*). The granular appearance of some fibers reflects the presence of ice crystals. Designations are based on localization of succinic dehydrogenase as well as ATPase activity according to criteria described by Burke et al. (9). Sections in *a* and *b* and in *c* and *d* are serial. Illustrations in *c* and *d*, from Gauthier (15). × 260.

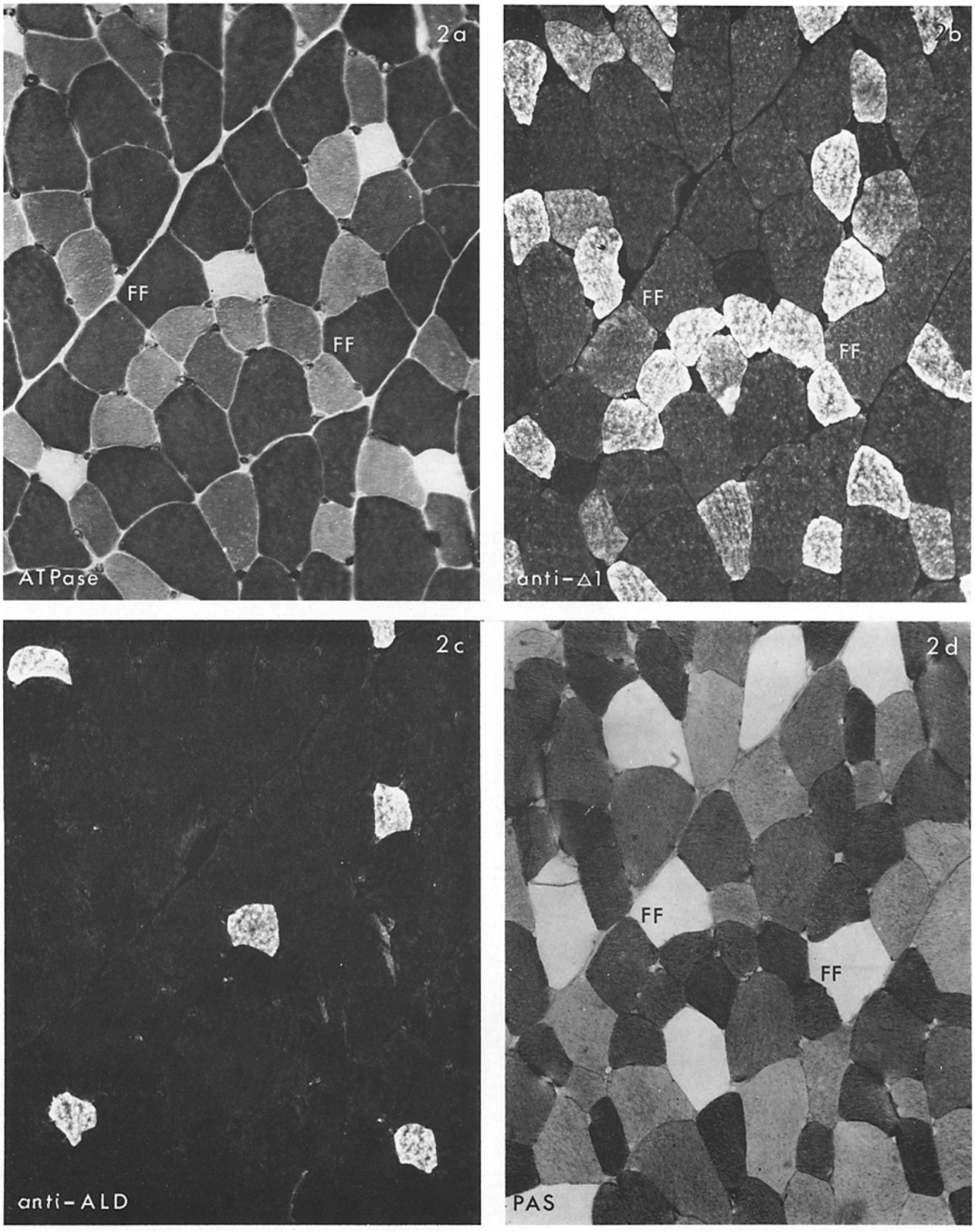

FIGURE 2 FDL, normal cat (#3293), including glycogen-depleted fibers belonging to an *FF* motor unit (*d*). Serial transverse sections. Fibers which comprise a single *FF* unit are depleted of their glycogen and hence are not stained by the PAS-reaction (*d*). Depleted fibers in *d*, two of which are labeled (*FF*), have high ATPase activity (*a*) and a moderate response to anti-Δ1 (*b*), but fail to react with antibody against slow myosin (anti-ALD) (*c*). Additional fast fibers with the same staining pattern correspond to other *FF* units that were not depleted. Fast fibers with moderate ATPase activity (*a*) react strongly with anti-Δ1 (*b*) but not with anti-ALD (*c*); these correspond to FR units. Slow fibers have low ATPase activity (*a*), and no response to anti-Δ1 (*b*), but react strongly with anti-ALD (*c*). × 260.

nearly homogeneous population of motor units and muscle fibers, in this case all type S (8). However, the type S units present are not identical to those present in the heterogeneous muscles that have been studied. SOL type S units tend to contract more slowly than other S units and many exhibit depression of twitch tension following a tetanus (posttetanic depression), while S units in heterogeneous muscles, including the FDL, tend to exhibit posttetanic potentiation of twitch tension (8, 9). The predominant muscle fibers (usually >95%) in the cat SOL have low alkali-stable ATPase activity (see Fig. 6*a*) and high oxidative activity, but they are larger than those of S units in heterogeneous muscles, and they differ in some histochemical properties (8).

Motor Units in Cross-reinnervated FDL and SOL Muscles

FDL muscles cross-reinnervated by SOL motoneurons were markedly different from normal muscles; wet weights and tetanic tensions were about half that of the normal, and isometric twitch contraction times were about double the normal value. In all of the cross-reinnervated FDL muscles in which there was no evidence of significant self-reinnervation by FDL axons, >90% of the muscle fibers were small in cross-section, exhibited low myosin ATPase activity and very high oxidative activity, and thus resembled type S fibers of the normal FDL. All of the 41 motor units studied in these muscles were type S by physiological criteria, and they exhibited isometric twitch contraction times in the same range found for type S units in the normal FDL. In addition, almost half of the sample showed the posttetanic depression which is common in normal SOL motor units but unusual in normal FDL type S units. Several units were studied by the glycogen-depletion method, and all had muscle fibers with the typical appearance of type S fibers. In summary, cross-reinnervation of the heterogeneous FDL muscle by SOL motoneurons produced dramatic and virtually complete transformation of the FDL muscle into slow twitch, type S units.

The results were different when the slow-twitch SOL muscle was reinnervated by largely fast-twitch FDL motoneurons. The wet weight, tetanic tension, and gross appearance of these muscles were normal, but the isometric twitch contraction times were about half those of normal cat SOL muscles. Despite this speeding of contraction, the histochemical appearance of FDL-reinnervated SOL muscles was virtually unchanged from that of the normal SOL. More than 95% of the fibers in cross-reinnervated SOL muscles were histochemically type S, and an absence of stainable neutral fat was the only detectable difference from normal SOL fibers. All of the 23 individual motor units studied in these muscles were type S by physiological criteria (absence of the "sag" property and marked resistance to fatigue; see references 8, 9), despite having faster twitch contraction times than normal SOL units. In addition, only 2 of the 23 cross-reinnervated SOL units exhibited the post-tetanic twitch depression found in many normal SOL units. Several units studied by glycogen depletion had the same type S histochemical characteristics as the majority of the fiber population. In summary, cross-reinnervation of the SOL muscle by predominately fast-twitch motoneurons produced speeding of contraction as observed by others (e.g., reference 4), but did not produce conversion of motor units to the fast-twitch type, based on physiological or histochemical criteria.

Myosin Isozymes in Normal FDL Muscle Fibers

Antibody against myosin from the anterior latissimus dorsi (ALD) of the chicken was used to localize slow myosin in transverse sections of the same muscles that were used to obtain the physiological data. Antibodies specific for the difference peptides (Δ1 and Δ2) unique to the alkali 1 and the alkali 2 light chains, respectively, from chicken pectoralis myosin were used as markers to identify fast myosin. The specificity of these antibodies is described elsewhere (18, 19). In addition, by use of a "Western" immunoblot, anti-Δ1 and anti-Δ2 were shown to cross-react with the alkali light chains of cat myosin. The FDL consists primarily of fast muscle fibers having high myosin (alkali-stable) ATPase activity (Fig. 1*a*), although the level of activity is somewhat lower in FR than in FF units (see above). The remaining few slow fibers have little or no alkali-stable activity. All of the fast fibers, but none of the slow fibers, reacted with antibody specific for the alkali 1 light chain of fast myosin (anti-Δ1). However, the intensity of immunofluorescence varied; it was relatively low in FF fibers but high in FR fibers (Fig. 1*b*). Fast fibers also reacted with antibody specific for the alkali 2 light chain of fast myosin (anti-Δ2), but there was again a difference in the degree of fluorescence. FR fibers, which stained more intensely with anti-Δ1 (Fig. 1*c*), usually reacted less with anti-Δ2 (Fig. 1*d*) than did FF fibers.

The pattern of distribution of myosin isozymes was verified by observation of muscle fibers belonging to individual motor units identified by glycogen depletion. When an FDL motoneuron is stimulated, the muscle fibers supplied by it are depleted of glycogen, and this can be recognized by the absence of staining following the PAS reaction (see above). The myosin composition of the same "marked" fibers can be examined in sections that are serial to those stained by the PAS procedure. It is evident, in the FF muscle unit illustrated in Fig. 2, that all PAS-negative (depleted) fibers (Fig. 2*d*) had a high level of ATPase activity (Fig. 2*a*), a moderate response to anti-Δ1 (Fig. 2*b*), and a negative response to antibodies against slow myosin (anti-ALD; Fig. 2*c*). The same fibers also reacted moderately with anti-Δ2 (not illustrated; see Fig. 1*d*). A similarly depleted FR muscle unit (not illustrated) had moderately high ATPase activity, and reacted strongly with anti-Δ1 but weakly with anti-Δ2 (see Fig. 1*d*). Fibers which had low ATPase activity (Fig. 2*a*) reacted strongly with anti-ALD (Fig. 2*c*), but failed to react with either of the antibodies to fast myosin (Fig. 2*b*). These are presumed to correspond to slow-twitch type S units.

Based on observations of serial sections in which ATPase and oxidative enzymic activities were localized, all fast fibers shown in Figs. 1 and 2 were identified as belonging to either FF or FR units. The third type of fast-twitch fiber, type F(int), is relatively uncommon in the normal cat FDL,[2] and was originally designated "unclassified" by Burke et al. (9). These fibers have ATPase activity resembling FF fibers, but oxidative activity is higher. In a few specimens of FDL muscle, this type of fiber was observed after staining with anti-Δ1. Response to this antibody was moderate, as in the FF fiber.

Four categories of muscle units can therefore be recognized by combined enzyme cytochemical and immunocytochemical procedures. The pattern of response to antibodies against myosin is comparable to that observed in four categories of muscle fibers in the rat (see Table I in references 15 and 18). These observations support our earlier suggestion that white, intermediate, fast red, and slow red fibers in the rat are

equivalent to FF, F(int), FR, and S muscle units, respectively, in the cat.

Myosin Isozymes in Cross-reinnervated FDL Muscle Fibers

After cross-reinnervation by SOL motoneurons, the FDL was converted to a population of predominantly slow muscle fibers that failed to react with anti-Δ1 (Fig. 3*b*) or anti-Δ2 (Fig. 3*c*), but which did react with anti-ALD (Fig. 3*d*). The level of ATPase activity in the majority of the population was significantly lower (Fig. 3*a*) than in the normal muscle (compare with Figs. 1*a* and 2*a*). The few fast fibers that were present served as useful "controls" for evaluating the staining pattern of the majority of the fibers. These fibers had high ATPase and oxidative activity, and they had the same response to the three antibodies that was observed for FR fibers in the normal FDL. They also resembled the occasional fast fibers observed in the normal SOL (see Fig. 6). The small angular fibers that stained with both anit-Δ1 and anti-ALD in Fig. 3, *b* and *d*, probably represent chronically denervated fibers, in which dual staining with antibodies to fast and slow myosin would be expected (16). Small numbers of similar fibers occurred in muscles that had been self-reinnervated (not illustrated).

It is not certain whether the relatively few fast fibers present in the cross-reinnervated FDL muscles (Figs. 3 and 4) were innervated by SOL or by FDL motoneurons. In the muscles selected for the present study, electrical stimulation of the chronically disconnected FDL nerve produced no detectable mechanical response in the FDL muscle, but the presence of a small degree of self-reinnervation was impossible to rule out. As noted above, most of these fast fibers had type FR characteristics but in one case (not illustrated), there were a few fast fibers with a staining pattern similar to that of a typical FF unit. Cross-reinnervation was assurred, however, in the cases where individual, functionally-identified SOL motoneurons innervated FDL muscle units.[2] Fibers belonging to such cross-reinnervated units were identified by glycogen depletion (Fig. 4*b*). In the unit illustrated in Fig. 4, depleted fibers reacted with anti-slow myosin, but not with either of the antibodies against fast myosin (see Fig. 4*a*).

The alteration produced by cross-reinnervation was a result of the new nerve supply and not an effect of reinnervation itself. When the FDL was deliberately reinnervated by its own nerve, the distribution of myosin in the self-reinnervated muscle was comparable to that of the normal control muscle. The majority of the fibers (fast-twitch) reacted, to variable degrees, with anti-Δ1 (Fig. 5*a*), and a minority (slow-twitch) reacted with anti-ALD (Fig. 5*b*) as in the normal FDL (Fig. 2). That the procedure for glycogen depletion also had no obvious effect on the myosin composition is illustrated in the control FDL with a depleted FF muscle unit. Depleted (PAS-negative) fibers (Fig. 2*d*) had the same myosin composition as did unstimulated (PAS-positive) fibers in the same category (Fig. 2, *b* and *c*).

Myosin Isozymes in Normal SOL Muscle Fibers

The normal cat SOL consists almost entirely of slow-twitch fibers with low myosin ATPase activity (see the predominant fiber type in Fig. 6*a*). These fibers reacted with anti-ALD (Fig. 6*d*) but not with either anti-Δ1 (Fig. 6*b*) or anti-Δ2 (Fig. 6*c*). The few fast fibers present in the SOL were useful in evaluating the specificity of the immunocytochemical procedures; hence they were deliberately included in Fig. 6. These fibers had high ATPase activity (Fig. 6*a*), and they reacted strongly with anti-Δ1 (Fig. 6*b*) and weakly with anti-Δ2 (Fig. 6*c*), but not with anti-ALD (Fig. 6*d*). Thus, they resemble FR fibers present in the normal FDL.

Myosin Isozymes in Cross-reinnervated SOL Muscle Fibers

Cross-reinnervated SOL muscles showed no change in the pattern of ATPase activity, even though their contraction times were faster than those of normal or self-reinnervated SOL. The majority of the fibers (>95%) in SOL muscles completely cross-reinnervated by FDL motoneurons had low ATPase activity (Fig. 7*a*). As in the normal muscle, these fibers reacted with anti-ALD, but did not react with anti-Δ1 (Fig. 7*b*). The small minority of fast-twitch fibers present in these muscles (Fig. 7*a*) reacted strongly with anti-Δ1 (Fig. 7*b*), although a few reacted less intensely and had somewhat lower ATPase activity as well.

Our analysis of myosins in cross-reinnervated SOL muscles included two examples in which there had been significant, albeit inadvertent, self-reinnervation by SOL motoneurons. These provided a convenient comparison, within a single muscle, of the physiological and cytochemical characteristics of cross- and self-reinnervated SOL fibers. For example, the records in Fig. 8 show that the isometric twitch of a dually reinnervated SOL muscle, produced by stimulation of the foreign FDL nerve, was about twice as fast as that produced by stimulation of the original SOL nerve. Despite the difference in contraction times, there was no detectable difference in the pattern of ATPase activity between the two groups of reinnervated fibers in these muscles. With the exception of the few fast fibers typical of the normal SOL, the population consisted of slow-twitch fibers having low ATPase activity (not illustrated), a negative response to anti-Δ1 (Fig. 9*a*), and a positive response to anti-ALD (Fig. 9*b*). However, the response to anti-Δ2 was significantly different from that of the normal muscle. In addition to the usual small population of fast fibers (Fig. 9*a*), a large number of fibers which had previously reacted only with anti-ALD (Fig. 9*b*) now reacted with anti-Δ2 (Fig. 9*c*) as well. These fibers, moreover, had been successfully cross-reinnervated, and this was demonstrated by stimulation of the whole FDL nerve. All muscle fibers cross-reinnervated by FDL motoneurons were depleted of their glycogen and therefore were not stained by the PAS procedure (Fig. 9*d*). These are the same fibers that reacted with anti-Δ2 (Fig. 9*c*). Those fibers that were not depleted, and which were therefore PAS-positive, had been reinnervated by the original SOL nerve (see Fig. 8). The contrasting appearance of cross- and self-reinnervated fibers demonstrated, furthermore, that a positive or negative response to an antibody did not reflect a nonspecific reaction or a failure of the staining procedure.

Fig. 10 illustrates material from a dually reinnervated SOL muscle in which an individual, functionally identified FDL motoneuron had been stimulated. Its muscle unit exhibited physiological properties typical of the sample of cross-reinnervated motor units (type S, with relatively fast contraction time; see above). The glycogen-depletion method was used to identify the fibers belonging to this unit (Fig. 10*d*). As in the dually reinnervated muscle described above (Fig. 9), depleted as well as undepleted fibers had low ATPase activity (Fig.

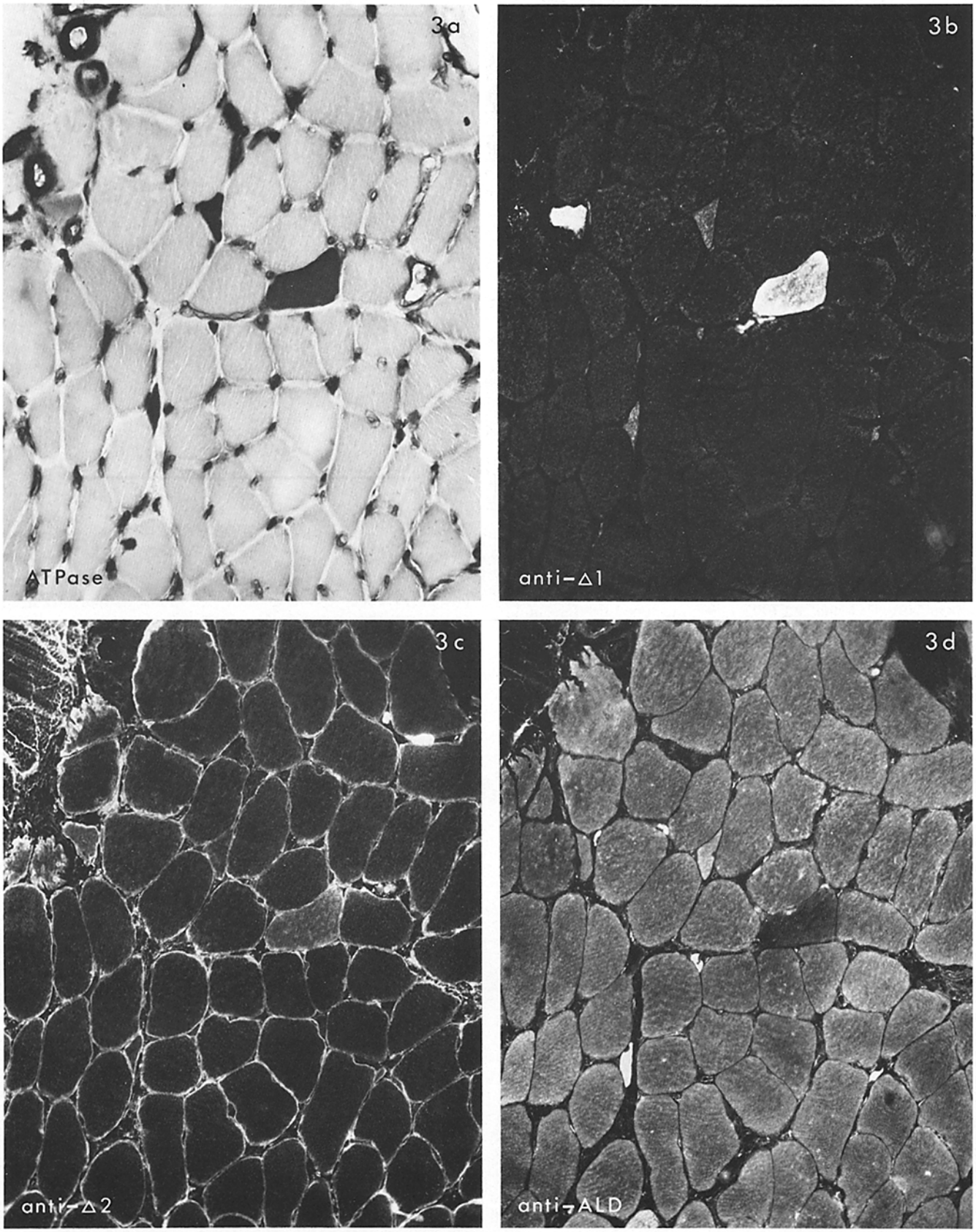

FIGURE 3 FDL, cross-reinnervated for 39 wk by SOL nerve (#3619). Serial transverse sections. One representative fast fiber has high ATPase activity (dark fiber in *a*), an intense response to anti-Δ1 (bright fiber in *b*), a weak response to anti-Δ2 (*c*), and negative response to anti-ALD (*d*). Small angular fibers having high ATPase activity which react with both anti-Δ1 and anti-ALD are probably denervated. Most fibers have low ATPase activity (*a*) and fail to react with either anti-Δ1 (*b*) or anti-Δ2 (*c*), but they do react with anti-ALD (*d*). Compare with the normal soleus in Fig. 6. × 260.

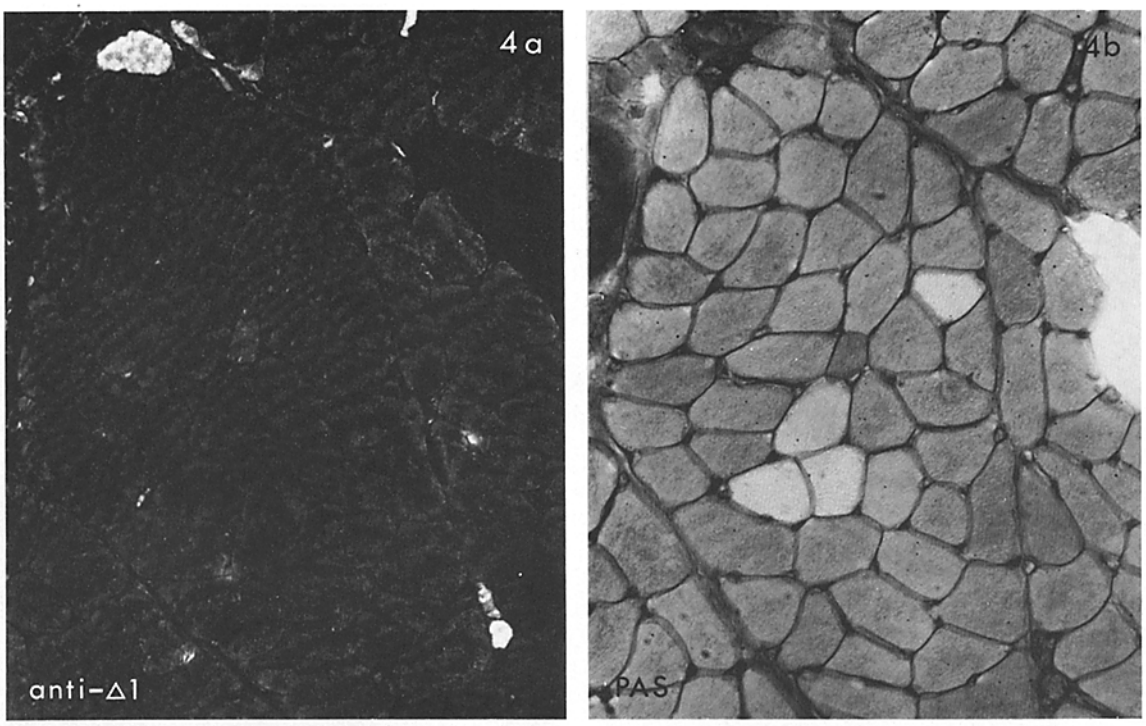

FIGURE 4 FDL, Cross-reinnervated by SOL nerve (#3619). This region of the same muscle as in Fig. 3 includes four glycogen-depleted type S fibers identified as innervated by a SOL motoneuron. Glycogen-depleted (PAS-negative) fibers (*b*) are representative of the majority, which have also been successfully cross-reinnervated. The majority of the population, including depleted fibers, react with anti-ALD (see Fig. 3*d*), but not with anti-Δ1 (*a*) or anti-Δ2 (see Fig. 3*c*). A fast fiber is included for comparison at the upper left (*a*). × 260.

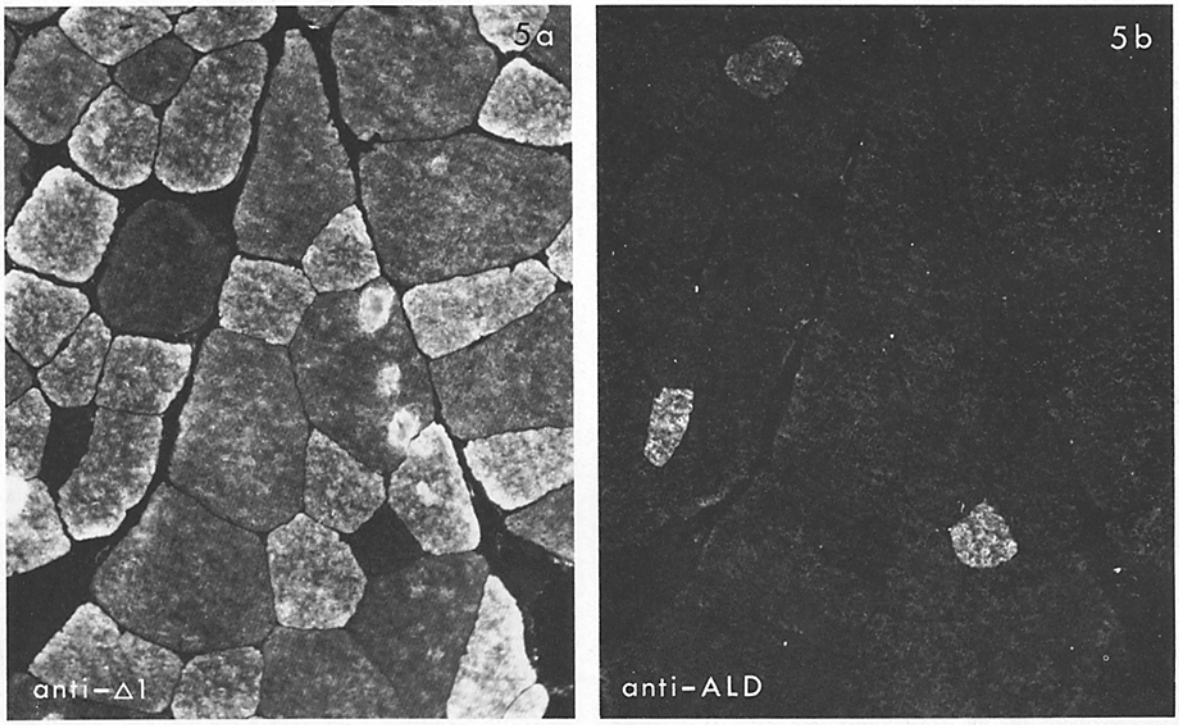

FIGURE 5 FDL, self-reinnervated for 15 wk (#3113). As in the normal cat (Figs. 1 and 2), the majority of the fibers (both FF and FR) react to variable degrees with anti-Δ1 (*a*), and a small minority (type S) react with anti-ALD (*b*). × 260.

10*a*), and a negative response to anti-Δ1 (Fig. 10*b*), but a positive response to anti-ALD. Also, as before, all depleted fibers (Fig. 10*d*) reacted with anti-Δ2 (Fig. 10*c*), but, in addition, other (undepleted) fibers, which were presumably innervated by other (unstimulated) FDL motoneurons, also reacted with anti-Δ2. It can be inferred that fibers not reacting with anti-Δ2 had been self-reinnervated by SOL motoneurons. The selective response to anti-Δ2 in cross-reinnervated

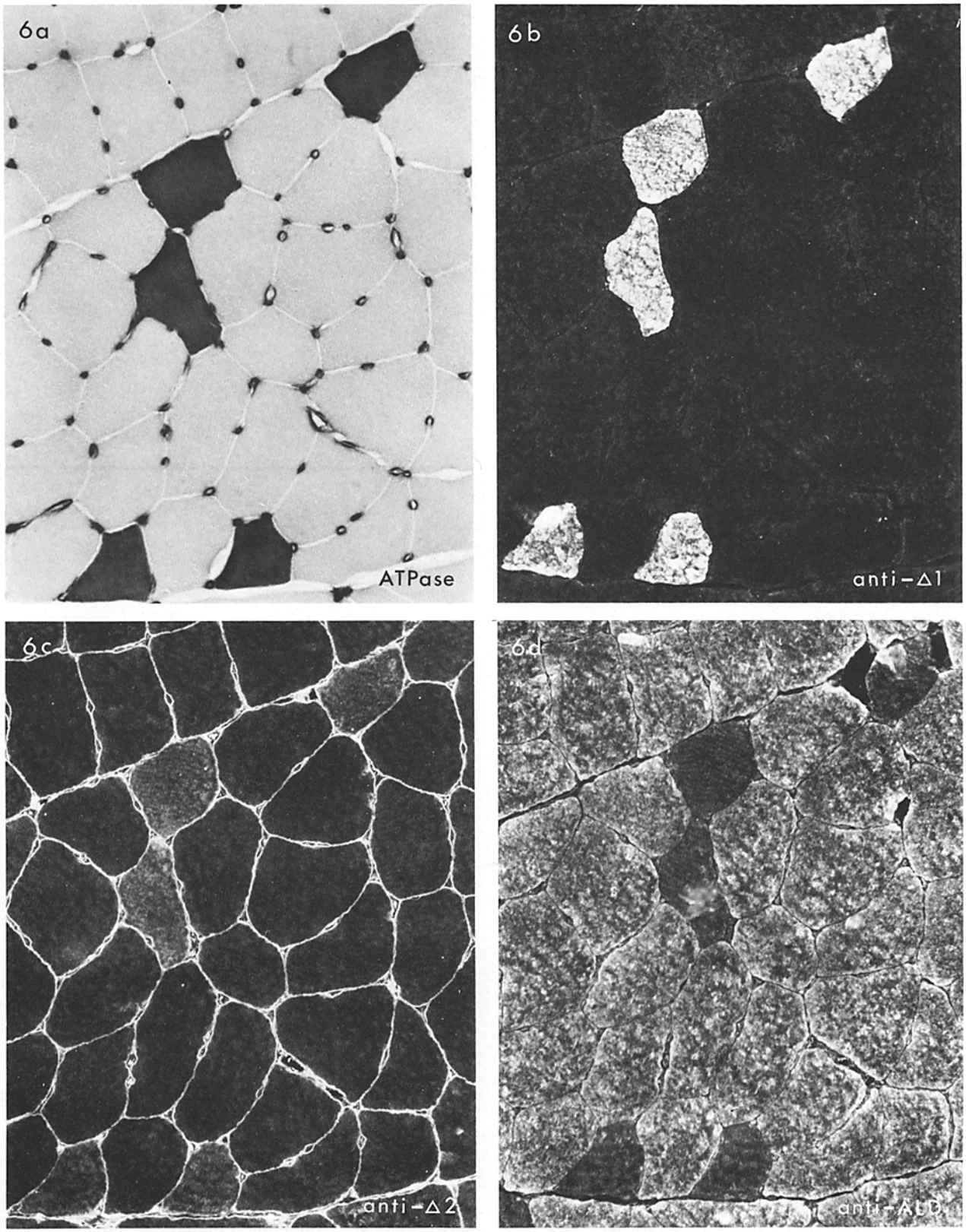

FIGURE 6 SOL, normal cat (#3333). Serial transverse sections. The large number of fast fibers (five) in this illustration is unusual for cat soleus, but they have been deliberately included for control purposes. They have high ATPase activity (dark fibers in *a*), and they react strongly with anti-Δ1 (bright fibers in *b*) and weakly with anti-Δ2 (*c*), but they fail to react with anti-ALD (*d*). Most of the fibers have low ATPase activity (*a*) and fail to react with either anti-Δ1 (*b*) or anti-Δ2 (*c*), but react strongly with anti-ALD (*d*). These are typical type S fibers (see also Figs. 1 and 2). Peripheral staining of otherwise unreactive fibers (*c*) may reflect cross-reactivity with some component of the muscle cell surface. × 260.

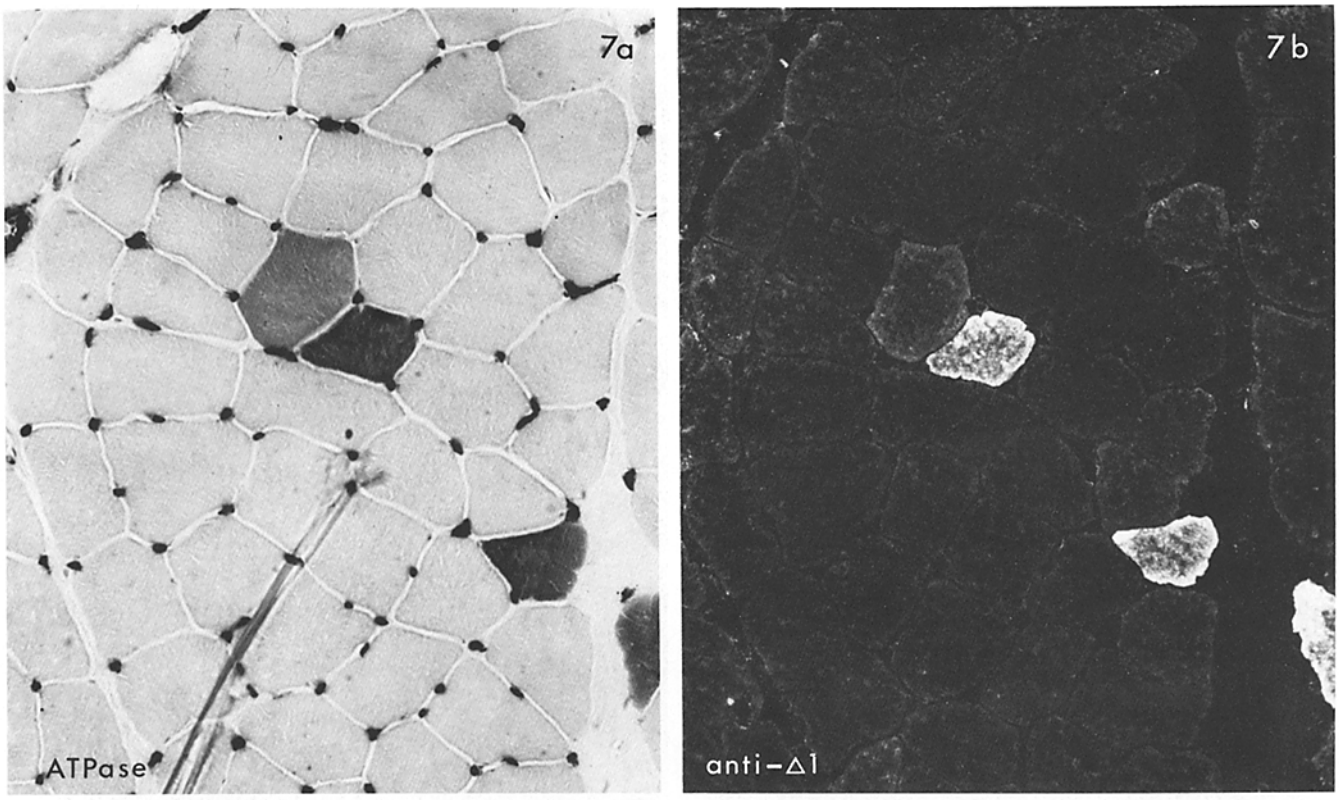

FIGURE 7 SOL, cross-reinnervated (completely) by FDL nerve for 64 wk (#3350). Serial transverse sections. Staining pattern is similar to that of the normal soleus (Fig. 6, *a* and *b*). Except for a few fibers with high ATPase activity (three in this illustration), the population consists of fibers with low ATPase activity (*a*) and no response to anti-Δ1 (*b*). × 260.

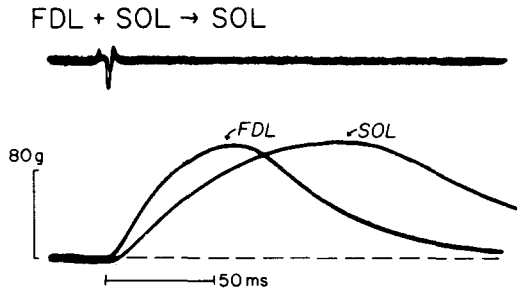

FIGURE 8 Isometric twitch response in SOL partly cross-reinnervated by FDL nerve and partly self-reinnervated for 64 wk. About half this muscle was inadvertently reinnervated by the original SOL nerve. Twitch contraction time for SOL muscle fibers that have been cross-reinnervated (*FDL*) is more than twice as fast as for the self-reinnervated fibers. Stimulation of both nerves together produced a twitch with shape and amplitude approximately equal to the algebraic addition of the FDL and SOL twitches, indicating that individual muscle fibers were not dually innervated. The same muscle was used to localize myosin (Fig. 9).

SOL fibers was not a consequence of the surgical procedure or of reinnervation *per se*. When the SOL was deliberately self-reinnervated entirely by its own nerve, there were no muscle fibers that reacted with anti-Δ2 except for the few fast (high ATPase) fibers which are normally present in this muscle (Fig. 11 *a*) and which are typically highly PAS-positive (Fig. 11 *b*). The positive response to anti-Δ2 also cannot be attributed to the prolonged stimulation used to achieve glycogen depletion, since fibers of a single motor unit which were depleted in the absence of cross-reinnervation (Fig. 11 *d*) did not react with anti-Δ2 (Fig. 11 *c*). Lack of response to this antibody did not reflect a failure of the procedure, since fast fibers in the same section showed the usual positive response (Fig. 11 *a*).

DISCUSSION

Identification of Distinctive Isozymes of Fast Myosin

Although the myosins within the population of fast fibers in a fast-twitch muscle are similar in their immunological cross-reactivity, there are significant differences. In the rat diaphragm, Gauthier and Lowey (18) showed a differential response to antibody specific for the alkali 2 light chain (anti-Δ2) of fast myosin by immunocytochemistry. Immunofluorescence was less intense in the "fast red" fiber than in other fast fibers, and a similar pattern was observed with antibody against the head (S1) region of myosin. They suggested that the myosin in this fiber was distinctive, but the possibility remained that the difference might reflect, instead, a variable distribution of the two fast myosin light chains, alkali 1 and 2.

The differential reactivity to immunological probes is strikingly illustrated by the intense response of FR fibers in the cat to antibodies against the alkali 1 light chain. This fiber appears to be equivalent to the "fast red" fiber present in the rat muscle (15, 18). The cat FF fiber, which is equivalent to the "fast white" fiber in the rat, stains only moderately with antibodies against either of the fast light chains. This is consistent with the presence of a distinctive isozyme in the

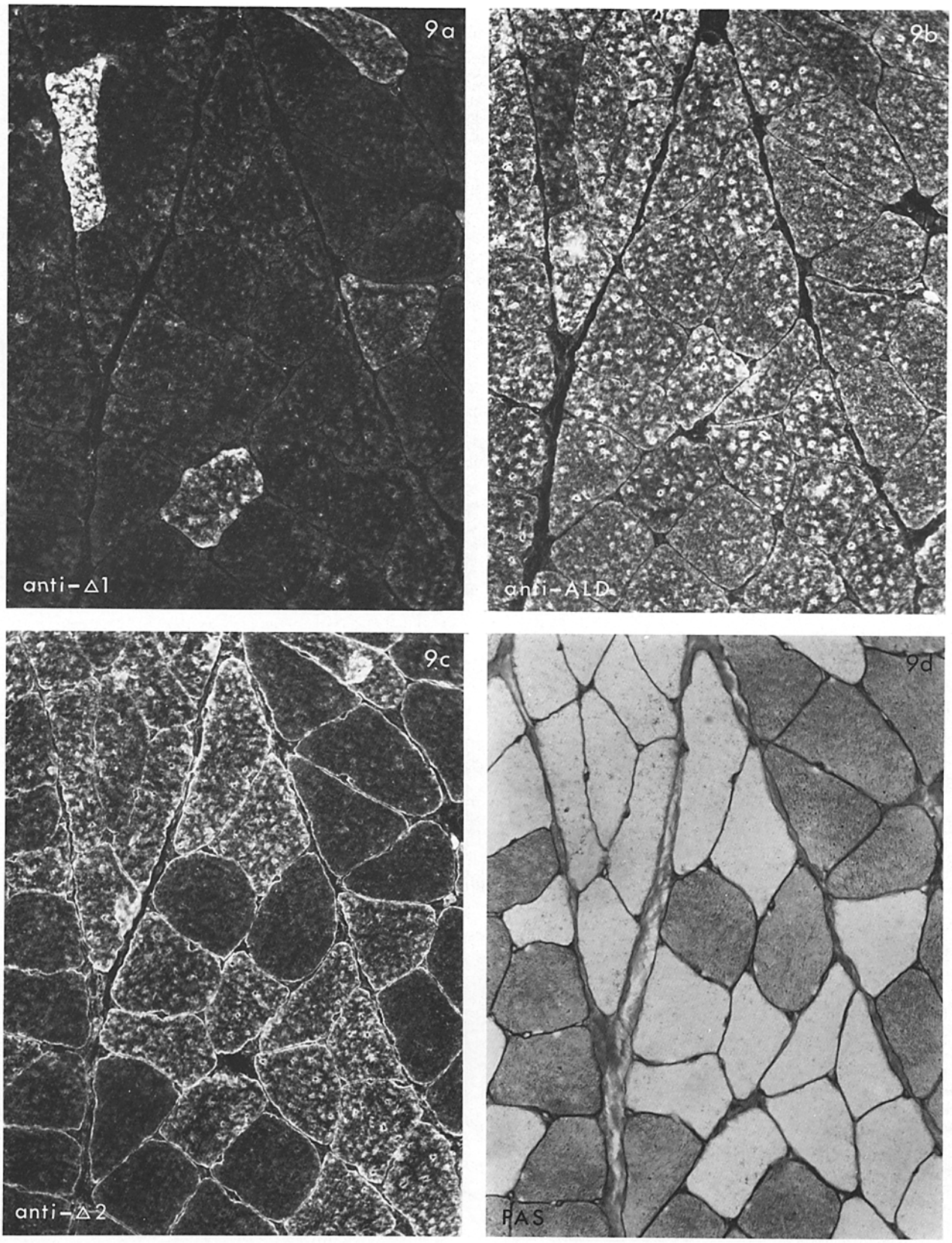

FIGURE 9 SOL, partially cross-reinnervated by FDL nerve and partially reinnervated by SOL nerve for 64 wk (#3329). Serial transverse sections. The same animal was used to record the twitch contraction times in Fig. 8. Fibers successfully cross-reinnervated by the FDL nerve are identified by glycogen depletion (*d*) following stimulation of the whole FDL nerve. Most fibers react with anti-ALD (*b*), but not with anti-Δ1 (*a*), as in the normal SOL. However, a substantial population of fibers react with anti-Δ2 (*c*), and comparison with *d* shows that the same fibers have been depleted of their glycogen (PAS-negative in *d*), which indicates successful cross-reinnervation by FDL motoneurons. PAS-positive (self-reinnervated) fibers (*d*) do not react with anti-Δ2 (*c*). × 260.

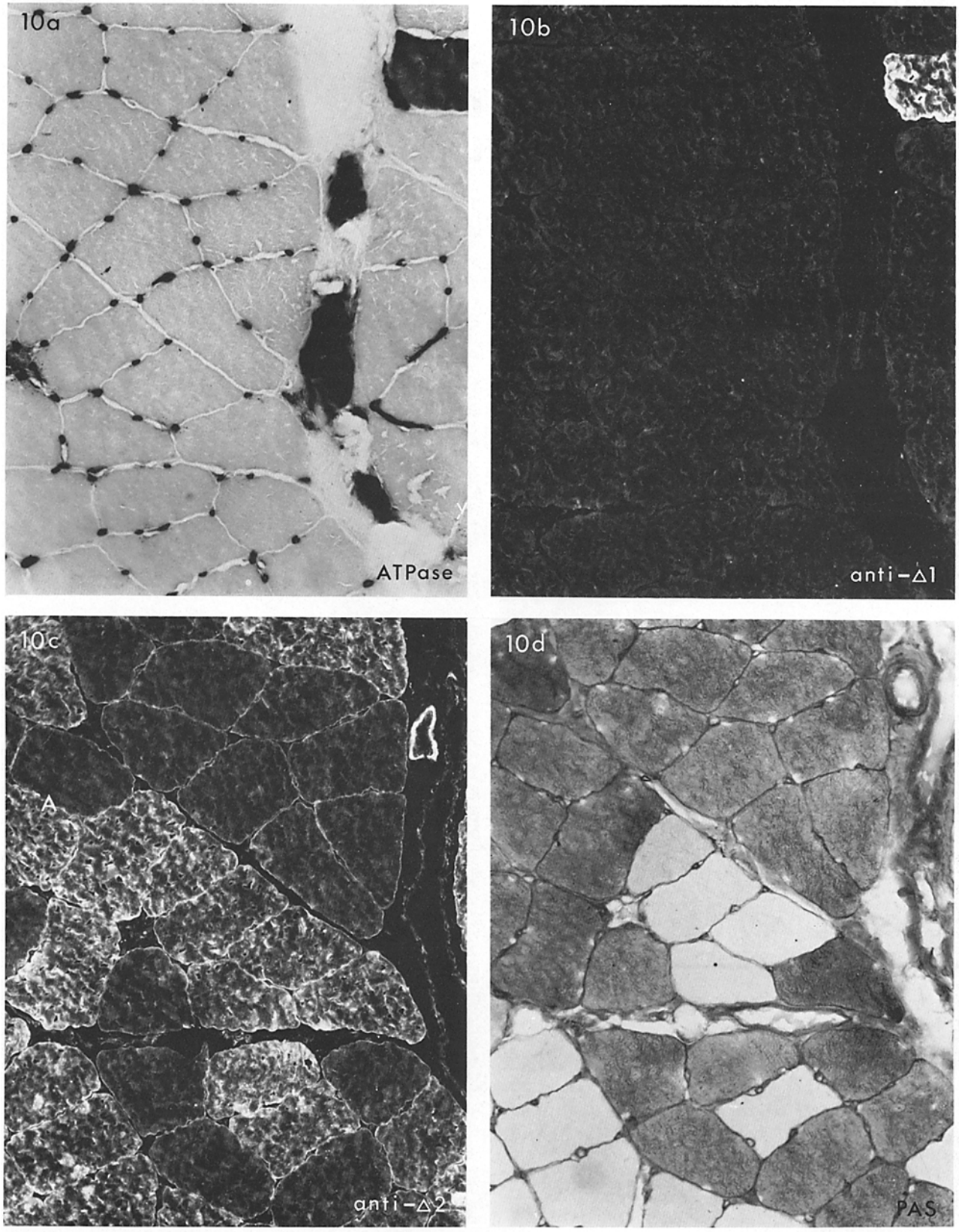

FIGURE 10 SOL, partially cross-reinnervated by FDL nerve and partially reinnervated by SOL nerve for 40 wk (#3266). A stimulated FDL-innervated type S motor unit is included. Serial transverse sections. Nearly all fibers (except for one at upper right) have low ATPase activity (*a*) and fail to react with anti-Δ1 (*b*). Many fibers react with anti-Δ2 (*c*). Fibers which have been depleted of their glycogen (PAS-negative in *d*) represent a single muscle unit supplied by an FDL motoneuron. These fibers all react with anti-Δ2. Additional fibers which are innervated by other (unstimulated) FDL motoneurons also react with anti-Δ2, but remain PAS-positive. Self-reinnervated fibers (also PAS-positive) do not react with anti-Δ2. Staining of the blood vessel at upper right (*c*) probably reflects cross-reactivity with vascular smooth muscle. × 260.

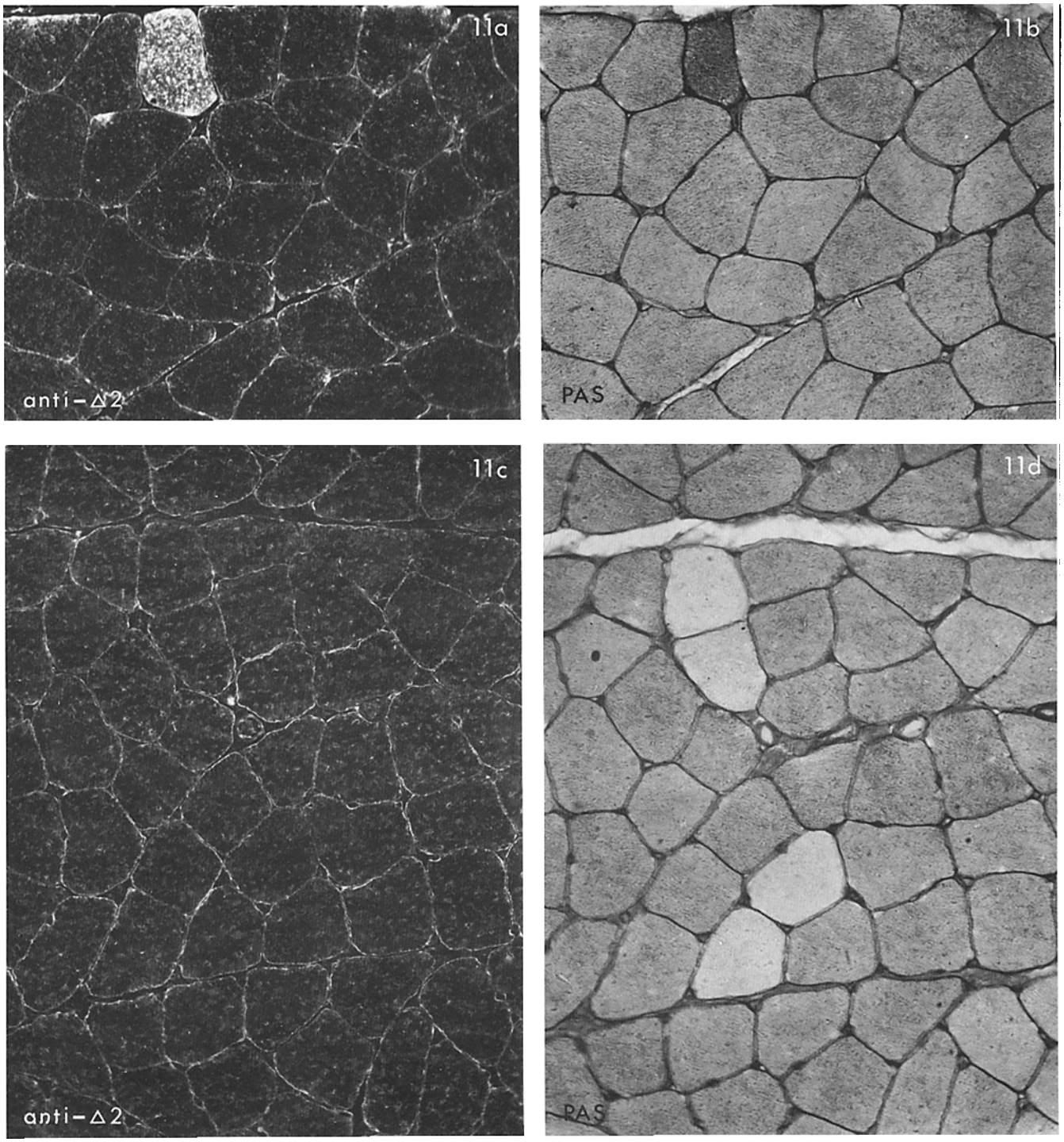

FIGURE 11 SOL, self-reinnervated for 44 wk (#3551), glycogen-depleted S unit included (*c* and *d*). Only an occasional fast fiber (upper left in *a* and *b*), which is characteristically PAS-positive (*b*), reacts with anti-Δ2 (a); all other fibers are unreactive. Glycogen-depleted (PAS-negative) fibers (*d*) likewise fail to react with anti-Δ2 (*c*). The single positive fiber (a) demonstrates that the negative response does not reflect failure of the staining procedure. Illustrations in *a*, *b*, *c*, and *d* are from the same section; regions included in *a* and *b* and in *c* and *d* are serial. × 260.

FR fiber. If the difference in staining of the two types of fast fibers were simply the result of different proportions of the two light chains, this would imply that very little of either fast light chain is present in the FF fiber, and this seems unlikely. Experiments with single muscle fibers indicate that both types of fast light chains are present in the same proportions in white and fast red fibers isolated from the tibialis anterior of the rabbit. (D. Pette and U. Seedorf, personal communication). There is no evidence to suggest that a particular light chain sequence would vary from one type of fiber to another, but this possibility cannot be excluded. However, there is evidence that the heavy chains are different in white and fast red fibers (D. Pette and U. Seedorf, personal communication). We therefore favor the explanation that the differential response to antibody against these light chains reflects a difference in the heavy chain in the two fast fibers of the rat and

cat as well. Such differences might well affect the light chain–heavy chain association such that antigenic determinants on the light chain are modified, resulting in altered reactivity with certain antibodies. A similar difference may also be apparent after alteration by cross-reinnervation (see below).

There is increasing evidence for the existence of distinctive isozymes of fast myosin. Chemical analysis of myosin from fast-twitch muscles of the rat suggests the existence of an isozyme that may either be unique to the rat or that may represent a "hybrid" molecule consisting of slow and fast heavy chains together with fast light chains (25). By comparing myosins from guinea pig muscles which consist primarily of either white or fast red fibers (tensor faciae latae and masseter), it has been shown that, although the light chains are similar, the heavy chain patterns of the two myosins are significantly different (11). In addition, antibodies against myosins from the tensor fasciae latae and masseter exhibit differential reactions with white and fast red fibers, respectively, in the extensor digitorum longus of the rat (32). In jaw muscles of the cat, there appears to be still another type of fast myosin having immunochemical properties, ATPase activity, and also a myosin light chain and heavy chain composition that differs from that of either the white or fast red fibers typical of limb muscles (33).

Influence of the Nerve Supply on Myosin Composition

The present observations are consistent with the long-standing notion that synthesis of an alternate type of myosin may be induced by changing the nerve supply (e.g., references 3, 4). The apparent completeness of transformation of FDL muscle units into type S subsequent to reinnervation by SOL motoneurons (7)[2] is now confirmed by immunocytochemical observations on the myosin composition of these cross-reinnervated units. In addition, a change from fast to slow forms of troponin has recently been observed in muscle fibers of the extensor digitorum longus of the rabbit following cross-reinnervation by the SOL muscle nerve (12).

In contrast to this complete conversion, our observations on the converse situation, namely reinnervation of the SOL muscle by FDL motoneurons, indicates that transformation is less complete, even after almost 15 months of postoperative survival. Although twitch contraction times were consistently speeded and the incidence of post-tetanic twitch depression decreased, FDL-innervated SOL muscle units remained type S by physiological criteria, and their appearance in conventional histochemical preparations was virtually unchanged from that of the normal or self-reinnervated SOL. The population of FDL motoneurons used to reinnervate the SOL does contain a small proportion of type S motoneurons,[3] but electrophysiological recordings from FDL motoneurons innervating SOL muscle units suggest that there was no selective reinnervation by type S cells. Thus, we conclude that the reinnervating motoneurons represented all of the motor unit types present in the normal FDL. It was therefore striking that all of the cross-reinnervated SOL muscle units studied individually had essentially the same physiological and cytochemical characteristics, namely those of type S units. However, we have shown here that the myosin of FDL-reinnervated SOL muscle units was indeed altered by cross-reinnervation. Unlike the normal or self-reinnervated SOL, cross-reinnervated SOL fibers reacted with antibody specific for the alkali a light chain of fast myosin anti-Δ2 (Figs. 9 and 10), and they also reacted with anti-slow myosin as in the normal muscle. The distinctive immunocytochemical properties of cross-reinnervated SOL fibers, together with their unaltered histochemical appearance, indicate that the newly synthesized myosin is not equivalent to that normally present in either the FDL or SOL.

It is possible that a different isozyme of myosin is synthesized by the altered SOL muscle. Although successfully cross-reinnervated SOL fibers do not react with antibody specific for the alkali 1 light chain of fast myosin, this does not necessarily imply that this light chain is absent. Its presence has been demonstrated by SDS gel electrophoresis in cross-reinnervated cat SOL (40), and the same fast light chain has been demonstrated by two-dimensional PAGE in cross-reinnervated rabbit SOL (34, 37). In addition, a distinctive myosin has been observed in cross-reinnervated SOL muscles of the rat (25) and rabbit (37). The negative response to antibody against the alakli 1 light chain, which was observed in the present study, probably reflects a change in the myosin heavy chain which, by virtue of its association with the light chain, may alter its immunological reactivity. However, until biochemical analysis of the light and heavy chains of cross-reinnervated cat muscles is undertaken, this interpetation of the antibody response must be considered speculative.

Functional Implications of the Type of Innervation

It is difficult to discuss the present results in relation to other studies in any detail, because of differences in species and methods. However, the apparent asymmetry in the effect of cross-reinnervation of the fast digit flexors (FDL and its neighbor, flexor hallucis longus [FHL]) and SOL in the cat has been found by others. Dubowitz (13), for example, noted that SOL-innervated FHL was largely "converted" to a slow muscle by histochemical criteria but that conversion of FHL-innervated SOL was less consistent. Buller and Mommaerts and their co-workers (5, 30) found a difference in response by these two muscles in biochemical studies of myosin ATPase activity. An asymmetrical influence of cross-reinnervation on cat FHL and SOL muscles was also observed after spinal cord section (20). On the other hand, Guth et al. (22) and, more recently, Lewis et al. (28), found substantial populations of fast fibers with high ATPase activity in FHL–reinnervated SOL muscles of the cat. Bagust et al. (1) also observed significant numbers of individual motor units with twitch contraction times shorter than any found in the present work. The reason for such discordant results is not clear.

The present results are consistent with prior data in that the isometric twitch contraction times of cross-reinnervated muscle units are altered in the directions appropriate to the identity of the innervating motoneurons, that is, FDL muscle units are slowed by SOL innervation and SOL muscle units speeded by FDL innervation. However, the role of specific types of myosin in this physiological transformation remains unclear. The well known relationship between speed of contraction and myosin ATPase activity (2) has focused attention on myosin, but this does not imply that other components of a fiber may not be of importance. For example, there is a

[3] Dum, R. P., M. J. O'Donovan, J. Toop, R. E. Burke, and P. Tsairis, manuscript in preparation.

shift from slow to fast forms of troponin in muscle fibers of the rabbit SOL following cross-reinnervation by axons in the lateral popliteal nerve (12). In addition, the calcium activation properties of rabbit SOL fibers are altered by cross-reinnervation (34). There is evidence also that the molecular composition of the sarcoplasmic membrane systems has a role in determining the speed of contraction (23). Nevertheless, as we have shown, the myosin composition is changed in fibers whose speed of contraction has been altered by cross-reinnervation. Recent evidence suggests that functional differences reflect the myosin heavy chain, since the light chain appears to have little effect on ATPase activity (39). In experiments with hybrid myosin molecules, the ATPase activity was determined primarily by the type of heavy chain present, and the particular light chain had no influence on the kinetic properties (38). It is therefore reasonable to expect that the change in physiological properties observed in the cross-reinnervated SOL might reflect a difference in the heavy chain rather than the light chain.

The lack of response by cross-reinnervated FDL fibers to antibodies against the light chains of fast myosin and the strong response to antibodies directed against slow myosin support the conclusion that fast muscle fibers can be completely converted to the slow-twitch type by reinnervation with slow-twitch motoneurons. Whether this conversion is accomplished by chemical "trophic" substances or by the markedly increased functional demand placed on the cross-reinnervated FDL (31) remains unclear. The positive response, by cross-reinnervated SOL fibers, to antibody against one of the fast light chains permits identification of a change in myosin to match the alteration in twitch contraction times. The apparent subtlety of the cross-reinnervation effect in the cat SOL seems to indicate the existence of some "resistance" by SOL fibers to reinnervation by a largely fast-twitch population of motoneurons, despite a marked reduction in the functional demand on the cross-reinnervated SOL (31).[4] This "resistance" is not necessarily a characteristic of all slow twitch muscle fibers, however, since self-reinnervation of heterogeneous muscles (27) produces often dramatic restructuring of the histochemical mosaic, which implies that many originally slow-twitch fibers must have been converted completely to fast-twitch types, and vice versa.

In conclusion, new information has been obtained by combining immunocytochemical with physiological data obtained from the same skeletal muscle fibers. Differences in the antigenic properties of normal FF and FR fibers provide evidence that the myosins of these two types of fast-twitch fibers are distinctive. Secondly, it has been possible to identify changes in cross-reinnervated SOL fibers that were not evident by conventional histochemical procedures. The distinctive properties of these SOL fibers suggest that synthesis of a new myosin isozyme may be induced by altering the pattern of innervation, but that the myosin is not completely respecified to that characteristic of fast-twitch fiber types. The present work has demonstrated these points in individual motor units, in which it was possible to study physiological, histochemical, and immunochemical properties of muscle fibers innervated by defined species of motoneurons.

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[4] O'Donovan, M. J., M. J. Pinter, R. P. Dum, and R. E. Burke, manuscript in preparation.

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