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MOTOR UNITS AND IMMUNOHISTOCHEMISTRY OF CAT SOLEUS MUSCLE AFTER LONG PERIODS OF CROSS-REINNERVATION

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SUMMARY

1. Cat soleus (slow twitch) was cross-reinnervated with nerve to flexor hallucis or digitorum longus muscle (fast twitch). More than 3 years later motor unit isometric contractions and muscle immunohistochemistry and histochemistry were investigated. All muscles differed from normal fast or slow muscle.

2. The motor units could be divided into two groups: one with fast twitches and low tetanic tension, the other with slow twitches and high tension. This is the reverse of the relation between motor unit twitch time and tetanic tension in normal muscle (fast or slow).

3. Motor unit twitch time to peak decreased with axonal conduction velocity, as in normal muscle, but so did tetanic tension, which is abnormal.

4. Twitch–tetanus ratio increased with twitch time to peak in the group of slow units but not in the fast group (although the range of ratios was as great).

5. A tetanus depressed the twitch tension of slow motor units and potentiated fast ones as in normal muscle but the potentiation was often accompanied by an abnormal prolongation of the twitch.

6. The mean conduction velocity of axons was slightly higher than at 6 months' reinnervation but below the normal value for fast muscle.

7. Antibody to slow myosin was bound strongly to Type I fibres but not to Type II fibres, confirming the histochemical division of fibres into Types I and II.

8. More than 95% of the fibres were oxidative, with Type I predominating over Type IIa in the ratio of about 2:1.

9. The higher tension of the slow motor units was the result of three factors: the number of fibres per motor unit was at least three times that in the fast; Type I fibres had cross-sectional areas little less than Type II (a and b together) and were estimated to develop more tension per unit area. All three findings were different from those in normal fast muscle.

10. One flexor hallucis longus muscle was self-reinnervated and examined histochemically. This muscle was abnormal in that a large majority of the fibres were Type I.

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INTRODUCTION

After a 6 month period of cross-reinnervation, some of the normal relationships between properties of axon and muscle fibres of motor units are re-established as in normal muscle, but many are not. Most notably, there is incomplete conversion of motor unit twitch contraction times in slow twitch (soleus) muscle reinnervated by the nerve from a fast twitch muscle. Another abnormality is the absence or reversal of the normal association of fast twitch properties of a motor unit with a large tetanic tension (Bagust, Lewis & Westerman, 1981).

We have, therefore, examined soleus muscles cross-reinnervated by nerves to flexor hallucis longus or flexor digitorum longus for periods of at least three years, to test whether any further motor unit reorganization had occurred. We have found that the pattern of motor unit properties were more consistent than after a shorter period of reinnervation, but the pattern was even further removed from that seen in any normal muscles. A preliminary report of this work has been presented (Lewis, Rowlerson & Webb, 1980).

METHODS

The nerve cross-union operations were performed under aseptic conditions on four cats supplied by the C.D.E., Porton Down, with body weights of 1·6 kg (S.D. 0·64; range 1·0–2·3) and aged about 4 months. The animals were anaesthetized with an alphaxalone–alphadolone mixture (Saffan; Glaxovet Ltd) injected into a forearm vein and maintained by halothane and penthrane in 70% nitrous oxide and 30% oxygen. The nerves to soleus and flexor digitorum longus were cross-united in two cats; soleus and flexor hallucis longus nerves were crossed in two others (flexor hallucis longus is the larger of this pair of fast muscles). In the opposite limbs the corresponding nerves were cut and self-united. Streptomycin sulphate (0·5 g) and long-acting penicillin (0·5 ml. Propen, Glaxovet) were given, and recovery of the animals was uneventful. The final experiments were performed 3 years 4 months (S.D. 0·2 months) after the initial operations under anaesthesia with pentobarbitone sodium (Sagatal; May & Baker): 40 mg/kg I.P. initially, supplemented I.V. as necessary. Body weights were 2·4 kg (S.D. 0·14) for the two female cats and 3·1 kg (S.D. 0·14) for the two males.

Motor units were isolated functionally from cross-reinnervated soleus muscles by splitting ventral roots, and properties were recorded as described by Bagust *et al.* (1981). Briefly, muscles were covered with liquid paraffin B.P.C. maintained at 37 °C. Length was adjusted to give maximal twitch tension, and held constant for all subsequent measurements. Contractions were analysed by a small digital computer (see Fig. 1). Whole muscle contractions were monitored at frequent intervals between motor unit observations. The one experiment in which tetanic tension fell by 10% from the initial value was terminated. In the others tetanic tension fell by less than 5% and twitch tension by 0–19%. At the end of the experiment, the muscles were removed, blotted dry for weighing, and frozen in melting isopentane at approximately the length used for recording motor units. One of the contralateral self-reinnervated muscles was also removed and frozen. The muscles were stored in dry ice for future histochemical and immunohistochemical staining. Cryostat sections (8 μm) were stained for myofibrillar ATPase activity with acid (pH 4·5 and 4·6) and alkaline (pH 9·4) preincubation (Brooke & Kaiser, 1970), for fluoresceine labelled antibody against the myosin of cat soleus (Lewis, Parry & Rowlerson, 1982). The preparation and specificity of this antiserum are described in greater detail by Rowlerson, Pope, Murray, Weeds & Whalen (1981).

A sample of 150–200 muscle fibres were typed from photographs of serial sections stained by the four histochemical methods for the two muscles reinnervated by flexor hallucis longus nerve and the self-reinnervated flexor hallucis longus. Only a few percent of these could not be classified as Type I, Type IIa or Type IIb. Areas were measured by tracing around camera lucida images of fibres stained for ATPase (alkaline pre-incubation) with a pen the position of which was digitized and analysed in a small computer programmed by T. J. Biscoe. Total muscle cross-sectional areas were measured from tracings of low power projections of the slides.

There was fibre type grouping (see Pls. 1 and 2) with considerable variation across the muscles. Estimates of the proportions of fibre types had, therefore, to be made from counts which spanned the whole of the section. This was done by scanning the slides at 450 or 270× magnification at about 0·5 or 1 mm intervals. All fibres were classified as light or dark in each field giving a total of 2100–2600 fibres per muscle. It was not thought necessary to type every fibre from serial sections in this procedure because of the clear cut results found with the smaller samples analysed more rigorously. So the proportions of dark and light fibres were counted for sections stained for ATPase with pre-incubation at pH 9·4 (dark = Type IIa+IIb; light = Type I) and at pH 4·6 (dark = Type

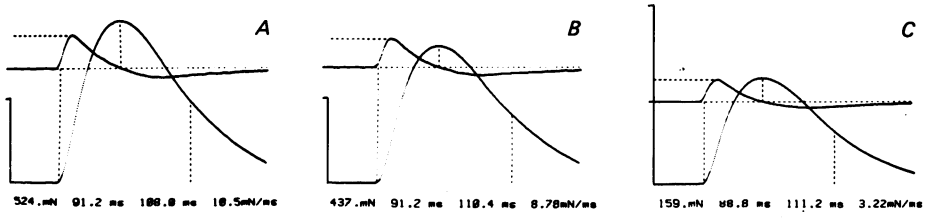

Fig. 1. Isometric twitch myograms from a soleus muscle cross-reinnervated by both its own nerve (*A*) and the nerve from flexor digitorum longus (*B*). Two large motor units contributed almost all the tension in *B*, and *C* is the twitch of one of these. The continuous lines in each trace are tension (below) and its first time derivative (above). The vertical lines (on the left of each myogram) are tension calibration pulses of 260 mN. Interrupted vertical lines indicate computer estimates of (i) latency, (ii) time-to-peak and peak tension and (iii) time-to-half-relaxation and the horizontal line the maximum rate of development of tension. Numerical values of the last four estimates are indicated below each myogram. (Note that the 'time constant' of the differential has been increased with the time to peak; the derivative at computer sample (t) was calculated from tension at $(t+n)-$ tension at $(t-n)$ where n was about one-twentieth of the number of samples between the latency and the peak.) The figures under each trace are part of the experimental record.

I+IIb; light = Type IIa). From the two sets of measurements proportions of all three types could be calculated. Sections were cut near the middle of the muscles where the greatest number of fibres are sampled. In one muscle a second set of sections was made in a region separated by one third of the muscle length in order to assess the adequacy of sampling. Estimates of the proportions of fibre types in the two regions were very close: Types I, IIa & IIb proportions were 62, 32 and 6·7% in one and 60, 35 and 5·5% in the other.

RESULTS

The two soleus muscles cross-reinnervated by flexor hallucis longus nerve received little (< 0·5%) or no contaminating reinnervation from soleus nerve. The other two muscles cross-reinnervated by flexor digitorum longus nerve could also be excited by stimulating soleus nerve indicating substantial contamination by self-reinnervation. One of the latter muscles was examined first in this series of experiments, and provided a clear indication of unexpected motor unit reorganization as seen from the isometric twitch myograms of Fig. 1. It can be seen that the twitch elicited from flexor digitorum longus nerve (Fig. 1*B*) was as slow as that from soleus nerve (Fig. 1*A*). The greater part of the tension elicited from flexor digitorum longus nerve was contributed by two large motor units, one of which is illustrated by Fig. 1 *C*. Both of these two large motor units were slow contracting. The small amount of residual tension was from an unknown number of fast contracting motor units, but their contribution was too small to have an appreciable effect on the total muscle twitch.

The myograms of the solei cross-reinnervated by flexor hallucis longus nerve (Fig. 2*A*) were similar to those of muscles with a shorter period of cross-reinnervation (e.g. Buller & Lewis, 1965) except that the relatively slow relaxation of this cross-reinnervated muscle was even more marked than at 6 months. This prolonged relaxation could have been produced by an increase in the ratio of slow to fast fibres

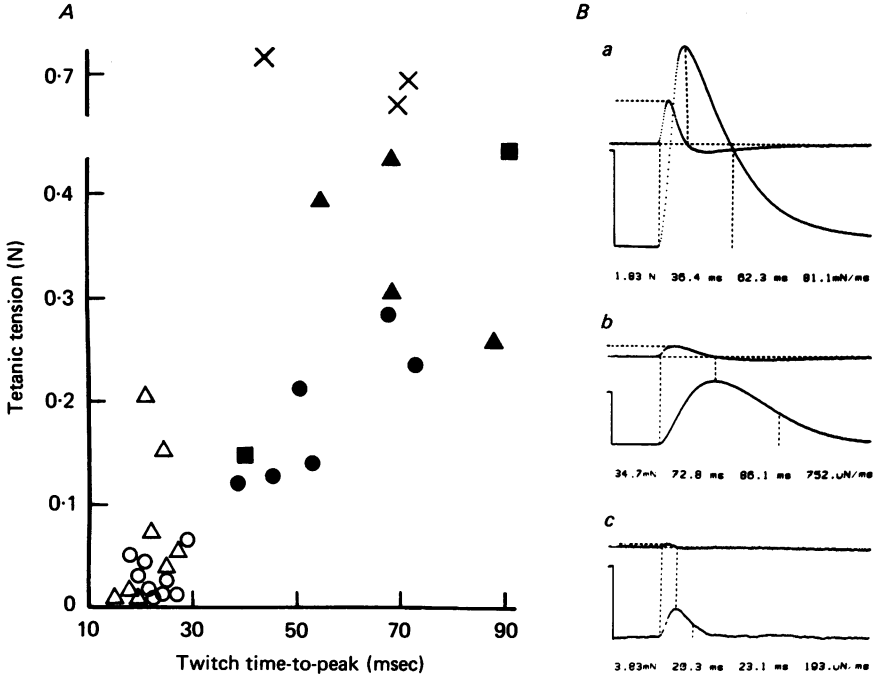

Fig. 2. *B*, isometric twitch myograms from (*a*) soleus muscle cross-reinnervated only by flexor hallucis longus nerve and (*b*, *c*) two of its motor units. Records as in Fig. 1 except calibration pulses are 100 mN (*a*), 30 mN (*b*) and 10 mN (*c*). *A*, relation between twitch time-to-peak and tetanic tension of individual motor units of reinnervated soleus muscles. Circles and triangles indicate motor units from two muscles cross-reinnervated by flexor hallucis longus nerve. Two other solei had mixed reinnervation, and those motor units with flexor digitorum axons are indicated by squares and those with soleus axons by crosses. Open symbols have been used for fast and filled symbols for slow motor units (see text). The relationships were significant for motor units in both flexor hallucis longus reinnervated muscles (circles: $r = -0{\cdot}94$, $n = 15$, $P < 0{\cdot}001$; triangles: $r = 0{\cdot}85$, $n = 13$, $P < 0{\cdot}001$).

similar to, but less marked than, that seen in the muscle of Fig. 1. As predicted, individual motor units from the two flexor hallucis longus reinnervated muscles showed the same trend as above: the slow motor units were large (Fig. 2*B*) and the fast ones were small (Fig. 2*C*).

The twitch times-to-peak and tetanic tensions of all the motor units are plotted in the graph of Fig. 2, and showed significant correlations (see legend to Fig. 2 for statistics), both for individual muscles and for the whole population. There appeared to be a division into two groups, one of large, slow contracting motor units (indicated by filled symbols in Fig. 2 and subsequent figures) and the other of smaller, fast

contracting units (open symbols). In the following paragraphs the motor units reinnervated by flexor hallucis and digitorum longus nerves will be described together whenever possible (but indicated by separate symbols as in Fig. 2). One exception is where tension is expressed as a percentage of whole muscle tension when only flexor hallucis longus motor units will be considered. Tension normalization prevents valid comparison between muscles with different numbers of axons: flexor hallucis longus has nearly twice the number of flexor digitorum longus.

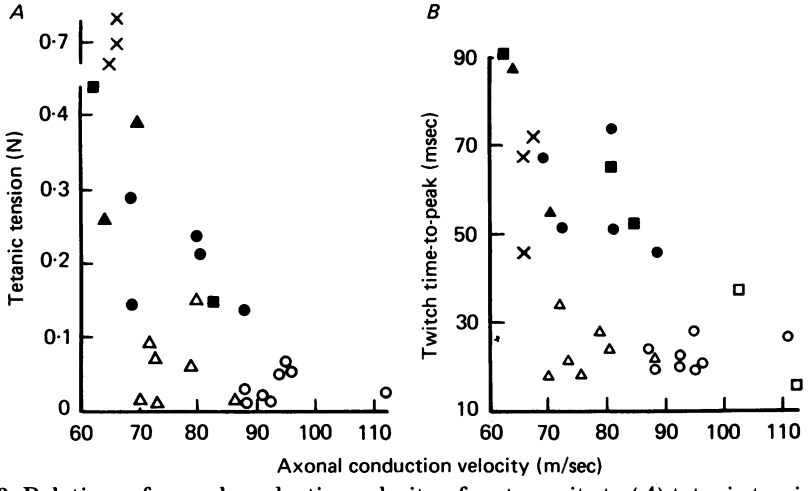

Fig. 3. Relations of axonal conduction velocity of motor units to (*A*) tetanic tension and (*B*) twitch time-to-peak. Symbols as in Fig. 2. Regressions were tested for the flexor hallucis longus innervated motor units. In *A* the regressions on conduction velocity were significant for one muscle (circles: $r = -0{\cdot}76$, $n = 13$, $P < 0{\cdot}01$) and for the total population ($r = -0{\cdot}44$, $P < 0{\cdot}05$) but not the other (triangles: $r = -0{\cdot}42$, $n = 9$). In *B* significant regressions were found for both muscles (circles: $r = -0{\cdot}72$, $n = 13$, $P < 0{\cdot}01$; triangles: $r = -0{\cdot}67$, $n = 9$, $P = 0{\cdot}05$) and the whole population ($r = 0{\cdot}49$, $P < 0{\cdot}05$). (Not all motor units had determinations made of axonal conduction velocity so numbers are smaller than in Fig. 2.)

Three motor units innervated by soleus nerve in the muscle of Fig. 1 were isolated: all were slow (time-to-peak 46–88 msec), produced large tensions (673–730 mN) and had low conduction velocities (65–66 m/sec). These have been indicated by crosses in appropriate figures, and are seen to fit the trends established for the cross-reinnervated motor units, except that the soleus units had tensions greater than the slow group of motor units innervated by the long flexor nerves.

The relationships of motor unit tension and twitch time-to-peak with axonal conduction velocity are shown in Fig. 3 and indicate that it was the tetanic tension of the motor units that was abnormal. Twitch time-to-peak decreased with increasing conduction velocity as in normal muscles (Fig. 3*B*). Tetanic tension also decreased with increasing velocity (Fig. 3*A*) which was the reverse of the trend seen either in normal soleus or any fast muscle.

Conduction velocity of flexor hallucis longus axons was $82{\cdot}6 \pm 11{\cdot}8$ m/sec (mean $\pm$ S.D., $n = 22$) which was slightly faster than that found after 6 months' cross union in flexor hallucis longus nerve ($74{\cdot}2 \pm 10{\cdot}1$ m/sec, $n = 38$; $t = 2{\cdot}9$, $P < 0{\cdot}01$) and possibly

in flexor digitorum longus nerve (78·5 ± 8·9 m/sec, $n = 62$; $t = 1·6$, $P = 0·1$), but slower than in normal flexor digitorum longus (94·2 ± 9·4 m/sec, $n = 205$; $t = 5·4$, $P < 0·001$). The conduction velocity of soleus nerve self-united for three years (67·9 ± 11·0 m/sec, $n = 73$ (Lewis & Owens, 1979) was less than that of the long-term cross-united flexor hallucis longus axons ($t = 5·5$, $P < 0·001$).

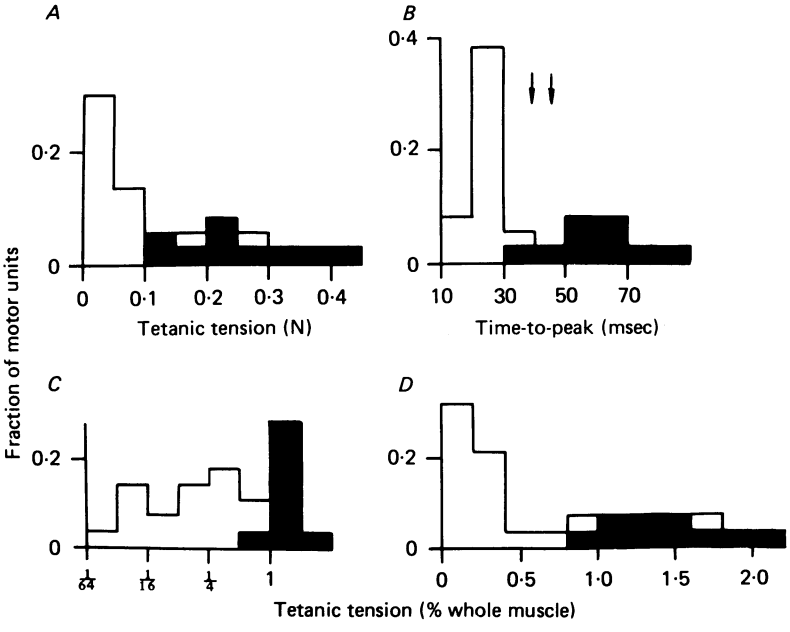

Fig. 4. Distributions of motor unit properties in two soleus muscles cross-reinnervated by flexor hallucis longus nerve (open and filled columns represent fast and slow motor units as in previous figures). In *A* the absolute tetanic tensions are plotted, in *B* the twitch times-to-peak and in *C*, *D* tetanic tension expressed as a percentage of whole muscle tension (recorded soon before and after the motor unit), *C* is plotted with a logarithmic scale to show clearly the distribution of small motor units. The arrows in *B* indicate the whole muscle twitch time-to-peak. (Motor units cross-reinnervated by flexor digitorum longus nerve are not shown, because they were not isolated randomly.)

In Fig. 4 are the distributions of tetanic tensions and twitch times-to-peak of flexor hallucis longus innervated motor units. (It is impossible to include the flexor digitorum longus innervated motor units because we biased the samples by trying to isolate the slow motor units.) The distribution of motor unit tetanic tensions is illustrated in Fig. 4*A*. The largest motor unit in the muscles reinnervated by flexor hallucis longus nerves developed 435 mN tension. The greatest tetanic tension in flexor digitorum longus reinnervated muscles was similar, being 440 mN: the motor unit shown in Fig. 1*C*. Normal and self-reinnervated soleus in cats of this size would be expected to have motor units developing up to 350 mN. The smallest motor unit developed 5·6 mN which is less than in normal soleus (estimated to be about 20 mN) but larger than in self-reinnervated soleus (0·64 mN: Lewis & Owens, 1979).

In Fig. 4*C* and *D* tetanic tension has been calculated as a percentage of the tension of the whole muscle, and in Fig. 4*D* there is the bimodal distribution indicating the

division into two groups of motor units. Bimodality was not seen in muscles cross-reinnervated for a shorter period (Bagust *et al.* 1981) although the distribution of tensions was skewed. The distribution of small motor units is seen most clearly in Fig. 4*C* which has logarithmically scaled bins. Normal soleus has a symmetrical distribution of tensions, but this is lost after self-reinnervation when there is a predominance of small motor units, with a few abnormally large ones.

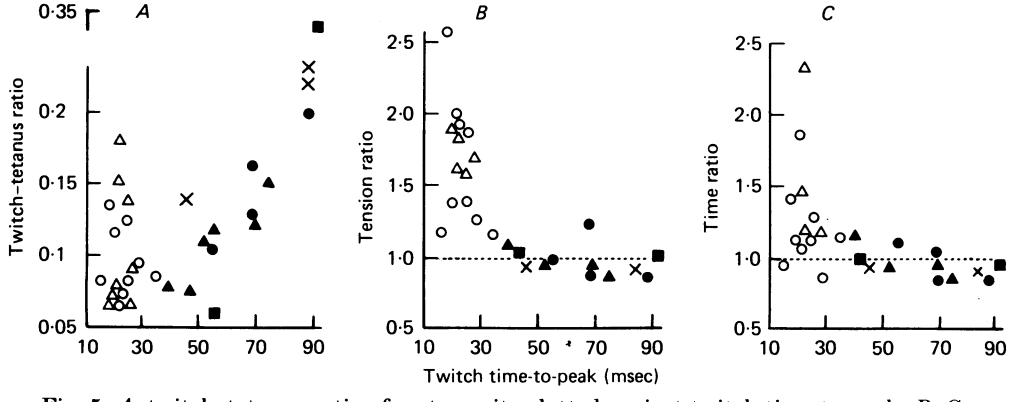

Fig. 5. *A*, twitch–tetanus ratio of motor units plotted against twitch time-to-peak. *B*, *C*, the effect of tetani on twitch tension (*B*) and twitch time-to-peak (*C*) of motor units; the ordinates are the ratios from a twitch tested 10 sec after the last of four tetani and a preceding twitch (tetani of 100/sec for 0·5 sec, tetani and twitches repeated 1/10 sec).

The mean normalized tetanic tension was 1·32 % for the group of slow motor units and 0·26 % for the fast group. The tension of all flexor hallucis longus motor units averaged 0·68 % (S.E. of mean 0·121 %, $n = 28$) of whole muscle tetanic tension, and the range was from 0·024 % to 2·07 %. After 6 months of cross-reinnervation with flexor hallucis longus nerve the mean (0·69 %, S.E. of mean 0·13 %) was not significantly different and the range (0·014–3·13 %) was similar. The mean tension of normal soleus motor units (0·69 %: Bagust, 1974) is higher than that expected for flexor hallucis longus which has almost twice as many α-motor axons.

The extremes of the twitch time-to-peak range were 15 and 88 msec (Fig. 4*B*). The lower end of this range is close to that found after 6 months cross-reinnervation (19 msec). The longest time-to-peak in the present series was longer than any seen at 6 months. Moreover, 29 % of motor units had times-to-peak longer than 50 msec compared with only 4 % at 6 months (difference significant: $P < 0{\cdot}025$, $\chi^2 = 6{\cdot}15$). The fastest motor unit (15 msec) had a twitch which was slower than the fastest found in normal flexor digitorum longus (11 msec time-to-peak) but faster than in normal soleus (24 msec). The slowest motor unit again fell between the slowest in normal flexor digitorum longus (47 msec) and that in soleus (130 msec).

In normal muscle it has been found that the ratio of the unpotentiated twitch tension to tetanic tension of a motor unit (the twitch–tetanus ratio) increases with twitch time-to-peak, although the regression lines for flexor digitorum longus (Bagust, Knott, Lewis, Luck & Westerman, 1973) and soleus (Bagust, 1974) do not coincide. This relationship is tested for the present results in Fig. 5*A*. The slow, large

motor units showed a clear increase of twitch–tetanus ratio with time to peak ($r = 0{\cdot}86$, $P < 0{\cdot}01$). The fast, small motor units had almost as large a range of twitch–tetanus ratios as the first group but with no correlation with time to peak ($r = 0{\cdot}24$).

Fig. 2*A* showed that relaxation of the whole muscle was prolonged compared with contraction, presumably because of the abnormally large contribution from slow motor units. This can be quantified by calculating the ratio of the time-to-peak to the time-to-half-relaxation which averaged 0·53 (S.D. 0·05) in the two muscles reinnervated by flexor hallucis longus nerve. The mean value for all the flexor hallucis longus nerve motor units was 0·904 (S.D. 0·130), and none of them gave a value less than 0·62. The mean ratio was only slightly larger for the fast motor units (0·93, S.D. 0·146) than for the slow (0·90, S.D. 0·089). These values may be compared with the mean ratio from a 6 month cross-reinnervated soleus of 0·907 (S.D. 0·117, $n = 41$). In normal muscles or their motor units this ratio is slightly greater than unity for fast and slightly less for slow ones.

Another feature of normal motor units of fast and slow muscle is that the rested twitch is potentiated or depressed by a conditioning tetanus, the direction of the change being related to twitch time-to-peak with a neutral region at about 35 msec (Bagust, Lewis & Luck, 1974). In the present experiments post-tetanic effects were measured as described in the legend to Fig. 5. The ratio of post- to pretetanic twitch tensions is plotted against twitch time-to-peak in Fig. 5*B*. The curved relationship is essentially similar to that found in normal muscles except that the neutral region was about 45 ms, but the conditions of measurement were different in the two series so such precise comparison is not justified. Another difference from normal is illustrated by Fig. 5*C* in which the ordinate is the ratio of post- to pretetanic twitch time-to-peak. In slow muscle or motor units post-tetanic depression is accompanied by shortening of time-to-peak. This is seen also in Fig. 5*C*. The responses of many of the fast motor units were normal also, showing only small changes in twitch duration. A number of the small fast motor units, however, showed a large increase in twitch time-to-peak after the tetanus; fast motor units of normal muscles show little change in time-to-peak even when potentiated to 2·5 times the rested twitch tension.

Within the groups of slow and fast motor units there were no statistically significant regressions between any of the variables, except for slow motor units in Figs. 5*A* and 3*B*.

Histochemical staining of one soleus muscle innervated by flexor hallucis longus nerve is illustrated in Pl. 1 with reactions for myofibrillar ATPase in Plate 1*A* and fluorescent labelled antibody to slow muscle myosin in Plate 1*B*. The extent to which Type I fibres bound anti-slow myosin antibody and the failure of Type II fibres to show more than background staining with the antibody were similar to that seen in normal cat muscles. Pl. 2 illustrates a wider range of histochemical stains in serial sections from the other soleus innervated by flexor hallucis longus nerve. Results from flexor digitorum longus cross-reinnervated muscles are not presented because of substantial self-reinnervation from soleus nerve, but the histochemistry was compatible with that seen in muscles cross-reinnervated by flexor hallucis longus nerve.

In the flexor hallucis longus innervated muscles the vast majority of fibres were oxidative with Type I predominating over Type IIa (Table 1, *A*). Despite an uneven distribution associated with fibre type grouping across the muscles, there were only small differences between the two muscles, especially as regards the division between Type I and Type II fibres. These findings are in complete contrast to those in normal flexor hallucis longus which contains a minority of Type I fibres (14% in three cats: Ariano, Armstrong & Edgerton, 1973), and glycolytic fibres predominate within the

Type II (Type II b 63 %). In normal and self-reinnervated soleus all fibres are Type I. In one cat we have also found 14·4 % Type I fibres in flexor hallucis longus and a very similar proportion (14·8 %) in the flexor digitorum longus, although the former muscle had a higher proportion of Type II b (51 % compared with 43 %). Cross-sectional areas of the fibre types are shown in Table 1*B*; part of the difference between the muscles was expected from differences in animal weight (see Table 1 legend). Within each muscle, however, there was little or no difference between average areas of Type I and II fibres and the variability of areas (Table 1, *B*) was no larger than that seen in normal cat fast muscles (e.g. Edjtehadi & Lewis, 1979).

Table 1, *H* estimates the specific tension (tetanic tension per unit area) of the two groups of motor units, making the assumption that slow motor units contain all the Type I fibres and no Type II. Intermediate steps in the calculations are shown in Table 1, *E–G* where these are of general interest, and the methods are explained in the legend. Similarly innervation ratios have been calculated for the Type I fibres/slow motor units and Type II fibres/fast motor units (Table 1, *I*).

Immunohistochemical studies were made on one of the contralateral flexor hallucis longus muscles which had been self-reinnervated at the same operation as the cross-union, and representative sections are shown in Pl. 1*C* and *D*. No detailed analysis of the sections is presented as no motor unit measurements were made, but it is of interest to note that the same fibre types were present as in the soleus muscles cross-reinnervated by flexor hallucis longus nerve and that the proportion of Type I fibres (80 %) was very much higher than in normal fast muscle. In contrast with the cross-reinnervated soleus, but similar to normal flexor hallucis longus, the areas of Type I fibres was less than half that of the Type II (about 2000 μm^2 compared with 5000 μm^2).

DISCUSSION

The present results show unambiguously what was suspected after six months of cross-reinnervation of soleus (Bagust *et al.* 1981): that the normal relationship between axonal conduction velocity and motor unit tension is reversed. Thus the inability of fast nerve to convert completely the mechanical (Buller & Lewis, 1965) and histochemical (Edgerton, Goslow, Rasmussen & Spector, 1980) properties of cat soleus muscle is not due to failure of conversion of individual fibres, but to the very small tension and numbers of the fast fibres which is a consequence of the abnormally small size of the fast motor units. Such an explanation would fit the more complete conversion of rat soleus (Close, 1969) after cross-reinnervation because rat fast muscles have few slow motor units (one in forty in extensor digitorum longus: Close, 1967). A single slow motor unit would have a small effect on the whole muscle contraction even if it were larger than normal.

The changes in the relation between tetanic tension and twitch time-to-peak are illustrated in Fig. 6, which compares soleus motor units after six months and three years of cross-reinnervation with flexor hallucis longus nerve. At both periods of cross-reinnervation there is a group of small, fast motor units. In contrast the large motor units form two separate groups at the two periods of cross-reinnervation: at three years the large motor units have slow contractions (time-to-peak 40–90 msec) but

TABLE 1. Fibre and tension measurements from two soleus muscles cross-reinnervated by flexor hallucis longus nerves. Note that cat 2 (male) weighed 1·4 times cat 1 (female). Mean values have been calculated where appropriate and recorded in column 3. Measurements for rows *A*, *B* & *C* are described in the Methods. Other rows have been calculated as indicated below. Row *D*, total number of fibres = muscle area/mean fibre area numbers of Types I & II = total number × proportion of Type (A). Row *E*, total fibre area = total number (*D*) × mean area (*B*), for each type. Row *F*, sum of individual motor unit normalized tension for the three sets (see Results). Row *G*, total motor unit tension = total muscle tension (*C*) × fraction of summed tension for slow or fast motor units (*F*). Row *H*, tension per unit area = total motor unit tension (*G*)/total fibre area (*E*). Calculation assumes that all slow motor units are composed of only Type I fibres and fast motor units are only of Type II. Row *I*, innervation ratio (mean number of fibres per motor unit) = Total number of Type I or II fibres (*D*)/Estimated number of slow or fast units (*G*). Same assumptions made as for *H*

	Muscle 1	Muscle 2	Mean (S.E. of mean)
(*A*) Percentage of fibres:			
Type I	61·8	59·7	60·8 (1·05)
Type II(a)	31·5	36·8	34·2 (2·65)
Type II(b)	6·7	3·5	5·1 (1·60)
Type II (all)	38·2	40·3	39·3 (1·05)
(*B*) Mean cross-sectional area (μm^2): (S.D. in parentheses)			
All	1540 (423)	4310 (729)	
Type I	1520 (407)	4100 (703)	
Type II	1580 (448)	4630 (765)	
Ratio I/II	0·96	0·89	0·92 (0·038)
(*C*) Whole muscle data:			
Tetanic tension (N)	13·8	24·1	
Weight (g)	2·03	4·18	
Cross-sectional area (mm^2)	27·6	75·6	
Tension/area (N/mm^2)	0·50	0·32	0·41 (0·091)
(*D*) Number of fibres (10^3):			
All	17·9	17·5	17·7 (0·19)
Type I	11·1	10·5	10·8 (0·30)
Type II	6·8	7·1	7·0 (0·11)
(*E*) Total fibre area (mm^2):			
Type I	16·8	42·9	
Type II	10·8	32·7	
Ratio I/II	1·56	1·31	1·43 (0·122)
(*F*) Sum of motor unit tensions (N): (Number of units in parentheses)			
All	1·45 (15)	2·06 (13)	
Slow (S)	1·18 (6)	1·40 (4)	
Fast (F)	0·27 (9)	0·66 (9)	
Ratio S/F	4·37	2·12	3·25 (1·12)
(*G*) Total tension of motor units (N): (Number of units in parentheses)			
Slow	11·2 (57)	11·4 (47)	
Fast	2·6 (86)	7·7 (105)	
(*H*) Tension per unit area (N/mm^2):			
Slow/Type I	0·66	0·38	0·52 (0·14)
Fast/Type II	0·24	0·24	0·24 (0·01)
All	0·50	0·32	0·41 (0·09)
(*I*) Innervation ratio:			
Type I/Slow	195	223	209 (14)
Type II/Fast	79	68	74 (5·5)

at six months have intermediate twitch times (20–40 msec) which are not clearly distinguishable from those of the small motor units. One interpretation of such data is that the two sets of axons of flexor hallucis longus nerve (with slow and fast conducting velocities) existed at all stages of cross-reinnervation. The slow conducting axons made connexions with an abnormally large number of muscle fibres. These large motor units, however, did not immediately reach a slow isometric twitch contraction

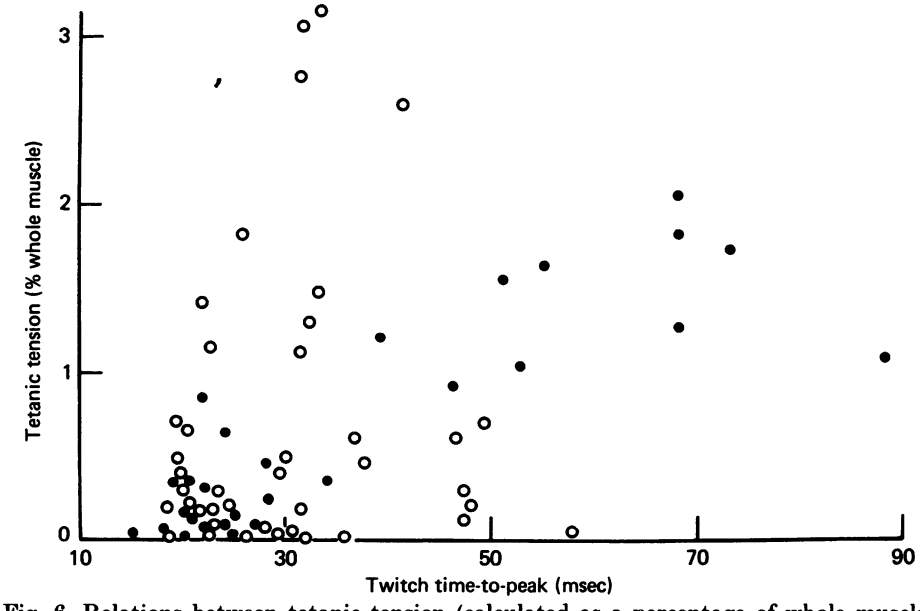

Fig. 6. Relations between tetanic tension (calculated as a percentage of whole muscle tetanic tension) and twitch time-to-peak for motor units of soleus cross-reinnervated by nerve to flexor hallucis longus. Filled circles, muscles reinnervated for 3 years (from Fig. 2); open circles, a muscle reinnervated for 6 months (Bagust *et al.* 1981).

but went through an initial, intermediate phase. The motor units with fast conducting axons were abnormally small but established their fast twitches at an early stage.

If such a hypothesis is adopted it offers an explanation of histochemical observations on cross-reinnervated muscle. Two reports (Edgerton *et al.* 1980; Burke, Dum, O'Donovan, Toop & Tsairis, 1979) have indicated a very high proportion of Type I fibres after periods of reinnervation of about one year, similar to that seen at three years. This high incidence of Type I fibres contrasts with the infrequency of slow twitch motor units at six months (Bagust *et al.* 1981). The hypothesis suggested above resolves the discrepancy if it is assumed that the slow myosin isoenzyme, and therefore the histochemical profile, of the motor units with slow conducting axons is established early whilst the isometric twitch is still in the initial, transient intermediate phase. The commonly found correlation between isometric twitch duration and histochemistry depends on a relation between the isometric twitch time-to-peak and the velocity of isotonic shortening, the latter depending on the type of myosin isoenzyme in the fibres. A reciprocal relation between isometric twitch and isotonic velocity is found for a large range of normal muscles (Close, 1965) but is not

universally true. It breaks down, for example, in the extrinsic eye muscles (Close & Luff, 1974) and following denervation (Kean, Lewis & McGarrick, 1974). The break-down is possible because, although both isotonic shortening velocity and rate of development of isometric twitch tension depend on the myosin isoenzyme type, the twitch duration will also depend on the rates of release and re-uptake of calcium following a muscle action potential. Moreover, the rate of tension development depends on the rate at which cross-bridges between actin and myosin are formed whereas the velocity of isotonic shortening is limited by the rate of breakage of cross-bridges. In most normal muscles the time course of the processes of release and re-uptake of calcium are matched to the speed of actomyosin ATPase, but clearly mismatching can occur (Close & Luff, 1974) or be induced (Kean *et al.* 1974). Therefore we can suggest that, following cross-reinnervation, the slow muscle fibres innervated by slow axons show a transient phase in which the myosin isoenzyme is of the slow type but calcium release and re-uptake in the twitch are relatively fast, and only slow down to match the reaction rate of the isoenzyme type after a long period of time.

We have suggested that the late changes in slow motor units of cross-reinnervated soleus (and possibly self-reinnervated flexor hallucis longus) are due to delayed modification of the mechanisms of calcium release and re-uptake. However, it is known that changes in muscle can occur very rapidly. For example, after denervation the twitch slows down within a few days (Finol, Lewis & Owens, 1981) and chronic stimulation affects the ATPase activity of the sarcoplasmic reticulum just as quickly (Heilmann & Pette, 1980). It is necessary, therefore, to argue that the delay in modification of calcium release–re-uptake which we have observed is due to a delay in the operation of the neuronal mechanism controlling this muscle property. A further consequence of this argument is that there is independent control of the processes of two muscle mechanisms: calcium release–re-uptake and protein isoenzyme type. The delayed response may be secondary to other changes in the reinnervated muscle. Motor and sensory innervation of muscle spindles is disturbed, and grossly abnormal afferent discharges are seen in afferent nerves from muscles up to twelve months after cross-reinnervation (J. E. Gregory, A. R. Luff & U. Proske, personal communication), which may result in abnormal motoneurone firing patterns. If sensory activity recovered over several years, this might result in late changes in the trophic effects of some motoneurones and, consequently, in the properties of their muscle fibres.

A prediction of the simple hypothesis above is that twitch–tetanus ratio would increase with time. Comparison of the two sets of motor units of Fig. 6 showed no such increase. Clearly our model is too simple: twitch–tetanus ratio depends not only on relative rates of calcium release and re-uptake but also on the total amount released which could be modulated independently by, for example, the extent of propagation of the membrane action potential along the T-tubules to the centre of the fibre.

One alternative explanation of our findings is that there would have been a transformation to Type I fibres with age without the reinnervation. Such a process does occur in rats and man but only to a small extent. It is unlikely to be more extensive in our cats which were relatively young (4 years) at the final experiments.

Although our hypothesis describes the sequence of effects in cross-reinnervation of soleus muscle and brings together several sets of results, it does leave a number of major questions. Most important is why the slow conducting axons take over a

larger number of muscle fibres than do the fast axons. Parallel conditions seem to occur early in muscle development in which the first myotubes to form and be innervated contain slow myosin (Rowlerson, 1980; Rubinstein & Kelly, 1981). The inference is that slow axons are the first to reach the muscle and innervate most of the primary myotubes. Fibres of the second generation of myotubes contain only fast myosin because, according to this hypothesis, they are innervated by the later arriving, fast axons. In reinnervated adult muscle there is no new fibre formation so the fast axons, if they grow in more slowly than the slow axons, would find few non-innervated fibres. The fast motor units, therefore, would be small. Such a hypothesis must be complicated by the fact that reinnervation is initially polyneuronal (Bernstein & Guth, 1961) so that withdrawal of excess terminals over the first few months makes remodelling possible. Moreover, although polyneuronal innervation has disappeared by six months (Bagust *et al.* 1981), there may also be some re-organization of the size of motor units up to three years after reinnervation (Lewis & Owens, 1979). However, the extent of polyneuronal innervation is less in adult reinnervation that in early post-natal development so the extent of remodelling may also be smaller.

There are other interpretations of the relationship between myosin isoenzyme type and maturity of myotubes. One possibility is that the nature of the muscle fibres is determined genetically or by sequence of development, and the motoneurone properties then modified by some retrograde axonal signal (Huizar, Kuno, Kudo & Miyata, 1977; Czéh, Gallego, Kudo & Kuno, 1978). Such a mechanism could operate in adult cross-reinnervation so that the slow muscle fibres have a retrograde effect on motoneurones (cf. Lewis, Bagust, Westerman, Webb & Finol, 1978).

In cross-reinnervated soleus, the increased contribution of the slow muscle fibres relative to that in fast muscle is due to three or possibly four factors. The three certain factors are increases in the number of fibres per motor unit (innervation ratio), the cross-sectional area of the slow fibres and their tension per unit area (specific tension). The fourth, uncertain factor, is an apparent increase in the proportion of slow motor units from 28 to 36%. (It should be noted that this does not represent an increase in the absolute number of slow motor units because there is a large fall in the total number of motor units from that found in normal flexor hallucis longus.)

The normal value of 28% quoted above has not been obtained directly because no data are available for normal flexor hallucis longus. It is the value for flexor digitorum longus which has a similar ratio of Type I to Type II fibres (see Results), and has 27% slow motor units (in a sample of 213: Bagust *et al.* 1973; D. M. Lewis, unpublished observations). Burke & Tsairis (1973) use a similar figure of 28% for cat medial gastrocnemius. The observed proportion of slow motor units in cross-reinnervated soleus was not significantly different from that in flexor digitorum longus ($\chi^2 = 1{\cdot}0$).

If it is necessary to assume there was sampling error in the ratio of slow to fast motor units in the present work, several corrections would have to be made in Table 1. The total tension (G) would have been smaller for the slow motor units and larger for the fast. This would lead to new estimates for specific tension (H) of 0·46 N/mm^2 for slow and 0·29 N/mm^2 for fast motor units. Specific tension must also be corrected for errors in the total number of muscle fibres. In both cross-innervated solei we found about 18,000 fibres in contrast with values of 22,000–28,000 for normal soleus by Clark (1931). Our estimates must be low because the fibres of soleus only extend 44% of the muscle length (Al-Amood & Pope, 1972) and, therefore, a mid-muscle section would only include 88% of fibres. Moreover our sections were not exactly mid-section. It is reasonable to use the figures of Clark (1931) to estimate both the total number of fibres (D) and the total area of the fibres (E) as 30–50% higher than the figures quoted in Table 1 (values for Types I and II fibres would be affected equally). This

would lead to a further revision of specific tensions to values of 0·36–0·31 N/mm² for slow motor units and 0·23–0·20 N/mm² for fast units.

Over-estimating the proportion of slow motor units and underestimating the total number of fibres would also affect innervation ratios. The values for slow and fast motor units of 209 and 74 (*I*) would become 270 and 65 after allowing maximum sampling error, and 410 and 100 after maximum correction for number of fibres.

Even after making maximum allowance for errors, it is clear that the specific tension of slow motor units is at least 50 % higher than that of fast units. This is the reverse of the normal state in which slow motor units and slow muscle have lower specific tensions than fast motor units and fast muscle. Edjtehadi & Lewis (1979) have calculated values of 0·29 and 0·39 N/mm² for slow and fast motor units in flexor digitorum longus. Kean *et al.* (1974) gave values of 0·25 and 0·38 N/mm² for soleus and flexor digitorum longus muscles. Thus it is certain that the relative specific tensions of slow and fast motor units are reversed in these long-term cross-reinnervated muscles, but in addition their absolute specific tensions may well be reversed too.

The same sort of comments may be made about innervation ratios. We have found these to be higher for the slow motor units than the fast (350–400 compared with 85–100). The slow motor units, therefore, have a higher number of fibres in the cross-reinnervated soleus than in normal flexor digitorum longus and the fast ones a smaller number. It may be expected that the motor unit sizes in flexor hallucis longus will be similar to those in flexor digitorum longus, in that it has about twice as many muscle fibres and 2·1 times as many motor axons (see Bagust *et al.* 1981). Normal soleus motor units have 185 muscle fibres; less than the cross-reinnervated slow motor units.

In normal cat flexor digitorum longus, innervation ratios can be calculated to be 75 and 195 for the slow and fast motor units respectively. These estimates are from the proportions of Types I and II fibres (Edjtehadi & Lewis, 1979), estimates of the total number of muscle fibres (Westerman, Lewis, Bagust, Edjtehadi & Pallot, 1974) and the proportion of fast to slow motor units. Burke & Tsairis (1973), by direct measurement of glycogen-depleted motor units in cat medial gastrocnemius, obtained mean values of 78 for slow and 385 for fast motor units. By indirect estimates, as above for flexor digitorum longus, Burke & Tsairis found approximately equal numbers of fibres in the two types of motor unit, but their calculations lead to very low values for specific tension and may not be the most reliable values to use.

The calculations above depend on the assumption that motor units in cross-reinnervated muscle contain only one type of fibre. If the assumption were untrue it would be impossible to estimate specific tensions. Some evidence for uniformity comes from the shape of the twitch contractions. The slow relaxation of the whole muscle contraction is characteristic of a mixed population (particularly one with two distinct groups of fibres). None of the motor units showed an abnormal shape as indicated by the ratio of twitch time-to-peak to time-to-half-relaxation. The absence of intermediate fibre types in the histochemistry supports the uniformity of motor units. It can be concluded that individual motor units are more uniform in fibre composition than the whole muscle. Since the ratio was almost the same as the value of 0·95 found for a motor unit population from normal fast muscle (Bagust *et al.* 1973) it is not unreasonable to assume that motor unit uniformity does not differ greatly between the normal and reinnervated muscles.

The number of reinnervating axons estimated from the mean tetanic tension was

147 which was very close to the figure of 145 calculated by Bagust *et al.* (1981) for a shorter period of reinnervation, suggesting that the regrowth of axons is substantially complete by 6 months (although in numbers less than in normal flexor hallucis longus). Self-reinnervated soleus has been calculated by Bagust & Lewis (1974) to receive about sixty-seven axons, again about two-thirds of the normal number in soleus (Bagust, 1974). It is, therefore, unlikely that late reinnervation and subsequent competition can account for changes in reinnervation pattern.

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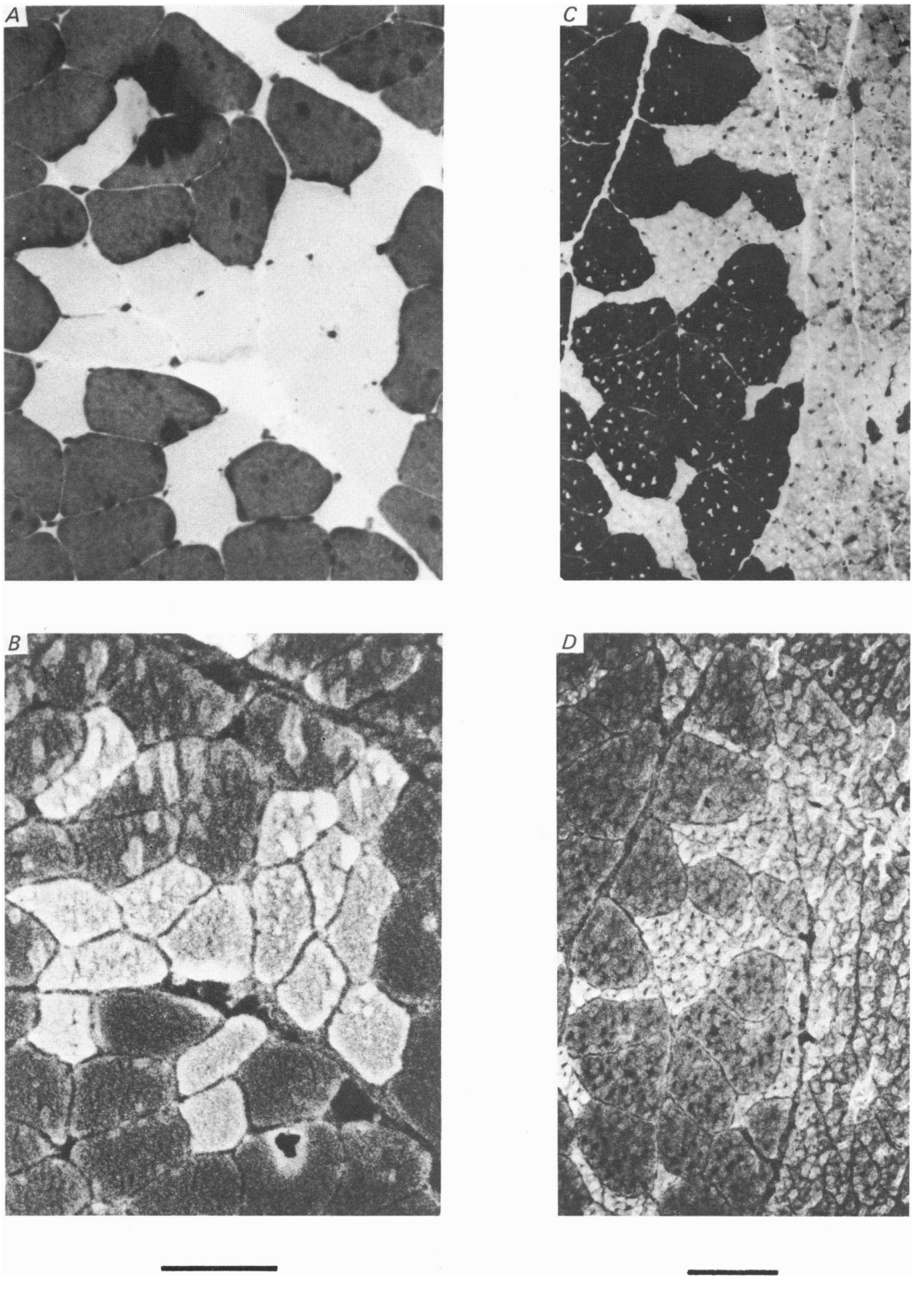
EXPLANATION OF PLATES

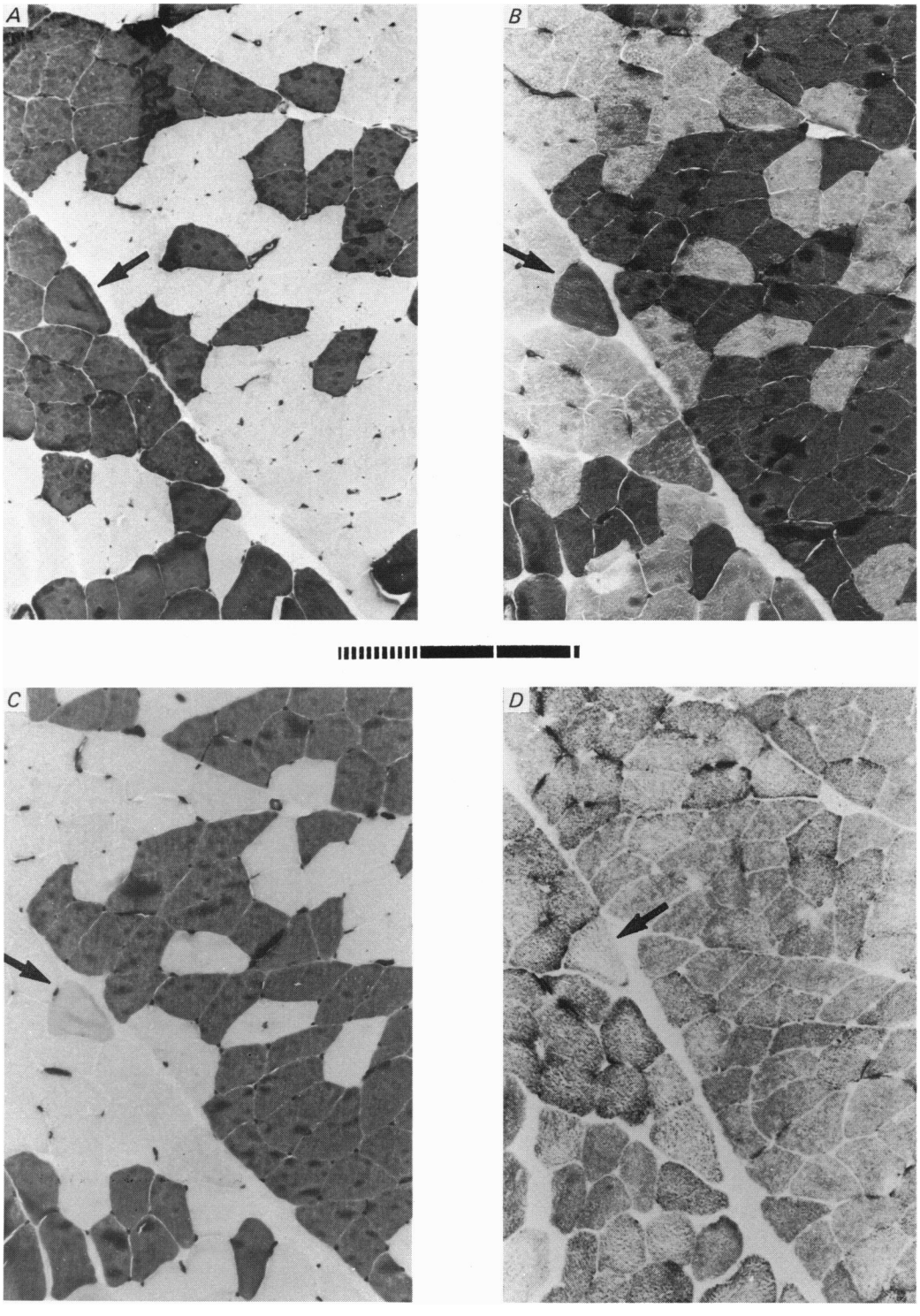
PLATE 1

Histochemistry of soleus cross-reinnervated by nerve to flexor hallucis longus (*A*, *B*, muscle 1 of Table 1) and self-reinnervated flexor hallucis longus (*C*, *D*). In *A* and *C* the muscles were stained for actomyosin ATPase (pre-incubation at pH 9·4). In *B* and *D* serial sections of the muscles were stained with fluoresceine labelled anti-slow muscle myosin. Sections cut at 8 μm. Calibration bars 100 μm.

PLATE 2

Serial sections of a soleus cross-reinnervated with flexor hallucis longus nerve (muscle 2 of Table 1). Sections cut at 8 μm and stained for actomyosin ATPase with pre-incubation at pH 9·4 (*A*), at pH 4·6 (*B*) and at pH 4·5 (*C*) or for succinic dehydrogenase (*D*). The arrows indicate a Type 2b fibre. Calibration grid 10 and 100 μm.


A
C
B
D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