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FIBRE SIZES AND HISTOCHEMICAL STAINING CHARACTERISTICS IN NORMAL AND CHRONICALLY STIMULATED FAST MUSCLE OF CAT

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SUMMARY

1. Normal and chronically stimulated peroneus longus muscles of the cat's hind limb were studied with respect to fibre size and staining properties for myofibrillar (myosin) adenosine triphosphatase (ATPase) and succinate dehydrogenase (SDH) activity. The intensity of staining for SDH activity was measured by microphotometry from the central portions of the muscle fibres ('core-SDH staining'). For comparison, histochemical properties were also studied in non-stimulated soleus muscles.

2. On account of the pH sensitivity of their myofibrillar ATPase, about 18% of the fibres in normal peroneus longus muscles were classified as type I, and about half of the remainder as IIA and IIB respectively.

3. In the normal peroneus longus muscles, the mean diameter of single muscle fibres generally varied between about 25 and 75 μm, whereby the average size of type I < type II.

4. In the normal peroneus longus muscles the staining intensity for core SDH varied over a wide range. The average heaviness of staining was clearly ranked in the order type I > type IIA > type IIB.

5. Chronic stimulation was given to the deafferented common peroneal nerve by aid of a portable and remotely controlled mini-stimulator. The stimulation was delivered in 'tonic' patterns ($\geqslant 50\%$ of total time taken up by activity) of 'fast' (20 or 40 Hz) or 'slow' (5 or 10 Hz) rates.

6. Prior to the period of long-term stimulation, the cats had been subjected to a dorsal rhizotomy and hemispinalization on the ipsilateral (left) side. In the absence of chronic stimulation, these operations had no evident effects on the sizes or staining properties of peroneus longus fibres.

7. After 8 weeks of treatment with tonic patterns of stimulation, the fibres of peroneus longus muscles clearly became more similar to each other with respect to their diameter as well as their staining for ATPase and SDH activity. With respect to ATPase staining, however, the chronically stimulated peroneus longus fibres had become more similar to non-stimulated soleus fibres than to non-stimulated type I fibres of peroneus longus. With respect to the staining for core SDH, the chronically stimulated fibres all became similar to normal IIA fibres of peroneus longus. The 'fast' and 'slow' patterns of chronic stimulation had the same effects on the staining properties.

8. Chronically stimulated peroneus longus muscles showed a decrease in fibre diameter which corresponded, roughly, to the concomitant decrease in muscle weight. The decrease in fibre size was significantly greater for the 'slow' patterns than for the 'fast' ones.

9. The results were discussed in relation to previously obtained physiological findings from the same muscles. The absence of an evident relationship between the pulse rate of chronic stimulation and the ATPase or SDH staining is in accordance with the finding that the present patterns of stimulation also all had the same effects on the isometric contractile speed and the contractile endurance of peroneus longus. The finding that the 'slow' patterns caused a greater decrease of fibre size than the 'fast' patterns is in accordance with differences between the effects of these patterns on contractile force. The relative decrease in cross-sectional area was, however, less than the relative decrease in maximum tetanic force.

INTRODUCTION

In a mixed hind-limb muscle, the various motor units differ markedly from each other with respect to their contractile speed, endurance and maximum force. The most easily recruited motoneurones of a pool often tend to be tonically active, and they are typically equipped with muscle units that are relatively slow, fatigue resistant and weak (Burke, 1981). Such mechanical properties may have developed in these units partly as a result of long-term effects of great amounts of activation, because it is known that several weeks of 'tonic' stimulation may make a fast muscle slower, weaker and more fatigue resistant (e.g. Salmons & Vrbová, 1969; Peckham, Mortimer & Van Der Meulen, 1973; Pette, Smith, Staudte & Vbrová, 1973; Salmons & Henriksson, 1981; Eerbeek, Kernell & Verhey, 1984). In a preceding paper, we have shown that, when given according to the same daily pattern, fast and slow rates of tonic activation had similar effects on muscle endurance, but different effects on maximum muscle force (Eerbeek *et al.* 1984). Furthermore, contrary to our own expectations, we found that the fast and slow stimulus patterns had similar long-term effects on isometric contractile speed. In the present complementary study, we used histological–histochemical methods for the further analysis of stimulation-evoked changes in contractile muscle properties. As a background for this analysis we also had to collect data concerning the normal fibre properties of the muscle employed, m. peroneus longus of the cat's hind limb. Physiological data concerning the normal motor unit properties of this muscle have recently been published elsewhere (Kernell, Eerbeek & Verhey, 1983).

In isometric recordings, differences in the speed of twitch contraction probably depend, to a great extent, on the combined effects of differences in (1) the properties of myosin, including the activity of myosin–ATPase (adenosine triphosphatase), (2) the properties of the sarcoplasmic reticulum, which regulate release and resorption of calcium ions (cf. Close, 1972). A correlate to the first one of these factors is relatively easily monitored by standard histochemical techniques: the pH sensitivity of myofibrillar ATPase. Earlier studies have shown that fibres with an acid-stable ATPase (type I) tend to be slow and fibres with an alkali-stable ATPase (type II) tend to be fast. Based on more detailed differences in pH sensitivity of the ATPase,

the group of fast fibres may be further subdivided into the groups IIA and IIB, which have been found to correspond to the fast fatigue-resistant (FR) and fast fatigue-sensitive (FF) categories of physiologically classified units in cat's muscles (Burke, 1981).

Differences in endurance between the various units of a muscle probably depend on the combined effects of a multitude of factors. In this context, the capability for oxidative metabolism is likely to be one of the relevant factors. A histochemical correlate is easily studied by standard methods: the activity of oxidative enzymes, such as succinate dehydrogenase (SDH). In mixed hind-limb muscles, the SDH staining tends to be more intense for fatigue-resistant fibres than for those belonging to more fatigue-sensitive muscle units (Burke & Tsairis, 1974; Kugelberg & Lindegren, 1979; Burke, 1981).

Differences in maximum tetanic force between the various units of a muscle are thought to depend on the combination of three factors: (i) the number of muscle fibres per unit, (ii) the cross-sectional areas of the respective fibres, (iii) their specific force, i.e. maximum force per unit cross-sectional area. In mixed muscles of the cat, the slow units, which tend to be weak, have thinner fibres than those of the fast units, which tend to be strong. Furthermore, indirect evidence suggests that also the specific force tends to be smaller for slow than for fast fibres of such muscles (McDonagh, Binder, Reinking & Stuart, 1980; Burke, 1981; Dum, Burke, O'Donovan, Toop & Hodgson, 1982).

The present studies were motivated by three specific questions.

(i) Did fast and slow tonic stimulus patterns have similar long-term effects on the myofibrillar-ATPase- and SDH-staining properties of muscle fibres?

(ii) Did fast and slow tonic stimulus patterns have different long-term effects on muscle fibre diameter?

(iii) Did the various fibres of a muscle become more similar to each other after being subjected to the same (fast or slow) tonic long-term pattern of activity?

Questions (i) and (ii) were inspired by our physiological results (Eerbeek *et al.* 1984). The answers could not, however, have been simply predicted from the physiological results themselves, because each one of the physiological variables studied (twitch speed, endurance, maximum force) depends on other mechanisms besides those directly related to the histological–histochemical parameters investigated in the present study (see above). Question (iii) concerns problems that could not be analysed by aid of our physiological measurements of whole-muscle contractions (Eerbeek *et al.* 1984).

As will be shown, the results tended to give an affirmative answer to all the three main questions.

METHODS

General experimental procedures. All results came from adult cats (body weight 2·3–4·7 kg). The chronically treated animals were the same cats as those used for illustrations and Tables in our preceding paper (Eerbeek *et al.* 1984), in which the procedures for long-term stimulation and physiological measurements are described in detail. In addition, muscles from normal, previously untreated animals were studied by the same histological–histochemical methods.

The chronically treated animals were, in short, subjected to the following procedures.

(1) In the first operation under general anaesthesia left-side hemispinalization (L1) and dorsal rhizotomy (L1–S2). As a result, the left hind-limb was insensitive to applied stimulation and subjected to little active use.

(2) In a second operation under general anaesthesia performed 2 weeks after the first one, stimulation electrodes were applied under the common peroneal nerve of the left hind limb, and a portable mini-stimulator was fixed to the back of the cat. The stimulator was remotely controlled by aid of infra-red light.

(3) During the 8 weeks following the second operation, most of the chronic animals were subjected to 'tonic' stimulus patterns (activity during $\geqslant 50\%$ of total time) at physiologically 'slow' (5 or 10 Hz) or 'fast' (20 or 40 Hz) pulse rates. The 5 Hz treatments were given continuously. In all other cases, long-term stimulation was given in patterns of 1 s bursts alternating with 1 s pauses for 24 h per day. Some of the operated animals were kept for a total of 10 weeks without any stimulation ('experimental controls').

(4) In a terminal acute experiment under general anaesthesia (pentobarbitone), m. peroneus longus was dissected free in both hind-limbs, attached to a force transducer, and subjected to a number of physiological measurements (see Eerbeek *et al.* 1984). During the measurements, the muscle was covered by liquid paraffin and kept at 37–38 °C. The general anaesthesia of the preparatory operations as well as of the terminal acute experiment was produced by aid of pentobarbitone (initial dose 40 mg/kg I.P.; additional doses I.V. as required).

(5) After the completion of physiological recordings from both sides, the peroneus longus muscle was quickly dissected free from both legs. The tendon was carefully cut away. The muscles were weighed, fixed to a rod in a slightly stretched position, and quickly frozen in melting isopenthane cooled by liquid nitrogen. Thereafter, the muscles were stored at −80 °C until further processing (typically 1–2 days). Besides peroneus longus, a number of other hind-limb muscles were also commonly removed and frozen for further histochemical studies (including, in many cats, m. soleus from the non-stimulated right leg). Sections of 12 μm were cut in a cryostat at −25 °C. Consecutive sections were stained for the activity of (1) SDH, (2) myofibrillar (myosin) ATPase after pre-incubation at pH 9·4, (3) ATPase after pH 4·5, (4) ATPase after pH 4·1. Sections from different muscles of the same cat were processed simultaneously and by identical methods. Unless otherwise stated, all measurements were performed on sections through the muscle belly.

Measurements concerning ATPase-related fibre types and distribution of fibre sizes. Myofibrillar-ATPase staining and fibre classification were performed by methods similar to those of Brooke & Kaiser (1970). Fibres of type I were light and type II dark after pre-incubation at pH 9·4. The opposite pattern of staining was seen after pre-incubation at pH 4·1 (cf. Fig. 1). Identification of types II A and II B could only be reliably performed by aid of serial sections: among fibres identified as type II at pH 9·4 or 4·1, the II A were light and II B dark after pre-incubation at pH 4·5.

For the estimates of fibre type composition and average fibre size we wished to ensure that our sample was as representative as possible for the respective muscle section. For this purpose, we fixed a plastic identification grid under the object slide of each such section. This grid ('cell finder culture slide', Microlab, Holland) was subdivided into alphabetically labelled squares with a side of 400 μm. By aid of the cell-finder grid, we could analyse the muscle composition within a number of evenly distributed measurement areas of 100×100 (sometimes 200×200) μm. The distribution of these measurement areas was in most cases such that we sampled 1/64 of all the fibres of a section (in a few cases, 1/16 was sampled). Sampling of 1/64 of the fibres led to the measurement of, on average, 270 ± 97 (mean $\pm$ S.D.) fibres per section through the muscle belly. Within measurement areas, each fibre outline was drawn at a scale of 2 μm/mm by aid of a split-beam drawing tubus. The staining intensity of each sampled fibre was noted (dark, intermediate, or light), and its mean diameter was calculated from its major and minor axes, as measured from the drawing at right angles to each other. Fibre diameters were only measured in ATPase-stained preparations that were appropriate for subdividing fibres into types I and II (i.e. not for the subdivisions II A and II B which, in our case, would require the processing of serial sections; see above).

Measurement of the intensity of 'core-SDH' staining. The staining for SDH activity was performed by a method similar to that of Pool, Diegenbach & Scholten (1979), using tetranitroblue-tetrazolium (TNBT). The incubation was carried out for 12 min at 37 °C (cf. Pool *et al.* 1979). Comparative measurements of the staining intensity for SDH in single muscle fibres were performed by aid of a microscope equipped with a Zeiss microscope–photometer SF coupled to a digital display. Within each section, 200 fibres from a randomly chosen area of the muscle belly were subjected to such measurements. Within each fibre, the staining intensity was ascertained within a central area of

12·5 μm diameter ('core-SDH staining'). The size of the measuring field was chosen in such a way that the irregular accumulations of sub-sarcolemmal mitochondria (i.e. sub-sarcolemmal SDH activity) did not influence the recording (cf. Kugelberg & Lindegren, 1979). A stabilized light source was used. During measurements, the field diaphragm was maximally closed. In the present study we used photometry as an aid for judging the density of SDH staining in a context of conventional histochemistry. In accordance with general practice in conventional histochemistry, we used white light for the photometric measurements. The end-product of the utilized technique (TNBT formazan) gives a black staining with a wide absorption spectrum (Pool *et al.* 1979). Control experiments confirmed that a group of muscle fibres would be ranked in practically the same way by our method as by that of Pool *et al.* (1979), who used a small-spot-scanning method at a wave-length around 660 nm.

The density (D) of core-SDH-staining was calculated according to the equation:

$$D = -100 \times \log (I_x/I_0),$$

where I_0 was equal to the background value read for light transmitted through glass slide plus mounting medium plus cover-slip just outside the muscle tissue, and I_x was equal to the value read for light transmitted through the centre of the muscle fibre.

In addition to our analysis of cat muscles, we also checked the validity of our histochemical methods by measuring the distribution of SDH staining density among ATPase-typed muscle fibres of the rat's extensor digitorum longus. These studies (see Fig. 4*B*) gave results very similar to those of Nemeth & Pette (1981), who estimated SDH activity by aid of measurements of maximum initial reaction rates.

Unless otherwise indicated, mean values in text and Tables are given ±S.D.

RESULTS

The normal peroneus longus muscle

Fibre types according to ATPase staining. The muscle fibres of peroneus longus could be clearly differentiated into types I and II respectively by aid of conventional staining methods for myofibrillar ATPase (Fig. 1). Practically the same percentages of type I fibres were obtained by counting dark fibres after acid (pH 4·1) pre-incubation as by counting light fibres after alkaline pre-incubation. The number of type II fibres belonging to types II A and II B respectively was, in three muscles, analysed by aid of the staining properties after pre-incubation at pH 4·5 (see Methods; Table 2). In the whole material of eight normal muscles, about 18 % (range 10–21 %) of the fibres belonged to type I. Among the type II fibres, about half were of type II A and half II B (see Table 2). All these figures refer to sections through the muscle belly. Studies at different proximo-distal levels suggested that the proportion of type I fibres generally tends to be lower at the most distal end of the muscle than in its more proximal portions (distal-most content of type I fibres in five muscles: mean 11 %, range 5–19 %). These tapering distal muscle portions did, however, contain relatively few fibres. Calculations indicated that the total percentage of type I fibres of the whole muscle might be 1–2 % lower than the value measured from the muscle belly. All further figures concerning the proportion of type I fibres refer to measurements from the muscle belly.

Fibre sizes. The majority of the individual fibres from normal muscles had mean diameters within the range 25–75 μm (cf. Fig. 2). Normal fibres of type I were systematically thinner than those of type II (Fig. 2, Table 1). On average, the diameter of type I fibres was about 70 % of that of type II fibres from the same muscle.

Core-SDH staining. The staining density for core SDH was measured in three

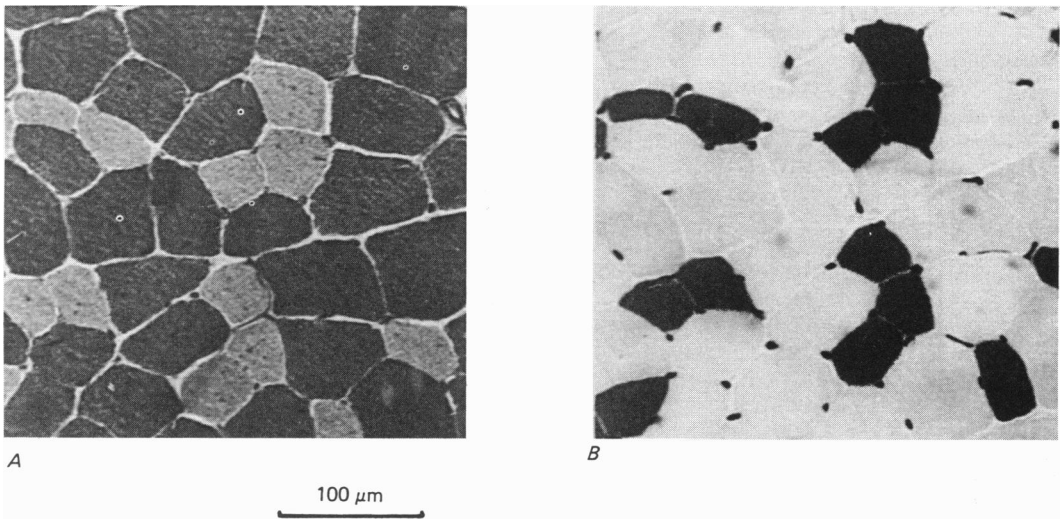

Fig. 1. Two consecutive sections from normal m. peroneus longus, stained for activity of myofibrillar ATPase after pre-incubation at pH 9·4 (*A*) and 4·1 (*B*) respectively. Fibres of type I are light in *A* and dark in *B*.

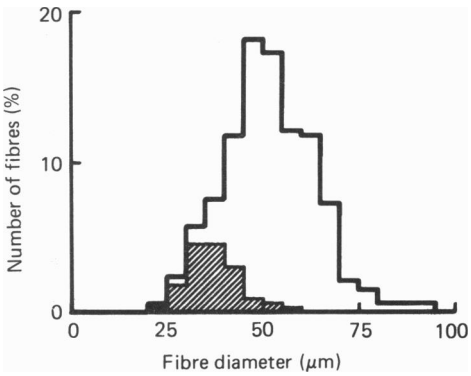

Fig. 2. Histogram showing the distribution of fibre diameters for a normal m peroneus longus. Hatched area: fibres of type I. Totally 329 measured fibres.

TABLE 1. Fibre diameters in peroneus longus muscles of normal cats: average and variability per muscle

	Type I		Type II	All
Average diameter (μm)	37·7 ± 4·1	*	53·3 ± 5·8	50·7 ± 5·7
Variability diameter (%)	16·4 ± 2·8	*	21·7 ± 2·0	24·5 ± 1·3
n	5		5	5

Means ± S.D. of values for diameter averages and diameter variabilities of peroneus longus muscles from five cats that had not been subjected to chronic treatment. Variability was, for each individual muscle, calculated as the ratio between the S.D. and the average for its fibre diameters. A '*' between adjoining columns indicates the presence of a statistically significant difference (*t* test, $P < 0.05$).

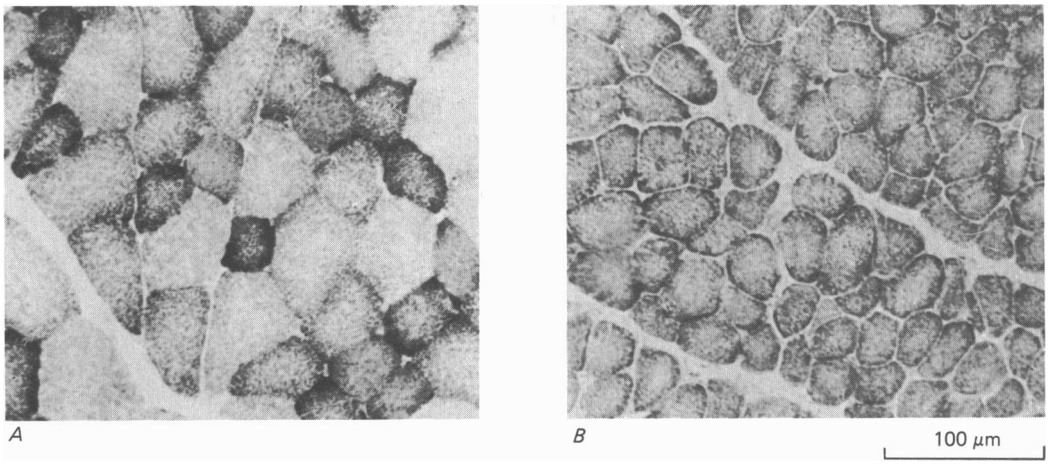

Fig. 3. Muscle fibres stained for activity of succinate dehydrogenase (SDH) in section from normal muscle (*A*) and from muscle subjected to chronic stimulation at a pulse rate of 40 Hz (*B*). Note that fibres of the stimulated muscle are relatively uniform with respect to the density of staining in central fibre regions ('core SDH').

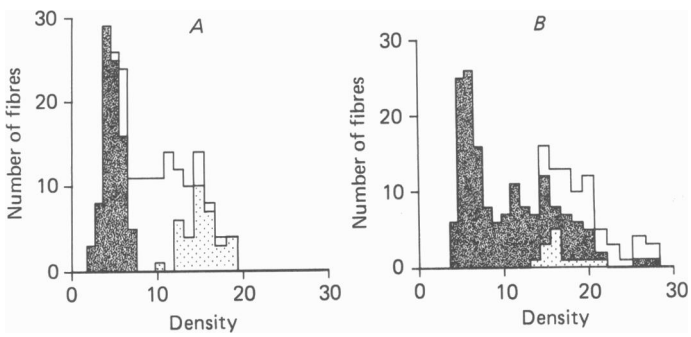

Fig. 4. Histograms showing the distribution of density of core-SDH staining among muscle fibres of a normal m. peroneus longus from cat (*A*) and a normal m. extensor digitorum longus from rat (*B*). Values for density calculated and measured (microphotometry) as described in Methods. Separate labelling of data for fibres of type I (stippled), IIA (open) and IIB (filled) respectively. In each muscle a total of 200 fibres were measured.

normal muscles for fibres that had been type classified by aid of ATPase staining. There was a continuous variation in staining density between separate fibres of the same muscle (Fig. 3*A*). As is demonstrated in Fig. 4*A*, individual fibres of types I, IIA and IIB showed some overlap with respect to their SDH staining (cf. Reichmann & Pette, 1982). There was, however, a fairly clear-cut separation between fibres of the three types with respect to their mean staining intensity (Fig. 4*A*, Table 2). Presumably for technical reasons, the absolute average values did, however, vary from experiment to experiment (e.g. experiment a *vs.* b in Table 2). In the present

context, we were primarily interested in relative values. In Table 3, a normalized intensity of core-SDH staining has, for each fibre type, been calculated in relation to the mean value for all the measured fibres from the same muscle. Furthermore, Table 3 also shows a quantitative estimate of the variability of core-SDH staining within different classes of fibres. The variability was, for (a class of) fibres of a given muscle, calculated as the ratio between the standard deviation and the corresponding mean value.

TABLE 2. Density of staining for core SDH in different types of muscle fibres of the normal peroneus longus

Experiment	All	Type I	Type II A	Type II B
a	12·6 ± 6·3 (200)	21·1 ± 3·3 (41)	14·0 ± 4·1 (79)	6·7 ± 2·3 (80)
b	7·9 ± 4·1 (200)	13·9 ± 1·3 (42)	8·1 ± 2·8 (82)	4·1 ± 1·0 (76)
c	8·7 ± 4·3 (200)	15·1 ± 2·1 (35)	10·1 ± 2·7 (79)	4·8 ± 1·1 (86)
Average	9·7	16·7	10·7	5·2

Mean ± S.D. (n) for density measurements of core-SDH staining in muscle fibres of three peroneus longus muscles that had not been subjected to chronic treatment. Within each muscle, differences between the mean values for types I, II A and II B respectively were statistically highly significant ($P < 0·001$). Bottom line shows averages of the respective mean values from the three different muscles. Classification of muscle fibres into types I, II A and II B by aid of ATPase staining (see Methods).

TABLE 3 Normalized density and variability of staining for core SDH in different types of normal muscle fibres of peroneus longus

	All		Type I		Type II A		Type II B
Normalized density (%)	100		173 ± 5	*	110 ± 7	*	53 ± 2
Variability (%)	51 ± 1	*	13 ± 3	*	30 ± 4	N.s.	27 ± 6
n	3		3		3		3

Normalized density of staining for a given fibre type: absolute density values normalized in relation to the average density for all measured fibres of the same section. Variability calculated, for each muscle and fibre type of Table 2, as (S.D./mean). Displayed values show the averages for normalized density and variability of staining, as calculated for the three muscles of Table 2. 'N.s.' between columns = difference not statistically significant (t test, $P > 0·05$); *, as in Table 1.

Peroneus longus muscles of animals subjected to tonic long-term stimulation

ATPase staining. Tonic long-term stimulation for 8 weeks had a very marked effect on the activity of myofibrillar ATPase: all fibres became similar to each other and there were no longer any dark fibres to be seen after alkaline pre-incubation (Fig. 5*C* and *E*). Thus, according to usual criteria there were no longer any fibres of type II in these muscles. The fibres of the stimulated muscles had, however, not acquired the same ATPase properties as those of type I fibres of the normal peroneus longus: the latter fibres were light after alkaline and dark after acid pre-incubation whereas the chronically stimulated fibres were about equally light after both types of pre-incubation. Furthermore, after pre-incubation at pH 9·4, the chronically stimulated fibres were not quite as lightly stained as the normal type I fibres of peroneus longus. With respect to these properties, the chronically stimulated fibres did, however, quite closely resemble the type I fibres of soleus. It has previously been

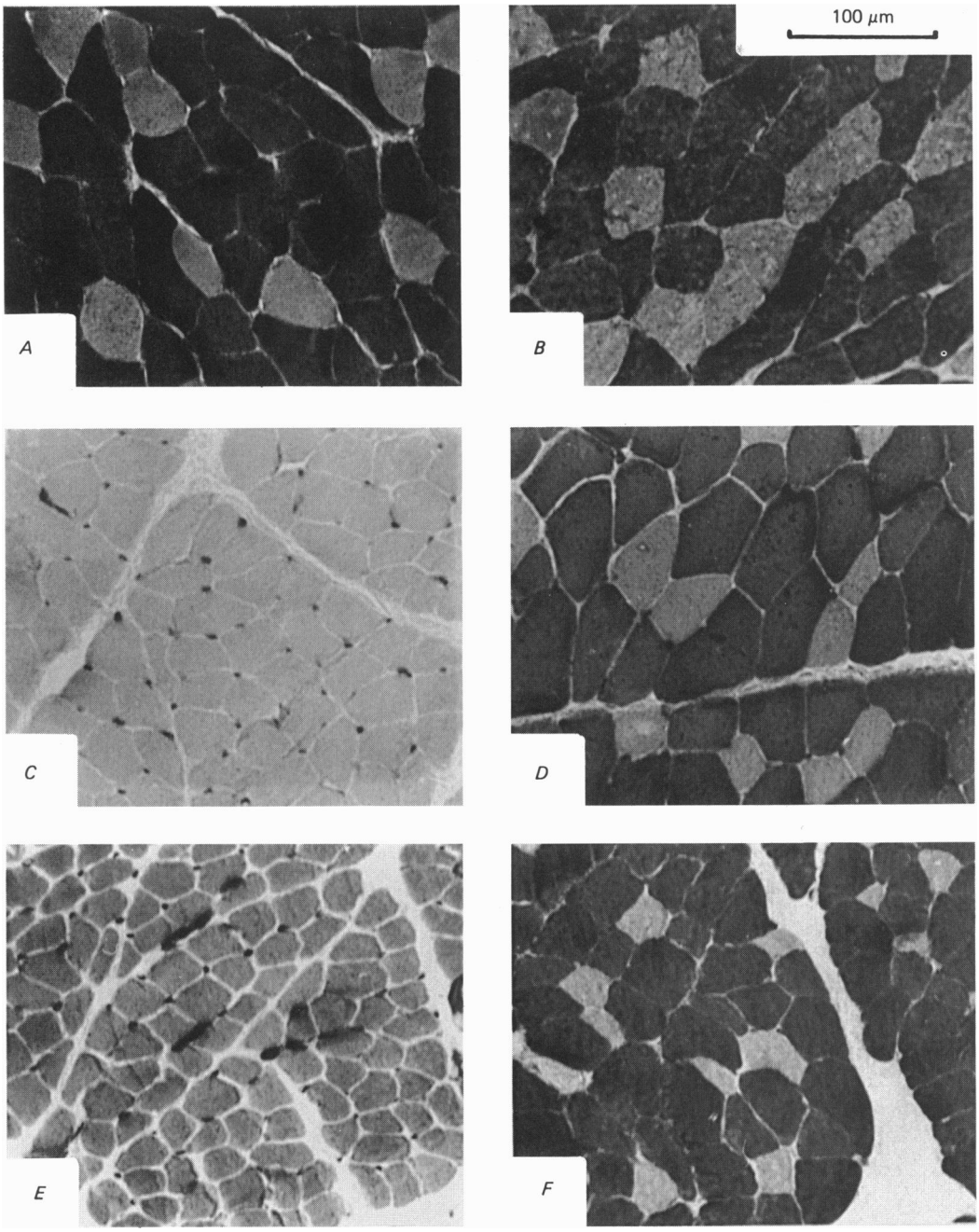

Fig. 5. Muscle fibres from cat's peroneus longus stained for myofibrillar ATPase after alkaline pre-incubation (pH 9·4). The respective sections were obtained from animals subjected to hemispinalization and deafferentation but no long-term stimulation (*A* and *B*), chronic stimulation at 40 Hz (*C* and *D*) and chronic stimulation at 10 Hz (*E* and *F*) respectively. Sections to the left (*A*, *C* and *E*) were from the experimental side and sections to the right (*B*, *D* and *F*) were from the contralateral control muscle.

reported that, after alkaline pre-incubation, the cat's soleus fibres show a somewhat darker ATPase stain than that of type I fibres of gastrocnemius (Burke & Tsairis, 1974). Furthermore, in the type I fibres of contralateral (right-side) soleus muscles we found the ATPase staining to be about equally light after pre-incubation at pH 9·4 and 4·1 respectively.

TABLE 4. Effects of chronic treatment on muscle fibre diameter

	Non-stim.		Slow stim.		Fast stim.	
Left-side mean (μm)	50±8	*	30±4	*	36±3	*
Left/right-all mean (%)	99±5	*	68±9	*	85±7	*
Left/right-I mean (%)	124±1	*	90±10	*	109±7	*
Left-side variability (%)	21±3	N.s.	17±4	N.s.	17±2	*
Left/right-all variability (%)	97±8	*	71±17	N.s.	79±12	*
n	4		5		6	

Data for fibre diameters, as measured in animals subjected to (i) left-side hemispinalization and dorsal rhizotomy but no chronic stimulation (non-stim.), (ii) the same operations plus left-side chronic stimulation of the common peroneal nerve at pulse rates of 5 or 10 Hz (slow stim.) or (iii) pulse rates of 20 or 40 Hz (fast stim.). Displayed values show averages of mean values or variabilities of fibre diameter, as obtained from the different individual muscles within the respective group. 'Left-side' = values for experimental muscles. 'Left/right-all' = ratio between values for experimental and contralateral muscles. 'Left/right-I' = ratio between value of experimental muscle *vs.* that for only the type I fibres of the contralateral control muscle. Symbols for statistical comparison (*, statistically significant, $P < 0{\cdot}05$; n.s., not statistically significant, $P > 0{\cdot}05$) to the right of column 'fast stim.' refer to comparisons between that column and the 'non-stim.' column; those between columns indicate significance of differences between those columns. For mean value and variability of fibre diameters in normal muscle, see Table 1.

In chronic cats that had not been subjected to long-term stimulation (experimental controls), the left-side muscles possessed normal properties with respect to ATPase staining as well as with regard to the distribution of fibre types. This was also true for the right-side non-stimulated peroneus longus muscles of chronically treated cats. We never saw any evidence for fibre-type grouping in non-stimulated muscles.

Fibre diameters. As all chronically stimulated fibres had become similar to each other in ATPase staining, these fibres could no longer be subdivided into different types. Hence, in chronically stimulated muscles we calculated the average properties (fibre diameter, core SDH) for all measured fibres together (cf. Tables 4 and 5).

In all the chronically stimulated muscles, average fibre size had become smaller than that of the respective contralateral control muscle (Figs. 6 and 7, Table 4). No such changes were observed in cats that had only been subjected to left-side hemispinalization and deafferentation (Table 4, non-stim.); hence, the decrease in fibre size of stimulated animals was indeed caused by the stimulation and not by other aspects of the chronic treatment.

The stimulation-evoked decrease in fibre diameter was significantly more marked after long-term tonic activation at slow pulse rates than after tonic treatment with fast rates (Table 4). After stimulation at slow rates the fibres became, on average, even thinner than the type I fibres of the contralateral control muscle (Fig. 6*A*, Table 4).

After 8 weeks of chronic stimulation the muscle fibres did not only become thinner, but they also became more similar to each other: on the stimulated side the variability in fibre size was 20–30 % lower than that found in the respective contralateral control muscles (Table 4). This effect was significant for slow as well as for fast rates of treatment (Table 4).

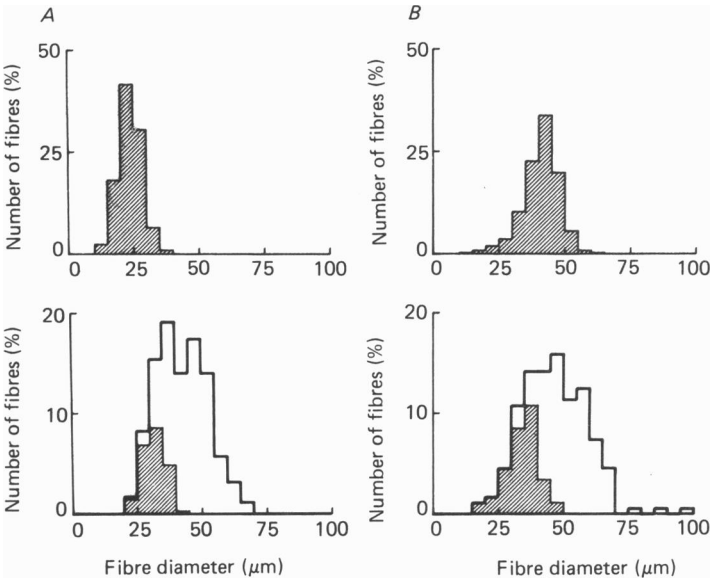

Fig. 6. Histograms showing the distribution of fibre diameters in four different peroneus longus muscles of cat. Hatched areas: fibres of type I. Upper graphs: muscles treated with chronic stimulation at 10 Hz (*A*) and 40 Hz (*B*) respectively. Lower graphs: non-stimulated muscles from the contralateral side of the same animals. Total number of measured fibres: *A* upper, 416 fibres; lower, 350 fibres. *B* upper, 358 fibres; lower, 176 fibres.

As one would expect, the marked stimulation-evoked decreases in fibre size were associated with a decline in muscle weight (Fig. 7*A*). For direct comparisons between these two parameters, fibre cross-sectional area is more adequate than fibre diameter. For the purpose of left-right comparisons, we computed an 'equivalent fibre area' from measured diameters by using the formula for a circle. If fibre length and fibre number stayed constant, the total weight of the muscle fibres themselves would be directly proportional to their mean cross-sectional area. As is seen in Fig. 7*A*, the relative decline in muscle weight was not consistently different from the relative decrease in fibre cross-sectional area.

Changes in fibre size vs. changes in force. If the number of fibres as well as their specific force remained constant, the maximum tetanic force of a muscle should vary in direct proportion to the average cross-sectional area of its muscle fibres. However, in all the chronically stimulated muscles, the relative decrease in maximum force was greater than the relative decline in fibre size (see left- *vs.* right-side comparisons of Fig. 7*B*). On average, the decrease in force was about 20 % greater than the decline in fibre cross-sectional area.

Core-SDH staining. The most evident effect of chronic stimulation on the staining

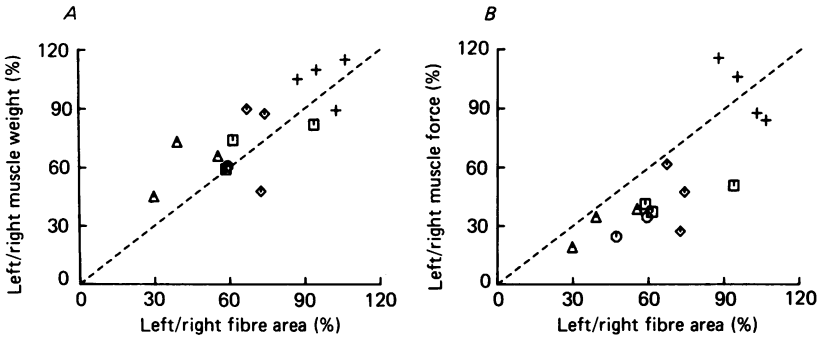

Fig. 7. Left/right (i.e. experimental/control) ratios for muscle weight (*A*) and maximum tetanic force (*B*) plotted *vs.* left/right ratio for fibre cross-sectional area of peroneal muscles from cats subjected to different types of chronic treatment: left-side hemispinalization and deafferentation but no chronic stimulation (crosses), chronic stimulation at 5 Hz (circles), 10 Hz (triangles), 20 Hz (squares) and 40 Hz (diamonds). Interrupted unity line drawn into each graph. For one of the muscles treated by 5 Hz, no muscle weight was available.

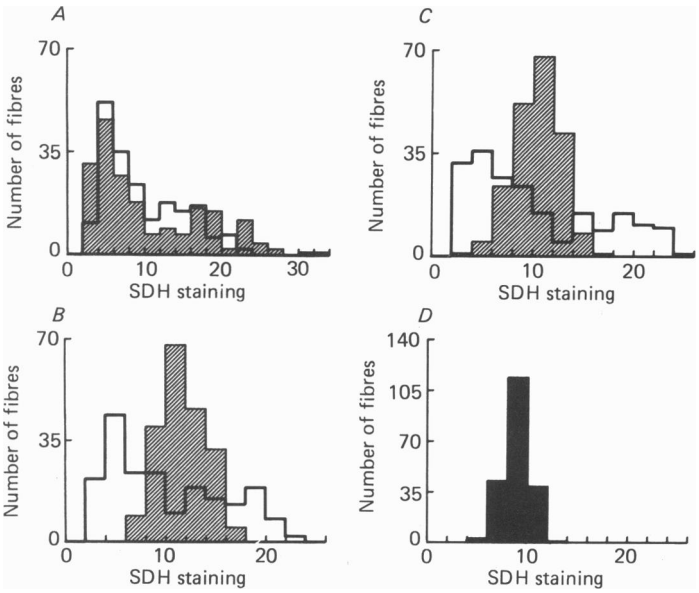

Fig. 8. Histograms *A–C* show the distribution of density of core-SDH staining among fibres of peroneus longus muscles from cats subjected to different types of chronic treatment. Hatched areas signify values from the experimental (left) side and open histograms are used for values from the contralateral (right) control side. Data from animals subjected to left-side hemispinalization and deafferentation but no chronic stimulation (*A*), chronic stimulation at 10 Hz (*B*) and chronic stimulation at 40 Hz (*C*). The plot of *D* shows corresponding measurements from the right-side soleus muscle of the same cat as *C*. Totally 200 fibres measured in each muscle.

for core SDH was that the fibres became more similar to each other (Fig. 3*B*). The average staining intensity remained about the same, but the variability decreased to less than half of that found in the respective contralateral control muscles (Fig. 8; Table 5). Thus, in chronically stimulated muscles, the mean and variability

TABLE 5. Effects of chronic treatment on density and variability of staining for core SDH

	Non-stim.		Slow stim.		Fast stim.	
Density left	11±2	N.s.	13±6	N.s.	10±2	N.s.
Density left/right (%)	102±16	N.s.	119±27	N.s.	103±15	N.s.
Variability left (%)	61±9	*	23±4	N.s.	28±6	*
Variability left/right (%)	123±22	*	37±7	N.s.	43±7	*
n	3		4		4	

Density and variability of staining for core SDH, measured and calculated as in Tables 2 and 3, for muscle fibres from the same types of experimental animals as in Table 4. 'Left/right' = ratio between mean values for experimental (left) and contralateral (right, control) muscles. The values for 'density left/right' should be compared to the 'normalized density' values of Table 3. *, n.s.: as in Table 4.

of core-SDH staining had actually become similar to that of normal IIA fibres (cf. Tables 5 and 3). Similar values for mean and variability were also found in non-stimulated soleus muscles (Fig. 8*D*). In five out of the eleven experiments of Table 5, core SDH was measured in the right-side soleus. For these five muscles, the mean density value was 8·6±0·7 and the variability 22±5% (values not significantly different from those of chronically stimulated muscles in Table 5; $P > 0{\cdot}1$).

DISCUSSION

Normal composition of the cat's peroneus longus

Our data concerning the fibre type composition of the cat's peroneus longus show a substantially greater percentage of type I fibres (mean 18%) than that reported in an earlier study (mean 6%; Ariano, Armstrong & Edgerton, 1973). The cause for this discrepancy is uncertain (different strains of cats?). The present histochemical results fit fairly well, however, with our physiological data for the same muscle (Table 6, 'proportion of slow motor units'). In several other mixed muscles it has been found that the fraction of muscle cross-sectional area taken up by type I fibres tends to be larger than the fraction of total muscle force supplied by slow units (McDonagh *et al.* 1980; Burke, 1981; Dum *et al.* 1982; cf., however, Edjetehadi & Lewis, 1979). This has led to the tentative conclusion that slow fibres of mixed muscles commonly tend to be intrinsically weaker than the fast fibres. In Table 6 we have performed similar calculations for peroneus longus, based on the present histochemical data in combination with our recent physiological observations (Kernell *et al.* 1983). The results are not unambiguously indicating that slow peroneal fibres would be intrinsically weak: the percentage of cross-sectional area supplied by fibres of type I lies in between the two different estimates for the percentage of force supplied by slow units. The latter two estimates refer to two different methods for performing the fast–slow classification within peroneus longus (see Kernell *et al.* 1983, for further details).

As is commonly the case within mixed hind-limb muscles of the cat, peroneal fibres of type I were markedly thinner than those of type II (Fig. 2, Table 1; cf., McDonagh *et al.* 1980; Burke, 1981).

The activity of core SDH, estimated by methods similar to the present ones ('conventional histochemistry'), have earlier been found to be well correlated to the

TABLE 6. Comparisons between muscle fibre and motor unit composition of m. peroneus longus

(a)	Proportion fibres of type I	18 (10–21) %
(b)	Proportion 'slow' motor units	21 or 29 %
(c)	Ratio cross-sectional diameters type I/type II	0·71 (0·64–0·75)
(d)	Calculated ratio cross-sectional areas type I/type II	0·49 (0·40–0·54)
(e)	Calculated proportion of muscle cross-section occupied by type I fibres	10 %
(f)	Proportion of maximum muscle force produced by 'slow' motor units	5 or 14 %

Owing to the usage of two alternative methods for fast–slow classification of motor units (see Kernell *et al.* 1983), two alternative figures appear at places concerning 'slow' peroneus longus units. The lower one of these estimates refers to classification according to the 'sag' method (cf. Burke, 1981). Motor unit data taken from Kernell *et al.* (1983). Percentage of muscle cross-section occupied by type I fibres (P) calculated according to equation:

$$P = (ab/(ab-a+100)) \times 100,$$

where 'a' is the percentage of type I fibres and 'b' is the ratio between the mean cross-sectional areas of fibres of type I and II. For other explanations, see text.

fatigue resistance of single motor units (fast muscle of rat; Kugelberg & Lindegren, 1979). In agreement with the identification, in the cat, of IIB fibres as those of fatigue-sensitive units (cf. Burke, 1981), we also found this fibre type, on average, to stain the least for core SDH (Fig. 4*A*; cf. Burke & Tsairis, 1974; Edjetehadi & Lewis, 1979; Reichmann & Pette, 1982).

Effects of chronic stimulation

General remarks. In our preceding physiological study (Eerbeek *et al.* 1984) we compared the effects of 'fast' and 'slow' patterns of tonic long-term stimulation on various contractile properties of the peroneus longus muscle. We then found that these two types of patterns had the same effects on contractile speed and endurance, but different effects on aspects of contractile force (maximum tetanic force, twitch: tetanus ratio). The present histological–histochemical results are consistent with the physiological findings: (1) the fast and slow patterns of tonic stimulation all had the same effects on the staining properties of myofibrillar ATPase (Fig. 5*C* and *E*), which is considered a histochemical correlate of contractile speed (cf. fast *vs.* slow motor units, Burke, 1981); (2) the fast and slow patterns of tonic stimulation had practically the same effects on the density of SDH staining (Fig. 8*B* and *C*; Table 5), which is considered a histochemical correlate of fatigue resistance; (3) the fast and slow patterns of tonic stimulation had different effects on fibre diameter (Fig. 6, Table 4), which is a histological correlate of maximum muscle force. In the latter case, the direction of the differences was also consistent: maximum tetanic

force as well as fibre diameter were better maintained after fast than after slow patterns of stimulation.

As has been the case in many preceding studies of chronic muscle stimulation, the present investigations concerned activation patterns that were covering a great proportion of total time per day (typically 50 %). Furthermore, our 'fast' rates were limited to 20–40 Hz (for motivation, see Eerbeek *et al.* 1984). It would be of interest to find out, in further experiments, whether the presently observed similarities in the effects of 'fast' and 'slow' patterns of stimulation would appear also if such patterns differed more in pulse rate and covered smaller daily amounts of time than was the case in the present study (investigation in progress).

In addition to our studies of mean fibre properties, we also analysed the variability of various fibre parameters. If the normally existing differences between muscle fibres were partly caused by differences in activation patterns, then all fibres should become more similar in properties if they were subjected to more similar amounts and patterns of activity. In the present study, chronic stimulation caused a marked increase in similarity (i.e. decrease in 'variability') between the fibres with respect to all the major properties investigated, namely: (1) ATPase staining (Fig. 5), (2) SDH staining (Figs. 3*B* and 8, Table 5), (3) fibre diameter (Fig. 6, Table 4). In the further discussion, these different properties will be dealt with separately.

ATPase staining. It is known from previous studies that slow patterns of long-term stimulation may cause all the fibres of a mixed (fast) muscle to acquire the ATPase staining properties characteristic for slow muscles (Pette, Müller, Leisner & Vrbová, 1976; Rubinstein, Mabuchi, Pepe, Salmons, Gergely & Sreter, 1978; Buchegger, Nemeth, Pette & Reichmann, 1984). Some fast patterns have also been reported to increase the percentage of type I fibres as well as promoting the appearance of slow-type myosin (Hudlická, Tyler, Srihari, Heilig & Pette, 1982; Sreter, Pinter, Jolesz & Mabuchi, 1982). It has not earlier been demonstrated, however, that even fast patterns may change the ATPase staining of *all* the fibres of a mixed muscle into that characteristic for slow muscle (cf. Fig. 5*C* and *E*). Such findings do not exclude the possible presence of some remaining differences in ATPase properties between the various stimulated fibres. However, it should be noticed that, in the stimulated muscles, we found uniform ATPase-staining properties at all the three different pH's employed for pre-incubation (9·4, 4·5 and 4·1).

In the absence of chronic stimulation, we found no evident effects of hemispinalization and deafferentation on the ATPase-staining or fibre-type composition of peroneus longus. This is in contrast to findings reported for muscles of hemispinalized rats (Caccia, Meola, Brignoli, Andreussi & Scarlato, 1978) and spinalized cats (Mayer, Burke, Toop, Walmsley & Hodgson, 1984). The reason for this discrepancy is unclear. The muscles of the experimental control animals, which seemed to be severely under-used, were still remarkably close to normal in their various histochemical and contractile properties (cf. 'non-stim.' category in Tables; see also Eerbeek *et al.* 1984). It should be stressed, however, that we do not yet know precisely how silent the respective motoneurones were. Furthermore, in muscles of the 'non-stim.' category, the maintenance of force and fibre size might have been favoured by the fact that the animals tended to keep the experimental muscle in a stretched position (ankle usually extended; cf. Gallego, Kuno, Núñez & Snider, 1979).

Core-SDH staining. It is known from several previous publications that long-term stimulation may cause muscle fibres to look more similar to each other with respect to their histochemical activity for oxidative enzymes (slow stimulus patterns: Pette *et al.* 1973, 1976; Pette, Ramirez, Müller, Simon, Exner & Hildebrand, 1975; Brown, Cotter, Hudlická & Vrbová, 1976; fast patterns: Sreter *et al.* 1982). In the present study, comparisons between normal and chronically stimulated fibres (Figs. 4*A* and 8*B* and *C*) suggested that the long-term activation left the core SDH practically unchanged in IIA fibres and caused it to increase in IIB and, unexpectedly, to decrease in type I fibres. As IIB fibres presumably correspond to the fatigue-sensitive fibres in cat's hind-limb muscles (FF units; Burke, 1981), such complex changes would still be consistent with the view that an increase in muscle endurance should be paralleled by an increase in the activity of oxidative enzymes. With respect to the apparent behaviour of the IIA and IIB fibres respectively, the present findings are in accordance with the observations of Pette & Tyler (1983), who estimated SDH activity by measuring maximum initial reaction rates (rabbit's tibialis anterior, 10 or 40 Hz patterns of chronic stimulation; type I fibres not investigated). On the other hand, in the study of Buchegger *et al.* (1984), it was noticed that chronic stimulation of a rabbit's tibialis anterior muscle for 84 days caused an increase of mean SDH activity among the muscle fibres while the range of variation remained as wide as normal. Also in this case, kinetic measurements of SDH activity were used (maximum initial reaction rates), and the authors made the interesting observation that such measurements could demonstrate differences between fibres which, in a conventional histochemical preparation, looked similar to each other. The reasons for this apparent discrepancy between the two techniques is not yet clear. In the present experiments, the similarity of SDH staining among the fibres of a chronically stimulated muscle could not easily be explained as being caused by some kind of saturation phenomenon: the *average* density of fibre staining was nearly the same for stimulated and non-stimulated muscles whereas the *variability* decreased after chronic activation (Fig. 8, Table 5).

In the present chronically stimulated muscles, the density of SDH staining increased in a prominent fraction of thick fibres (IIB) whereas it decreased within a less numerous group of thin fibres (I). Such results would be consistent with an over-all increase in the SDH activity of the muscle as a whole, as expressed per unit weight. Biochemical measurements on whole fast muscles have generally shown an increase by a factor of two or more of the activity of oxidative enzymes after a few weeks of chronic stimulation at low or high pulse rates (Pette *et al.* 1973, 1975; Henriksson, Galbo & Blomstrand, 1982; Buchegger *et al*, 1984; Hudlická, Aitman, Heilig, Leberer, Tyler & Pette, 1984).

Fibre diameters. With respect to slow patterns of tonic long-term stimulation, the present findings confirm earlier reports that such types of chronic activation may cause muscle fibres to become thinner and more similar to each other in size (Pette *et al.* 1975; Brown *et al.* 1976; Salmons & Henriksson, 1981). To these earlier results we add the new observations that (i) a decrease in diameter and size variability was caused also by chronic stimulation at physiologically 'fast' pulse rates, (ii) the decrease in diameter produced by 'fast' patterns was less than that caused by 'slow' patterns. It should be noted that this latter difference could not easily be explained

as an effect of different amounts of activation. Treatment at 40 Hz produced less fibre shrinkage than that of 10 Hz ($P < 0{\cdot}02$; cf. Fig. 7) in spite of the fact that both these patterns were given during 50% of total time per day and, hence, the total number of impulses per day was four times as great for the 40 Hz pattern as for the 10 Hz pattern. The difference between the effects of fast and slow patterns on fibre diameter and force might have been related to the strength of the contractions produced by the chronic stimulation. The higher rates of the fast patterns would produce stronger contractions than those of the slow patterns, and a higher amount of 'force stress' might favour the maintenance of factors of relevance for contractile force.

In our present investigation of 8-week-stimulated muscles, we observed no signs indicating that the activation-evoked decline in fibre diameter and force should be regarded as essentially pathological reactions (cf. Gambke, Maier & Pette, 1985). The muscle fibres showed no histological signs of degeneration, and the muscles displayed an excellent resistance to fatigue (Eerbeek *et al.* 1984).

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