J. Physiol. (1987), **392**, *pp.* 377–395
With 8 text-figures
Printed in Great Britain

THE ROLE OF FREQUENCY IN THE EFFECTS OF LONG-TERM INTERMITTENT STIMULATION OF DENERVATED SLOW-TWITCH MUSCLE IN THE RAT

BY W. S. AL-AMOOD AND D. M. LEWIS
From the Department of Physiology, University of Bristol, Bristol BS8 1TD

(*Received 10 February 1987*)

SUMMARY

1. Rat soleus muscle was denervated by sciatic transection and electrically stimulated for periods of between 3 and 9 weeks with intermittent 1 s bursts of pulses. Most of the bursts were either repeated every 90 s and pulses within them had frequencies between 10 and 100 Hz, or had a frequency of 50 Hz and were repeated at intervals between 60 and 600 s. Comparisons were made with continuous stimulation at 10 Hz.

2. At the end of the period of stimulation, isometric twitches and tetani were measured and, in a proportion, also isotonic shortening velocity.

3. Isometric twitch duration (contraction and relaxation) decreased with time of stimulation. Very similar effects were seen in all animals in which intermittent stimulation had been used. There was a significant relationship between the change in twitch duration and the frequency used within the bursts of chronic stimulation, with slightly larger effects at frequencies of 40 and 60 Hz. The lowest burst repetition rate produced the largest effects.

4. It was confirmed that similar changes were found in denervated muscles that were not stimulated, although these changes were smaller and developed more slowly.

5. The extreme loss of tetanic tension induced in the muscle by denervation was reduced by chronic stimulation, with no significant difference between different regimes, although there were small differences which showed the same patterns of effectiveness described for twitch durations.

6. Continuous stimulation at 10 Hz maintained the twitch contraction and relaxation phases at the values found 3 weeks after denervation, that is it prevented secondary shortening of the twitch. Continuous stimulation reduced tension loss but was, perhaps, less effective than intermittent stimulation.

7. Twitch–tetanus ratio increased with denervation with little spontaneous reversal later. Stimulation at all frequencies reduced the ratio, but it did not reach normal values.

8. Isotonic shortening velocity was measured in many of the muscles. Maximum velocity was estimated and normalized by muscle length. Results were variable but the largest increases in velocity were seen as frequently in muscles chronically stimulated intermittently at 10 Hz as in muscles in which higher frequencies were used.

9. It is concluded that the effect of intermittent stimulation is almost independent of the frequency used within the bursts of pulses. This independence of frequency reinforces a proposal made earlier that the effect of intermittent chronic stimulation is to enhance the synthesis of protein, but not to switch that synthesis from slow to fast myosin which, in rat soleus at least, is induced by denervation. Continuous stimulation (at 10 Hz) prevents the transformation from slow to fast muscle after denervation.

INTRODUCTION

The trophic effect of nerve on mammalian skeletal muscle is mediated, in part at least, by activity induced by patterns of nerve activity. Two types of pattern have been shown to produce different effects by imposing long-term electrical stimulation. Salmons & Vrbová (1969) found that low-frequency continuous stimulation of the nerve changed fast muscle to slow. The reverse change, from slow to fast, has been seen in denervated slow muscle activated by intermittent bursts of stimulation, the pulse frequency within the bursts being set at 100 Hz (Lømo, Westgaard & Dahl, 1974; Lømo, Westgaard & Engebretsen, 1980). It has been suggested from these and other experiments that the transformation of slow to fast muscle is brought about by imposed or natural activity stimulating the muscle at high frequencies (100 Hz). An alternative hypothesis about the trophic effect of activity has arisen from investigations of fast to slow transformations by Sreter, Pinter, Jolesz & Mabachi (1982). They found that fast muscle was changed to slow over a range of stimulation frequencies up to 60 Hz as long as the activity was nearly continuous. Sreter and his colleagues suggested that the degree of conversion was dependent on the total number of stimuli delivered to the muscle. It is impossible to compare these two hypotheses directly because the two sets of experiments examined different transformations (slow to fast and fast to slow) and different states of innervation (normal and denervated) as well as being in different species.

The experiments we report here investigate the transformation of denervated rat slow muscle reported by Lømo *et al.* (1974) and ask how the change is related to stimulation characteristics. Two variables were explored. In one series bursts of pulses of different frequencies (10–100 Hz) were delivered with 90 s between bursts; in this both frequency and number of pulses varied. In the second series bursts of pulses at 40 Hz were given at different intervals (60–600 s); here only the number of pulses was changed. If the frequency of activation of muscle were important we would expect maximal slow to fast transformation with the 100 Hz bursts, less change at lower frequencies with none when the frequency was 10 Hz. If the number of impulses were important, the regimes with the highest frequencies and the shortest interburst intervals would be expected to be most effective in keeping the muscle slow and therefore allow minimal conversion to fast muscle. Our results showed very little or no effect from either of the stimulus parameters despite various statistical manipulations to reveal underlying trends. We discuss the hypothesis that it is the total amount of time during which the muscle is contracted that maintains (or produces) slow-contracting muscles and the presence of substantial rest periods that allows a slow muscle to become fast. Our data are compatible with the suggestion by Al-Amood, Finol & Lewis (1986) that the transformation of slow to fast muscle is a

response to denervation rather than a specific effect of the stimulation, as long as the stimulation was intermittent. A preliminary account of this work has been presented by Al-Amood, Lewis & Rowlerson (1985).

METHODS

Mature male Wistar rats aged from 6 to 8 months and weighing between 360 and 575 g (mean 445 g) had one sciatic nerve sectioned in mid-thigh under full anaesthesia (sodium pentobarbitone, 60 mg/kg, injected I.P. and supplemented as necessary) and aseptic conditions. Both sciatic stumps were tied and the central one was drawn through the denervated hamstring muscles to delay reinnervation beyond the period of stimulation. Electrodes were placed to stimulate soleus. In most animals one electrode was a stainless-steel plate placed under the deep surface of the muscle and the other a stainless-steel multistrand wire wrapped around the Achilles tendon. Animals were housed singly in large plastic-bottomed cages, and they fed and slept normally. Body weight was maintained.

Other details of electrodes and external connections have been described by Al-Amood *et al.* (1986). One improvement was the use of a miniature jack plug which allowed free rotation with little twisting of the external cable but which was retained by a locking nut (Farnell).

A stimulator was designed by Mr J. Croker which allowed pulses of alternating polarity to be delivered and was powered entirely by rechargeable batteries. The design included a safety circuit which caused the output to be cut off if the pulses were prolonged to more than 2 ms. This would have prevented damage to the muscle if the output transitors should have failed in a way that produced a d.c. voltage at the muscle. (In practice this safety mechanism never was needed.)

One or two days after the denervation and implantation of electrodes stimulation was begun. Most animals (forty-six) were stimulated with bursts of pulses with a rest interval between bursts. Two main series of experiments were performed, varying the frequency within the bursts in one and interval between bursts in the other. For each combination of frequency and interval at least three rats were used, with totals of twenty-one and seventeen rats in the two series (seven were in common), and in both series stimuli of 1 ms duration were delivered in bursts lasting 1 s. In the first series the bursts were repeated every 90 s and frequency was set at either 10, 20, 40, 60 or 100 Hz In the second series a frequency of 40 Hz was chosen (because it appeared to be causing the best response) and the bursts were repeated at intervals of 60, 90, 180, 300 or 600 s. In preliminary experiments we tried a few other combinations of frequency and interval in order to find the optimal final protocol, and the data from these are included in the Figures.

In addition we did one other set of experiments (four rats) designed to mimic more closely the conditions used by Lømo *et al.* (1974, 1980). The electrode arrangement was different, with the steel wire wound around the proximal as well as the distal tendon. Stimulus pulses were only 0·1 ms in duration, which required stronger stimuli for maximal responses (10–15 mA compared with 3–5 mA for the main series using 1 ms pulses). Another set of conditions was used in four rats, and, although these do not fit in with the two main series, they are reported in this paper because the results are of general interest. In this set of pairs of pulses with an interval of 5 ms (200 Hz) were repeated every 90 s. Finally three rats were stimulated continuously at 10 Hz.

Stimulation was applied throughout the day to avoid the period of decreased threshold which was seen for up to an hour after inactivity when stimulation was restricted to part of the day (Al-Amood *et al.* 1986). It was therefore possible to compare the two daily regimes, and some data from Al-Amood *et al.* (1986) have been included in the Figures for this purpose. The data were selected from those animals with a comparable period of denervation (averaging 48 days). A rigorous comparison is still not possible because, although Al-Amood *et al.* (1986) used 1 s bursts of pulses at 100 Hz, they varied the repeat interval between 10 and 100 s. Even when we selected animals in which the interval was at least 50 s, so that the average interval of this set was 69 s, the coefficient of variation was as high as 36%. These two criteria only allow us to present data which can be compared in a general way with the present data, and this has been done visually for interest, although formal statistical comparisons have not been thought worth while.

In the final experiments the animals were anaesthetized fully with pentobarbitone (*v.s.*) and isometric twitches and tetani were recorded by the methods described by Finol, Lewis & Owens (1981). The important features are that measurements were made *in vivo* in liquid paraffin at

36 °C with the muscle held at the length producing maximal twitch tension. Stimuli were applied between the proximal and distal tendons which were freed from surrounding tissue to prevent stimulus spread. Pulses were of 0·5 ms duration and 1·3–1·5 times maximal. Some muscles were then removed from the body and measurements confirmed in bicarbonate–phosphate-buffered Ringer solution bubbled with 5% CO_2–95% O_2 mixture. In a proportion isotonic shortening velocity was recorded (Al-Amood *et al.* 1986), but in the remainder the extra of tetanic stimulation was avoided because the muscles were to be examined by histochemical and immunocytochemical techniques (Al-Amood *et al.* 1985).

We tried to maintain stimulation for between 50 and 60 days, a time at which Al-Amood *et al.* (1986) thought changes in dynamic properties were complete. In some cases stimulation failed because internal wires snapped, and we recorded from these animals within 24 h of the failure.

RESULTS

The changes in the isometric twitches are illustrated in Fig. 1. Twitches are shown from two muscles that had been denervated and stimulated intermittently for 7–8 weeks (*e* and *f*). Muscle *f* had been stimulated at a frequency of 10 Hz within the bursts, and it can be seen to be almost identical in time course with the twitch of muscle *e* which had been subjected to a frequency of 100 Hz within stimulus bursts. It can also be seen that there is little difference between these twitches and that of a denervated fast muscle (*d*). The analysed twitch data from all our experiments are illustrated in Fig. 2. There was a progressive shortening of the twitch between 20 and about 48 days in nearly all the muscles stimulated intermittently. The frequency of stimulation within the bursts is indicated by different symbols (see legend to Fig. 2), and it appears that there was no obvious difference related to this frequency. In contrast muscles stimulated continuously at 10 Hz (crosses in Fig. 2) had twitch times to peak longer than normal solei. Note that with intermittent stimulation the lowest (10 Hz) frequency (open circles) and the highest (100 Hz) frequency (squares) showed no systematic differences. One set of muscles (filled triangles) was stimulated differently from the rest, using high longitudinal currents and 0·1 ms pulses (to reproduce more closely the parameters used by Lømo *et al.* 1980), but again these are indistinguishable from the muscles subjected to other stimulation regimes. The final value reached by the stimulated, denervated muscles was close to the mean of soleus cross-reinnervated by the nerve from a fast-twitch muscle and subsequently denervated (vertical cross, far right; Baker & Lewis, 1984).

The one exception to this consistency of response was the set of muscles subjected to minimal stimulation – a pair of pulses 5 ms apart and repeated every 90 s (left-pointing triangles). Two of these muscles fall within the trend described above but the other two had twitches very similar to the continuously stimulated muscles.

For comparison Fig. 2 shows average data (filled squares with bars indicating S.D.) from a previous series (Al-Amood *et al.* 1986) in which the muscles were stimulated intermittently at 100 Hz but for only part of the day. These data are very similar to the new material. Figure 2 also includes data from muscles subjected to denervation without stimulation (filled circles). It can be seen that denervation prolonged the twitch, reaching a maximum duration at about 20 days. Later the twitch became briefer, matching control values at 6 weeks and by 11 weeks was faster than normal soleus. Although the average twitch of denervated muscles was longer than that of intermittently stimulated muscles, there was overlap between the two sets. Figure 1*c*

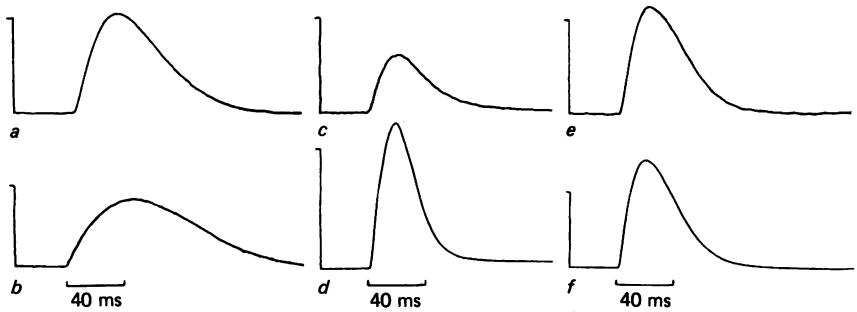

Fig. 1. Isometric twitch myograms of rat muscles. *a*, control innervated soleus (slow twitch). *b*, soleus denervated for 20 days. *c*, soleus denervated for 63 days (average of thirty-two contractions). *d*, extensor digitorum longus (fast twitch) denervated for 20 days for comparison. *e*, denervated soleus stimulated for 8 weeks by 1 s bursts of pulses of frequency 100 Hz with 90 s between bursts. *f*, denervated soleus stimulated similarly except frequency was 10 Hz and duration 7 weeks. Note: all are recorded at the same sweep speed. The tension calibration pulses were 500 (*a*), 125 (*b*), 25 (*c*), 255 (*d*) and 80 mN (*e* and *f*).

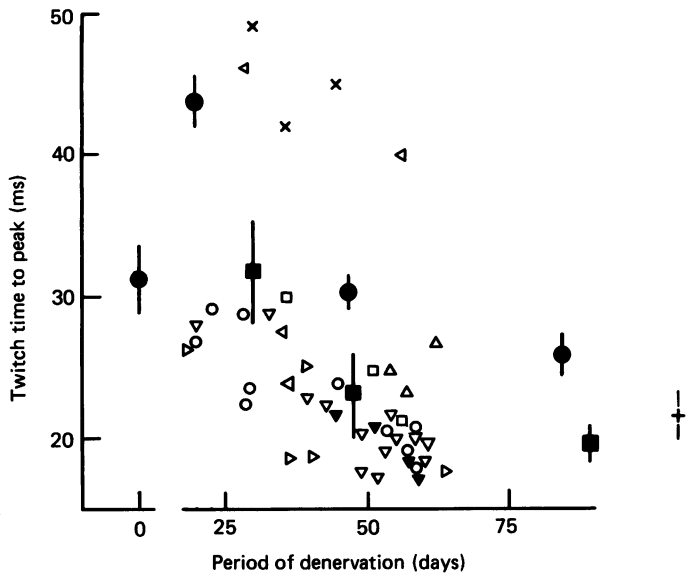

Fig. 2. Twitch time to peak of rat soleus muscle plotted against the time after denervation. Individual points show single muscles stimulated in bursts of pulses every 60–600 s. Frequencies within the bursts were 10 (○), 20 (△), 40 (▽, ▼), 60 (▷) and 100 (□) Hz for 1 s or 200 Hz for 5 ms (◁). Three muscles were stimulated continuously at 10 Hz (×). Pooled data (means and S.D. bars) are from previously published work: denervated unstimulated soleus and control soleus (●) and denervated cross-innervated soleus (+) are taken from Finol *et al.* (1981) and Baker & Lewis (1984). Denervated soleus stimulated at 100 Hz intermittently for part of the day (■) from Al-Amood *et al.* (1986). The abscissa shows time after denervation; stimulation was for 1 or 2 days less.

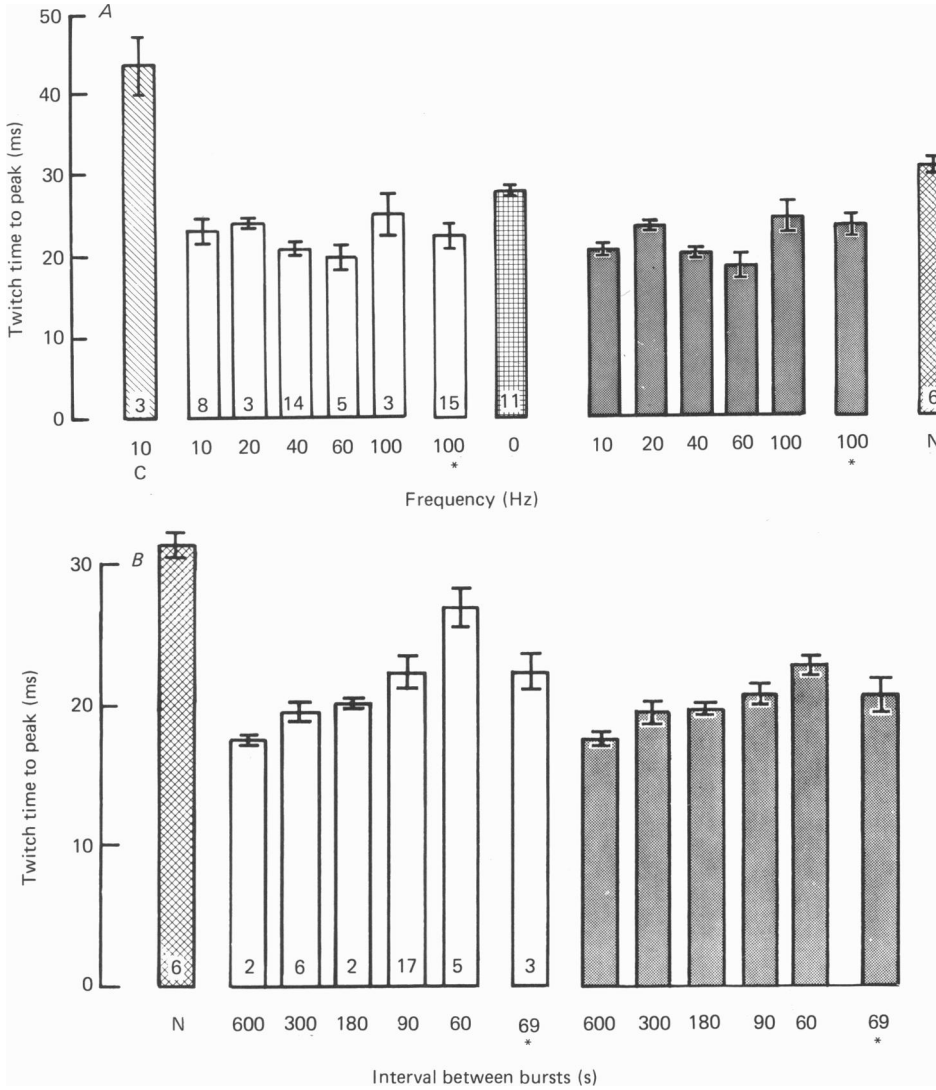

Fig. 3. Histograms showing mean (±S.E. of mean) twitch time to peak for different groups of denervated rat solei; numbers of animals are indicated within columns. In *A*, the muscles were grouped according to the frequency of stimulation (Hz) which is shown below each column, *C* indicates continuous rather than intermittent stimulation, the asterisk shows data from Al-Amood *et al.* (1986), 0 indicates unstimulated 48 day denervated muscles and N normal solei. The open columns are averages of raw data. The stippled columns were obtained after normalization to 48 days stimulation by assuming that all muscles would have shown a change in time to peak of −0·21 ms/day up to 46 days (the regression fitted to the points of Fig. 2). After normalization the difference between the 40 Hz and 100 Hz stimulated groups was significant with $P < 0{\cdot}01$ ($t = 3{\cdot}4$) and between the 60 and 100 Hz sets with $P < 0{\cdot}05$ ($t = 2{\cdot}7$). In *B* the muscles are grouped according to the interval between bursts (shown in seconds below each column), other labels as in Fig. 3*A*. The interval used for Al-Amood *et al.* (1986), indicated by *, represents an average; values between 10 and 100 s were used. Row 1 of Table 1 corresponds to the open columns and row 4 to the stippled ones.

also illustrates this point, showing the twitch of a soleus denervated for 7 weeks that had a contraction briefer than the average of the stimulated, denervated solei.

It may be noted that the continuously stimulated muscles (crosses, Fig. 2) had twitch characteristics very similar to denervated unstimulated muscles at 20 days, when the twitch was maximally prolonged (Baker & Lewis, 1983, see also Fig. 1*b*).

Two methods of analysis of the data are presented below. In one all data were grouped together according to either frequency within the bursts or interval between bursts, and the results are presented as histograms in subsequent Figures. This method puts together all the data but has the problem that many of the groups are inhomogeneous in relation to one variable. Therefore two subsets of data were selected in which only one of the two parameters was varied: (1) within-burst frequency with a fixed interval of 90 s between bursts and (2) the interval between bursts of 40 Hz. The total number of animals was reduced by this selection but a more formal testing by analysis of variance was possible, and the results are presented in Table 1.

Figure 3*A* shows the means and standard errors calculated for the times to peak of all the data displayed as open-column histograms. Statistical comparison of the means by a two-tailed *t* test revealed no differences between any of the intermittently stimulated sets either of the current series or of data from Al-Amood *et al.* (1986) in which muscles were stimulated with 100 Hz bursts (columns marked with asterisk). The effects of frequency within bursts on twitch times to peak were also subjected to an analysis of variance the results of which are presented in row 1 of Table 1. No significant variation could be ascribed to frequency with the initial analysis.

In contrast the muscles stimulated continuously at 10 Hz were significantly slower. The unstimulated denervated solei were statistically different to both intermittently and continuously stimulated muscles, but much closer to the former.

The fact that we were unable to maintain all animals for a similar period of time increased the variation in such averages. The scatter of data was such that it was difficult to be sure of the time course of the changes with time. Al-Amood *et al.* (1986, see Fig. 4*a*) had concluded that the changes were substantially complete by 50 days. Some confirmation of this was obtained from the present data by dividing into two arbitrary groups with different devervation times: less than 46 days and more than 47 days (no denervations happened to have lasted 46 or 47 days). The fall in time to peak with time up to 46 days (Fig. 2) was significant statistically ($n = 18$, $r = -0{\cdot}70$, $P < 0{\cdot}05$), and the slope of the regression was calculated as $-0{\cdot}21$ ms/day after omitting the two grossly aberrant muscles that had been stimulated by only two pulses. The regression for periods of more than 47 days was not significant ($r = 0{\cdot}34$, $n = 23$). This estimate of the rate of change of the twitch time to peak was used to normalize the data to a standard time of 48 days, assuming that all muscles were changing linearly and equally over 20–46 days and that the change was complete by 46 days (i.e. no adjustments were applied after that time). The normalization was made to 48 days because this was the mean period of denervation for one set of unstimulated muscles of Fig. 2, and also of one set of 100 Hz stimulated muscles reported by Al-Amood *et al.* (1986). Averages of these normalized data are shown by the shaded histograms of Fig. 3*A*. Normalization reduced the variability of the data by an average of about 40%. Further, significant difference are now seen between the

TABLE 1. The results of analysis of variance on two series of experiments in which either the interval between bursts or frequency of stimuli within bursts was varied

	Frequency within bursts varied					Interval between bursts varied				
	Mean	Variance between sets	d.f. (residual)	Variance ratio	P	Mean	Variance between sets	d.f. (residual)	Variance ratio	P
Twitch time to peak (ms)										
(1) All data	22·7	25·7 (4)	10·9 (16)	2·4	n.s.	20·8	29·9 (4)	4·88 (12)	6·1	< 0·01
(2) Data > 47 days	21·8	29·6 (3)	5·2 (6)	5·7	< 0·05	19·4	3·4 (4)	2·0 (8)	1·7	n.s.
(3) All data regressed	22·0	39·7 (4)	5·9 (16)	6·7	< 0·005	21·0	7·6 (4)	1·68 (12)	4·5	< 0·025
(4) Regressed < 46 days	21·3	32·7 (4)	7·3 (16)	4·5	< 0·025	20·0	11·2 (4)	2·1 (12)	5·2	< 0·025
Twitch half-relaxation (ms)										
(5) All data	26·3	42·0 (4)	34·9 (16)	1·2	n.s.	24·4	109·0 (4)	9·7 (12)	11·2	< 0·005
(6) Data > 47 days	23·7	29·3 (3)	1·4 (6)	2·1	n.s.	22·3	25·8 (4)	4·5 (8)	5·7	< 0·025
Tetanic tension (N)										
(7) All data	0·38	93·0 (4)	48·0 (16)	1·9	n.s.	0·49	140·0 (4)	50·0 (12)	2·8	n.s.
(8) Data > 47 days	0·43	38·0 (3)	53·0 (6)	0·7	n.s.	0·54	509·0 (4)	63·0 (8)	0·8	n.s.
Twitch-tetanus ratio										
(9) All data	0·31	0·008 (4)	0·0119 (16)	0·7	n.s.	0·31	0·0068 (4)	0·0050 (12)	1·4	n.s.
(10) Data > 47 days	0·30	0·015 (3)	0·0071 (6)	2·1	n.s.	0·30	0·00161 (3)	0·0041 (8)	0·4	n.s.
Days denervation	43·9	283·0 (4)	95·0 (16)	3·0	= 0·05	48·6	172·0 (4)	84·0 (12)	2·0	n.s.

Analysis has been done on four contractile properties and on the period of denervation of the muscles. For each of the first four variables the analysis was done (i) for all muscles examined (comparable to all open histogram columns in the Figures) and (ii) for those muscles that had been denervated for more than 47 days. For twitch time to peak, the data were also normalized to estimate the change that would have been expected if the period of denervation had been 48 days in all animals. Normalization used the linear regressions fitted to the data, and two normalizations were tried. In the first all data were regressed (row 3); in the second normalization was applied only to data from animals denervated for less than 46 days. The latter therefore corresponds to the stippled histogram columns in the Figures. d.f., degrees of freedom; n.s., not significant.

40 and 100 Hz stimulated muscles and also between the 60 and 100 Hz sets (see legend to Fig. 3). Analysis of variance (row 4 of Table 1) confirmed that this normalization revealed a significant ($P < 0{\cdot}025$), although small, effect of frequency on twitch time to peak.

The normalization used above clearly depends on an arbitrary division of the data, so a further procedure was used, in which a linear regression was fitted to the whole data. The resultant slope ($-0{\cdot}225$ ms/day) was not very different to that used for periods less than 47 days. The second regression was applied to all the data to normalize them to the same time (also 48 days), and this analysis is presented in row 3 of Table 1. Again a significant effect was seen ($P < 0{\cdot}025$).

The two methods of normalization shown in Table 1 rows 3 and 4 also suffered from the assumption that the time to peak changed linearly throughout the period, whereas it is likely that the change has a time course that is roughly exponential in shape. A final analysis was therefore done on data obtained only from rats denervated for more than 47 days (row 2 of Table 1). This assumed that changes were substantially complete by that time, as shown by the lack of any statistically significant regression of time to peak after 47 days (*v.s.*). The resultant analysis of variation is presented in row 2 of Table 1 and it also shows a possible effect of frequency on twitch time to peak ($P < 0{\cdot}05$).

All three methods used to try to reduce time-dependent effects suggested that there was significant variance introduced by using different frequencies within bursts of stimuli. We would conclude, therefore, that the effects of stimulation on twitch time to peak do depend on frequency. The analysis of variance does not indicate the nature of an effect, only its presence, but the histograms of Fig. 3 suggest that it is that the middle range (40 and 60 Hz) of frequencies that is more effective. It should also be remembered that the shape of the histograms suggests that stimulation intermittently at 10 Hz was just as effective as 100 Hz bursts. Further, the effect of changing frequency was small when compared with the fall in twitch time to peak observed between 20 and 50 days of denervation.

In addition to varying the frequency of stimuli within the bursts, we used intervals between bursts of between 60 and 600 s. The data were analysed by grouping according to interburst interval, and these results are displayed in Fig. 3*B*. Analysis of variance was also carried out as for frequency effects (Table 1). Three of the four analyses indicated that interval between bursts had significant effects on twitch time to peak ($P < 0{\cdot}025$ and $< 0{\cdot}01$). The histograms of Fig. 3*B* indicate that the effect was greatest with the use of the longest intervals. Note too that the selected data from Al-Amood *et al.* (1986, shown in Fig. 3*B* as columns labelled 69*) in which the interburst interval averaged 69 s showed greater change than current data for 60 s intervals perhaps because the stimuli were only delivered for about one-third of the day by Al-Amood *et al.* (1986) and for 24 h/day in the present experiments. A final analysis was done on the current data by grouping the data further; intervals of more than 100 s gave a mean to peak of 19·2 (S.E. of mean = 0·44) ms whilst those stimulated more frequently had a mean of 21·0 (S.E. of mean = 0·85) ms and this difference approached the levels of significance ($t = 1{\cdot}91$, $n = 32$, $P < 0{\cdot}1$). Only this coarse division has been presented in subsequent Figures.

The relaxation phase of the twitch was also modified by denervation and stim-

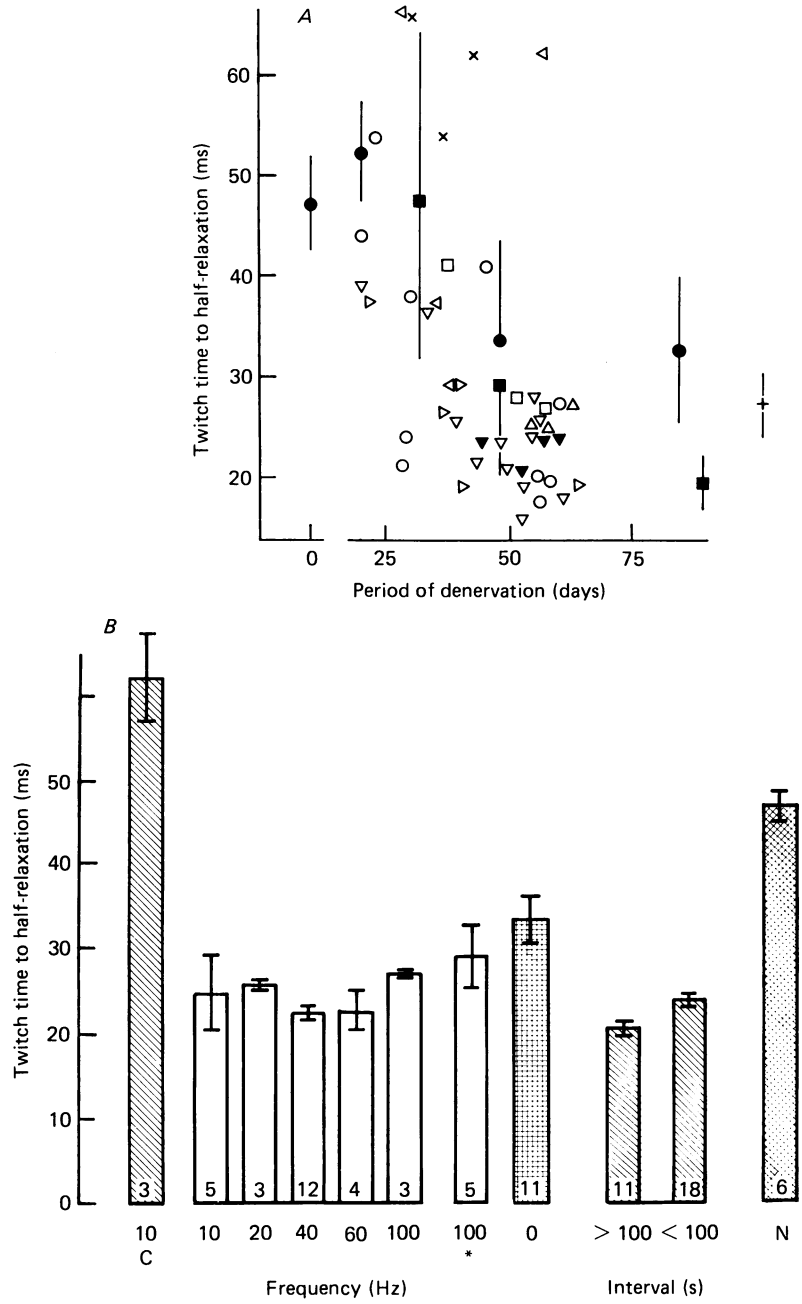

Fig. 4. Twitch time to half-relaxation (time from peak to half-peak tension) in denervated and stimulated muscles. In *A* data of individual animals and group means are presented using the same symbols as in Fig. 2. In *B* the histograms show means ± S.E. of mean, and are labelled in the same way as in Fig. 3. Original data have been used in calculating the statistics but all muscles denervated for less than 40 days have been excluded. The mean interburst intervals have been calculated for only two groups: those stimulated at intervals of less than 100 s (mean value = 24·0, S.E. of mean = 0·80 ms, $n = 14$) and those with intervals more than 100 s (20·6 ± 1·05 ms, $n = 10$).

ulation (Fig. 4). Denervation alone, as with the contraction phase of the twitch, produced an initial prolongation which was reversed after 20 days (Finol *et al.* 1981) unless continuous stimulation at 10 Hz was imposed (Al-Amood *et al.* 1986). Intermittent stimulation at all frequencies between 10 and 100 Hz produced a greater decrease in the time of relaxation than did denervation alone. The response to stimulation seemed to be faster than that seen in the contraction phase, and to be complete by 40 days when relaxation was a little faster than that of cross-reinnervated and denervated solei (+ on Fig. 4 *A*). Means were calculated for all muscles recorded more than 40 days after denervation, and the results are presented in Fig. 4*B*. The relationship between mean half-relaxation time and stimulus frequency appeared to follow the same pattern found with the contraction time, but none of the differences between frequencies were significant statistically. The muscles stimulated less often (intervals of more than 100 s) had half-relaxation times shorter on average by about 10%, and the difference was statistically significant ($t = 2{\cdot}60$, $n = 24$, $P < 0{\cdot}02$). A formal analysis of variance was also calculated. To make this comparable with the time to peak statistics of Table 1, two analyses of relaxation time are presented: one for all the data (corresponding to the open columns of Fig. 4*B*) and one for data obtained at periods of denervation of more than 47 days; these are presented in rows 5 and 6 of Table 1 (and correspond in method of selection to rows 1 and 2). Visually the means of this second set were indistinguishable from those of Fig. 4. As with time to peak, significant effects of interburst interval were seen on the relaxation time ($P < 0{\cdot}005$ and $< 0{\cdot}025$ for the two rows). No effects of frequency within bursts were detected, however.

Tetanic tension showed considerable variation but most muscles were partially maintained by intermittent stimulation, although there were no clear trends with time (Fig. 5*A*). There were apparent differences in the means relating to frequency and interburst interval (Fig. 5*B*), and these appeared to mirror the effects on twitch time course (middle frequencies and long intervals producing the largest tensions). Except for the comparison between non-stimulated muscles and all the rest, none of the differences were significant statistically. Analysis of variance (rows 7 and 8 of Table 1) also indicated that there was no significant effect of either stimulation parameter. It should be noted that continuous 10 Hz stimulation was least effective in preventing tension loss.

Denervation reduces twitch tension less than tetanic tension, so that the twitch–tetanus ratio increases from a normal value of about 0·23 in soleus to 0·37 at 20 days after axotomy (Baker, 1983). Intermittent stimulation seemed to reverse this change in the ratio although no time dependence was apparent. Our overall mean was 0·295 (S.E. of mean 0·0169, $n = 29$) which was just significantly lower than the value for 20 days denervated muscles of 0·372 (S.E. of mean 0·068, $n = 8$; $t = 2{\cdot}20$, $P < 0{\cdot}05$). Continuous stimulation increased the twitch–tetanus ratio to 0·359 (S.E. of mean 0·0099; $t = 4{\cdot}1$, $P < 0{\cdot}001$). There were consistent (but non-significant) trends which indicated that lower frequencies of intermittent stimulation produced the greatest decrease in twitch–tetanus ratio, and that long interburst intervals were more effective than short (mean ratios for intervals more than 100 s were 0·227 compared with 0·305 for shorter intervals). Analysis of variance (rows 9 and 10 of Table 1) indicated, however, that there were no significant effects on twitch–tetanus ratio of either burst frequency or interval.

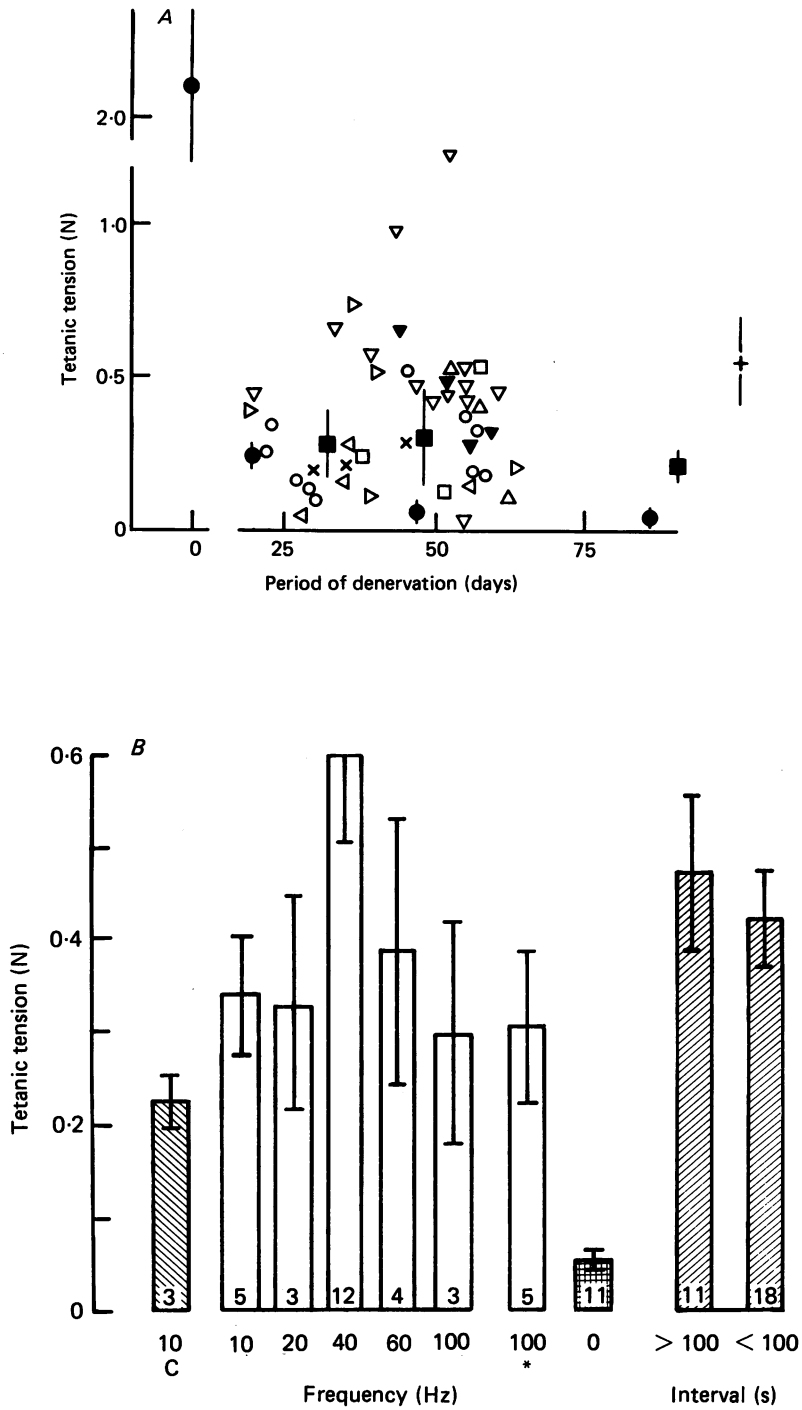

Fig. 5. Maximum tetanic tension of denervated and stimulated muscles. Individual data and means (±S.D.) from previous studies are shown in *A*; means (±S.E. mean) are presented as histograms in *B*. Conventions as in Fig. 4.

The effects of a 800 ms tetanus on the twitch were measured at 5 s after the tetanus. In most muscles there was only a small post-tetanic potentiation of 5–10%. In all muscles with a twitch time to peak of 40 ms or more the effect was depression of twitch tension. Of the faster contracting muscles two also showed depression (times

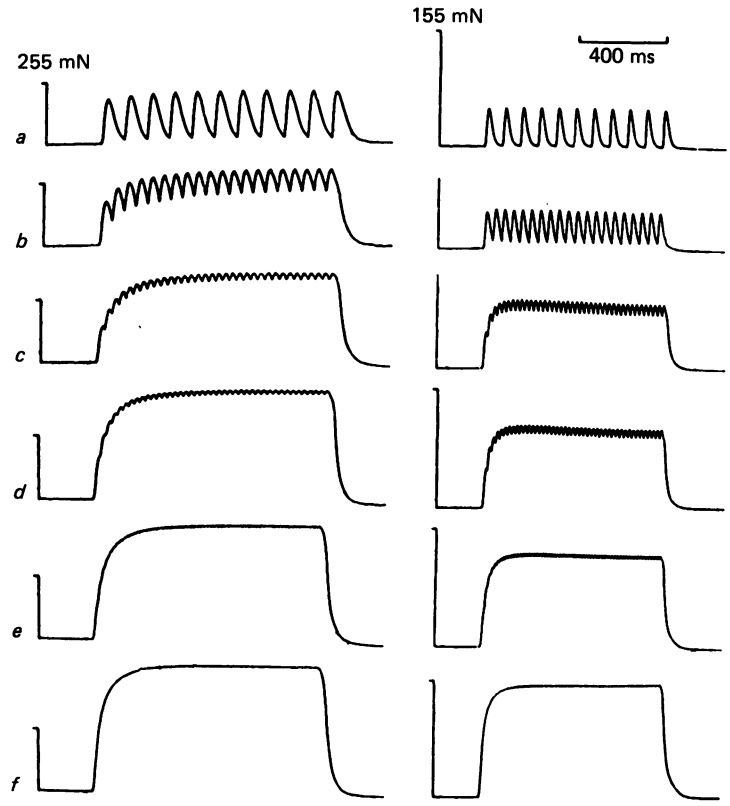

Fig. 6. Genesis of tetanus in two denervated muscles stimulated intermittently at 10 Hz (right, same muscle as Fig. 1*f*) and at 100 Hz (left, denervated 6 weeks). The muscles have been selected to illustrate also two ends of our range: that on the right had the shortest twitch time to peak and on the left is one of the slowest twitches from muscles denervated for more than 45 days. The timescale applies to all myograms, and the initial tension calibration bars are also identical (except where they have been truncated to avoid overlap. The frequencies illustrated in *a*, *b*, *d*, *e* and *f* were 10, 20, 40, 60 and 100 Hz, and are those used in the chronic phase of these experiments. The interpulse interval in *c* was 125% of twitch time to peak (see Burke *et al.* 1971).

to peak near 25 ms), five were unaffected and fourteen were potentiated. Only one exceeded 13% potentiation and its twitch was increased by about one-third. These usually small and variable effects are much less than would be seen in normal fast muscle, although denervation abolishes most post-tetanic potentiation (Finol & Lewis, 1976).

The genesis of tetanus was examined in several muscles and, as would be expected, fusion frequency increased as the twitch became briefer. Two examples are presented

in Fig. 6. The muscles had been stimulated for 6–7 weeks, and have been chosen from either end of the range of twitch times to peak. Figure 6 also illustrates the degree of fusion involved with the stimulus patterns used during the chronic phase of our experiments. It can be seen that at 10 Hz individual contractions showed no sum-

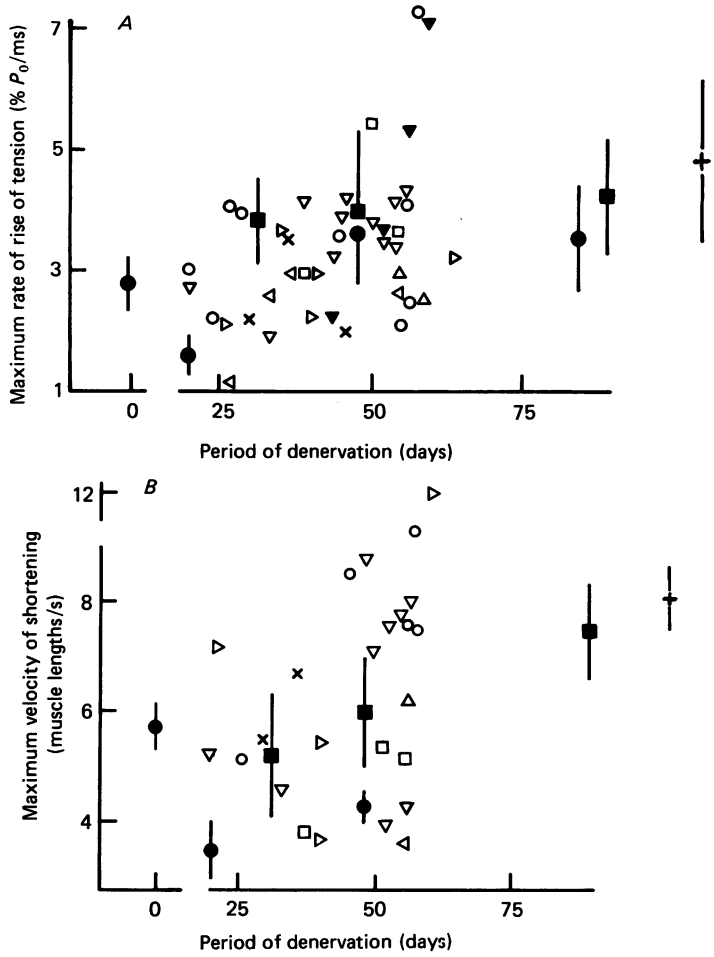

Fig. 7. Rate of tetanic contraction measured under isometric (*A*) and isotonic (*B*) conditions. Maximum rates have been measured in both cases and normalized by maximum tetanic tension (P_0, *A*) or muscle length (*B*). Plotted as in earlier Figures.

mation while at 100 Hz tetanic tension was close to that in a fully fused tetanus, and there was little difference in the degree of fusion in tetani of the two muscles.

Most tetani showed the continuously rising tetanus characteristic of slow-twitch muscle (Cooper & Eccles, 1930) or a flat plateau. In some the tension declined after an initial peak. To emphasize such differences we also used unfused tetani elicited with interpulse intervals equal to 125% of twitch time to peak. Burke, Levine, Zajac, Tsairis & Engel (1971) found this interval best to distinguish between fast and slow motor units. They described the falling tension seen late in the tetanic myogram

of a fast unit as 'sag'. We saw sag only in muscles with a twitch time to peak less than 22 ms. Neither the development of this tetanic sag nor the emergence of post-tetanic potentiation was obviously related to stimulus frequency. Examples of two muscles, only one of which shows sag, are shown in Fig. 6.

Higher rates of stimulation were used to measure the maximum rate of rise of

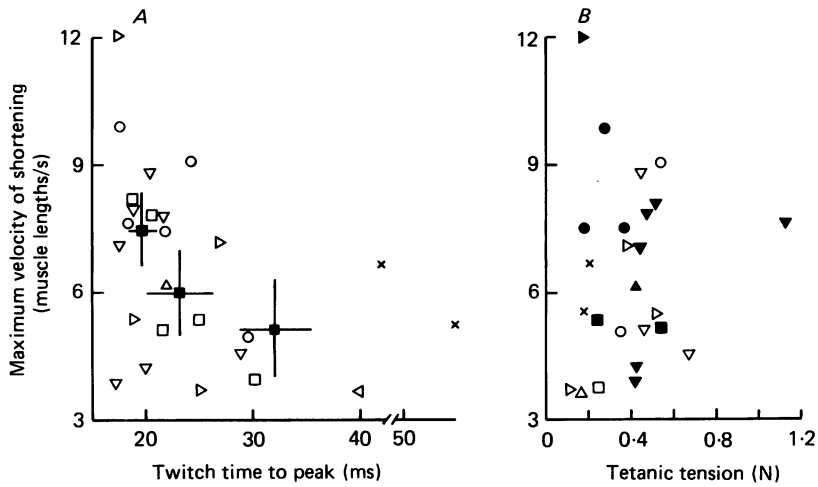

Fig. 8. Relationship between normalized maximum velocity of shortening and isometric twitch time to peak (*A*) or maximum tetanic tension (*B*). The symbols in *A* have the same significance as in all other Figures, but in *B* the open symbols indicate denervation and stimulation of less than 46 days duration and filled symbols more than 47 days.

tension in the tetanus; this was normalized by dividing by the maximum tetanic tension and plotted in Fig. 7*A*. As reported by Finol *et al.* (1981) the normalized maximum rate of tension development initially falls after denervation alone, but rises again and becomes slightly greater than normal at 7 and 11 weeks, although not as high as seen in cross-reinnervated and subsequently denervated soleus (+ in Fig. 7*A*) or in denervated fast muscle (Baker, 1983). Chronically stimulated muscles had, on average, rates of rise of tension close to those of denervated muscle, although some were much higher and in the range for normal fast muscles. This parameter is difficult to measure accurately in denervated muscles with low active tension, partly because of stimulus artifacts, and this is possibly the reason for the large amount of variability relative to the difference between fast and slow muscle. There was no effect of frequency of chronic stimulation on the extent of the change in maximum rate of rise, but this is difficult to interpret especially as the results from continuously stimulated muscles were also indistinguishable.

The maximum velocity of isotonic shortening was estimated in many of the muscles in which isometric tetanic tension was sufficiently high for sensible measurements. This velocity was normalized by dividing by muscle length (Fig. 7*B*). Again there was a lot of difference between muscles, and some appeared to be unaffected by stimulation. It was clear, however, that at 8 weeks several muscles, including four stimulated intermittently at 10 Hz, had velocities of shortening as high as those

previously observed in soleus following chronic stimulation at 100 Hz (Al-Amood *et al.* 1986) or after cross-reinnervation with or without subsequent denervation (Baker & Lewis, 1984).

The change in maximum velocity has been related to that in the twitch time to peak by Al-Amood *et al.* (1986, Fig. 7*A*). The trend in the present data does not seem so clear but the regression was significant ($r = -0{\cdot}62$, $n = 23$, $P < 0{\cdot}01$). There was no relationship between maximum shortening velocity and tetanic tension (Fig. 8*B*). In none of the three graphs involving shortening velocity was there any indication that the frequency of the preceding chronic stimulation had any influence on the shortening velocity of the muscle.

DISCUSSION

It is clear that, if short bursts of impulses are given intermittently to denervated rat soleus, the effect is largely independent of frequency over the range of 10–100 Hz which covers most of the frequencies occurring physiologically. It is only when the 10 Hz frequency is given continuously that the muscle is induced to remain slowly contracting. Sreter *et al.* (1982) and Eerbeck, Kernell & Verhey (1984) came to comparable conclusions in chronic stimulation of innervated fast muscle. Sreter *et al.* (1982) found that rabbit fast muscle could be converted to slow with stimulation at a rate as high as 60 Hz when it was delivered almost continuously (2·5 s every 10 s, a very much shorter interburst interval than those used in the present work). Further, Eerbeck *et al.* (1984) found that fast to slow transformation in the cat was also almost independent of stimulation rate over the range of 5–40 Hz when that stimulation was delivered either continuously or intermittently with bursts of 1 s repeated every 2 s. We conclude that it is the time allowed between muscle contractions rather than the frequency of stimulation inducing the contraction that is crucial in determining the effect on contractile properties, in both slow to fast as well as fast to slow transformation.

We do disagree with a further interpretation placed on their data by Sreter *et al.* (1982). They suggest that it is the number of stimuli which initiates the fast to slow transformation. In our experiments it is clear that the number of stimuli does not affect the response (i.e. 10 Hz bursts had the same effect as 100 Hz ones). A more attractive alternative is that the intervals of rest between contractions allow the concentration of some chemical to fall below a level that would induce a fast to slow transformation. Such an hypothesis would explain both our results and those of Streter *et al.* (1982) and Eerbeck *et al.* (1984). It seems unlikely that the chemical involved is the cytosol calcium, the levels of which are likely to be much lower in the unfused response to 10 Hz stimulus than in the fused tetanus at 100 Hz (see Fig. 6). Perhaps some product of anaerobic metabolism is responsible.

An additional conclusion that we would draw is that the only specific effect of long-term stimulation on the speed of denervated rat slow-twitch muscle is the effect of continuous stimulation which maintains the production of slow myosin and, probably, other contractile proteins. We have argued (Al-Amood *et al.* 1986) that the change in gene expression in the rat which allows fast myosin to be formed in all fibres of a once slow-twitch muscle is primarily brought about by denervation. This

results in transformation which is slow and incomplete because there is little new protein synthesis in denervated muscle. Intermittent long-term stimulation reduces atrophy by increasing the rate of formation of new proteins, and these are the proteins of fast muscle because of the switch of gene expression already induced by the denervation. The present observations are compatible with the hypothesis of Al-Amood *et al.* (1986) because the extent of the transformation is independent of the frequency of stimulation. It requires almost continuous activity to prevent the slow to fast transformation induced by denervation or to convert fast- to slow-twitch muscle.

The stimulation patterns used were, of course, unphysiological in that all fibres were activated simultaneously and the bursts were at high uniform frequencies throughout the 1 s of stimulation. For this reason we looked at the effects of short bursts. It is known that many contractions begin with a pair of nerve impulses at short intervals. The interval of 5 ms was chosen because it was compatible with values seen in natural contractions and also because it is the interval which gives maximal summation of contractions (Buller & Lewis, 1965). We also felt that limiting the burst to two pulses would minimize ischaemia due to synchronous activation of the muscle. The results from these experiments were inconsistent but it was clear that in two out of four muscles a pair of pulses could induce changes as big as a 1 s burst. The other two muscles not only did not respond but showed contractions which were slower than those of unstimulated denervated muscles and close to those stimulated continuously. The numbers used were small and clearly this anomaly needs further investigation.

There were small differences between the effects of different frequencies within the bursts used in chronic stimulation and, although the differences were not significant for all variables, the same pattern was seen. The common pattern was that the middle range of frequencies (40–60 Hz) and long intervals ($>$ 100 s) between the bursts were most effective in producing changes. Lømo *et al.* (1980) also suggested that less frequent bursts produced larger effects. Similarly in athletic training maximal isometric contractions need only be repeated a few times a day to induce a full increase in muscle force (e.g. McDonagh, Hayward & Davies, 1983). Such small differences do not affect our earlier arguments, which need only be extended to say that 40 Hz or the longest intervals were marginally more effective in stimulating new protein synthesis and so inducing larger changes in muscle speed.

The observations on isotonic shortening were more variable, but they indicate that the contractile proteins could be equally affected by 10 and 100 Hz chronic stimulation. This view is supported by current collaborative work with A. Rowlerson which suggests that fast-muscle isozymes appear with 10 Hz stimulation as well as with 100 Hz or denervation alone (Al-Amood *et al.* 1985 and unpublished observations).

Our data differ from those of Lømo *et al.* (1974, 1980) who reported greater increases of tension and decreases in shortening of the twitch contraction. In the majority of experiments we did not use the same stimulating conditions as Lømo and his colleagues, but we tried to reproduce his parameters as closely as possible in one set (indicated by ▼ in our Figures), but the data are indistinguishable from the main body of results. The only difference remaining in this set was that Lømo used currents

about 1·5–2 times those we used, but we could not increase our stimulation intensity without causing distress to our animals. Lømo *et al.* (1974, 1980) also stimulated at different interburst intervals to us (either 15 or 67 s or 2 h), but there is overlap between the two ranges and the absence of any large effect of interburst interval on the degree of conversion makes it very unlikely that this is a major cause of the difference between results from the two groups. There may be other differences in methods of stimulation but no major ones that are likely to produce substantial differences in responses are apparent from the written reports.

Although Lømo reports considerably more conversion in twitch time to peak than we see, the difference between the two groups may be quantitative rather than qualitative. Lømo also obtains higher tensions, and the hypothesis set out above predicts that if more new protein formation is stimulated then the ratio of fast to slow protein will increase and the muscle will have a faster contraction. Thus Al-Amood *et al.* (1986) found that twitch time to peak decreases with increased tetanic tension, and Lømo's data may be considered as an extension of such a relationship.

The muscles in Lømo's experiments might be alternatively considered to have shown a qualitatively different change, having twitch characteristics very similar to those of cross-reinnervated slow muscle (Close, 1969). We have been unable to produce muscles with twitch contractions shorter than those of denervated fast muscle or denervated cross-reinnervated slow muscle (Baker & Lewis, 1984). A further difference was that Lømo *et al.* (1974) reported that stimulation decreased the twitch–tetanus ratio to 0·07–0·18, values within the range of normally innervated muscles. We have seen only a much smaller decrease. If there is a qualititatively different effect in Lømo's experiments, it may be that there is a reversal of the twitch potentiation seen a few days after denervation (Finol *et al.* 1980). If so we have not seen such an effect. We are unable to offer any explanation for the differences between the two laboratories.

This work was supported by a grant from the Medical Research Council. W. S. Al-Amood was supported by the Medical Research Council.

REFERENCES

Al-Amood, W. S., Finol, H. J. & Lewis, D. M. (1986). Chronic stimulation modifies the isotonic shortening velocity of denervated rat slow-twitch muscle. *Proceedings of the Royal Society* B **228**, 43–58.

Al-Amood, W. S., Lewis, D. M. & Rowlerson, A. (1985). The effects of chronic stimulation at low rates on denervated rat soleus muscle. *Journal of Physiology* **361**, 36*P*.

Baker, A. J. (1983). *Neural control of muscle contraction speed*. Ph.D. Thesis, University of Bristol.

Baker, A. J. & Lewis, D. M. (1983). The effects of denervation on isotonic shortening velocity of rat fast and slow muscle. *Journal of Physiology* **345**, 56*P*.

Baker, A. J. & Lewis, D. M. (1984). The effect of cross-reinnervation on the isotonic shortening velocity of rat soleus persists after subsequent denervation. *Journal of Physiology* **349**, 39*P*.

Buller, A. J. & Lewis, D. M. (1965). The rate of tension development in isometric tetanic contractions of mammalian fast and slow skeletal muscle. *Journal of Physiology* **176**, 337–354.

Burke, R. E., Levine, D. N., Zajac III, F. R., Tsairis, P. & Engel, W. K. (1971). Mammalian motor units: physico-histochemical correlation in three types in cat gastrocnemius. *Science* **174**, 709–712.

CLOSE, R. (1969). Dynamic properties of fast and slow skeletal muscle of the rat after nerve cross-union. *Journal of Physiology* **204**, 331–346.

COOPER, S. & ECCLES, J. C. (1930). The isometric responses of mammalian muscles. *Journal of Physiology* **69**, 377–385.

EERBECK, O., KERNELL, D. & VERHEY, B. A. (1984). Effects of fast and slow patterns of tonic-term stimulation on contractile properties of fast muscle in the cat. *Journal of Physiology* **352**, 73–90.

FINOL, H. J. & LEWIS, D. M. (1976). Post-tetanic effects in denervated rat skeletal muscle. *Journal of Physiology* **259**, 45–46*P*.

FINOL, H. J., LEWIS, D. M. & OWENS, R. (1981). The effects of denervation on contractile properties of rat skeletal muscle. *Journal of Physiology* **319**, 81–92.

LØMO, T., WESTGAARD, R. H. & DAHL, H. A. (1974). Contractile properties of muscle: control by pattern of muscle activity in the rat. *Proceedings of the Royal Society* B **187**, 99–193.

LØMO, T., WESTGAARD, R. H. & ENGEBRETSEN, L. (1980). Different stimulation patterns affect contractile properties of denervated rat soleus muscle. In *Plasticity of Muscle*, ed. PETTE, D., pp. 297–309. Berlin: de Gruyter.

MCDONAGH, M. J. N., HAYWARD, C. M. & DAVIES, C. T. M. (1983). Isometric training in human elbow flexor muscles: the effects on voluntary and electrically evoked forces. *Journal of Bone and Joint Surgery* **65** B, 355–358.

SALMONS, S. & VRBOVÁ, G. (1969). The influence of activity on some contractile characteristics of mammalian fast and slow muscles. *Journal of Physiology* **201**, 535–549.

SRETER, F. A., PINTER, K., JOLESZ, F. & MABUCHI, D. (1982). Fast to slow transformation of fast muscle in response to long-term phasic stimulation. *Experimental Neurology* **75**, 95–102.