

# Induction of a fatigue-resistant phenotype in rabbit fast muscle by small daily amounts of stimulation

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**Lopez-Guajardo, Ana, Hazel Sutherland, Jonathan C. Jarvis, and Stanley Salmons.** Induction of a fatigue-resistant phenotype in rabbit fast muscle by small daily amounts of stimulation. *J Appl Physiol* 90: 1909–1918, 2001.—We have shown that fatigue resistance can be induced in rabbit tibialis anterior (TA) muscles without excessive power loss by continuous stimulation at low frequencies, such as 5 Hz, and that the same result is obtained by delivering a 10-Hz pattern in equal *on/off* periods. Here we ask whether the same phenotype could be produced with daily amounts of stimulation that would be more appropriate for clinical use. We stimulated rabbit TA muscles for 6 wk, alternating fixed 30-min *on* periods of stimulation at 10 Hz with *off* periods of different duration. All patterns transformed fast-glycolytic fibers into fast-oxidative fibers. The muscles had fatigue-resistant properties but retained a higher contractile speed and power production than muscles transformed completely to the slow-oxidative type. We conclude that in the rabbit as little as one 30-min period of stimulation in 24 h can result in a substantial increase in the resistance of the muscle to fatigue.

adaptation; skeletal muscle; type transformation; implantable stimulator

FUNCTIONAL ELECTRICAL STIMULATION of skeletal muscle lends itself to three broad areas of clinical application. One area consists of those applications in which the muscle is stimulated *in situ* to restore functions lost through stroke or spinal cord injury. In patients with paraplegia, for example, muscles of the lower limb may be stimulated to eliminate drop foot (32) and to permit periods of standing and walking (2); in patients with quadriplegia, muscles of the upper limb may be stimulated to provide two modes of grasp (37); and in patients with high cervical lesions of the spinal cord, in whom the diaphragm is paralyzed but the lower motor neurons are intact, ventilatory support may be provided by pacing one or both phrenic nerves (12, 15). In a second area, stimulation of muscles *in situ* could potentially provide the forces needed to correct skeletal deformities such as club foot or scoliosis. A third area includes applications in which the muscle is partially

mobilized and redeployed to perform a different function. For example, a pedicle graft of the latissimus dorsi muscle can be transferred into the chest to assist a failing heart (40). This is achieved either by wrapping it around existing structures, the ventricles of the heart in cardiomyoplasty (14), and the ascending or descending aorta in aortomyoplasty (48), or by forming it into an auxiliary pump or skeletal muscle ventricle (1, 47). A further example is the use of the gracilis muscle to construct a neosphincter in cases in which normal anal sphincter function is absent by reason of anorectal pathology or congenital malformations of the distal bowel (50).

In muscles that are activated under these conditions, the orderly recruitment of motor units that is a feature of voluntary contraction is replaced by inverted, or at best disorderly, recruitment. Furthermore, motor neurons that would fire asynchronously at moderate frequencies under physiological conditions have to be excited synchronously at higher frequencies by electrical stimulation to produce the equivalent force. As a consequence, electrical stimulation is much more likely to induce muscle fatigue than normal voluntary activity. Although there have been various attempts to establish a more physiological recruitment order in electrically stimulated muscles, these measures are of limited value in patients with longstanding spinal cord injury, in whom the paralyzed muscles will have reverted more or less completely to a fast, glycolytic character that is highly susceptible to fatigue (16).

Fortunately there is a solution to this problem, which is to exploit the remarkable adaptive capability of skeletal muscle (41). This phenomenon allows the muscle to respond to a change in demand by developing properties that are more appropriate to the task, a process often referred to in clinical circles as “conditioning.” The nature of this process has been investigated extensively in laboratory animals. For example, a rabbit tibialis anterior (TA) muscle that is stimulated supramaximally and continuously at 10 Hz acquires, over a period of weeks, a more highly developed capacity for the generation of energy from oxidative path-

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ways and undergoes changes in the expression of a number of genes, including those encoding myosin isoforms (for reviews see Refs. 31, 38, and 41). If the conditioning process is allowed to go to completion, the entire muscle is transformed to a slow-twitch, fatigue-resistant (type I) phenotype. This degree of transformation is not optimal for the majority of clinical applications because of the associated loss of mass, force, contractile speed, and power (39). A muscle profile better suited to these applications is the fast, fatigue-resistant (type IIa) phenotype, which can be established and maintained stably in the rabbit by the application of continuous stimulation at the lower frequency of 2.5 Hz (28, 35, 45). In a laboratory setting, continuous, 24-h stimulation has the advantage that it enables the conditions to be manipulated by changes in a single variable, namely frequency. In a clinical setting, however, stimulation should interfere as little as possible with the patient's normal daily activities, and to meet this requirement conditioning regimes have been used that deliver stimulation for short daily periods or overnight (see Ref. 2, for example). Until now, the response of the muscle to such patterns has not been subjected to systematic scrutiny. In this study, we systematically varied the percentage of time for which a rabbit fast muscle was subjected to stimulation (the duty cycle). This was achieved by alternating fixed periods of stimulation at 10 Hz (*on* periods) with periods of recovery (*off* periods) of different duration. We ask the question: how does variation of the duty cycle affect the response of a muscle to chronic stimulation?

## MATERIALS AND METHODS

**Animals.** Experiments were conducted on New Zealand White rabbits of either sex, with body weights in the range 2–3 kg. All procedures were conducted in strict accordance with the Animals (Scientific Procedures) Act of 1986, which governs the experimental use of animals in the United Kingdom.

**Stimulation.** The stimulation patterns were generated by microcontroller-based programmable miniature stimulators (17). The memory of each device contained 12 stimulation patterns from which the desired pattern, or the *off* condition, could be selected remotely after implantation by optical communication through the skin and subjacent tissues. The leads consisted of PVC-insulated multistranded stainless steel (Cooner Wire, Cooner Sales, Chatsworth, CA) terminating in bared loop electrodes mounted on Dacron velour pads. The output pulses, which had a duration of 0.2 ms and an amplitude of 3.2 V, produced supramaximal stimulation. The devices were sterilized in ethylene oxide for 24 h before implantation.

The devices were implanted intraperitoneally under strictly aseptic conditions, and the electrodes were conducted subcutaneously to the left leg. The active electrode was placed under the common peroneal nerve and the indifferent electrode on the adjacent surface of the gastrocnemius muscle. A week was allowed for full recovery of the animal from surgery and anesthesia, after which the selected stimulation pattern was initiated by transmitting the appropriate code through the skin via a series of light pulses from a strobe. The patterns of stimulation used had a total cycle time of 24, 12, 4, or 2 h, during which the muscles of the anterior

compartment of the lower limb were stimulated at 10 Hz for an *on* period of 30 min, corresponding to duty cycles of 2.1, 4.2, 12.5, or 25%, respectively. Each of these four patterns was applied in four rabbits. It was possible to extend some of the results by including data obtained with the 30 min on, 30 min off (50% duty cycle) pattern from the previous study (33). This was justified because the studies were performed soon after each other in identical fashion on a similar group of animals, and control values for the contralateral TA muscles showed no significant differences from the control population in the present study.

**Physiological measurements.** After 6 wk of stimulation, terminal experiments were performed as described previously (33) for the measurement of isometric and isotonic contractions in each stimulated TA muscle and its contralateral counterpart.

The following variables were recorded under isometric conditions and at the muscle length that corresponded to maximum developed twitch tension ( $L_o$ ): amplitude, time to peak, time from peak to half-relaxation of the twitch, and maximum tetanic force. A series of isotonic contractions were used to construct force-velocity curves from which the following measurements were taken: maximum velocity of shortening, maximum power, and velocity corresponding to maximum power. Specific force was calculated by dividing maximum tetanic force by an estimate of the physiological cross-sectional area based on the muscle mass divided by  $L_o$ . Such estimation is common physiological practice and is justified here because the density of muscle tissue is close to unity and because the angle between the predominant direction of the fibers and the tendon axis in the unipennate rabbit TA muscle is small enough for its cosine to approximate to unity.

On completion of the physiological measurements, a fatigue test was performed. The muscles of both sides were set to contract isometrically at  $L_o$  and were stimulated with a 40-Hz impulse train of 330-ms duration once per second for 5 min (6).

**Harvesting of tissue.** At the end of the fatigue test, the animal was killed by intravenous injection of pentobarbitone sodium (200 mg/kg, Euthatal TM, Rhône Merieux, Essex, UK). The TA muscles were dissected free and weighed. Transverse blocks were cut from the widest part of the muscle, transferred to cork disks, and frozen in melting isopentane for subsequent cryostat processing. The rest of the muscle was frozen immediately in liquid nitrogen and stored at  $-77^\circ\text{C}$  for myosin extraction and enzyme assays.

**Enzyme analysis.** Homogenates were prepared from muscle samples stored at  $-77^\circ\text{C}$ . Each sample was assayed in duplicate for every enzyme, and appropriate standards were carried through the separate procedures. The individual enzymes were assayed as described previously (20), using as a direct measure of activity the coupled production of fluorescence by NAD or the reduced form NADH. Enzyme activity was calculated as fluorometric units produced over 60 min at  $25^\circ\text{C}$  and expressed as moles per hour per kilogram noncollagen protein in the homogenate.

**Protein assay.** Protein was measured by the Bradford method. For enzyme assays, a noncollagen protein determination was carried out by incubating homogenates overnight in 0.08N NaOH, after which alkali-soluble protein in the supernatant protein was measured.

**Myosin light chains.** Myosin was extracted and purified as described previously (35). Equal amounts of protein from the different samples were analyzed by SDS-PAGE in a resolving gel containing 14.3% acrylamide and 0.125% bisacrylamide. Protein bands were visualized with Coomassie blue R250 and

identified by their molecular weights. The portion of the gel containing the myosin light chains (MLCs) was scanned densitometrically (Ultrascan XL Laser Densitometer; LKB, Pharmacia). The protein peaks were integrated with the software package GelScan XL (LKB, Pharmacia), and each band was expressed as a percentage of the total absorbance of the light chain bands. Unstimulated TA and soleus muscles were included in the gels as fast and slow controls.

**Myosin heavy chains.** Myosin heavy chains (MHCs) were separated from the same preparations as the light chains according to Talmadge and Roy (46) on an 8% SDS-polyacrylamide gel containing 30% glycerol. Approximately 1.5 µg of protein were loaded in each lane, and electrophoresis was carried out for 20 h at a constant 70 V. Protein bands were stained with Coomassie blue R250, and the density of the bands was scanned and expressed as for the light chains.

**Histochemistry and immunohistochemistry.** Serial 10-µm frozen sections of TA muscle were cut in a cryostat at -21°C and stained as follows. NADH dehydrogenase was used as a marker of the mitochondrial oxidative capacity of the fibers. NADH was added fresh to a solution of 0.1% nitroblue tetrazolium in 0.2 M Tris buffer (pH 7.4) at a ratio 1:1,000 wt/vol. The solution was pipetted onto thawed sections and incubated at 37°C for 30 min. The slides were then dehydrated through graded concentrations of alcohol to xylene and mounted in DPX (BDH Chemicals).

Myofibrillar ATPase was demonstrated by a modification of the method of Tunell and Hart (49), in which the formaldehyde incubation was conducted for 7.5 min and the final incubation for 50 min. This method clearly distinguished three fiber types in a single section: dark (type IIb or IIx), intermediate (type IIa), and light (type I). For this reason it was used as the basis of fiber type analysis.

Some sections were also processed as described previously (45) for the demonstration of fast or slow myosin isoforms by immunohistochemistry with specific monoclonal antibodies. The monoclonal antibody to the type IIa MHC (SC-71 α2AMHC) was the kind gift of Dr. J. D. Rosenblatt.

**Point-counting morphometry.** Stained sections were covered with a transparent grid consisting of 1-mm squares. Ten squares were chosen for quantitation; these were distributed systematically to ensure representative sampling of the entire cross section. For each of the 1-mm sample squares, the number of fibers of each type contained within the boundaries was counted, with the usual convention that fibers were included if they crossed the upper and left boundaries and excluded if they crossed the bottom and right boundaries. The proportions of each fiber type were calculated by pooling the data from all squares counted in each section. Type IIb fibers could not be distinguished from IIx with the techniques used, and the two are reported as a single class, IIx.

**Statistical analysis.** For all parameters, differences between the groups of stimulated muscles and the pooled contralateral controls were examined by analysis of variance (ANOVA) followed by a Tukey-Kramer multiple-comparison posttest.

**Reagents.** Enzymes were obtained from Boehringer Mannheim (Sussex, UK). Reagents and chemicals for which a source is not stated in the text were purchased from Sigma Chemical (Dorset, UK) or Merck (Leicester, UK).

## RESULTS

**Rationale of the experimental design.** The time course of the transformation induced in the rabbit TA muscle by continuous stimulation at 10 Hz is well documented, and this frequency was therefore chosen as the base frequency for the patterns in this study. The effects of continuous stimulation at 10 Hz are substantially complete by 6 wk; this provided a useful basis for comparison, and all the patterns were therefore applied for 6 wk. The unstimulated muscle of the opposite hindlimb was used as a source of control data because we have never observed any evidence of contralateral effects of stimulation for the variables measured here (see, for example, Refs. 5, 10, and 20). A previous study (33) showed that the intracellular signaling events triggered by stimulation are very rapid, with a time course of only tens of seconds. An *on* period of 30 min was therefore chosen in the knowledge that this period would not be too short to have an effect. The 30-min *on* period was alternated with an *off* period of 23.5, 11.5, 3.5, or 1.5 h, to give cycle times of 24, 12, 4, or 2 h, corresponding to duty cycles of 2.1, 4.2, 12.5, or 25%. For some measurements it was possible to extend this range to duty cycles of 50% by including data from a group stimulated for 30 min on, 30 min off from the previous study (33).

**Myosin isoforms.** An increased proportion of slow MLC isoforms and a corresponding reduction in fast MLCs were seen in all the intermittently stimulated groups of muscles (Table 1). Those stimulated with duty cycles of 25 and 50% showed a more marked, and significant, induction of MLC<sub>2s</sub>. The changes are most easily visualized by plotting the combined fast and slow isoforms (Fig. 1A). Only MLCs of the fast type were expressed at detectable levels in the contralateral TA muscles and only MLCs of the slow type were seen in a typical slow muscle, soleus (Fig. 1A). Figure 1A

Table 1. Percentage composition of myosin light chains

	Control TA (n = 12)	2.1% (n = 4)	4.2% (n = 4)	12.5% (n = 3)	25% (n = 3)	50% (n = 4)
LC1f	38.1 ± 3.4	32.2 ± 7	36.7 ± 5.6	30.5 ± 6	28 ± 7.3	39 ± 4.5
LC2f	48.5 ± 3	43.8 ± 1.5	42.1 ± 3.7	40.1 ± 2	42.0 ± 5.4	26.8 ± 7.7†
LC3f	13.5 ± 0.9	7.9 ± 2.6	10.7 ± 2	11.1 ± 2.6	6.6 ± 1.8	7.1 ± 1.5
LC2s	0 ± 0	10.9 ± 3.8	7.3 ± 4.2	9.9 ± 2.8	19.5 ± 9.8†	18.1 ± 1.9†
LC1s	0 ± 0	3.9 ± 1.1†	2.2 ± 1.3	4.5 ± 1.4†	2.6 ± 0.2	6.6 ± 0.6‡
LC1s'	0 ± 0	2.1 ± 0.9	1.5 ± 0.9	3 ± 1.5*	1.1 ± 0.5	2.3 ± 0.9

Values are means ± SE obtained by densitometric scanning of gel electrophoretograms from tibialis anterior (TA) muscles stimulated at 10 Hz with duty cycles of 2.1, 4.2, 12.5, 25, 50, and 100%, corresponding to 30 min in every 24, 12, 4, 2, and 1 h, and unstimulated contralateral muscles included in the gels as reference lanes. LC, light chain. Results for muscles stimulated with the 50% duty cycle are from the previous study (Ref. 33). \*, †, ‡Departures from control values that were significant at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively.



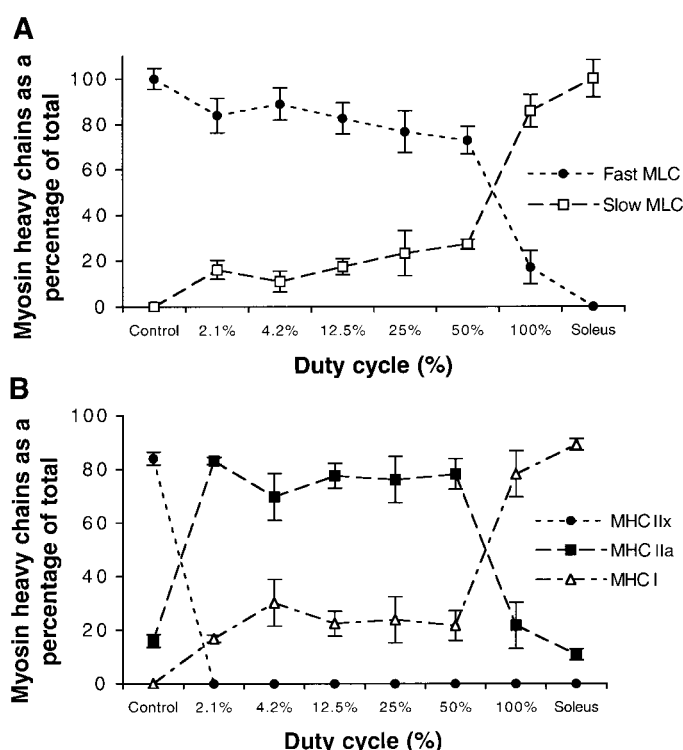


Fig. 1. Results of gel densitometry for the myosin isoform composition of rabbit tibialis anterior (TA) muscles stimulated at 10 Hz with duty cycles of 2.1, 4.2, 12.5, 25, 50, and 100%, corresponding to 30 min in every 24, 12, 4, 2, and 1 h and continuous stimulation. *A*: myosin light chains (MLC), grouped into fast and slow isoforms and expressed as a percentage of total light chains ( $n = 3-4$ , each group, see Table 1). *B*: myosin heavy chains (MHC), expressed as a percentage of total heavy chains ( $n = 4$ , each group). In both *A* and *B*, isoform compositions of unstimulated rabbit TA ( $n = 12$ ) and soleus muscles ( $n = 12$ ) are included for comparison. Data for 50% duty cycle taken from Ref. 33. Values are means  $\pm$  SE. Broken lines are included to clarify trends and do not imply a time course.

allows a further comparison to be made between the MLC compositions of the five intermittently stimulated groups of TA muscles and a group from the previous study (33) that was stimulated continuously at 10 Hz for 6 wk. The MLC composition of this group shows a significantly larger induction of slow isoforms than the intermittently stimulated muscles; in fact it does not differ significantly from that of the soleus muscle.

Changes in MHC isoform composition are shown in Fig. 1*B*. In agreement with the corresponding changes in light chain composition, this illustrates a moderate induction of MHC<sub>1</sub>, which was significant in all but the group stimulated with a 2.1% duty cycle. More striking, however, is the fact that MHC<sub>2X</sub>, which constituted  $84.0 \pm 2.4\%$  of MHC isoforms in the control TA muscles, was no longer detectable in any of the intermittently stimulated groups, whereas MHC<sub>2A</sub>, present at  $16.0 \pm 2.4\%$  in the control TA muscles, had risen significantly to over 70%. Again, both the continuously stimulated group and the control soleus muscles show a much higher proportion of MHC<sub>1</sub> and a correspondingly low level of MHC<sub>2A</sub>.

In terms of both MLC and MHC composition, the intermediate level of muscle transformation produced

by the phasic patterns contrasts markedly with the complete transformation elicited by continuous stimulation at 10 Hz.

**Fiber type composition.** Fiber type composition was assessed by staining for the demonstration of myofibrillar ATPase. In the control muscle group there was a preponderance of fast type IIx ( $60.6 \pm 2.6\%$ ), and type IIa ( $32.0 \pm 2.1\%$ ) fibers and a small proportion of slow type I fibers ( $5.5 \pm 1.0\%$ ). Each of the four intermittent stimulation patterns produced a highly significant departure from the control fiber type composition ( $P < 0.001$ ), with no significant differences between the groups. Most of the fibers in these muscles were of the fast-oxidative IIa type ( $91.8 \pm 2.4$ ,  $90.1 \pm 0.8$ ,  $90.4 \pm 1.2$ ,  $92.6 \pm 0.8\%$ , respectively, in the groups stimulated with on/off cycles of 2, 4, 12, and 24 h); the rest of the fibers were of the slow-oxidative type I (Figs. 2 and 3).

These results were consistent with the MHC analysis, if account is taken of the known limitations of the two techniques. Thus the difference between the proportions of type I fibers and MHC<sub>1</sub> in the stimulated muscles can be attributed to coexpression of MHC<sub>1</sub> in partially transformed type 2 fibers at levels too low to produce a detectable change in staining characteristics. Conversely, the histochemical method registers the presence of type I fibers in the control muscles even though the corresponding amounts of MHC<sub>1</sub> are below the detection limit of the electrophoretic technique.

Cryostat sections were also stained for NADH dehydrogenase activity, which is associated largely, although not entirely, with the electron transport chain in the mitochondria. In control muscles, staining was light or absent in the type IIx fibers, in which energy is derived mainly from anaerobic glycolysis. Type IIa fibers, which have a more aerobic metabolism, stained with intermediate density, with a tendency for dye deposition in the periphery of the fibers, suggestive of a

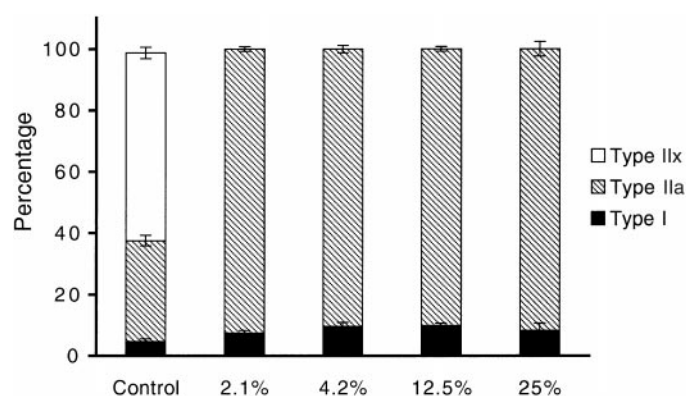


Fig. 2. Fiber type composition of rabbit TA muscles stimulated at 10 Hz with duty cycles of 2.1, 4.2, 12.5, and 25%, corresponding to 30 min in every 24, 12, 4, and 2 h ( $n = 4$ , each group), determined from sections stained by the Tunell and Hart (49) method for demonstrating myofibrillar ATPase. Control (pooled unstimulated contralateral) TA muscles ( $n = 16$ ) showed a predominance of type IIx, with the balance consisting of type IIa and a very small contribution from type I. Muscles in all the stimulated groups showed a predominance of IIa, together with a small proportion of type I; fibers of type IIx were completely absent. Values are means  $\pm$  SE.

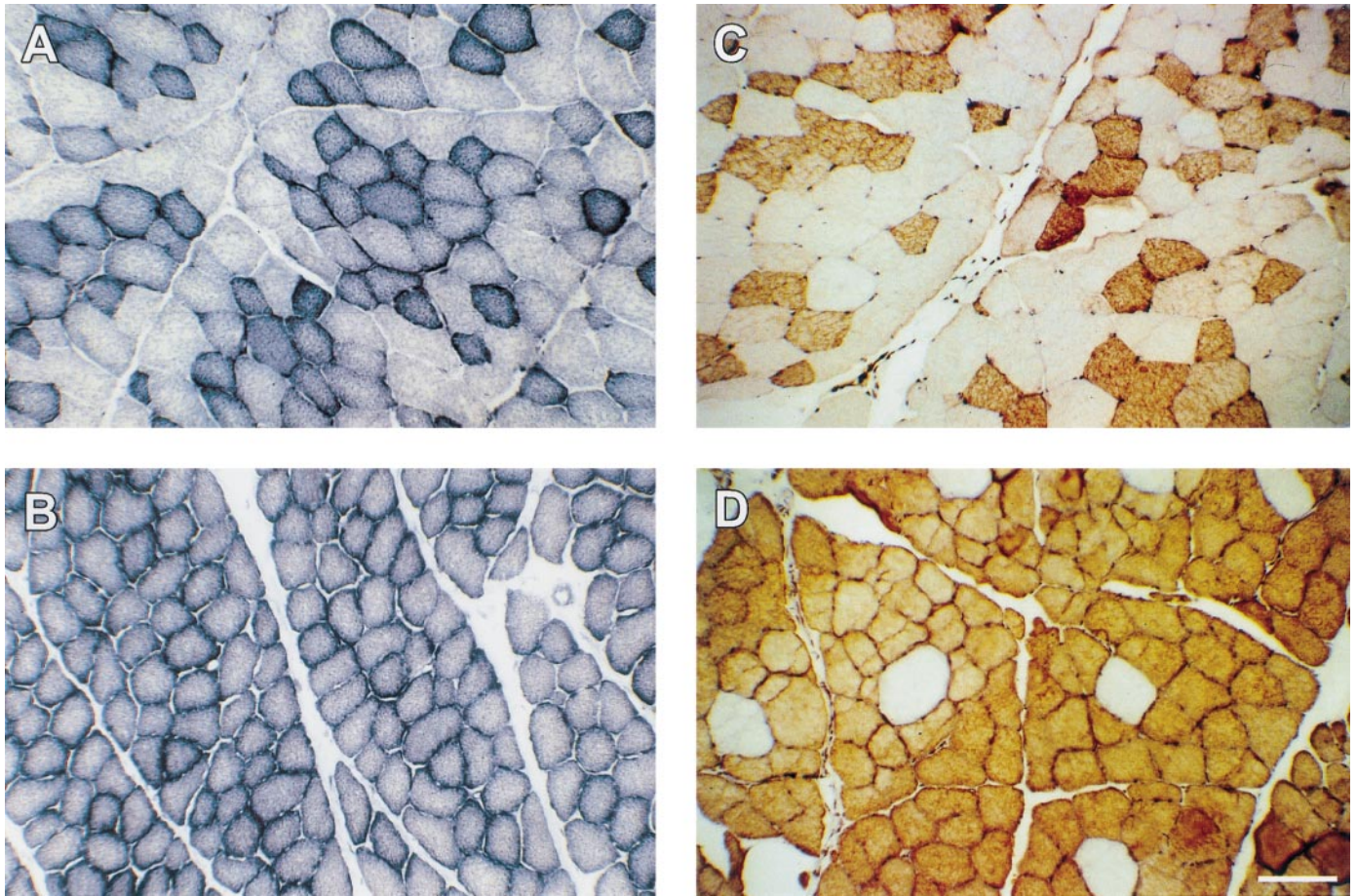


Fig. 3. Sections from rabbit TA muscles stained histochemically for the demonstration of NADH tetrazolium reductase (A and B) and immunohistochemically for MHC<sub>2A</sub> (C and D). A and C: contralateral, unstimulated TA muscle. B and D: TA muscle after 6 wk of stimulation at 10 Hz for 30 min in every 24 h. Scale bar, 200  $\mu$ m. Unstained fibers in D are type I fibers; all the other fibers express MHC<sub>2A</sub>.

correlation with subsarcolemmal concentrations of mitochondria. The highly oxidative type I fibers showed a uniformly dense staining. In the stimulated groups of muscles, there were no lightly stained fibers: all of the fibers were stained with a density between intermediate and dark (Fig. 3B). Point-counting morphometry was not carried out in these sections because gradation in the staining made it difficult to classify the fibers, but the overall picture was one of a substantial increase in oxidative activity in all stimulated fibers.

**Enzymes of energy metabolism.** Biochemical assays were conducted on some enzymes representative of major pathways of energy metabolism. Succinate dehydrogenase served as a marker of the mitochondrial tricarboxylic acid cycle. 3-Hydroxyacyl-CoA dehydrogenase was used as a marker of fatty acid oxidation, which is an important pathway for substrate supply to the tricarboxylic acid cycle. Capacity for anaerobic glycolysis was assessed by measuring the activity of lactate dehydrogenase in the cytosol. The batch of homogenates for assay always included samples from both the stimulated muscles and the contralateral unstimulated control muscles. The enzymatic activity of each stimulated muscle could then be expressed as a per-

centage of that in the unstimulated contralateral muscle. The results are shown in Fig. 4.

SDH showed a significant increase in activity in all stimulated muscles, with levels that were 200–350% those of the contralateral control muscles. HAD underwent similar, but apparently more marked, changes, with levels of activity 270–500% of control; however, variation was greater for this assay, and only the group stimulated with the 25% duty cycle showed departures from control that were significant (at  $P < 0.01$ ).

No change in the activity of LDH could be discerned for any of the stimulated groups.

**Physiological measurements.** Muscle mass appeared to be preserved more effectively in muscles stimulated with the lowest duty cycles. This trend was not, however, reflected in maximum tetanic force (Table 2). Thus changes in the twitch-tetanus ratio reflected mainly changes in maximum isometric twitch force. Both were significantly greater than control in all the stimulated groups of muscles to an extent that appeared to increase with increasing duty cycle (Table 2,  $P < 0.01$ ). None of the stimulated groups of muscles showed any significant change in specific force (Table 2).



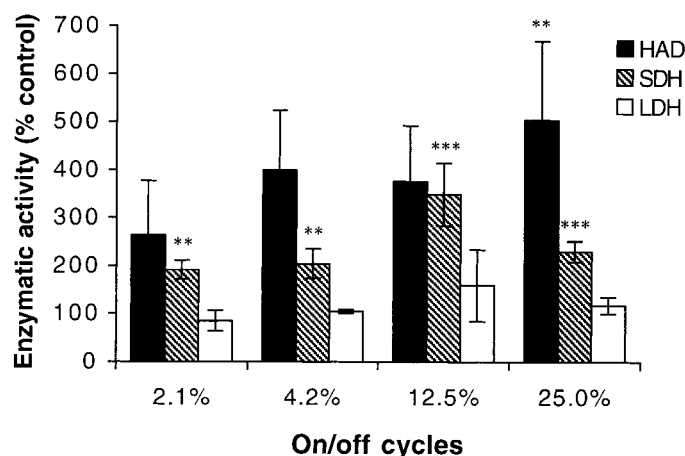


Fig. 4. Enzymatic activity of succinate dehydrogenase (SDH), 3-hydroxyacyl-CoA dehydrogenase (HAD), and lactate dehydrogenase (LDH) in rabbit TA muscles stimulated at 10 Hz with duty cycles of 2.1, 4.2, 12.5, and 25%, corresponding to 30 min in every 24, 12, 4, and 2 h ( $n = 4$ , each group). For each animal the activity determined in the stimulated TA muscle was expressed as a percentage of that in the contralateral unstimulated muscle. Values are means  $\pm$  SE.

The time to peak contraction and the time from peak to half-relaxation of the isometric twitch for muscles stimulated with duty cycles of 2.1 and 4.2% did not differ from control values. As the duty cycle increased to 12.5, 25, and 50%, however, these times increased considerably to values that differed significantly from both the contralateral control group and the other two stimulated groups ( $P < 0.01$ ; Fig. 5 and Table 2). Figure 5 includes data, taken from the previous study, for the contraction and half-relaxation times of muscles after 6 wk of continuous stimulation at 10 Hz. These times are significantly slower than either the unstimulated control ( $P < 0.001$ ) or any of the intermittently stimulated muscle groups ( $P < 0.001$ ).

The maximum velocity of shortening also showed a significant reduction in the muscle groups that were stimulated with the highest duty cycles of 12.5, 25, and 50% (Table 2); the reductions for duty cycles of 2.1 and 4.2% did not achieve significance. As a result, maximum power, which is a function of both force and

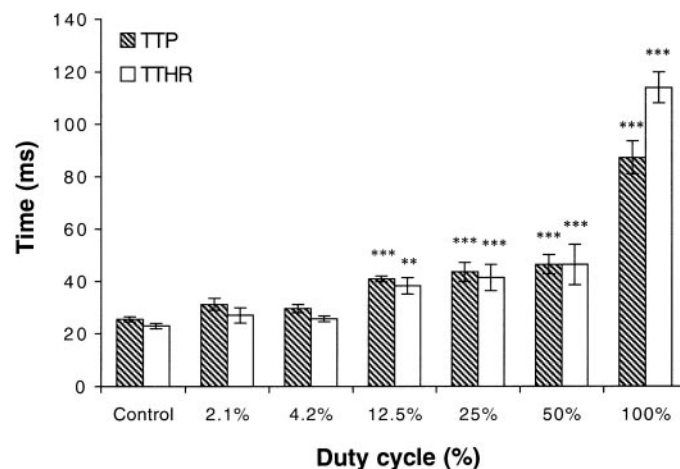


Fig. 5. Isometric twitch characteristics of rabbit TA muscles stimulated at 10 Hz with duty cycles of 2.1, 4.2, 12.5, 25, 50, and 100%, corresponding to 30 min in every 24, 12, 4, 2, and 1 h and continuous stimulation ( $n = 4$ , for each group). Control values are for the pooled unstimulated contralateral muscles ( $n = 20$ ). Data for 50% duty cycle taken from Ref. 33. TTP, time to peak twitch contraction. TTHR, time from peak twitch to half-relaxation. Values are means  $\pm$  SE. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  for comparisons with control.

shortening velocity, decreased progressively from  $71 \pm 17\%$  of control for the 2.1% duty cycle to  $69 \pm 18\%$ ,  $47 \pm 13\%$ ,  $44 \pm 19\%$ , and  $43 \pm 21\%$ , for duty cycles of 4.2, 12.5, 25, and 50%, respectively. The reduction in power at the highest three duty cycles was significant.

**Fatigue.** The fatigue tests yielded a fatigue index, defined conventionally as the ratio between the isometric force developed after 5 min to that in the first contraction. On this scale, a muscle with a fatigue index of unity would be considered to show no fatigue. All the stimulated groups of muscles had fatigue indexes of between 0.85 and 0.95, whereas the contralateral control muscles, which were stimulated under the same conditions and at the same time, had a fatigue index of  $\sim 0.5$ . Thus stimulation brought about a marked and highly significant increase in the resistance to fatigue for all the duty cycles examined (Fig. 6).

Table 2. Physiological measurements

	Control TA ( $n = 20$ )	2.1% ( $n = 4$ )	4.2% ( $n = 4$ )	12.5% ( $n = 4$ )	25% ( $n = 4$ )	50% ( $n = 4$ )
Contraction time, ms	$25.9 \pm 1.1$	$31.2 \pm 2.2$	$29.6 \pm 1.6$	$40.9 \pm 1.1^\dagger$	$43.5 \pm 3.7^\dagger$	$46.4 \pm 3.7^\dagger$
Half-relaxation time, ms	$22.9 \pm 1.1$	$27.0 \pm 2.9$	$25.7 \pm 1.1$	$38.2 \pm 3.2^\dagger$	$41.3 \pm 5.0^\dagger$	$35 \pm 18^\dagger$
Twitch force, N	$2.8 \pm 0.2$	$4.6 \pm 0.5^*$	$5.3 \pm 0.5^\dagger$	$5.8 \pm 0.5^\dagger$	$6.2 \pm 0.6^\dagger$	$6.2 \pm 0.9^\dagger$
Maximum force, N	$24.9 \pm 1.0$	$20.1 \pm 1.8$	$19.2 \pm 1.4$	$19.0 \pm 1.6$	$16.7 \pm 2.4^*$	$18.7 \pm 2.9$
Twitch-tetanus ratio	$0.12 \pm 0.01$	$0.23 \pm 0.02^*$	$0.28 \pm 0.03^\dagger$	$0.31 \pm 0.02^\dagger$	$0.39 \pm 0.04^\dagger$	$0.3 \pm 0.03^\dagger$
Maximum velocity, mm/s	$443 \pm 18$	$364 \pm 25$	$372 \pm 40$	$307 \pm 14^*$	$293 \pm 25^\dagger$	$299 \pm 30^*$
Maximum power, mW	$1,035 \pm 87$	$733 \pm 105$	$718 \pm 114$	$484 \pm 49^*$	$455 \pm 79^*$	$442 \pm 83^*$
Muscle mass, g	$3.07 \pm 0.13$	$3.18 \pm 0.23$	$3.03 \pm 0.28$	$2.39 \pm 0.20$	$2.39 \pm 0.25$	$2.5 \pm 0.4$
Specific force, N/mm <sup>2</sup>	$0.61 \pm 0.03$	$0.46 \pm 0.05$	$0.46 \pm 0.03$	$0.55 \pm 0.04$	$0.47 \pm 0.05$	$0.53 \pm 0.06$
Fatigue index	$0.52 \pm 0.04$	$0.90 \pm 0.05^\dagger$	$0.97 \pm 0.01^\dagger$	$0.89 \pm 0.07^\dagger$	$0.93 \pm 0.03^\dagger$	$0.8 \pm 0.04^*$

Values are means  $\pm$  SE from muscles stimulated at 10 Hz for 30 min with duty cycles of 2.1, 4.2, 12.5, 25, 50, and 100%, corresponding to 30 min in every 24, 12, 4, 2, and 1 h, and for the pooled unstimulated contralateral control muscles. Results for muscles stimulated with the 50% duty cycle are from the previous study (Ref. 33). \*,  $P < 0.01$ ,  $P < 0.001$ , respectively, for comparisons with control muscles.

## DISCUSSION

In the clinical sphere, electrical stimulation is used to restore the function of a paralyzed muscle or to activate a muscle that has been redeployed to fulfill a new role (see introduction). Conditioning of the muscle induces phenotypic changes that enable the muscle to cope with the new working conditions. The main goal of conditioning is to produce a muscle whose vascular supply and oxidative metabolism are adapted to sustained use. However, some conditioning regimes achieve this goal only at the expense of a serious loss in both the force-generating capacity and the contractile speed of the muscle. This can be avoided if the end point of transformation is a muscle with a predominantly fast-oxidative, IIa fiber type composition, which offers a substantial increase in endurance without an unacceptable reduction in power. Our previous work in rabbit fast muscle has shown that this can be achieved by stimulating continuously at a low frequency, such as 2.5 Hz (28), or by partitioning stimulation at a higher frequency, such as 10 Hz, into equal *on* and *off* periods (33). The present study extends these observations to unequal *on* and *off* periods and shows that a substantial gain in fatigue resistance may be achieved by stimulating for as little as 30 min/day.

From a clinical perspective, the clear advantage of a conditioning regime based on 30 min/day is that it can be acceptably incorporated into a patient's normal daily activities. This type of regime has been used previously for conditioning paralyzed muscles in spinal cord-injured subjects preparatory to the use of functional electrical stimulation (2). The present study puts the practice on a firmer scientific foundation by showing that the desired physiological and biochemical changes can indeed be induced to a sufficient extent under these conditions. It is, however, necessary to be clear about the limitations of the present results as a basis for extrapolation to humans. First, this study was conducted on rabbits. Previous studies indicate that a similar degree of stimulation-induced transformation can be achieved in larger mammals with lower daily amounts of stimulation (26). Second, the low frequency

of the stimulation patterns used in these experiments was chosen to facilitate comparison with previous experiments. In a therapeutic situation it would be desirable to use higher frequencies, because the higher contractile forces that are generated preserve more effectively the mass and force-generating capacity of the stimulated muscle (13, 27). Finally, it should be pointed out that this study was concerned only with the induction of phenotypic changes in normal muscles. In patients with spinal cord injury of several years' standing, disuse of the muscles will have resulted in atrophy and acquisition of a predominantly fast, fatigue-susceptible profile (16). Stimulation of such muscles elicits first and foremost a reversal of the disused condition, and the transformation follows a time course that is concerned initially with the restoration of normal mass, force-generating capacity, fiber type composition, and endurance (34, 44). These processes are not involved in the present study.

*Transformation depends on aggregate impulse activity.* Earlier studies (8, 9, 13, 25, 28, 29, 35, 45) have provided strong evidence that the extent of the myosin type transformation induced in muscles by chronic stimulation depends primarily on the aggregate number of impulses delivered to the muscles rather than the frequency of stimulation during the *on* phase. This evidence was extended by a recent study in which stimulation at 10 Hz alternating with an *off* time of the same duration induced changes indistinguishable from continuous stimulation at 5 Hz, although the equal *on/off* periods ranged from 30 s to 12 h (33).

The design of the present study may therefore be interpreted as delivering progressively smaller aggregate amounts of impulse activity while maintaining the actual frequency of stimulation constant at 10 Hz. The most intense pattern, based on a 50% duty cycle, would be expected to have effects equivalent to continuous stimulation at 5 Hz. Previous work (33, 45) indicates that such a pattern is close to the activity threshold for induction of the slow MHC isoform MHC<sub>1</sub>. The 25% duty cycle would be equivalent to continuous 2.5 Hz, which as we have shown previously results in induction of MHC<sub>2A</sub> with little induction of MHC<sub>1</sub> (28, 35, 45).

Such equivalency was substantially confirmed by the present study. The major change observed in the myosin isoform composition of the intermittently stimulated muscle groups was a complete disappearance of MHC<sub>2X</sub> and its replacement mainly by MHC<sub>2A</sub>, with a small increase (up to ~20%) in the proportion of slow myosin heavy and light chains. In contrast, slow myosin isoforms constituted about 80% of the total in muscles that had been stimulated continuously at 10 Hz.

The threshold for induction of MHC<sub>2A</sub> evidently corresponds to quite low levels of activity, because this isoform effectively replaced MHC<sub>2X</sub> in all the intermittently stimulated muscles, even those stimulated for as little as 30 min in 24 h. The quantitation of fiber type proportions in histochemically stained sections corroborated these biochemical findings. It is worth empha-

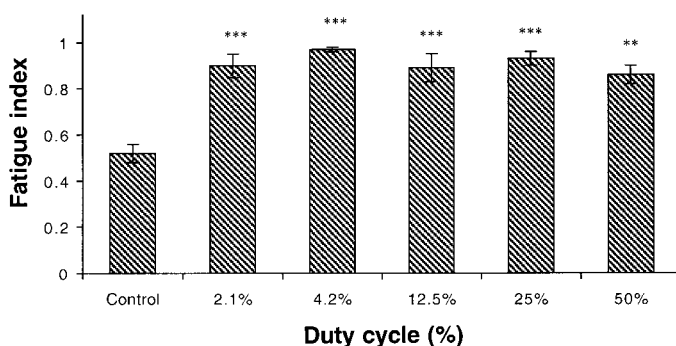


Fig. 6. Fatigue index, defined as the fraction of initial tension remaining after 5 min, for rabbit TA muscles stimulated as in Fig. 5 ( $n = 4$ , each group) and for a control group ( $n = 20$ ) consisting of the pooled unstimulated contralateral muscles. Data for 50% duty cycle taken from Ref. 33. \*\*\* $P < 0.001$ , \*\* $P < 0.01$  for comparisons with control.

sizing here that the entire muscle was activated under the conditions of supramaximal electrical stimulation used in this experimental study. Under conditions of voluntary activation, motor units would be recruited in a much more selective way, and this explains why human muscles subjected to endurance exercise would normally show a less homogeneous adaptation.

*Physiological measurements.* Changes in MHC composition are usually reflected in the maximum velocity of shortening. The maximum velocity of shortening was in fact significantly lower for stimulation patterns with the highest duty cycles of 12.5, 25, and 50%, but the reduction for duty cycles of 2.1 and 4.2% was smaller and did not attain statistical significance. This is suggestive of subtle gradations in the myosin isoform transitions that may have failed to emerge as clearly in the overall biochemical results as in the physiological data.

Significantly higher values for the twitch-tetanus ratio were observed for all the stimulated muscle groups than for either contralateral control fast-twitch muscles ( $0.12 \pm 0.01$ ,  $n = 20$ , this study) or a typical slow-twitch muscle ( $0.21 \pm 0.01$ ,  $n = 15$ , figure for rabbit soleus from Ref. 42). On its own, a decreased maximum velocity of shortening would be expected to reduce, rather than increase, the fraction of tetanic force attained during a twitch contraction. This effect was presumably more than offset by an increased duration of calcium activation. This combination of a reduced shortening velocity and a prolonged activation time would then account for the observed lengthening of the times to peak and half-relaxation of the isometric twitch. The known decrease in the capacity of stimulated muscle for active sequestration of cytoplasmic free calcium (42, 43) has been attributed to both a reduction in the extent of the sarcoplasmic reticulum (11) and changes in the proteins responsible for calcium uptake and storage (4, 18, 19, 22, 23, 30, 36). These changes in the kinetics of calcium transport occur at an early stage of the response to continuous stimulation at 10 Hz (21). Therefore by analogy with the MHC<sub>2X</sub>-to-MHC<sub>2A</sub> myosin isoform transition, for example, they could also be expected to develop during the more prolonged application of less intensive stimulation patterns. When fast muscles are stimulated at 10 Hz for much longer periods than those in the present study (45), shortening velocity decreases still further and the twitch-tetanus ratio then declines to values more typical of rabbit slow muscles.

The muscles stimulated with the lowest duty cycles of 2.1 and 4.2% showed no sign of the reduction in mass that is usually associated with continuous stimulation at 10 Hz. Changes in mass are the net result of the effects of stimulation on global protein synthesis and degradation (7) and are probably regulated by signaling pathways distinct from those that trigger individual molecular transitions. The observations indicate that stimulation with the lowest duty cycles produced an increase in protein synthesis that was equal in duration or effect to any associated increase in the rate of protein degradation.

Examination of histological sections from the muscles did not reveal evidence of stimulation-induced damage, and the absence of significant changes in specific force confirms that any such damage can only have been of very limited extent.

*Energy metabolism.* Sections from the stimulated muscles that were stained histochemically for the demonstration of NADH tetrazolium reductase showed the mitochondrial density to be high in all muscle fibers. This was true for all the patterns, including the lowest duty cycle, which delivered stimulation for only 30 min in 24 h.

The histochemical picture was borne out by biochemical assay of enzymes representative of the tricarboxylic acid cycle and fatty acid oxidation. The closest data with which these results could be compared are those obtained from muscles stimulated continuously for 6 wk at 2.5 Hz (35). The latter regime would deliver the same total number of impulses as the 25% duty cycle in the present study. The increases in enzymatic activity corresponding to these two patterns were in fact broadly similar.

No change in LDH activity was observed in any of the stimulated muscle groups. Comparison with the data of Mayne et al. (35) would suggest that, for the equivalent number of impulses, a decline of 20% might have been anticipated. LDH activity also shows no consistent response to endurance training (41), so the absence of change in the present study could possibly be related to the intermittent nature of the stimulation.

*Fatigue resistance.* Muscles in all of the stimulated groups retained between 85 and 95% of their initial tension after 5 min of a standard fatigue test, whereas the contralateral control muscles lost ~50% of their initial tension under identical conditions. This increased resistance to fatigue is a physiological reflection of the observed changes in oxidative enzyme activity and the transformation of the muscles to a predominantly fast-oxidative, IIa fiber type composition. Previous studies based on more continuous stimulation regimes (3, 24) would suggest that these changes could well have been accompanied by an increase in capillary density and perfusion of the muscle. Arguably one of the most important factors determining the ability of the muscles to accommodate sustained activity was the uniform and marked increase in mitochondrial density, revealed in sections stained histochemically for NADH tetrazolium reductase, because this would increase the efficiency with which oxygen could be extracted from blood supplied to the muscle.

Fatigue resistance is not an absolute measure but depends on the level of metabolic demand made on the muscle under the test conditions. It is not suggested, therefore, that the stimulated groups all had the same resistance to fatigue. Nonetheless the fatigue test used in this study (6) has proved to be a good index of the endurance capabilities that can be expected from the muscles under a variety of conditions. On this basis, it can be concluded that stimulation for as little as 30



min/day was sufficient to bring about changes that underpinned a striking increase in fatigue resistance.

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