J. Anat. (1973), **116**, 1, pp. 45–55
With 3 figures
Printed in Great Britain

The effect of immobilization on the longitudinal growth of striated muscle fibres

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(*Accepted* 26 *June* 1973)

INTRODUCTION

It is known that during postnatal growth the skeletal muscles of most mammals increase in length as a result of the longitudinal growth of their component muscle fibres (Kitiyakara & Angevine, 1963; Bridge & Allbrook, 1970) and that increase in fibre length is due mainly to an increase in the number of sarcomeres in series (Goldspink, 1968; Williams & Goldspink, 1971). The aim of the present study is to investigate the effects of immobilization on the sarcomere number in muscle fibres of both young and adult mice.

Immobilization of young muscles in the shortened position diminishes the growth of the muscle (Alder, Crawford & Edwards, 1959) and experiments using single teased fibres have demonstrated that this reduced growth results from the fibres failing to develop the normal number of sarcomeres in series (Williams & Goldspink, 1971). In young muscle diminished growth resulting from immobilization can be compensated for by a rapid addition of sarcomeres when the restriction is removed (Williams & Goldspink, 1971), but it is not known whether muscles which have undergone very long periods of immobilization have the same ability to recover. It was therefore decided to immobilize mouse muscles in the shortened position for different lengths of time and, again using single teased fibres, to investigate their ability to recover.

Haines (1931–2) considered that immobilization does not affect the length of the fibres of adult muscles, but recent work on cat muscle (Tabary *et al.* 1972) has shown that immobilization of adult muscles in the shortened position results in a loss of sarcomeres in series which is then followed by a return to normal levels once the restriction is removed. In the present study a further investigation has been made of the effects of immobilization on adult mouse muscle.

Immobilization of a very young muscle in its lengthened position results in reduced longitudinal growth (Williams & Goldspink, 1971), but immobilization of adult cat muscle in the same position results in an increase in the number of sarcomeres in series (Tabary *et al.* 1972). A more detailed investigation has therefore been undertaken of the effect of immobilization in the lengthened position, using muscles from mice of ages ranging from newborn to adult.

It is generally considered that during postnatal increase in muscle fibre length new sarcomeres are added on serially at the ends of the fibres (Holtzer, Marshall & Fink, 1957; Ishikawa, 1965; Mackay, Harrop & Muir, 1969). Williams & Goldspink

(1971) supported this theory by showing that when ^3H adenosine is injected into young mice more of the radioactive label is incorporated into the end than into the middle regions of the muscles. This technique has now been applied to muscles which have undergone periods of immobilization in order to ascertain where the radioactive label is incorporated during the subsequent period of rapid recovery.

During the postnatal development of normal muscle fibres there is a large increase in the number of muscle fibre nuclei (Enesco & Puddy, 1964; Williams & Goldspink, 1971). In the present study the effect of immobilization on the rate of proliferation of muscle fibre nuclei has been investigated using individual teased fibres.

Tenotomy of one muscle may produce effects on the synergist which are similar to immobilization, since after tenotomy the synergistic muscle may be held in an extended position by the stronger action of its antagonists (Schiaffino & Hanzlikova, 1970). An experiment was therefore carried out, again using single teased fibres, to determine whether tenotomy of a muscle affects the number of sarcomeres in fibres from the synergistic muscle, and to compare the results with those of immobilization of a muscle in an extended position.

MATERIALS AND METHODS

Animals

The mice used were normal heterozygous males of the strain 129/Re. The animals were reared in the Department of Zoology, University of Hull, from a colony originally obtained from Jackson Memorial Laboratories, Bar Harbor, U.S.A. They were fed on Pilsbury's Special Breeding Diet with food and water available at all times. The m. soleus was chosen for this study on account of its relatively simple fusiform structure with fibres which run from tendon to tendon and which are all approximately the same length.

Immobilization of soleus muscle

Plaster casts were used to hold the whole hind limb in either the extended position (that is, with the soleus in its shortened position) or in the flexed position (that is, with the soleus in its lengthened position). Mice were anaesthetized with intraperitoneal injections of Nembutal and plaster casts were applied, using Gypsona Plaster of Paris bandage. In each animal the cast was put on one hind limb; the contralateral leg served as the control. When dry, the casts were coated with several layers of adhesive, which helped to prevent fraying caused by the animals biting their casts. Casts were changed weekly. The presence of the cast did not cause any decrease in the growth of the bones.

Determination of sarcomere number

The method used to determine the number of sarcomeres along the length of single, teased muscle fibres has been described in detail already (Williams & Goldspink, 1971).

The mice were killed and the hind limbs were pinned out so that the soleus muscles were in the lengthened position. The soleus muscles were exposed and fixed *in situ* by glutaraldehyde. After fixation the muscles were placed in 30 % HNO_3 to hydrolyse

the connective tissue. They were then stored in 50 % glycerol to remove the soluble proteins and make the myofibrils more clearly visible. Individual whole fibres were teased out from the region of the centre of the muscles and mounted in glycerine jelly. Using a Leitz projecting microscope the number of sarcomeres along the length of each fibre was counted.

Recovery of muscle following long periods of immobilization

The soleus muscles of young mice (4 g) were immobilized in the shortened position until the mice weighed 30 g (3 months old). The plaster casts were then removed. One mouse was killed immediately and the rest were left for periods of 1–4 weeks before being killed. In all animals the numbers of sarcomeres in fibres from the experimental and contralateral soleus muscles were determined.

Immobilization of adult muscle in the shortened position

Plaster casts were used to immobilize the hind limbs of mice weighing approximately 30 g. At intervals of 1–3½ weeks after immobilization the mice were killed and the numbers of sarcomeres in fibres from experimental and contralateral soleus muscles were determined. One mouse had its cast removed after a 3½-week period of immobilization and, after 4 weeks recovery, was killed; the sarcomere number was then determined.

Immobilization, in the lengthened position, of soleus muscles of different ages

In one experiment mice weighing 4, 6, 9, 12 and 15 g had their soleus muscles immobilized in the lengthened position until the mice weighed 20 g. The numbers of sarcomeres in immobilized and control muscle fibres were then determined. In a second experiment adult mice (28–30 g) had their soleus muscles immobilized in the same position for 1–3 weeks. The sarcomere numbers were then determined. Two of the mice which had undergone a 2½ week period of immobilization were allowed to recover for 4 weeks before the sarcomeres were counted.

Location of sarcomeres produced during recovery from immobilization

The soleus muscles of mice weighing 4 g were immobilized in the shortened position for 3 weeks. The plaster casts were then removed. After a 2 day interval the animals were given 4 daily intraperitoneal injections of 25 μCi tritiated adenosine in an attempt to label the newly formed actin and ribosomes (Griffin & Goldspink, 1973) and hence the new sarcomeres laid down during the recovery period. Experimental and contralateral hind limbs were removed and the soleus muscles glycerol-extracted. The muscles were dissected out and each was divided into five portions using the method described by Williams & Goldspink (1971). The portions of muscle tissue were then prepared for scintillation counting and the radioactivity in each sample was expressed as DPM/mm^3 tissue. In this way the amount of radioactivity in different regions of experimental and control muscles could be determined.

Effect of immobilization on the postnatal addition of muscle fibre nuclei

Soleus muscles of 4 g mice were immobilized in the shortened position by means of plaster casts. Mice from one group were killed at intervals of 2 weeks. The soleus muscles from the experimental and contralateral limbs were exposed and fixed in Carnoy's fixative. They were then removed and immersed for 2 days in gallocyanin in order to stain the nuclei. Single fibres were teased out and mounted in glycerol jelly; the number of nuclei in each fibre was then counted. Mice from a second group had their casts removed when the mice weighed 18–20 g. They were allowed to recover for 3–4 weeks before being killed. The number of nuclei per fibre was then determined.

The effect of tenotomy of the gastrocnemius on sarcomere number in the soleus

Mice weighing 12 g were anaesthetized by intraperitoneal injections of Nembutal. The common insertion tendon of the gastrocnemius and the soleus (tendo Achillis) was exposed and the gastrocnemius insertion tendon was cut just proximal to its junction with the soleus tendon. The incision in the skin was then sutured. This procedure was carried out on both hind limbs of the experimental mice. Control animals were 12 g mice which had undergone a sham operation consisting of incision and suturing. At intervals of 1, $1\frac{1}{2}$, $3\frac{1}{2}$ and 11 weeks after the operations, the mice were killed and the number of sarcomeres in the soleus muscle fibres of experimental and control animals was determined in the usual way.

RESULTS

Recovery of muscle following long periods of immobilization

When plaster casts were removed from limbs which had been immobilized in the shortened position until the mice weighed 30 g (approximately 16 weeks) the number of sarcomeres along the length of the soleus muscle fibres then rapidly increased, so that after 4 weeks the sarcomere numbers in experimental and control muscle fibres did not differ significantly (Fig. 1).

Immobilization of adult muscle (shortened position)

The effect of immobilization of adult soleus muscles in the shortened position was to reduce the number of sarcomeres along the length of the muscle fibres. A significant reduction in sarcomere number occurred after a $3\frac{1}{2}$ week period of immobilization (Fig. 2). Removal of the cast was followed by a return to almost the normal level.

Immobilization, in the lengthened position, of soleus muscles of different ages

Immobilization of the soleus muscles of the 4 g, 6 g and one of the 9 g mice resulted in a reduction of the normal increase in sarcomere number, whereas immobilization of the muscles of the other 9 g mouse and the 12 g mice resulted in an increase in the rate of sarcomere addition (Table 1). Immobilization of adult muscles in the same position resulted in an increase in sarcomere number followed by a return to almost normal levels when the restriction was removed (Fig. 2).

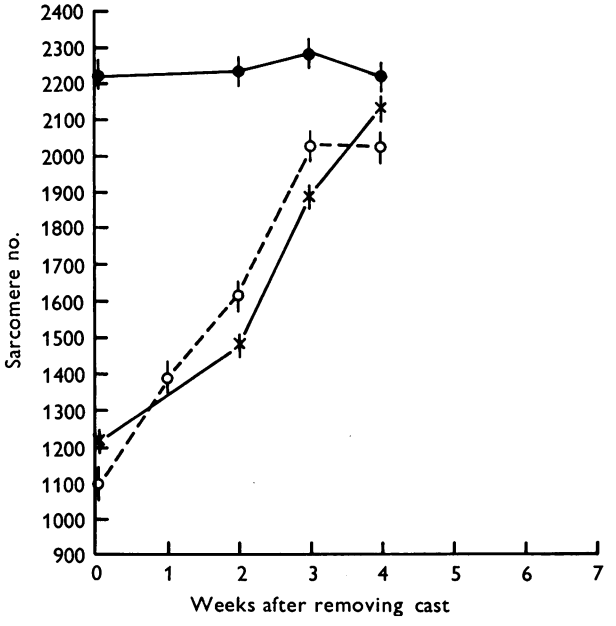

Fig. 1. Sarcomere numbers in soleus muscle fibres recovering from very long periods of immobilization. The muscles had been immobilized from the first week after birth until the mice were 3 months old. The plaster casts were then removed and the sarcomere number determined during the subsequent recovery period. ×, experimental muscle fibres; ●, contralateral fibres. The broken line shows the recovery of muscle fibres from 5 week old mice. The results are expressed as the means of at least three readings ± the standard errors of the means.

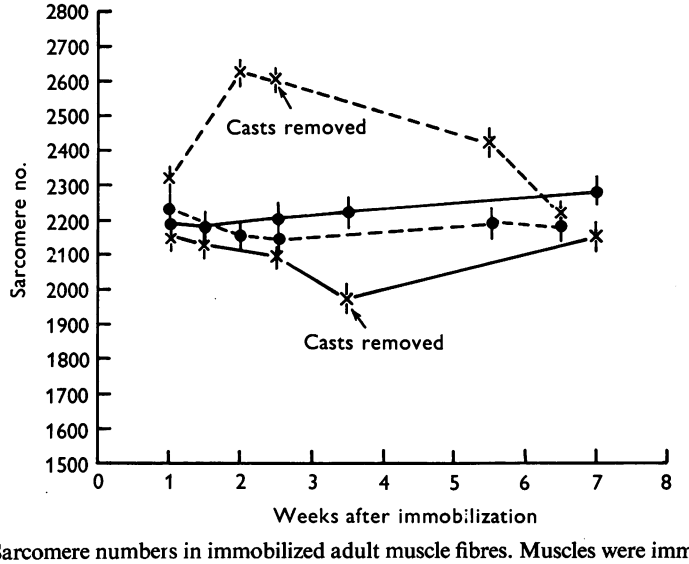

Fig. 2. Sarcomere numbers in immobilized adult muscle fibres. Muscles were immobilized in the lengthened (----) and shortened (——) positions. The ability of the muscles to recover following removal of the casts (↑) was also followed. ×, experimental and ●, contralateral muscle fibres. Each point is the mean of at least three observations ± standard error of the mean.

Table 1. *Immobilization, in the lengthened position, of soleus muscles of different ages*

(Each datum is the mean of five observations. Data in brackets are not significantly different from each other).

Weight when immobilized (g)	Weight when killed (g)	Mean sarcomere no. Experimental muscles	Mean sarcomere no. Control muscles
		S.E.	S.E.
4	18	1326±35	1973±37
4	19	1532±34	2201±46
6	18	1655±32	1986±53
6·5	20	1668±49	2189±30
9	19·5	[1894±51	1999±40]
9	20	1893±64	2136±46
12	19·5	2324±48	1963±25
12·5	20·5	2482±30	2072±42

Table 2. *Amount of radioactivity incorporated into different regions of soleus muscles recovering from immobilization*

(The end regions of the muscles are contained in vials 1 and 5, the distal end in each case being in vial 5. A Fischer Berman test showed that in experimental muscles 1, 2 and 3 there was more radioactive incorporation into the ends than into the middle regions of the muscles.)

		Experimental muscles			Contralateral muscles		
Muscle pairs	Vial no.	d.p.m.	Volume of muscle tissue in vial (mm^3)	d.p.m./mm^3 tissue	d.p.m.	Volume of muscle tissue in vial (mm^3)	d.p.m./mm^3 tissue
1	1	120	0·31	387	123	0·32	384
	2	151	0·42	360	106	0·50	212
	3	251	0·52	494	98	0·60	163
	4	260	0·42	620	141	0·55	256
	5	326	0·28	1164	170	0·31	548
2	1	120	0·28	429	96	0·31	290
	2	124	0·43	288	107	0·41	261
	3	212	0·54	293	63	0·57	110
	4	148	0·48	308	81	0·47	172
	5	281	0·25	1124	64	0·39	164
3	1	132	0·27	488	51	0·28	182
	2	78	0·37	211	51	0·40	127
	3	97	0·46	212	63	0·54	117
	4	95	0·39	244	68	0·43	158
	5	119	0·25	476	54	0·39	139
4	1	59	0·26	227	51	0·28	203
	2	81	0·36	225	109	0·50	218
	3	80	0·46	174	77	0·55	140
	4	107	0·37	289	33	0·42	79
	5	52	0·22	236	31	0·27	115
5	1	140	0·24	585	58	0·28	207
	2	222	0·35	634	87	0·37	235
	3	189	0·43	440	102	0·49	208
	4	253	0·29	872	84	0·42	200
	5	192	0·27	711	74	0·30	247

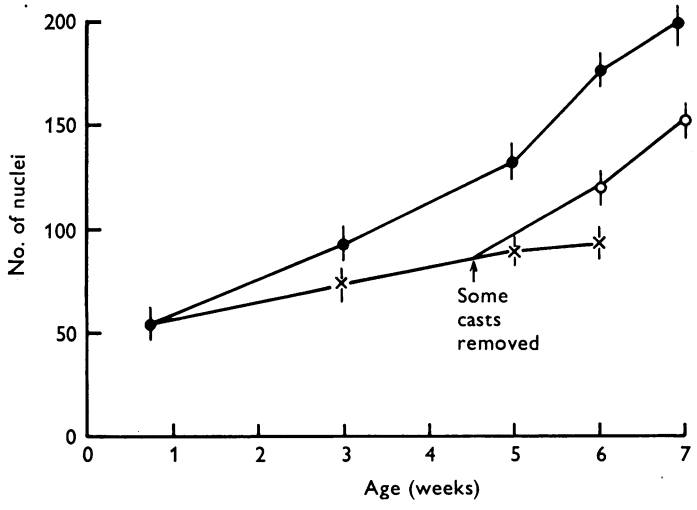

Fig. 3. Numbers of nuclei in immobilized muscle fibres and in fibres from muscles recovering from immobilization. ×, immobilized fibres; ●, contralateral fibres; ○, fibres from muscles recovering from immobilization. Each point is the mean of three observations ± standard error of the mean.

Table 3. *The effect of tenotomy of the gastrocnemius on sarcomere numbers in soleus muscle fibres*

(Each datum is the mean of at least three readings.)

Weeks after tenotomy	Sarcomere no. Experimental muscles		Sarcomere no. Control muscles (left only)
	Left	Right	
1	2332 ± 28	2170 ± 59	1782 ± 12
1	2411 ± 17	2140 ± 32	1820 ± 13
1½	2210 ± 15	2208 ± 63	1866 ± 34
3	2274 ± 12	2288 ± 13	2205 ± 15
11	2181 ± 39	2127 ± 18	2191 ± 15

Location of the sarcomeres produced during recovery from immobilization

The results of these experiments are given in Table 2. The table shows the amount of radioactivity, expressed as DPM/mm³ tissue in different regions of the five experimental and contralateral muscles. In experimental muscles 1, 2 and 3, more radioactivity was incorporated into the ends than into the middle regions of the muscles and the distal end was the more heavily labelled end of the muscle. These results suggest that during the period of recovery from immobilization, new actin and ribosomes (and hence new sarcomeres) are often added to the ends of the muscle fibres, and that growth occurs mainly at the distal end of the muscle.

Effect of immobilization on the number of muscle fibre nuclei

After a 2 week period of immobilization fibres from immobilized muscles contained significantly fewer nuclei than fibres from the contralateral muscles (Fig. 3). When the plaster casts were removed the number of nuclei increased, so that after 4 weeks of recovery the numbers of nuclei in experimental and contralateral fibres did not differ greatly.

The effect of tenotomy of the gastrocnemius on sarcomere number in the soleus

During the $1\frac{1}{2}$ weeks following tenotomy of the gastrocnemius, soleus muscle fibres showed an increase in sarcomere number compared with fibres from control muscles (Table 3). This result is similar to that which occurred when soleus muscles from mice of the same age were immobilized in the extended position (Table 1). After $1\frac{1}{2}$ weeks, however, the sarcomere number in tenotomized animals was found to return quite rapidly to the normal level.

DISCUSSION

In the limbs of growing animals there seems to be a correlation between range of movement and muscle length (Haines, 1931–2; Crawford, 1954; Comer, 1956; Alder, Crawford & Edwards, 1959). When the range of movement of the hind limbs of young mice is restricted by immobilization in either the extended or the flexed position the rate of growth in length of the soleus muscles is reduced due to a decrease in the rate of the postnatal addition of sarcomeres (Williams & Goldspink, 1971), and when the restriction is removed the sarcomere number increases to the normal level. It can be seen from Fig. 1 that even muscles which have undergone extremely long periods of immobilization have the same ability to recover as younger muscles; there would seem to be no point in development beyond which the muscle cannot recover from the effects of immobilization by producing more sarcomeres in series. This ensures that it operates at an optimum length in relation to range of limb movement.

Immobilization of a muscle in the shortened position results in a reduced number of sarcomeres, not only in young but also in adult muscle. It can be seen from Fig. 2 that when adult muscle was immobilized in the flexed position there was an initial loss of sarcomeres in series, followed by a return to normal numbers when the restriction was removed. It may be that a muscle must contract throughout its normal range of movement, not only for normal growth to occur, but also for the sarcomere number to be maintained in the adult stage. On the other hand, as suggested by Tabary *et al.* (1972), when a muscle is immobilized in the fully shortened position its functional length is decreased; in this position the sarcomeres are fully shortened and maximum contractile strength cannot be developed since there is not an optimum overlap of myosin and actin filaments. It may be that, to counteract this effect of immobilization, the fibres lose sarcomeres until the sarcomere length is optimum.

In the case of immobilization in the lengthened position, different results were obtained according to the age of the animals used (Fig. 2 and Table 1). Muscle fibres from adult animals and all animals which were more than $2\frac{1}{2}$ weeks old when their limbs were immobilized had more sarcomeres in series than fibres from control

animals. This is probably because the extended position of the muscle increases its functional length, so that the sarcomeres would be too long if others were not added on to give optimal sarcomere length. In the very young mice, on the other hand, although the immobilized muscle was constantly held in the lengthened position both by the flexion of the limb and by bone growth, the fibres contained fewer sarcomeres in series than those from control animals. This would suggest that during the first few weeks following birth the factor of overriding importance controlling sarcomere addition is the contraction of the muscle throughout its normal range.

The experiments involving the incorporation of ^{3}H adenosine indicate that the sarcomeres which are produced during the period of recovery from immobilization are located at the ends of the muscle fibres (Table 2), just as they are during normal postnatal growth. It is interesting that the distal end of the muscle appears to be the more active end during recovery from immobilization. Possible explanations of this might be that the distal end has a richer blood supply or that it undergoes more tension per cross-sectional area.

Not all muscles showed heavy labelling of the end relative to the middle regions. There appear to be two reasons for this. In the first place it was noticed that in some animals the hind limbs remained very stiff for several days after removal of the plaster cast: the soleus muscle was thus still effectively immobilized, and this would explain why in muscle 4 there seems to have been very little growth in length immediately following removal of the plaster cast. Secondly, since the muscles were increasing not only in length but also in diameter, in muscle 5 the incorporation due to hypertrophy might have masked the incorporation due to increase in length.

The effect of immobilization on sarcomere number in young mice is paralleled by the effect on nuclear number. In single fibres from immobilized muscles the addition of nuclei fell short of that in normal muscle fibres. This presumably results from a slowing down in the proliferation of satellite cells, which are thought to be the source of additional nuclei during postnatal growth (Moss & Leblond, 1970, 1971).

Tenotomy of the gastrocnemius was found to affect sarcomere number in the soleus in a way similar to immobilization. After the operation the animals walked with their lower hind limbs in a flexed position, that is to say with the soleus in a lengthened position, probably because the soleus muscle was not strong enough to balance the action of the antagonistic muscles. The sarcomere number increased at a much greater rate than in control muscles (Table 3) and this increased rate was similar to that found when muscles of the same age were immobilized in the lengthened position. It would seem probable that in both cases the functional length of the muscle was increased, so producing an increase in sarcomere number. When, after 1–2 weeks, the animals started to walk with a more or less normal gait, the soleus muscle fibres had possibly undergone sufficient hypertrophy to be able to compensate for the loss of the gastrocnemius and thus balance the antagonists. (Certainly their diameter was much greater than that of normal soleus fibres.) At this stage the sarcomere number started to return to normal in a manner resembling the recovery of muscle from immobilization.

The experiments described above illustrate the adaptability of muscle tissue of all ages. In very young muscle, where immobilization appears to cause a slowing down in the normal postnatal growth of the fibre, recovery in both sarcomere number and

nuclear number can occur even after very long periods of immobilization. In adult muscle sarcomere number can be adapted, possibly in order to adjust sarcomere length, when the functional length of the muscle is changed, either by immobilization or by tenotomy of a synergistic muscle. The molecular events involved in this adaptation of muscle fibres have yet to be determined.

SUMMARY

The effect on sarcomere number of immobilizing muscles in lengthened and shortened positions has been investigated in both young and adult mice. Immobilization of the soleus muscle in the shortened position resulted in a decrease in the postnatal addition of sarcomeres in young muscles and a loss of sarcomeres in adult muscles. In both there was a return to normal sarcomere numbers when the restriction was removed.

Immobilization in the lengthened position resulted in a decrease in the postnatal addition of sarcomeres in very young muscles, but an increase in muscles from animals more than 2½ weeks old. It is suggested that immobilization alters the functional length of the muscle, and that, whereas adult muscles can adapt to an increase or decrease in the functional length by gaining or losing sarcomeres, in young muscle the factor of overriding importance controlling sarcomere addition is the contraction of the muscle throughout its normal range of movement: if movement is restricted sarcomere production is decreased whatever the position of immobilization.

The ability to recover from immobilization is not affected either by the length of the period of immobilization or by the stage in development during which recovery is made.

By injecting ^{3}H adenosine into mice recovering from immobilization, an attempt was made to label the sarcomeres which are laid down during this period of longitudinal growth. It appears that, as during normal growth, the muscle fibres were adding sarcomeres mainly at their ends.

Immobilization of young muscle was found to result in a reduction of the postnatal increase in muscle fibre nuclei. Removal of the restriction was followed by a rapid increase in the number of muscle fibre nuclei.

Following tenotomy of the gastrocnemius there was an increase in the number of sarcomeres in the soleus muscle fibres. It is suggested that in the absence of the gastrocnemius the soleus is not able to balance its antagonistic muscles and is thus effectively immobilized in the lengthened position, resulting in increased sarcomere production.

This work was supported by a grant from the Spastics Society.

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