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Quantitative studies of the regeneration of rat myelinated nerve fibres: variations in the number and size of regenerating fibres after repeated localized freezings

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INTRODUCTION

Since the early observations of Mayer (1881), Vanlair (1888) and Cajal (1928), the few studies devoted to peripheral nerve regeneration after repeated injuries have produced conflicting morphological and physiological results. Thus, some studies seemed to indicate that the regenerative effects were lessened (Duncan & Jarvis, 1941, 1943; Sabaino & Solerio, 1952), some that they were unchanged (Holmes & Young, 1942; Duncan & Jarvis, 1943; Falin, 1961) and some that they were enhanced (Gutmann, 1942, 1948; Abercrombie & Santler, 1957; Thomas, 1968, 1969, 1970; Ducker, Kempe & Hayes, 1969; McQuarrie & Grafstein, 1973). These divergencies prompted the author to undertake a quantitative study of the variations in the number and size of regenerating myelinated nerve fibres following repeated nerve injuries.

The present author has reported several quantitative studies of the regeneration of rat myelinated fibres in the nerve to the medial head of the gastrocnemius muscle following a single experimental injury to the sciatic nerve. Injuries were produced in one of five ways: localized crushing, section and immediate suture, section without suture, resection of a 10 mm segment (Mira, 1976*b*) and localized freezing (Mira, 1972*a*, 1977*a*). Normal controls were also studied (Mira, 1976*a*).

Results were recorded from 10 to 720 days following the nerve damage. After localized freezing or crushing the number of regenerating myelinated nerve fibres returned to normal during the fourth week, but only during the second month after section followed by immediate suture, and during the fourth month after section without suture. Except for resection, where only 20% of regenerating myelinated nerve fibres succeeded in bridging the gap, fibre numbers increased up to a mean of 115% after crush, 124% after freezing, 130% after section without suture and 150% after section and suture. The mean diameter of all myelinated nerve fibres returned to control values at the end of the first year after freezing, but did not exceed 80% after crush, 50–60% after section and 40% after resection. Fibre size distribution became bimodal from the sixtieth day after freezing and the ninety seventh day after crush. It was usually unimodal after section, and always unimodal after resection. Fibre diameter histograms of regenerating and control nerves were superimposable by the 330th day after freezing; they were almost superimposable during the second year after crush, but never after section and resection.

On the whole, results observed after a single localized freezing were more consistent than after crush or section. This is why the freezing procedure was chosen for the

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present study of the effects of repeated injuries on myelinated nerve fibre populations. A preliminary communication was published on this subject (Mira, 1977*b*).

MATERIALS AND METHODS

Experiments were carried out on 19 albino rats (13 males and 6 females) weighing from 90 to 220 g at the time of the first freezing, and from 170 to 500 g when they were killed under general anaesthesia. In all cases, repeated localized freezings were performed *in situ* on the left sciatic nerve before it branches out into the tibial, peroneal and sural nerves. Results were recorded at standardized levels in the nerve to the the medial head of the right and left gastrocnemius muscles (RGM and LGM nerves). This nerve is a small branch of the tibial nerve which comes off 30–40 mm below the point where the lesion was produced in the main nerve.

Surgical procedure

To carry out the freezings, a previously described liquid nitrogen cryode was used (Mira, 1977*a*). The active point of this probe was directly applied to the sciatic nerve at three successive points on the same nerve circumference and quickly frozen at about −180 °C. The site of the virtual circle formed by the three points was indicated by a suture of non-resorbable flexilon thread (diameter: 1·5 decimal) placed in the perineurium and marking the approximate centre of the frozen area. The total duration of freezing and thawing never exceeded 30–40 seconds.

After a single freezing, all the myelinated nerve fibres in the frozen area exhibited ultrastructural changes indicating local necrosis of axons and disorganization of their myelin sheaths. However, there was no disruption of the nerve connective tissue sheaths, the blood vessels supplying the nerve trunk or the basal lamina surrounding each nerve fibre (Mira, 1971, 1972*b*).

Periodicity of nerve injuries

In order to determine the optimal period between successive localized nerve injuries, a single freezing was performed on the sciatic nerve of four rats. Using the Bielschowsky–Gros silver impregnation technique the first regenerating nerve endings were detected in the middle part of the gastrocnemius muscle between the sixteenth and the eighteenth post-operative days. Consequently, it was decided to freeze at intervals of 3 weeks.

Histological procedure

RGM and LGM nerves were removed from each operated rat under general ether anaesthesia. They were fixed for 1½ hours with 2·5% glutaraldehyde in 0·2 M Sorensen phosphate buffer (pH 7·2–7·4) and post-fixed for 1 hour with 2% osmium tetroxide in 0·2 M Palade buffer (pH 7·2–7·4). They were then dehydrated in graded concentrations of ethyl alcohol and finally embedded in Araldite.

For conventional light microscopy, 30–40 semi-serial 1 μm thick transverse sections of the same nerve were stained with toluidine blue.

Mathematical data

Only perfect transverse sections of each nerve were selected and photographed. Myelinated nerve fibre counts and measurements were made on the best 2–3 photographic prints at a magnification of × 1000. Under an oil-immersion objective, each

fibre was simultaneously identified in the corresponding and adjacent transverse section, special attention being paid to the finest. The total diameter of each fibre was measured over the contour of its myelin sheath, using a series of graduated circles printed on rhodoid. These measurements allowed the study of fibre size distribution for each nerve. The mean diameter of all myelinated fibres was also calculated. Results presented here are necessarily expressed in highly summarized form (Tables 1 and 2).

In an earlier work (Mira, 1976*a*), the number and size of myelinated fibre populations were determined in RGM and LGM of 24 normal, unoperated rats. Calculations comprised arithmetical averages (absolute and relative values) and statistical averages (standard deviation, variance, Student–Fischer test and correlation coefficient). All results were comparable for both sides of the same rat and no significant differences were observed in any mean values for the number and size of myelinated nerve fibres; possible sources of errors in measurements were also discussed.

Groups of operated rats

Repeated localized freezings were performed using the same experimental procedure as for a single freezing, but in two different ways:

In a first group, the left sciatic nerve in 12 rats was frozen from one to five times at 23–25 days intervals. Results for each animal were investigated about 1 month after the final freezing.

On the basis of the results for this group, a second experimental series was initiated in which the left sciatic nerve of 7 rats underwent three successive localized freezings, and the animals were killed from 3 to 18 months later.

RESULTS

The number and size of myelinated fibre populations were determined in the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles of the rats operated on the left side only.

Results were nevertheless determined in two different ways. Thus, rats in Group I underwent from 1 to 5 freezings, and measurements were made about 1 month after the final one, whereas measurements on Group II animals were carried out 1, 3, 6, 12 and 18 months after the third and final freezing.

Variations in the number and size of myelinated nerve fibres in Group I rats

Controls were made on both RGM and LGM nerves of 12 rats (4 males and 8 females) killed under general anaesthesia 1 month after the last freezing of their left sciatic nerve. Results are shown in Table 1.

Variations in the number of myelinated nerve fibres

Twenty two and 31 days after a single localized freezing, the relative numbers of regenerating myelinated nerve fibres reached mean values of 101% and 117% respectively, i.e. in the LGM regenerating nerve there were 1% and 17% more myelinated nerve fibres than in the RGM contralateral nerve used as a control.

About 1 month after the last of repeated localized freezings, all regenerating nerves contained a larger number of myelinated fibres than control nerves. An average of 150% fibres were found 28 days after the second freezing, 212% 30 days after the

Table 1. *Mean values of the number and size of myelinated fibres in the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles of rats whose left sciatic nerve underwent 1 to 5 freezings*

* Mean weight at first freezing (FF) and killing (K)

Number of localized freezings	Time since the last freezing	Rats: Number and sex	Rats: Mean weight* (g) FF	Rats: Mean weight* (g) K	Mean number of myelinated nerve fibres: RGM	Mean number of myelinated nerve fibres: LGM	Mean % of regeneration	Mean diameter (μm): RGM	Mean diameter (μm): LGM	Mean % of recovery
1	22 days	2♀	95	170	274	277	101·1	7·9	3·9	49·4
1	31 days	2♀	215	230	289	337	116·6	8·5	4·3	50·6
2	28 days	2♂	110	295	271	418	154·2	8·3	3·8	45·8
3	30 days	2♀	100	260	267	566	212·0	8·1	3·3	40·7
4	28 days	2♀	100	280	276	599	217·0	8·4	3·1	36·9
5	34 days	2♂	110	305	277	605	218·4	9·7	3·5	36·1

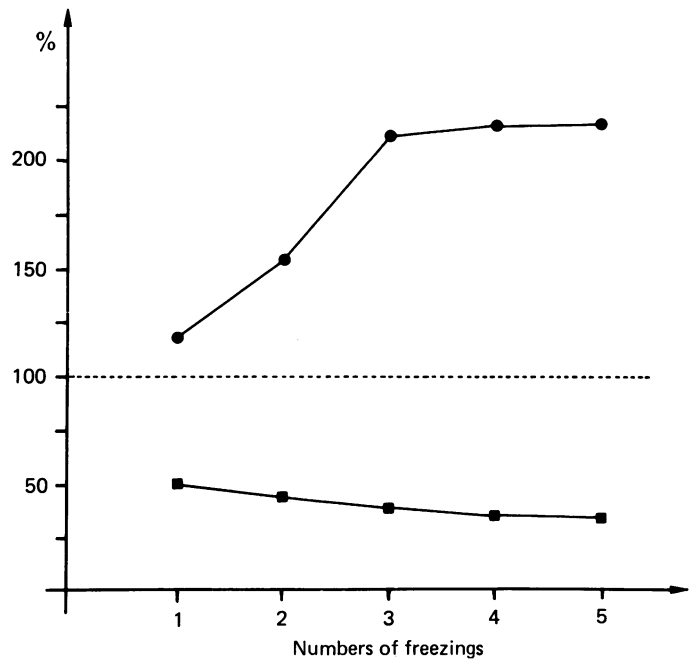


Fig. 1. Mean values of the number (●) and size (■) of the regenerating myelinated nerve fibres, as a function of contralateral uninjured nerve values and the number of freezings.

third one, 217% 28 days after the fourth and 218% 34 days after the fifth one. Concomitantly, results for the different animals in this group showed that the cross sectonal nerve area gradually increased (Figs. 3–8). Graphs for these results are given in Figure 1.

Consequently, the number of myelinated fibres in the nerve to the medial head of the left gastrocnemius muscle gradually increased after the three first freezings of the left sciatic nerve. The fourth and fifth cold injuries did not seem to bring about noticeable changes, the number of regenerating myelinated fibres remaining at about 220% of the control value determined in the contralateral nerve.

Variations in the size of myelinated nerve fibres

Fibre size distribution. In both RGM and LGM nerves of normal, unoperated rats, size distribution of myelinated fibres was always bimodal, and two different fibre populations were constantly detected and characterized by their respective peaks. The peak of the small myelinated fibres was located at 3–4 μm, and of the large ones at 10–12 μm. Since these characteristics only varied slightly, both from one animal to another and within the symmetrical nerves of the same rat, the mean diameter of all myelinated fibres was used to distinguish between the two populations in a given nerve. According to this criterion, the average number of myelinated fibres in each population for most of the rats studied was noticeably similar on both sides (40 % for small and 60 % for large fibres) and, with very few exceptions, histograms for RGM and LGM nerve fibre diameters were superimposable (Figs. 3, 9).

In the uninjured RGM nerve of the operated rats, used as a control in the experiments, size distribution of myelinated fibres was always bimodal and averaged 37·5 % for small and 62·5 % for large fibres.

In all LGM regenerating nerves, myelinated fibres were very small and their size distribution was always unimodal about 1 month after the last freezing of the left sciatic nerve. Only one myelinated nerve fibre population was found in which the diameter of most fibres ranged from 3 to 5 μm. After one freezing (Figs. 4, 10) or two freezings (Figs. 5, 11), diameters ranged from 1 to 8 μm, with a single peak at 3 μm and there were 1 % large fibres. After three (Figs. 6, 12), four (Figs. 7, 13) or five (Figs. 8, 14) freezings, fibre diameters varied from 1 to 7 μm, peak ranges being respectively 2–5, 2–5 and 2–6 μm; there were no large fibres.

Mean diameter. In the RGM nerve of unoperated rats, the mean diameter for all myelinated fibres was 8·1 μm. For large fibres only, it was 10·5 μm, i.e. 129·6 % of the overall value (Mira, 1976*a*).

One month after a single localized freezing, regenerating myelinated nerve fibres had already grown to half their normal size (Table 1) (Mira, 1977*a*).

One month after the last of repeated freezings, a gradual reduction in fibre size was observed, namely to 50 %, 46%, 41 %, 37% and 36 % after one, two, three, four and five cold injuries (Table 1); there were no large fibres. The fifth freezing did not therefore appear to enhance the decrease in fibre size in regenerating nerves. These results are plotted in Figure 1.

Variations in the number and size of myelinated nerve fibres in Group II rats

Controls were also made in both RGM and LGM nerves of 7 male rats between 3 and 18 months after the third and final freezing of their left sciatic nerve. Results are shown in Table 2.

Variations in the number of myelinated nerve fibres

One month after the third freezing, the number of regenerating myelinated nerve fibres reached 212 % of the control value (Table 1).

Three, 6, 12 and 18 months after a third and final freezing, these fibre numbers were respectively 183 %, 182 %, 188 % and 188 %. Concomitantly, cross sectional areas of regenerating nerves gradually increased (Figs. 15–18). These results are plotted in Figure 2.

Between the first and third months after the third freezing, myelinated nerve fibres therefore decreased in number by about 30 %. From then on, no further changes

Table 2. *Mean values of the number and size of myelinated fibres in the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles of rats whose left sciatic nerve underwent 3 localized freezings*

Time since the 3rd freezing	Rats: Number and sex	Rats: Mean weight* FF	Rats: Mean weight* K	Mean number of myelinated nerve fibres: RGM	LGM	Mean % of regeneration	Mean diameter (μm): RGM	LGM	Mean % of recovery
1 month	2♂	100	260	267	566	212·0	8·1	3·3	40·7
3 months	2♂	165	345	263	481	182·9	9·0	4·6	51·7
6 months	2♂	170	390	269	489	181·8	9·1	5·6	61·5
12 months	2♂	185	490	276	519	188·0	9·7	6·5	67·0
18 months	1♂	180	460	296	556	187·8	9·8	6·9	70·4

(* See legend to Table 1.)

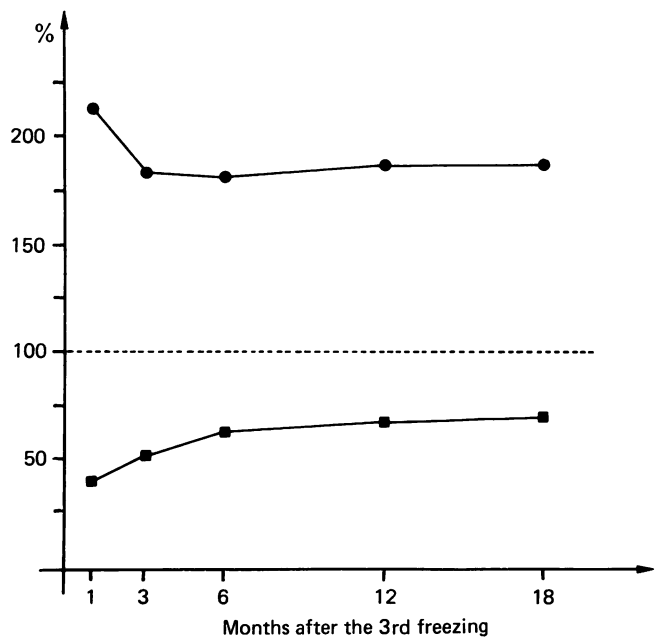


Fig. 2. Mean values of the number (●) and size (■) of the regenerating myelinated nerve fibres, as a function of contralateral nerve values and the time interval since the third and final freezing.

occurred and their number remained at about 190% of the normal contralateral value.

Variations in the size of myelinated nerve fibres

Fibre size distribution. In the uninjured RGM nerve of the operated rats, size distribution of myelinated fibres was always bimodal and averaged 39% for small and 61% for large fibres.

In the regenerating LGM nerve, the size distribution and number of large myelinated fibres varied as the time interval grew following the third localized freezing

of the left sciatic nerve. One month afterwards, fibre size distribution was unimodal and fibre diameters ranged from 1 to 7 μm, with a single very high peak at 3 μm; there were no large fibres (Figs. 6, 12).

From the third month onwards, fibre size distribution became bimodal. After 3 mouths had elapsed, myelinated fibre diameter ranged from 2 to 9 μm, with a first high peak at 3 μm and a second hardly visible peak at 7 μm; about 1 % large fibres were found (Figs. 15, 19). At the sixth month, fibre diameter ranged from 2 to 12 μm, with peaks at 3–4 and 9–10 μm, and there were about 18 % large fibres (Figs. 16, 20). At the twelfth month, fibre diameter was between 2 and 14 μm, with peaks at 3–4 and 8 μm, and 20 % large fibres (Figs. 17, 21). Finally, at the eighteenth month, fibre diameter ranged from 2 to 16 μm, with peaks at 3–5 and 12–13 μm, and about 29 % large fibres (Figs. 18, 22).

However, for this longest regeneration period in the present experiments, peaks did not have the same value on both sides (4 and 14 μm on the right side, and 3–5 and 12–13 μm on the left), although fibre size distribution in the regenerating nerve (2–16 μm) was comparable to that observed in the uninjured contralateral nerve (3–17 μm). In addition, fibre diameter histograms for RGM and LGM were not superimposable.

Mean diameter. Between the first and eighteenth months after the third and final freezing of the sciatic nerve, a regular increase in fibre size was observed in the LGM nerve. It grew from 41 % after 1 month to 52 % after 3, 62 % after 6, 67 % after 12 and 70 % after 18 months (Table 2). These results are plotted in Figure 2.

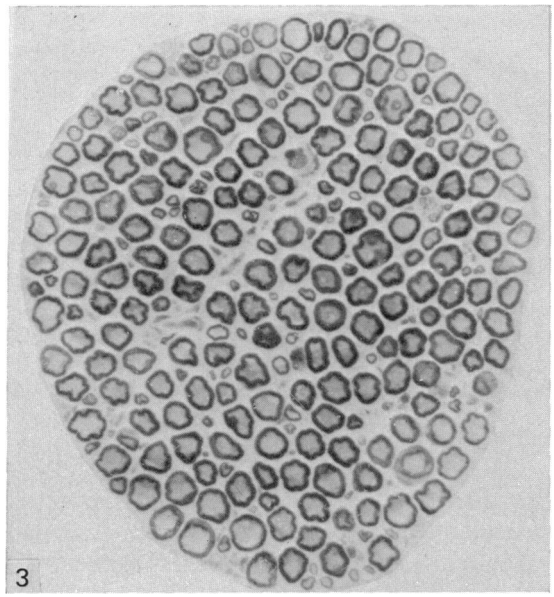
In the final stage of this study the mean diameter of all myelinated nerve fibres remained very much below control value. There were no large fibres after 1 month, but after 3 months the diameter of such fibres equalled the mean diameter of all RGM myelinated nerve fibres (100 %), and reached 106 % at 6 months, 118 % at 12 and 128 % at 18 months. At the latter time, the size of large regenerated myelinated nerve fibres had practically returned to normal (129 %).

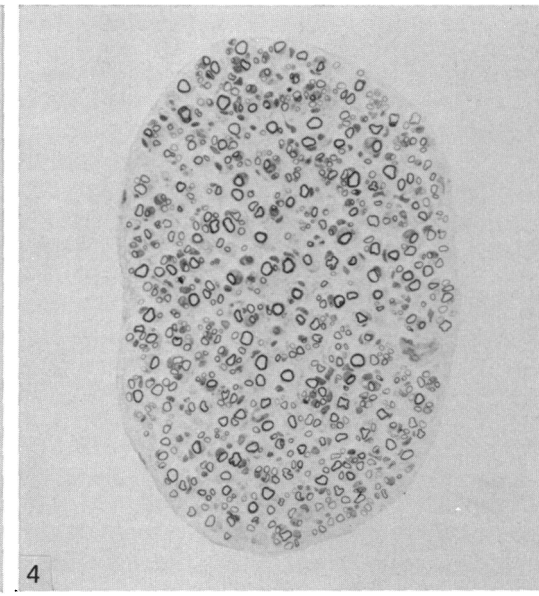
DISCUSSION

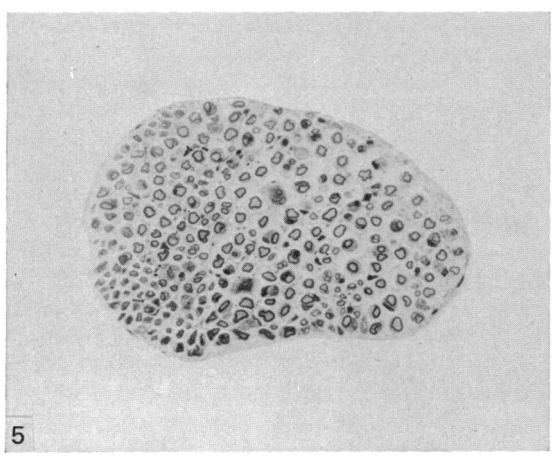
When axons are disrupted by a lesion of the nerve trunk they put out numerous new branches. This applies even when the basal laminae are left intact, as was the case under our experimental conditions. In the present work we only took into account the new branches which acquired a myelin sheath, and not those which remained unmyelinated. Accordingly, we tried to arrive at an accurate quantitative determination of myelinated nerve fibre regeneration in the rat. Our investigation was performed on the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles after repeated localized freezings of the left sciatic nerve.

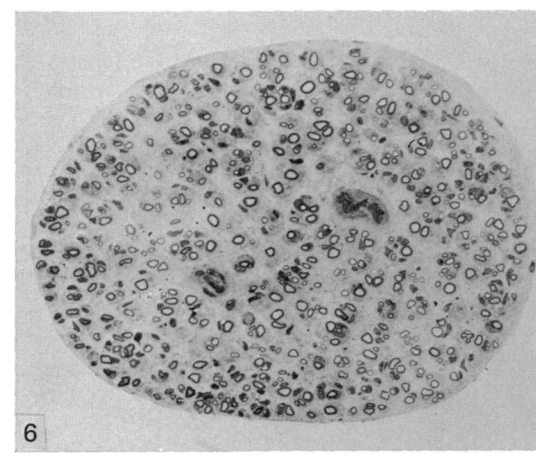
Localized freezing was used to study the variations in the number and size of myelinated nerve fibres after repeated localized nerve injuries. This technique was selected because anatomical and electrophysiological recovery is faster and more complete after freezing than after crush or transection.

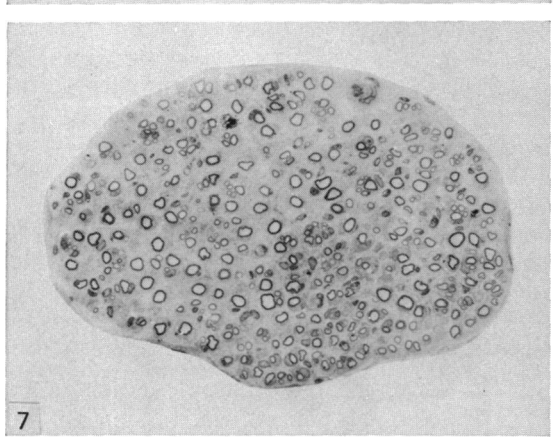
Thus, after a single localized freezing, nerve conduction is immediately blocked if stimulation is applied above the site of lesion, and compound action potentials are recorded below it. Functional recovery is complete about 5–6 months after the cold injury (Mira & Pecot-Dechavassine, 1971). More recently, the regeneration rate of myelinated fibres was measured in the sciatic nerve, using a chamber filled with mineral oil at 37 °C, and containing 70 transversal platinum electrodes 1 mm apart.

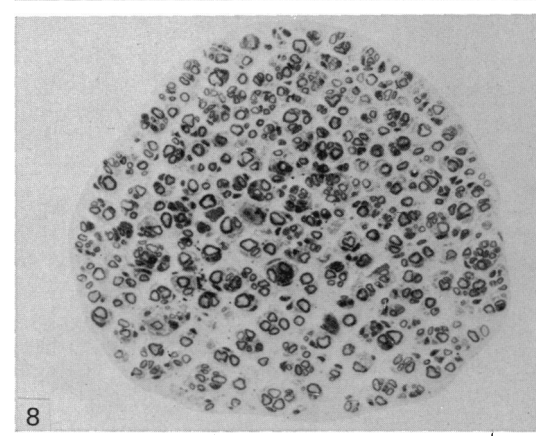












Figs. 3–8. Semi-serial transverse sections of the nerve to the medial head of the left gastrocnemius muscle of rats whose left sciatic nerve was locally frozen 1 to 5 times every 3 weeks. Toluidine blue. × 250.

Fig. 3. Normal, unoperated rat (N25) weighing 420 g. There were 304 myelinated fibres in the right nerve (RGM) and 298 in the left (LGM). Mean diameters were respectively 8·2 and 8·0 μm.

Fig. 4. Female rat (R413) weighing 170 g, subjected to 1 freezing. RGM: 272 myelinated fibres; LGM: 283. Mean diameters were respectively 7·9 and 3·9 μm.

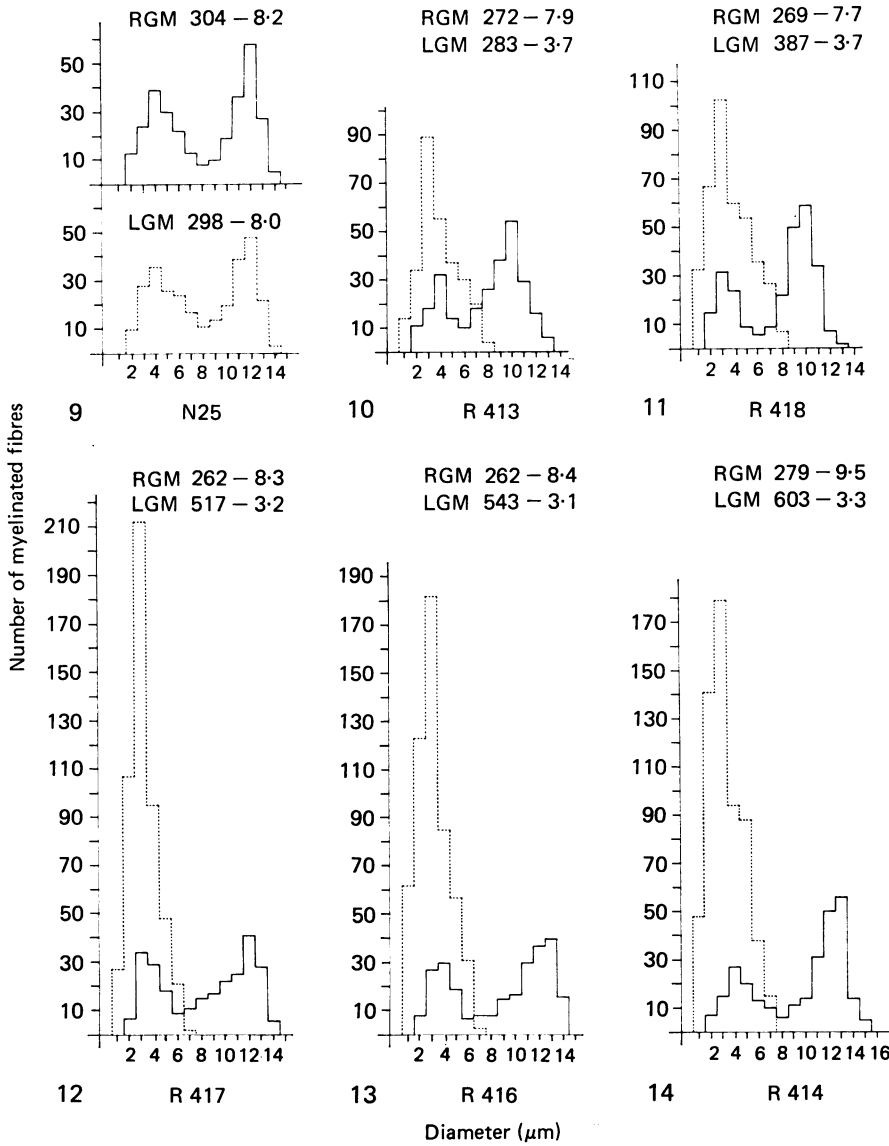


Fig. 5. Male rat (R418) weighing 225 g, subjected to 2 freezings. RGM: 269 myelinated fibres; LGM: 387. Mean diameters were respectively 7·7 and 3·7 μm.

Fig. 6. Female rat (R417) weighing 320 g, subjected to 3 freezings. RGM: 262 myelinated fibres; LGM: 517. Mean diameters were respectively 8·3 and 3·2 μm.

Fig. 7. Female rat (R416) weighing 230 g, subjected to 4 freezings. RGM: 262 myelinated fibres; LGM: 543. Mean diameters were respectively 8·4 and 3·1 μm.

Fig. 8. Male rat (R414) weighing 255 g, subjected to 5 freezings. RGM: 279 myelinated fibres; LGM: 603. Mean diameters were respectively 9·5 and 3·3 μm.

Figs. 9–14. Nerve fibre diameter histograms of myelinated fibres of the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles of rats whose left sciatic nerve was locally frozen 1 to 5 times every 3 weeks. Full lines: histogram of the RGM contralateral nerve used as a control; dotted lines: histogram of the LGM experimental nerve.

Fig. 9. Male rat (N25), unoperated.

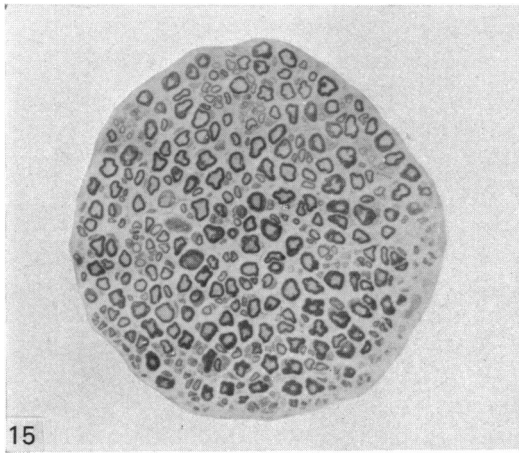
Fig. 10. Female rat (R413), 1 freezing.

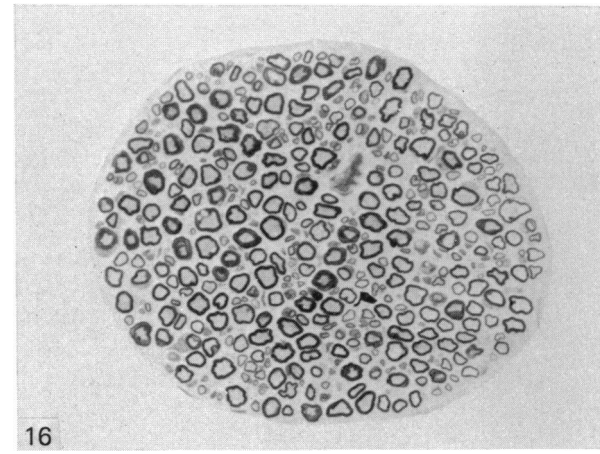
Fig. 11. Male rat (R418), 2 freezings.

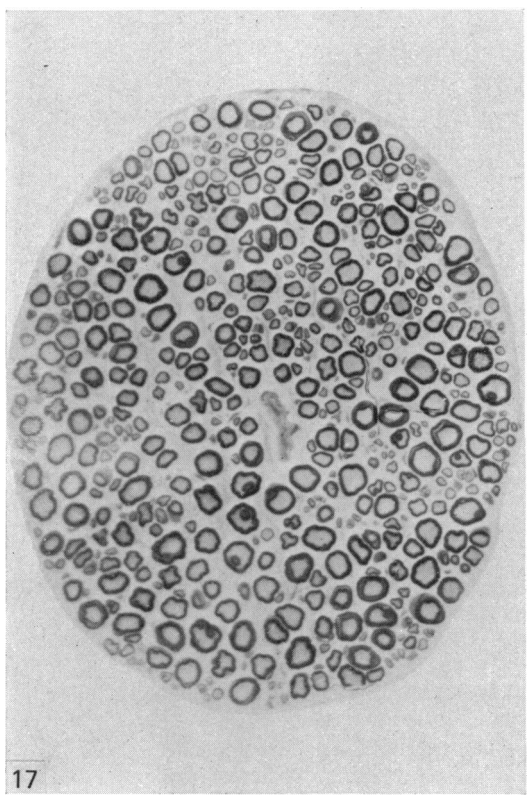
Fig. 12. Female rat (R417), 3 freezings.

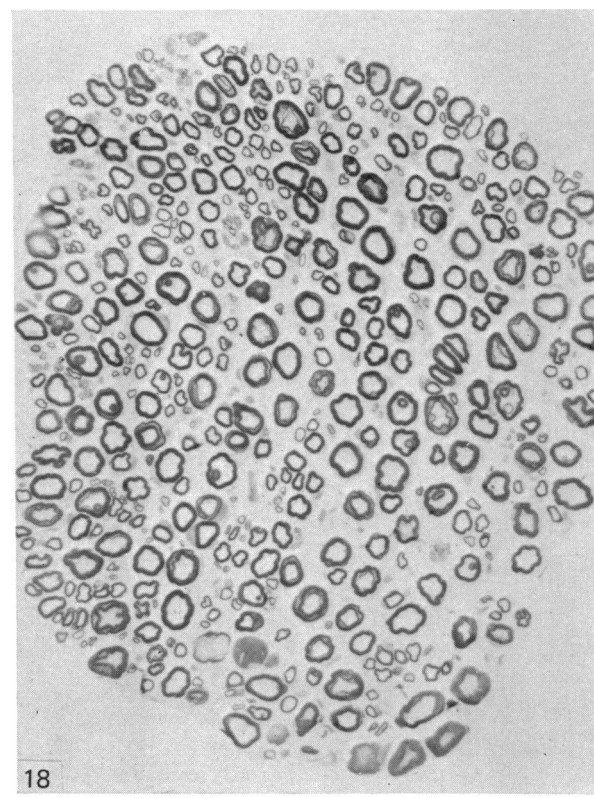
Fig. 13. Female rat (R416), 4 freezings.

Fig. 14. Male rat (R414), 5 freezings.









Figs. 15–18. Semi-serial transverse sections of the rat nerve to the medial head of the left gastrocnemius muscle, removed from 3 to 18 months after the third and final freezing of the left sciatic nerve. Toluidine blue. ×275.

Fig. 15. Male rat (R437) weighing 280 g and killed after 3 months. There were 248 myelinated fibres in the right nerve (RGM) and 405 in the left (LGM). Mean diameters were respectively 9·3 and 4·6 μm.

Fig. 16. Male rat (R434) weighing 395 g and killed after 6 months. RGM: 267 myelinated fibres; LGM: 536. Mean diameters were respectively 9·0 and 5·4 μm.

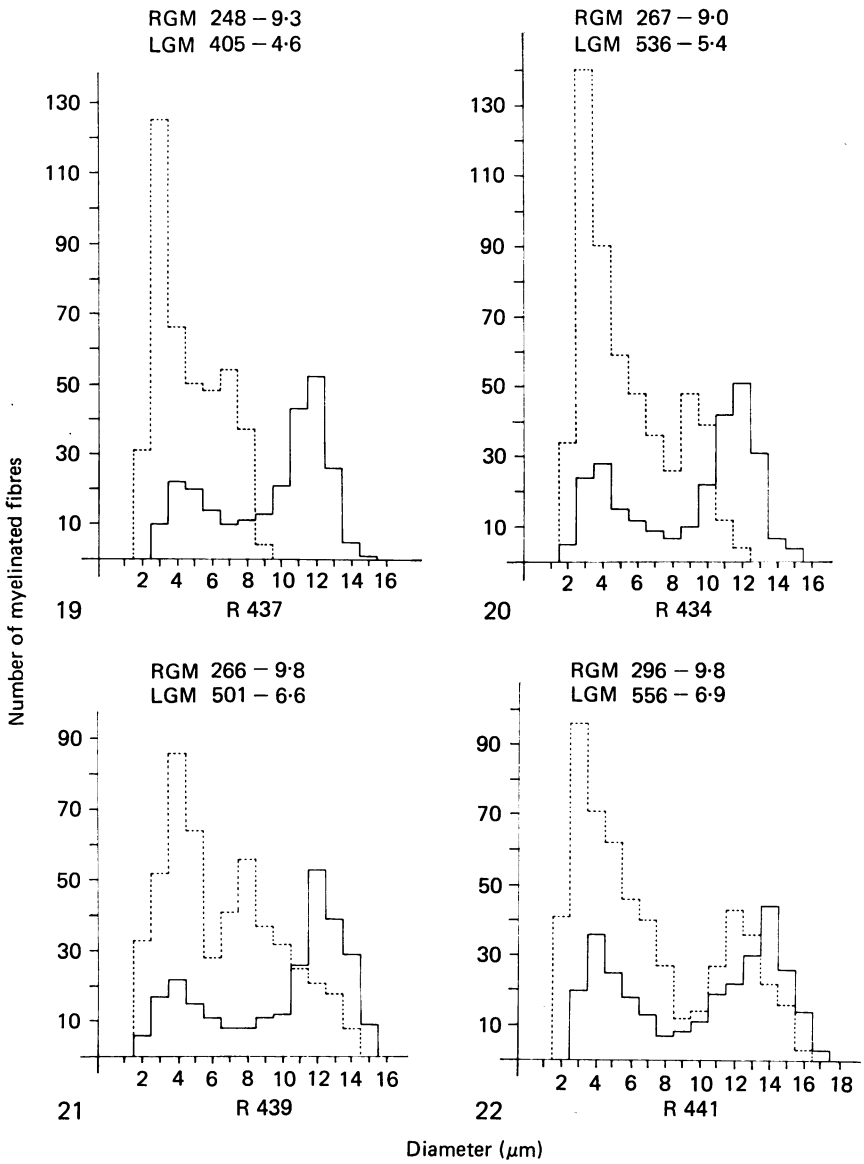


Fig. 17. Male rat (R439) weighing 480 g and killed after 12 months. RGM: 266 myelinated fibres; LGM: 501. Mean diameters were respectively 9·8 and 6·6 μm.

Fig. 18. Male rat (R441) weighing 460 g and killed after 18 months. RGM: 296 myelinated fibres; LGM: 556. Mean diameters were respectively 9·8 and 6·9 μm.

Figs. 19–22. Nerve fibre diameter histograms of rat myelinated fibres in the nerve to the medial head of the right (RGM) and left (LGM) gastrocnemius muscles, removed from 3 to 18 months after the third and final freezing of the left sciatic nerve. Full lines: histogram of the RGM contralateral nerve used as a control; dotted lines: histogram of the LGM experimental nerve.

Fig. 19. Male rat (R437) killed after 3 months.

Fig. 20. Male rat (R434) killed after 6 months.

Fig. 21. Male rat (R439) killed after 12 months.

Fig. 22. Male rat (R441) killed after 18 months.

Electrophysiological data from 12 electrodes surrounding the supposed position of the regenerating cone were automatically collected and stored in a computer in order to reduce the duration of recordings to 90 seconds. Between the third and tenth days after a single localized freezing the rate of regeneration was very nearly 4·4 mm/day (Jahan & Mira, unpublished observations). These results were confirmed by Legrain (1977) who studied electrophysiological recovery of rat sciatic nerve after section-suture, crush and cold injury.

Moreover, electron microscopy of the directly frozen area revealed ultrastructural changes in all myelinated and unmyelinated nerve fibres immediately after freezing and thawing. These changes indicate necrosis of the axons and disorganization of the myelin sheaths, with minimal damage to other nerve components. Thus, the continuity of connective tissue sheaths, of blood vessels supplying the nerve trunk and of the basal lamina surrounding each nerve fibre was not disrupted (Mira, 1971, 1972*b*).

In addition, the freezing procedure performed *in situ* in our experiments was perfectly controllable and reproducible, and all its results are much more consistent than those for crush and transection (Mira, 1976*b*–1977*a*; Legrain, 1977). Since a preliminary study showed that regeneration after a single localized freezing took place within 3 weeks in the middle part of the gastrocnemius muscle, the individual freezings in each of the present series were separated by approximately 3 weekly intervals ensuring that muscle re-innervation had just begun at the time of the next freezing.

When measurements were completed about 1 month after the last of repeated freezings (Group I), a regular increase was observed in the number of LGM regenerating myelinated nerve fibres, from 20% after the first freezing to 120% after the third, fourth or fifth ones. On the other hand, the size of these fibres diminished from 51% to 36% of the control value. This mean increase was far greater than after all the various types of single nerve injury performed in previous experiments, for which it was only 24% after a single freezing, 15% after crush, 30% after section without suture and 50% after section and immediate suture. In the present work, a gradual increase was observed in the cross section area of the LGM regenerating nerve, indicating a growth in the latter's internal volume.

When measurements were made 1–18 months after a third and final freezing (Group II), fibre numbers reached a mean value of 210% after 1 month, i.e. the increase recorded was 110% in relation to the contralateral value. A drop of about 30% occurred between the first and third months, followed by stabilization at about 190% of control value. Concomitantly, fibre size gradually increased, but did not exceed 70% of the control value at the eighteenth month. Here too, a progressive increase was observed in the cross section area of the LGM regenerating nerve.

A similar growth in the cross section area of regenerating nerves has been reported previously by others and variously attributed to an increase in the quantity of nerve fibres, growth of the cell population in the nerve trunk and expansion of endoneurial spaces. Cell proliferation occurs mainly in the Schwann cells, but a contribution from mononuclear blood cells is not impossible (Gutmann, 1942; Abercrombie & Santler, 1957). Thomas (1968, 1969, 1970) confirms a gradual increase in the number of nuclei ($\times$ 8) and in nuclear density ($\times$ 4) following the ninth crush of rabbit peroneal nerve. There was an approximately linear relationship between the changes in the nuclear counts and the number of crushes. Proliferation was mainly observed in Schwann cells. Moreover, following repeated experimental Wallerian degeneration

with intervening regeneration, nerve transverse sections showed extensive nerve fibre systems composed of groups of several myelinated and unmyelinated axons with associated Schwann cells and collagen fibrils. Each of these clusters was the product of a single Büngner band which gradually grew larger and was subdivided by the repeated degeneration and regeneration of multiple axonal sprouts. These clusters resemble those sometimes observed in chronic neuropathy associated with repeated axonal degeneration and regeneration. Similar 'hyperneurotization' of Büngner band was also noted by Schröder (1968) in sciatic nerves of rats with experimental izoniazid neuropathy. A large increase in the endoneurial collagen content was also observed after repeated tourniquet injuries which strongly impeded axon and myelin regeneration (Dyck, 1969; Pleasure & Towfighi, 1972). By contrast, Duncan & Jarvis (1941, 1943), who studied regeneration of the motor branches of the cat facial nerve, reported that recovery was apparently achieved about one month after each of five successive sections, eight successive crushes and nine successive chemical treatments (e.g. destruction of nerve fibres with 10 % benzyl alcohol in sweet almond oil). Moreover, 3 months after the last nerve injury the number of fibres peripheral to the site of lesion was greatly reduced in the sectioned nerves, slightly reduced in the crushed nerves, and definitely increased in chemically treated nerves. Duncan & Jarvis concluded that, by the methods they employed, small motor branches were capable of repeated regenerations without reducing the vigour of the reaction and without demonstrable effects on parent cell bodies.

Holmes & Young (1942) considered that the power of a central stump to send out new fibres was not reduced if it was injured a second time, either within a week of the first lesion or after an interval as long as a year. Gutmann (1948) found that repeated injury of the same nerve did not hinder axonal regenerative capacity; repeated crushes ($\times$ 6–8) of a nerve led to functional recovery, the rate of regeneration being almost normal even after the eighth crush. Gutmann concluded that the nerve cell was able to regenerate and reinnervate the muscle repeatedly and successfully. Gutmann & Holubar (1951) observed that secondary crushing of the central stump of a sectioned nerve maintained the nerve in better condition, because conduction velocity and height of the action potential were greater than on the control side. Accordingly, the number of nerve fibres and the thickness of their myelin sheaths were greater in the central stump where the crush had been performed.

In our experiments, the increase in the number of regenerating myelinated nerve fibres was extensive and relatively stable. However, even when the number of freezings increased, the quantity of nerve fibres did not grow indefinitely, and their size diminished concomitantly. These observations may be explained by the simultaneous action of central, local and/or peripheral factors, even if, at the present time, it is impossible to determine the precise part played by each of them. It is well known that all regenerating fibres result from the sprouting of the normal proximal part of the initial fibres. It is quite possible that the sprouting power of myelinated fibres may be affected by repeated nerve injuries. This power can be limited by the relatively confined space in which axonal sprouts grow, by the degeneration of some sprouts, and by the relative depletion in motoneurons of the metabolic components necessary for regeneration. The axon is dependent on the trophic influence of the nerve cell body, the source of which is located in the perikaryon. In fact, the neuronal cell body is absolutely necessary to the maintenance of the axon, and its own integrity may be affected by repeated removal of a considerable part of its distal processes. Gutmann & Holubar (1951) suggested that 'prophylactic' crushing of the central

stump of an interrupted nerve stimulates the nerve cells to greater metabolic activity during nerve fibre regeneration within the peripheral stump. Ducker *et al.* (1969) indicated that axonal growth proceeds faster after the second lesion than after the first, provided there is a 2–3 weeks interval between the lesions; however, in the spinal cord, the neuron cannot respond a second time with maximal metabolic effort. McQuarrie & Grafstein (1973) reported that the growth-enhancement of the priming lesion was due to changes in the perikaryon and its environment, but their contribution to the enhancement of axonal outgrowth remains to be evaluated. Moreover, in the double lesion, the onset of outgrowth did not significantly change. Consequently, while the rate of regeneration may be centrally controlled, its initiation may depend on local changes at the site of the lesion (alteration of plasma membranes. mobilization of lysosomes, etc). Droz & Leblond (1962, 1963) were the first to indicate that protein synthesis in neurons appeared to be confined to the perikaryon. Accordingly, there is a marked increase in proteosynthesis in neuronal cell bodies of injured nerve fibres to ensure their survival and provide the metabolic environment necessary for them to regenerate and sprout one or more new distal axons. Though the exact upper limit of the perikaryal metabolism is still not known, the number of distal axonal sprouts which regenerate after repeated nerve injuries may exceed the metabolic capacity of motoneurons. Thus, a certain number of axonal sprouts may degenerate secondarily. Moreover, some regenerating nerve fibres may fail to make an effective connexion with their end organs, and this fact should also lead to a reduction of the axonal branches. On the other hand, the degree of nerve fibre myelination peripheral to the site of lesion is dependent on the fibres' axonal size. After repeated localized freezings, it is much below the normal value and the thickness of the myelin sheath formed around small axonal sprouts from Schwann cells will consequently be less than normal. These considerations may explain the 'plateau' observed after the third freezing (Group I) and the 30% drop in the number of fibres recorded between the first and third months after the third freezing (Group II). Finally, from the site of lesion to the periphery, the internal volume of the nerve trunk may increase, but its dilation will still be limited by its environment and collagenous sheaths. Accordingly, a very large increase may appear in the number of myelinated fibres in regenerating nerves, but an accompanying reduction in their size will be observed concomitantly.

One month after the last localized freezing (Group I) only small myelinated fibres were found in the LGM regenerating nerve. This is not surprising if one remembers that the regenerating fibres had just begun their myelination before again being interrupted by the next cold injury. In the case of a single freezing, axonal sprout myelination in fact begins on the eighth to ninth day afterwards in the directly frozen segment of the sciatic nerve, before it branches out into the tibial, peroneal and sural nerves (Mira, 1972*b*). In the proximal part of the nerve to the medial head of the gastrocnemius muscle, at the point where the present measurements were made (30–40 mm below the site of lesion), the first regenerating myelinated fibres were observed between the twelfth and fifteenth days. In addition, the first regenerating nerve endings were detected in the middle part of the muscle itself from the sixteenth to eighteenth days, using silver impregnation. Consequently, one month after the last of repeated freezings of the sciatic nerve all myelinated fibres in the LGM nerve were still far from having accomplished their myelination and were thus small in diameter.

When measurements were made between 1 and 18 months after a third and final

freezing (Group II) the data were comparable, although regeneration time was not confined to 1 month but was sometimes as much as 18 months. Myelination and maturation of regenerated fibres were progressive and an increase in the number of fibres paralleled an increase in the cross sectional area of the nerve. Nevertheless, even at the eighteenth month, the number of large myelinated nerve fibres was far below the normal contralateral value, and only 29 % were found in the LGM nerve compared to 61 % in the RGM control nerve; the mean diameter of all LGM myelinated fibres was about 70 % compared to that of the RGM nerve; by contrast, the mean diameter of small and large myelinated fibres had nearly returned to normal (48 % instead of 49 % and 128 % instead of 129 % respectively).

Studies in detail of the muscular changes induced in the gastrocnemius and soleus muscles by the different nerve injuries performed in our experiments will be published soon after this report appears. A preliminary communication has been published already (Mira & Fardeau, 1978). Moreover, in addition to the present quantitative study of myelinated nerve fibre regeneration, a parallel investigation of variations in axonal size, myelin sheath thickness, and unmyelinated nerve fibre population has been commenced.

After various experimental injuries to the rat sciatic nerve, our conclusions regarding changes in the number and size of myelinated fibres in the nerve to the medial head of the gastrocnemius muscle may be summarized as follows:

After a single localized freezing or crushing the results are very different from those observed after section or resection, and much more constant. However, anatomical recovery is more complete after freezing than after crush. Thus, using the contralateral nerve as a control, the number of regenerating myelinated fibres averaged 115 % after crush, 124 % after freezing, 130 % after section without suture, 150 % after section and immediate suture and only 20 % after resection of a 10 mm nervous segment. Mean fibre diameter gradually returned to normal after freezing, but remained much below it after crush (80 %), section (50–60 %) and resection (40 %). In regenerating nerves, size distribution of myelinated fibres was mostly unimodal after section, but became bimodal after crush and freezing. Nerve fibre diameter histograms of regenerating and control nerves were superimposable after freezing, and to a smaller extent after crush, but never after section. In all cases the population of small myelinated fibres was larger than the normal contralateral value (+5% after freezing, +15 % after crush and +50–60 % after section; after resection, all fibres were small).

After repeated localized freezings the results may be separated into two different series. When measurements were made 1 month after the last of two to five freezings, the number of regenerating myelinated fibres increased considerably after the three first operations, reaching 220 % of the normal value, but did not vary much after four or five freezings. Mean fibre diameter diminished after each freezing and dropped to only 36 % of the normal value after the fifth operation; all the myelinated fibres were small and their size distribution remained unimodal in all the nerves studied. When measurements were made betwen 1 and 18 months after a third and final freezing, 210 % myelinated fibres were found in the regenerating nerves at the end of the first month; afterwards, only 190 % were found. Mean fibre diameter increased with the regenerative period, but did not exceed 70 % of the normal value. Fibre size distribution became bimodal from the third month, but, even at the eighteenth month, small myelinated nerve fibres were very numerous (71 % instead of 39 %).

These observations show that repetition of an experimental nerve injury causes

a large and lasting increase in the number of myelinated fibres, but an accompanying reduction in their size. Moreover, localized freezing seems the only experimental nerve injury allowing full anatomical and physiological recovery, because this technique affords particularly favourable conditions for nerve fibre regeneration, and recovery of former fibre terminations at the periphery.

SUMMARY

The number and size of myelinated nerve fibres were determined in the nerve to the medial head of the gastrocnemius muscles of rats whose left sciatic nerve was repeatedly frozen (one to five times at three weekly intervals). The contralateral nerve was used as a control. Results varied according to the number of freezings performed and, for a given number of freezings, according to the period of regeneration.

When measurements were completed 1 month after the last of several localized freezings, the number of regenerating myelinated nerve fibres increased regularly up to the third freezing, reaching to about 220% of the control value, but no higher values were recorded after four or five freezings. The nerve fibre distribution was unimodal in all the nerves studied. The mean diameter of all myelinated fibres decreased with the number of freezings from 50% of the control value after the first to 36% after the fifth.

When measurements were made 1, 3, 6, 12 and 18 months after the third and final freezing, the number of regenerating myelinated nerve fibres decreased by about 30% between the first and third month and then stabilized at 190% of the control value. Nerve fibre distribution became bimodal from the third month onwards, and the mean diameter of all myelinated fibres increased regularly. However, by the eighteenth month, the size of regenerated myelinated nerve fibres had only reached 70% of the normal contralateral value.

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REFERENCES

ABERCROMBIE, M. & SANTLER, J. (1957). An analysis of growth in nuclear population during Wallerian degeneration. *Journal of Cellular and Comparative Physiology* **50**, 429–450.

CAJAL, S. R. y. (1928). *Degeneration and Regeneration of the Nervous System* (translated by R. M. May). London: Oxford University Press.

DROZ, B. & LEBLOND, C. P. (1962). Migration of proteins along the axons of the sciatic nerve. *Science* **137**, 1047–1048.

DROZ, B. & LEBLOND, C. P. (1963). Axonal migration of proteins in the central nervous system and peripheral nerves as shown by autoradiography. *Journal of Comparative Neurology* **121**, 325–346.

DUCKER, T. B., KEMPE, L. G. & HAYES, G. J. (1969). The metabolic background for peripheral nerve surgery. *Journal of Neurosurgery* **30**, 270–280.

DUNCAN, D. & JARVIS, W. H. (1941). A study of repeated regeneration of facial nerve branches in cats. *Anatomical Record* **79**, Suppl. 2, 20.

DUNCAN, D. & JARVIS, W. H. (1943). Observations on repeated regeneration of the facial nerve in cats. *Journal of Comparative Neurology* **79**, 315–325.

DYCK, P. J. (1969). Experimental hypertrophic neuropathy. Pathogenesis of onion-bulb formations produced by repeated tourniquet applications. *Archives of Neurology* **21**, 73–95.

FALIN, L. I. (1961). Regeneration of nerve fibres after repeated injuries of the nerve trunk (in Russian). *Voprosy Nejrokhirurgii* **25**, 5–12.

GUTMANN, E. (1942). Factors affecting recovery of motor function after nerve lesions. *Journal of Neurology, Neurosurgery and Psychiatry* **5**, 81–95.

GUTMANN, E. (1948). Effect of delay of innervation on recovery of muscle after nerve lesions. *Journal of Neurophysiology* **11**, 279–294.

GUTMANN, E. & HOLUBAR, J. (1951). Atrophy of fibres in the central stump following nerve section and the possibilities of its prevention. *Archivio internazionale di studi neurologici* **1**, 314–324.

HOLMES, W. & YOUNG, J. Z. (1942). Nerve regeneration after immediate and delayed sutures. *Journal of Anatomy* **77**, 63–97.

LEGRAIN, Y. (1977). Méthode de comparaison de la vitesse de régénération des fibres du nerf sciatique de rat. *Journal de Physiologie* **73**, 13–22.

MCQUARRIE, I. G. & GRAFSTEIN, B. (1973). Axon outgrowth enhanced by a previous nerve injury. *Archives of Neurology* **29**, 53–55.

MAYER, S. (1881). Über Vorgänge der Degeneration und Regeneration in unversehrten peripherischen Nervensystem. Eine biologische Studie. *Zeitschrift für Heilkunde* **2**, 154–258.

MIRA, J. C. (1971). Maintien de la continuité de la lame basale des fibres nerveuses myélinisées après une congélation localisée. *Compte rendu de l'Académie des sciences, série D* **273**, 1836–1839.

MIRA, J. C. (1972*a*). Variations du nombre et du calibre des fibres nerveuses myélinisées après une 'section' des axones par congélation localisée. *Compt rendu de l'Académie des sciences, série D* **275**, 979–982.

MIRA, J. C. (1972*b*). Effets d'une congélation localisée sur la structure des fibres nerveuses myélinisées et leur regénération. *Journal de Microscopie* **14**, 155–168.

MIRA, J. C. (1976*a*). Etudes quantitatives sur la régénération des fibres nerveuses myélinisées. I. Variations du nombre et du calibre des fibres myélinisées dans les nerfs de rats normaux. *Archives d'anatomie microscopique et de morphologie expérimentale* **65**, 209–229.

MIRA, J. C. (1976*b*). Etudes quantitatives sue la régénération des fibres nerveuses myélinisées. II. Variations du nombre et du calibre des fibres régénérées après un écrasement ou une section du nerf. *Archives d'anatomie microscopique et de morphologie expérimentale* **65**, 255–284.

MIRA, J. C. (1977*a*). Etudes quantitatives sur la régénération des fibres nerveuses myélinisées. III. Variations du nombre et du calibre des fibres régénérées après une congélation localisée. *Archives d'anatomie microscopique et de morphologie expérimentale* **66**, 1–16.

MIRA, J. C. (1977*b*). Variations du nombre et du calibre des fibres nerveuses myélinisées régenérées après des congélations itératives du nerf sciatique de rat. *Compte rendu de l'Académie des sciences, série D* **284**, 2357–2360.

MIRA, J. C. & FARDEAU, M. (1978). Modifications dans la distribution des types de fibres musculaires provoquées par des congélations localisées itératives du nerf sciatique de rat. *Compte rendu de l'Académie des sciences, série D* **286**, 1367–1370.

MIRA, J. C. & PECOT-DECHAVASSINE, M. (1971). Effet d'une congélation localisée d'un nerf périphérique sur la conduction de l'influx nerveux, au cours de la dégénérescence et de la régénération. *Pflügers Archiv* **330**, 5–14.

PLEASURE, D. E. & TOWFIGHI, J. (1972). Onion-bulb neuropathies. *Archives of Neurology* **26**, 289–301.

SABAINO, D. & SOLERIO, L. (1952). Sul comportamento numerico delle fibre rigenerate in tronchi nervosi sottoposti a ripetute neurotomie. Richerche sperimentali. *Archivio di scienze medicinale* **93**, 463–474.

SCHRÖDER, J. M. (1968). Die Hyperneurotisation Büngnerscher Bänder bei der experimentelle Isoniazid-Neuropathie: Phasenkontrast- und elektronenmikroskopische Untersuchungen. *Virchows Archiv, Abt. B, Zellpathologie* **1**, 131–156.

THOMAS, P. K. (1968). The effects of repeated regenerative activity on the structure of peripheral nerve. In *Research in Muscular Dystrophy*, pp. 413–419. London: Pitman.

THOMAS, P. K. (1969). The influence of repeated crush injuries on the nuclear population of peripheral nerve. *Journal of Physiology* **201**, 69*P*.

THOMAS, P. K. (1970). The cellular response to nerve injury. 3. The effect of repeated crush injuries. *Journal of Anatomy* **106**, 463–470.

VANLAIR, C. (1888). Sur la persistance de l'aptitude régénérative des nerfs. *Bulletin de l'Académie royale des Sciences belges* **16**, 93–110.