A New Approach Toward Analyzing Peripheral Nerve Fiber Populations. II. Foreshortening of Regenerated Internodes Corresponds to Reduced Sheath Thickness

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Abstract. The new approach used in this study is based on the concept that axon caliber is not the only factor affecting the thickness of the myelin sheath. It is necessary to consider the entire geometric proportions of the internode, since sheath thickness corresponds to the relationship between axon caliber and the length of the internode. This type of analysis was applied to the regenerated internodes in rat sciatic nerves. Survival periods of 4, 9, 18 and 36 weeks were studied after lesions had been placed in young adult rats. The data show significantly thinner sheaths for regenerated fibers as compared with normal nerves, consistent with previous observations. This reduction in sheath thickness, however, corresponded quantitatively to the degree of foreshortening of internodes in the regenerated nerves. An average reduction of 10 in the quotient internode length/fiber caliber corresponded to a reduction of about 0.015 in the relative thickness of the sheath (quotient axon diameter/fiber diameter). This means that regenerated myelin sheaths are not truly hypoplastic; rather, they are adapted to the reduced internode length, and have the same relationship found for normal fibers. In partially damaged nerves there was a clear distinction in terms of sheath thickness between regenerated fibers and undamaged fibers. Demonstration of this phenomenon by scatter diagrams opens new possibilities for the quantitative assessment of neuropathies.

Key Words: Internode length; Myelin thickness; Nerve regeneration; Neuropathy; Peripheral nerves.

INTRODUCTION

The preceding paper in this journal (1) describes minor variations in the sheath thickness of normal sciatic fibers, using a semi-automatic method which permits the assessment of large numbers of fibers. These data were compared with measurements in teased fibers. It was found that the thickness of the myelin sheath does not merely increase with the caliber of the axon; the relative length of the internode is also important. Short internodes of a given caliber have slightly thinner sheaths than long internodes, as shown previously in electron micrographs of isolated nerve fibers (2).

The present paper extends this study to regenerated sciatic nerve fiber populations. If a nerve regenerates following transection, its original long internodes are replaced by a new population of greatly shortened internodes. The sheaths of these regenerated fibers are also thinner than those of normal fibers of equal caliber. These two phenomena are well known and are amply documented in the literature, yet, they were never considered interrelated. The data obtained in this study show that the thickness of the myelin sheaths of regenerated fibers follows the same pattern of organization

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found in normal fiber populations. The foreshortened internodes possess slightly thinner sheaths than internodes of normal length, and the degree of reduction in sheath thickness corresponds to the degree of internode foreshortening just as in normal nerves.

MATERIALS AND METHODS

The left sciatic nerve was exposed under deep anesthesia with Rompun-Ketanest and was mobilized carefully to minimize traction or mechanical injury. A small suture was attached to the perineurium to mark the site of the lesion. A Cryoprobe (Leonhard Klein, D-6900 Heidelberg) was brought in touch with the nerve which at this point was completely free from the adjoining muscle tissue. The nerve was frozen for two minutes (min) at −10 to −12°C just distal to the suture. Our experience with this method agrees with Mira (3), who obtained excellent regeneration for up to five successive freezings. Compared to crush lesions, optimal alignment is preserved in freezing lesions; there was also no penetration of axon sprouts beyond the perineurium as one may see following mechanical injury. The rats used were commercially obtained young male adults of 240 to 260 g weight. Survival periods after freezing were 4, 9, 18 and 36 weeks (wk). Data from the one-year-old rats of the preceding study served as controls. The 36 wk regenerates were approximately one and a half years old. In addition, scatter diagrams were obtained from three-month-old male rats weighing 180 to 190 g for comparison with the 12-month-old rats. The methods of tissue preparation and morphometry were described in the preceding paper.

RESULTS

Sheath Thickness in Regenerated Fibers

Scatter diagrams of the g-ratio (quotient axon diameter/fiber diameter) for 4, 9, 18 and 36 wk regeneration (Fig. 1) showed a slow increase in axon calibers. The curves show 1,474, 1,952, 1,442 and 922 fibers, respectively. At four wk survival there were few fibers having axons in excess of 6 μm. By nine and 18 wk the thickest axons measured about 9 μm and by 36 wk they had reached diameters of 12 μm, comparable to the thickest fibers found in the normal adult rat sciatic nerve. Throughout this period, the mean values for the g-ratio were higher than in normal nerves: 0.77 at four wk; 0.74 at nine wk; 0.76 at 18 wk; 0.77 at 36 wk (in comparison to 0.69 for normal nerves (Fig. 4)). These means are of limited significance, however, since they represent a regression of the g-ratio with fiber caliber in which the mean depends greatly on the number of fibers present in each caliber class. When scatter diagrams for normal and for regenerating fiber populations were superimposed, there were two separate regressions with very little overlap, particularly for the axons thicker than 5 μm (Fig. 7). For the latter, regenerated fibers were readily distinguished from normal fibers by the difference in the g-ratio.

The axons below 6 μm formed a cluster which slightly overlapped with the cornucopia-shaped scatter diagrams of normal nerves, yet the mean was about 0.1 higher for the regenerated fibers. The scatter diagrams showed that many of the fibers which had grown thicker at later stages of regeneration were part of this population of thin fibers during early growth. At the early phases of regeneration, these thin fibers and the thicker fibers seemed to fall into one single regression. At 36 wk, however, there was separation of a group of axons of about 3 μm with a ratio of above 0.8, similar to the thin, thinly myelinated fibers found in normal nerves.

A feature not found in normal nerves was extremely thin fibers with axons below 2 μm and extremely thick sheaths with g-ratios between 0.65 and 0.2, forming a "tail" at the left end of the scatter diagrams. These "low g" fibers were numerous at nine wk, but at 36 wk only a few persisted. Such fibers may undergo atrophy and involution after failing to achieve contact with their target organ.

Fig. 1. Scatter diagrams of sheath thickness of regenerating nerves show the regrowth of fiber calibers by the extension of the curves toward the right. The thickness of the myelin sheaths is shown by the g-ratio, i.e. the quotient axon diameter/fiber diameter. Sheaths are thinner at four wk than at 36 wk (higher g-ratio), but all regenerated nerves have distinctly thinner sheaths than normal nerves (compare Figs. 4 and 7). The "tail" of the left side of the curves shows the presence of abnormal, very thin fibers having excessively thick sheaths. "Axon diameter, circular" refers to the diameter of a circle of equivalent circumference.

The scatter diagrams of the g-ratio find their counterparts in the curves obtained when plotting sheath thickness against axon diameter, area of myelin against area of the axon profile, or the area of myelin against fiber diameter (Fig. 2). All three types of curves showed distinctly lower values for sheath thickness than normal sciatic nerves. Similar to the curves for the g-ratio (Fig. 1), there was an overlap between normal fibers and regenerates below 5 µm, the difference becoming progressively greater with increasing fiber calibers.

In the results for non-circularity (Fig. 3), there was a variable trend toward more shrunken, thin fibers, but this was seen only in the later phases of regeneration. There was no indication that regenerated fibers were more apt to shrink than normal ones.

Data on Young Rats

Because the preceding scatter diagrams involve regrowth of fibers in young adult rats, it was desirable to compare these findings with scatter diagrams showing normal fiber growth in three-month-old immature rats. The largest fibers of these animals had barely reached an axon diameter of 9 µm, the diameter being similar to that of the nine to 18 wk regeneration, in comparison to the 12 µm found in the adults. The mean g-ratio was 0.69 (0.77 for regenerates) and the shape of the curve for the g-ratio resembled that of adult rats, only more compressed, and with a similar cornucopia pattern (Fig. 4). If the curves for the three-month-old rats were superimposed with those of four or nine wk regenerates of adults, they showed the same range of fiber calibers with some overlap in g-ratios. However, the curves of the regenerated nerves had generally higher g-values than those of immature normal rats. This was also true when comparing sheath thickness or myelin area with the

dimensions of the axis cylinder. The curves for immature rats had the same slope as those for adults, and both differed from regenerating nerves. There were no differences in non-circularity for the immature rats.

Proportions of Internodes in Regenerated Nerves

Internode length in regenerated nerves was determined at $261 \pm 75 \mu m$ for four wk; $314 \pm 90 \mu m$ for nine wk; $310 \pm 68 \mu m$ for 18 wk; and $297 \pm 72$ for 36 wk. This agrees with the generally accepted observation that regeneration of peripheral nerve fibers involves replacement of the original long internodes with short ones measuring approximately 300 \mu m.

The length of the regenerated internodes did not change significantly during regeneration, but fiber diameter increased progressively. Mean values were $4.0 \pm 1.1 \mu m$ at four wk, $6.0 \pm 1.5 \mu m$ at nine wk, $7.8 \pm 2.3 \mu m$ at 18 wk, and $8.2 \pm 2.3 \mu m$ at 36 wk. The proportions of internodes, therefore, depended largely upon axon regrowth, and the mean values for the L/d quotient decreased from 67 to 55 to 43 to 40, respectively. If the L/d quotient was plotted against fiber diameter, there was
no clear-cut trend by the fourth wk, when only thin axons were present. Thereafter, the quotient decreased with increasing fiber caliber, the slope of the regression being similar to that found in normal nerves (Fig. 5). Values for l/d were generally much lower than for normal nerves, on average by about 60.

Plotting the absolute length of the internode against fiber caliber did not show the regression seen in normal nerve, internode length being, essentially, constant with increasing caliber of the fibers (Fig. 6). A minor increase in internode length with fiber caliber was seen at four wk. The difference was slight and the correlation coefficient was only 0.41. A similar difference was found when plotting the l/d quotient against internode length (not shown). If there were such a slight difference in initial internode length at very early phases of regeneration, it would imply some degree of prematching of internode length to prospective fiber calibers; however, the existence of this phenomenon is not clear from our data.

**Volume of Myelin per Internode**

The volume of myelin per internode may be calculated from the curves relating fiber diameters to the area of myelin (Fig. 2) and from the corresponding values for the l/d quotient (Fig. 5), including a linear shrinkage factor of 1.18 for the former. Regenerated internodes contained much less myelin than normal internodes of comparable calibers. For fibers of 5 μm caliber, the difference amounted to 0.42, and

![Graph showing non-circularity vs axon diameter for different weeks.](image)

**Fig. 3.** The degree of fiber shrinkage (non-circularity) is shown for four and 36 wk. There is no evidence that regenerated fibers shrink more than normal ones.

![Graph showing g-ratio vs axon diameter for different age groups.](image)

**Fig. 4.** Scatter diagrams of young, three-month-old rats are compared with adults. Young rats have thinner fibers than adult rats so that the scatter diagrams appear compressed toward the left side. Fibers of young rats have about the same caliber as those of four or nine wk regenerates (Fig. 1), but the young rats have thicker sheaths (higher g-ratio) similar to normal adult rats. The shape of the scatter diagrams for normal nerves and for regenerates (Fig. 1) are quite different.
Fig. 5. The length of the internodes is shown in these scatter diagrams in terms of multiples of fiber diameter (l/d quotient). While axons regrow, their internodes retain a constant length of approximately 300 μm (Fig. 6). It follows that the thicker fibers have relatively foreshortened internodes with lower l/d quotients.

for those of 10 μm, the difference was 0.23. Mean volumes of myelin were for a 5 μm regenerated fiber 2,400 μm³ compared to 5,660 μm³ for a normal fiber, and for a 10 μm regenerated fiber 8,580 μm³ myelin compared with 38,180 μm³ for a normal one.

Observations in Partially Damaged Nerves

The distribution of the g-ratio in normal rats was superimposed on that of the 36 wk regenerates (Fig. 7). The difference in the sheath thickness of the two fiber populations is evident, particularly for the fibers of medium and thick calibers. This diagram provides a frame of reference for the interpretation of pathological fiber populations.

Damage to the sciatic nerve was incomplete in several experiments due to variation in the application of the Cryoprobe. The damage found in these nerves affected preferentially the thin fibers, with relative persistence of thicker fibers contrary to the type of damage reported for non-freezing cold injury (4). In these nerves, the surviving normal fiber populations were distinguished from the regenerated fibers at a glance and the extent of damage was readily assessed (Fig. 7). This interpretation of the scatter diagrams of the g-ratio was confirmed by the distribution of the l/d quotient in teased fibers of partially damaged nerves. There were two regressions: one of the short internodes with low l/d and one of the persisting long, normal internodes with much higher l/d quotients.

DISCUSSION

The most important factor determining the length of an internode in a mammalian peripheral nerve is the increase in body size or the elongation of the nerve subsequent to the onset of myelination. This principle was recognized by Boycott (5), Hiscoe
Fig. 6. These curves show the conventional method of plotting internode length against fiber caliber. The length of regenerated internodes varies around 300 μm, but mean length does not increase during regeneration. Fiber caliber increases with regeneration so that there is no constant relationship between the length of a regenerated internode and the caliber of its fiber.

(6) and Vizoso and Young (7), who attributed differences in internode length between thick and thin fibers to differences in the onset of myelination. The dependence of internode length on body growth was particularly evident from experiments on nerve regeneration at different stages of development (7, 8). The earlier the regeneration occurred in very young rats, the longer the regenerated internodes tended to be. The degree of elongation of the nerves after the onset of regeneration caused corresponding variations in internode length. Beyond these elegant experiments, there are numerous reports confirming the foreshortening of regenerated internodes in transected nerves (6, 9, 10), in experimental neuropathies (11, 12), in human disease (13), or for the intercalated internodes found after segmental demyelination (14, 15). The initial phases of myelination and the initial phases of regeneration may involve some degree of remodelling of nerve structure in terms of the elimination of very short, redundant internodes (16); these phenomena, however, do not seem to alter the principles described above.

Apart from this information on internode length, there is also a consensus that regenerated myelinated nerve fibers have somewhat thinner sheaths than normal fibers (17, 18). Indeed, a comparatively thin sheath is widely accepted as a criterion for identifying a regenerating fiber (19–26). This phenomenon is usually thought to indicate insufficient or incomplete regeneration, causing a state of "hypoplasia" of the myelin sheaths. This presumed disturbance was attributed to an impaired regenerative capacity of the Schwann cell for myelin formation, or an ineffective transmission of a signal from the axon to the Schwann cell (27). Data on regeneration in central tracts imply that myelination differs, depending whether the axon comes in contact with the oligodendroglia for the first or the second time (28). More recently, sheath thickness of regenerated fibers was reinvestigated by Smith et al (29) using

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Fig. 7. A. In this scatter diagram, the computer printout of the curves for 36 wk regeneration (Fig. 1) was superimposed with that for adult rats (Fig. 4). Differences in sheath thickness are readily evident, particularly for fibers thicker than 5 μm. B. This curve shows partially damaged nerves in which the regenerated fibers are distinguishable, at a glance, from the persisting fibers, by having thinner sheaths. C. This scatter diagram shows the internode proportions in the same nerve. The two populations of long, normal internodes and of foreshortened regenerated internodes are readily apparent. Comparison of the two curves shows the same reciprocal trends for internode length and for sheath thickness in the normal and in the regenerated populations.

electron microscopic analysis of single fiber preparations. Their data imply that sheath thickness in regenerated fibers is determined by the quotient between the axolemmal surface area covered by the sheath cell and the total area of the myelin leaflet as described by Friede and Bischhausen (30). The validity of the interpretation of Smith et al (29) is questionable, since comparison of thin regenerates with thick
control fibers cannot show the subtle difference in sheath thickness such as those seen in Figure 2. Their study, therefore, offers no understanding of the relative "hypoplasia" of sheaths in regenerated nerves.

The present data demonstrate the known foreshortening of internodes in regenerated fibers. The lesions were placed in young adult rats, and the length of time allowed for regeneration carried them through about half of their normal life span. Average internode length was about 300 μm with an upper range of 500 μm, compared with a mean length of 850 μm and an upper range of 1,800 μm in normal adult sciatic nerves at the age of one year. Cragg and Thomas (9) found an internode length of large fibers of about 600 μm for rabbit regenerated nerves. Such internodes must inherently have lower l/d quotients than normal internodes, even when previous calibers are not fully regained (3). In considering internode proportions, one should bear in mind that the l/d factor is the result of variation in either internode length or in fiber caliber, or concurrent variations in both. There are fiber populations, e.g. ventral spinal roots, for which fiber caliber is uniform, while internode length varies greatly from one root to the other. The variation of l/d in root fibers, therefore, is mainly a function of internode length (31, 32). The reverse is true for regenerated fibers; the length of their internodes is uniform, but the caliber of the regrown fibers varies considerably and approaches normal values (7, 9, 18, 33). Variation of l/d in regenerated nerves, therefore, becomes mainly a function of fiber caliber.

We may now compare the degree of reduction in sheath thickness of regenerated fibers with their internode foreshortening. For each set of data, a lower l/d quotient corresponded to higher values for the g-ratio. Moreover, the slopes of the regressions of l/d and g showed reciprocal changes in every given fiber population. These trends were identical to those observed in normal fiber populations. This shows that the reduced sheath thickness of regenerated fibers is not from insufficient or incomplete regeneration. Incomplete regeneration may occur during initial regrowth of sheaths, e.g. after four wk. Between 18 and 36 wk there are no longer any significant changes in sheath thickness, internode proportions have stabilized, and the thinner sheaths of the regenerated fibers are clearly quantitative adjustments to the degree of internode foreshortening.

The exact magnitude of the change in sheath thickness caused by changes in internode length is still subject to review. Electron microscopic measurements of isolated internodes had shown a decrease in the g-ratio by 0.006 for every change of 10 in the l/d quotient (2). Values obtained in the present series of measurements were greater. The g-ratios of regenerated fibers were on average about 0.09 higher than those of normal fibers, and the l/d quotient had decreased by 60. This corresponds to a ratio of 0.015 per 10. In normal cat sciatic nerves one may estimate the relation between l/d and sheath thickness from the well-defined slope of the fibers between 5 and 15 μm (1). The g-ratio of this population decreased by 0.03 for every change of 10 in the l/d quotient. Hence, the sheath thickness found here for regenerated fibers actually lies between the normal values obtained in two studies using different methods of preparation. Such variation may be attributable to the fact that these measurements require an extremely high degree of precision, since calculation of the g-ratio has to be carried to the third decimal.

The reduced thickness of the myelin sheaths of regenerated fibers is also shown by the more customary methods of assessing sheath thickness, namely: comparing sheath thickness with axon diameter; myelin area with axon area; or myelin area with fiber diameter. All three of these curves clearly showed that regenerated fibers had thinner sheaths than normal fibers (Fig. 2).
Comparison of different stages of regeneration shows a progressive increase in fiber caliper. Maximum calibers attained by the age of 36 wk are near those of normal fibers. These large fibers have higher g-values and form a separate regression with little overlap with normal fibers. This clear-cut distinction between normal and regenerated fibers has great potential for analyzing pathological nerve populations. This was shown in partially damaged nerves where the population of surviving normal fibers was easily distinguished from the regenerated ones. The distinction between normal and regenerated fibers is more difficult at the initial phases of regeneration, when the thin regenerating fibers overlap with the normal population of thin, thinly myelinated fibers. Fibers below 5 μm, therefore, may either be persisting normal fibers or regenerating fibers in an early phase of regrowth.

Another phenomenon of interest is the occurrence of very thin nerve fibers with excessively thick sheaths (18, 21, 34). These fibers form a "tail" in the left lower part of the scatter diagrams for the g-ratio, and this tail is particularly evident during the early phases of regeneration. Such fibers are a minority, which is consistent with the assumption that they represent atrophic regenerated fibers which failed to reach their target organ. The excessive thickness of the sheaths may be understood as the consequence of the collapse of the myelin sheaths upon a shrinking axon (35). This interpretation of the "low g" fiber population is still hypothetical since it implies that these fibers are eliminated at a time when their distal ends had barely reached their target organs.

The data shown in the preceding paper (1) indicate that sheath thickness adjusts to the geometric proportions of the internode in such a way as to optimize conditions for the transmission of impulses from one node to the next. One may ask, therefore, in which way the geometry of regenerated internodes relates to the known physiological properties of regenerated fibers. The physiological properties of regenerated nodes of Ranvier differ from those of normal fibers (36). Regenerated nodes possess potassium channels, absent from normal nodes, with the consequence of having a lower safety factor. The reduced length of the regenerated internodes would tend to increase the safety factor, giving a functional advantage compensating for the changed nodal characteristics of the fibers. The contribution of potassium channels to impulse conduction remains stable in regenerated internodes contrary to their decrease during early normal development (37), a point which may also relate to the developmental changes in internode geometry described earlier in this discussion.

The present data show that the sheath thickness of regenerated fibers may be considered the result of adaptation to the changes in internode geometry and the physiological properties as well. Demonstration of these sheath changes by scatter diagrams opens new perspectives in the interpretation of diseased nerve fiber populations.

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REFERENCES

2. Friede RL, Bischhausen R. How are sheath dimensions affected by axon caliper and internode length? Brain Res 1982;235:335–50
5. Boycott AE. On the number of nodes of Ranvier in different stages of the growth of nerve fibres in the frog. J Physiol (Lond) 1904;30:370–80
7. Vizoso AD, Young JZ. Internode length and fibre diameter in developing and regenerating nerves. J Anat (Lond) 1948;82:110–34
9. Cragg BG, Thomas PK. The conduction velocity of regenerated peripheral nerve fibres. J Physiol (Lond) 1964;171:164–75
17. Sanders FK, Whitteridge D. Conduction velocity and myelin thickness in regenerating nerve fibres. J Physiol (Lond) 1946;105:52–74

33. Gutmann F, Sanders FK. Recovery of fibre numbers and diameters in the regeneration of peripheral nerves. J Physiol (Lond) 1943;101:489–518

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