The Use of Fixed and Stained Sections in Quantitative Studies of Peripheral Nerve

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SUMMARY

A technique which has been widely used in quantitative studies of peripheral nerve involves fixation in Flemming's fluid, embedding in paraffin wax, and staining with haematoxylin. An appraisal of the size changes induced by a standardized form of this technique has been made. Observations have been confined to changes in the thickness and external diameter of the internodal compact myelin sheath of the non-fasciculated nervus gastrocnemius medialis of the adult rabbit.

For a comparison of external diameters, sections from adjacent parts of the chosen nerve were prepared (*a*) by the standardized Flemming-Wolter technique; (*b*) by the rapid freezing technique, previously shown to reproduce accurately the dimensions present in fresh teased unfixed nerve-fibres. There was no significant difference in distribution of external diameters between sections prepared in the two ways.

For a comparison of sheath thicknesses, a regression line of 2 × myelin sheath thickness on external diameter was derived from each type of preparation. As external diameter was not appreciably affected by processing, a direct comparison of these regression lines could be made. It was found that regardless of external diameter or original sheath thickness, processing caused a reduction of sheath thickness of some 20% as a result of increase in internal diameter. This latter increase ranged from about 50% to under 20% with increasing fibre size.

Single frozen sections were passed through the series of reagents used in the Flemming–Wolter technique and photographed after each of seven stages. The principal changes in sheath thickness occurred in the first and last stages of processing. External diameter was not appreciably affected whereas the internal margin of the sheath was extremely labile. Observations suggest that slight inconsistencies in technique may be accompanied by relatively gross errors when measurements are undertaken. All stages need careful standardization, the most important in this respect being fixation, differentiation, final dehydration, and photographic exposure.

INTRODUCTION

MOST quantitative studies of peripheral nerve have been based on sections which have been fixed and stained. Such methods of preparation are generally assumed to be accompanied by some degree of shrinkage and distortion (Baker, 1958), which will vary with the particular tissue component and reagents used.

It has been shown that the proportion of compact myelin to axoplasm in peripheral nerve-fibres varies appreciably not only with external fibre diameter (Taylor, 1942; Sanders, 1948; Evans and Vizoso, 1951), but also with the type of nerve studied (Wendell-Smith and Williams, 1958). It is possible that the size changes of axoplasm and myelin induced by a variety of reagents are comparable and proportionate so that the relation between them is unaffected. However, in view of the marked difference in the ultrastructure of

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these two components, this possibility seems remote, and on *a priori* grounds differential effects may be postulated.

A technique which has been widely used involves fixation in Flemming's fluid, embedding in paraffin wax, and staining with haematoxylin. Much important comparative work has been carried out by a standardized form of this technique. The error involved has proved unimportant in this context. Moreover, most workers have limited their studies to external fibre diameter, and as will be shown this dimension is not appreciably affected by the technique. However, modern theories of conduction (Rushton, 1951) concern themselves in part with the ratio between the diameter of the axon and the thickness of the ensheathing structures, so that for their verification a knowledge of the effect of the standardized technique on these dimensions is necessary. Thus attention is focused not only on possible changes in the external diameter of the compact myelin sheath but more particularly on changes in internal diameter. Further, the validity of assuming that estimates of internal diameter performed on fixed and stained preparations provide a reliable index of axon diameter (e.g. Gasser and Grundfest, 1939) must be considered. In addition the importance of internal diameter measurements is stressed in relation to the observed differences between nerve-fibres of muscle and skin of the same external diameter, and to possible variations in the ratio of thickness of the myelin sheath to diameter of the axon along the length of the fibre (Evans and Vizoso, 1951; Sunderland and Roche, 1958).

The literature dealing with the size changes during processing has been reviewed by Sunderland and Roche (1958). A number of different techniques have been assessed by as many methods, but the majority of workers have confined their studies to changes in external diameter. Sanders (1948) studied the changes in both internal and external diameter as a result of a modified Weigert technique (Gutmann and Sanders, 1943). The principal stages of this technique are similar to those considered here. However, the nerve studied was the peroneal nerve of the rabbit, in which there are nerve-fibres of both muscle and skin. His basis of reference was the freshly teased nerve-fibre, and he made no allowance for the magnifying effect of the myelin lens system (Williams and Wendell-Smith, 1958).

Material and Method

The nervus gastrocnemius medialis (N.G.M.) of the rabbit was used throughout the present investigation. Adult rabbits were used; no account was taken of breed, sex, or weight. The use of lipid-soluble inhalation anaesthetics was avoided and intravenous nembutal was used. The appropriate part of the sciatic nerve and its branches was removed in one piece. Sections from a short segment including the N.G.M. at about 3 cm proximal to its muscle were prepared in two ways.

(i) 5- or 6-μ sections of the fresh nerve were prepared by a rapid freezing technique (Williams, 1959). The sections were irrigated with a standard volume of normal saline and photographed immediately without a cover-slip,

10 sec. Ilford R. 20 quarter-plates were developed in I.D. 11 fine grain developer for 6 min at room temperature, and subsequently enlargements ×1,000 and ×2,000 diameters were made on Kodak W.S.G. 2. S. paper and developed in D. 163 for 2 min. After processing, the prints were allowed to dry between sheets of Fotonic paper. A squeegee was not used. A micrometer scale was photographed and enlarged at each session.

The optimum exposure times were determined by a comparison between the dimensions of the direct visual image and the dimensions of the photographic images produced after different exposure times. Ten fibres between 11 and 21 μ in external diameter were selected, and measurements of internal and external diameter made directly on the slide with a screw ocular micrometer to the nearest 0·125 μ, and indirectly with a ruler to the nearest 0·25 μ on photographic enlargements after exposures of $3\frac{1}{2}$, 5, 7, 10, 15, and 20 sec. The ratio of internal to external diameter was used as a basis for comparisons by the χ^2 test.

Comparison of external diameters. The non-fasciculated part of the N.G.M. was removed as above, and carefully cut into two segments. Sections from the two adjoining cut surfaces were prepared by the freezing technique and the Flemming–Wolter technique respectively. Estimates of external diameter were made by two methods. A Perspex disk inscribed with concentric circles 2 mm apart was used to allocate the magnified images of fibres to size-categories differing in diameter by 2 μ steps. This method was chosen initially because it has frequently been adopted by other workers in this field. Since minor differences might not be apparent when a 2 μ grouping was used, a more accurate estimation was made for one N.G.M. by taking the mean of two measurements at right angles made with a 0·5 mm scale. Histograms were constructed and a comparison of the distributions made by the χ^2 test.

Comparison of sheath thicknesses. Sections were prepared by the two techniques and estimates of external and internal diameter were made on photographs at final magnifications of 1,000 diameters (10 to 20 μ fibres) and 2,000 diameters (< 10 μ fibres). For each fibre the estimator was the mean of two measurements made at right angles. The maximum error of the measuring technique was $\pm 4\%$.

For each type of preparation a scatter diagram was constructed by plotting external diameter against 2 × sheath thickness. Regression lines and standard errors of estimate were calculated.

The results from frozen sections and fixed stained sections were compared and the changes in sheath thickness and internal diameter demonstrated graphically.

Illustration of lability of sheath dimensions during processing. Frozen sections were prepared and photographed after each of the following successive stages:

1. Physiological saline.
2. Flemming's fluid (15 min).
3. 70%, 90%, 95%, and absolute alcohol (15 min each).

4. Cedarwood oil (15 min).
5. Molten paraffin wax (15 min) and return to xylene (15 min).
6. Through the alcohols to water.
7. Wolter's haematoxylin staining; dehydration, clearing, and mounting in Canada balsam.

Twenty fibres, all approximately 15μ in external diameter, were followed

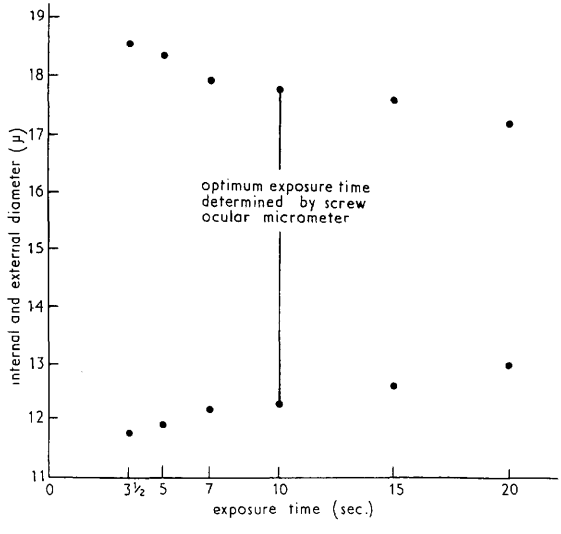

FIG. 1. Internal diameter and external diameter plotted against exposure time. Each point represents the mean measurement for the same 10 fibres after the specified exposure time had been used in the production of a negative.

through the successive stages by measurements of internal and external diameter. The mean changes in dimensions were used to construct a diagram.

RESULTS

Exposure time. It was found that the thickness of the myelin sheath decreased with increasing exposure time; that this decrease was regular and due to changes in both internal and external diameter (fig. 1). The ratios of internal to external diameter for the 10 fibres as determined with the screw ocular micrometer were taken as the best estimates. The ratios as determined on photographic images after 10 sec exposure did not differ significantly from those estimates. This and other results are given in table 1.

Comparison of external diameters. It was found that there was no significant

difference in distribution between sections prepared by the two techniques (table 2).

TABLE 1

Significance tests for difference between direct measurements and those on photographs

Exposure in seconds	χ^2	*Degrees of freedom*	*Probability*
3·5	60·21	9	<0·001
5·0	37·31	9	<0·001
7·0	21·36	9	<0·02
10·0	11·67	9	>0·20
15·0	31·47	9	<0·001
20·0	80·75	9	<0·001

TABLE 2

Significance tests for difference between fresh and fixed-stained fibre distributions

Nerve	*Grouping* (μ)	χ^2	*Degrees of freedom*	*Probability*
N_1	2	14·70	9	0·10
N_2	2	9·39	9	0·40
N_3	2	11·41	9	0·25
N_1, N_2, and N_3	2	35·50	29	0·20
N_1	1	21·61	18	0·25
N_1	0·5	40·62	35	0·25

Histograms for the N.G.M. which was examined in more detail are shown in fig. 2.

Comparison of sheath thicknesses. It having been demonstrated that external diameter was not appreciably different after processing, a direct comparison of the regression lines of sheath thickness on external diameter gave a measure of reduction in sheath thickness.

Fig. 3, A is a scatter diagram of 2×sheath thickness against external diameter for 204 fibres from fresh frozen sections. A calculated regression line and relevant statistics are given in fig. 3, B. A similar scatter diagram and regression line for 172 fibres from the fixed and stained preparation are given in fig. 4, A, B.

The regression lines for fresh frozen sections and for fixed stained sections are compared in fig. 5. Comparison of these lines shows that processing resulted in a reduction of sheath thickness of some 20% regardless of external diameter (fig. 6, A) or original sheath thickness (fig. 6, B). This reduction in sheath thickness was the result of an increase in internal diameter. It will be noted that within the range of fibres studied, as fibre diameter increased, the increase in internal diameter changed from some 50% to under 20% (fig. 6, C).

Lability of sheath dimensions. The results of this investigation are illustrated in fig. 7. The principal changes in sheath thickness occurred in the first and last stages of processing. External diameter was not appreciably affected, whereas the internal margin of the sheath was extremely labile. A typical 15-μ fibre as seen in a section prepared by the standard Flemming–Wolter technique is included for comparison.

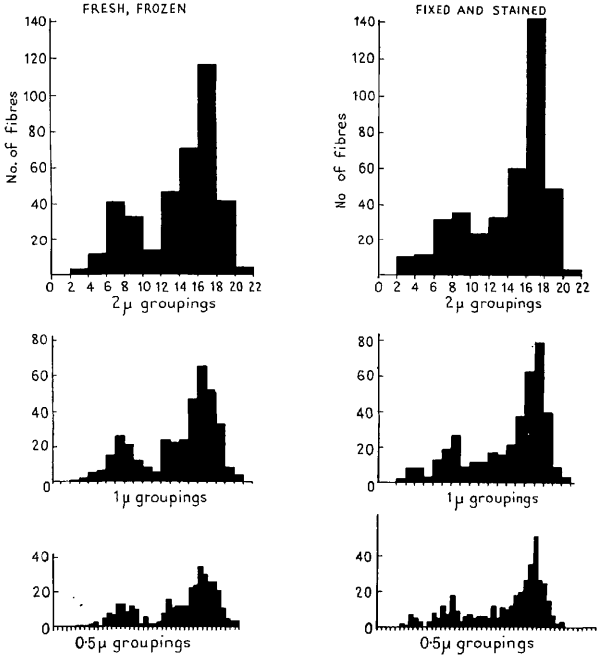

FIG. 2. Histograms of an N.G.M. of the rabbit, prepared by the two methods from adjacent portions of the nerve, presented in the usual 2-μ groupings and also in 1-μ and 0·5-μ groupings. The essential similarity of the distributions persists throughout the three groupings.

DISCUSSION

It is axiomatic that for quantitative histology a standardized technique is necessary. Different aspects of the technique requiring standardization will be considered.

The selection of a particular nerve and site is important in an investigation of this type. As has been pointed out, a mixed population of fibres to muscle

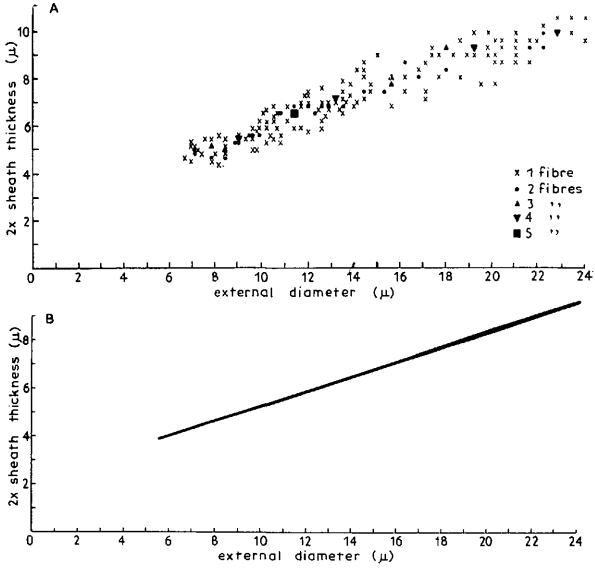

FIG. 3. A, scatter diagram of 2× myelin sheath thickness on external diameter for 204 fibres from frozen sections of the N.G.M. of the rabbit. B, calculated regression line for data from fig. 3, A. Correlation coefficient, 0·9291; regression coefficient, 0·3099; standard error of estimate of sheath thickness from external diameter, 0·5287μ.

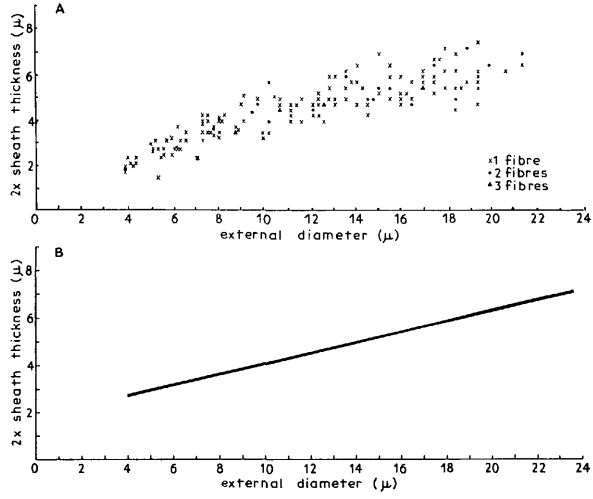

FIG. 4. A, scatter diagram of 2× myelin sheath thickness on external diameter for 172 fibres from Flemming–Wolter preparations of the N.G.M. of the rabbit. B, calculated regression line for data from fig. 4, A. Correlation coefficient, 0·9500; regression coefficient, 0·2292; standard error of estimate of sheath thickness from external diameter, 0·4154μ.