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In vivo observations on mature myelinated nerve fibres of the mouse

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INTRODUCTION

Current views of the morphology of mature, myelinated nerve fibres are derived from studies on fixed material, or on unfixed, excised specimens after teasing in saline or frozen-sectioning; all techniques with inherent errors of shrinkage and distortion (Wendell-Smith & Williams, 1959; Williams & Wendell-Smith, 1960). The dimensional lability of the myelin sheath during processing has further been accentuated by X-ray diffraction analysis and electron microscopy (Finean, 1954, 1958). It is, therefore, clearly desirable to be able to monitor the *in vivo* situation in the assessment of the relative quantitative and qualitative merits of all *in vitro* methods.

Surprisingly few attempts have been made to compare the morphology of conventionally prepared tissue with that of its living counterpart. Such attempts that have been made were usually dependent upon transillumination procedures, since the early classic studies by Basler (1917) and Knisely (1936) incorporating internal reflection systems with curved glass or fused quartz rods. In this way, information has been obtained on the circulation of the lung (Wearn *et al.* 1934), in urodele endocranial vessels (Roofe, 1935) and in the spleen (Knisely, 1936). However, tissue density, thickness and the dislocation involved have always precluded the transillumination of substantially undisturbed mature mammalian nerve trunks. The observation of living nerve fibres has previously been limited to small diameter cutaneous afferents in the amphibian lymph sac (Schwalbe, 1882) or to equally fine, early developing or regenerating fibres, e.g. in the tadpole tail (Speidel, 1933); in transparent ear chambers (Clark, Clark & Williams, 1934) or in cultured explants (Murray, 1965). Many of the problems of large, normal, mature nerve fibre morphology and dynamics cannot be answered by these experimental techniques. Equally unconvincing are the transillumination attempts of Singer (1969) in which all limb tissues including bone are removed from a segment of a limb and single fibres are isolated throughout this part of their course by teasing.

With oblique incident illumination it is, however, possible to examine hitherto inaccessible structures with minimal tissue damage for prolonged periods of time with subsequent recovery of the experimental animal. This paper describes the technique involved and the results of examining optical sections of fibres in the sural and sciatic nerves of the adult mouse *in vivo*. It has in particular been directed towards certain aspects of fibre morphology; the inter-relationships of the constituent fibres in the whole nerve trunk; the appearance of the typical uncomplicated internode and

of the paranodal apparatus; the existence or otherwise of the controversial Schmidt–Lanterman incisures as visible discrete zones in the internodal myelin and the possibility of observing dynamic alterations in fibre contour similar to those seen in culture (Murray & Herrmann, 1968).

MATERIALS AND METHODS

Adult mice were anaesthetized with intraperitoneal nembutal, the skin of the upper thigh shaved and a longitudinal incision made. The edges of the wound were gently sutured back and muscle bellies carefully separated to display a nerve bundle (preferably $< 1{\cdot}0$ mm diameter) crossing the floor of the resulting intermuscular cleft. The animal was positioned on cork blocks on the large stage of a Leitz Orthoplan microscope and the intermuscular cleft filled with warmed, isotonic saline into which the immersion objective was lowered (Figs. 1 and 2). Illumination was provided by a high-intensity xenon discharge lamp fitted with filters. The water immersion objectives used were either $55\times$ (N.A. 0·84, working distance 0·57 mm) or $75\times$ (N.A. 0·90, working distance 0·45 mm) and were surrounded by ring condensers, the optimal setting of which depended upon the surface structure under observation. Suitable adjustment of the illumination train and progressive focussing allowed the examination of successive optical sections of the nerve until the central fibres of the bundle were reached. Additional information was often obtained after the insertion of a half-circle stop to produce oblique incident illumination. Photographs were taken with a Leitz Orthomat fully automatic microscope camera, using Ilford Pan F film (A.S.A. 50, DIN 18). After observation, the wound was closed and the animal allowed to recover for periods from 10 d to 3 weeks during which time it was observed for any signs of motor disturbance. Subsequently, samples of nerve were prepared for light microscopic examination by the method of Swank & Davenport (1935) and for routine electron microscopy.

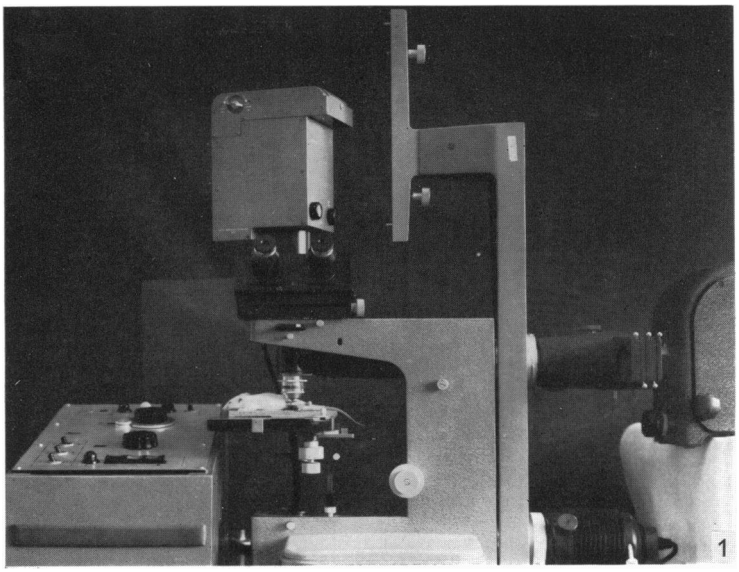
RESULTS

Observations on nerve fibres were made by progressive focussing through the nerve sheaths, adipose tissue and vasculature; hence the initial structures encountered were irregular sheets of connective tissue and small perineural blood vessels. The blood flow through these vessels was extremely rapid and any obvious interference in blood supply was infrequent. In exceptional cases, where a number of capillaries were seen to be persistently empty, or to contain stagnant corpuscles, or where large numbers of extravasated corpuscles were present in the immersing saline, the animal was discarded. The nerve bundle, because of its high refractility, appeared brightly illuminated, and this assisted initial location. Surrounding the bundle were prominent ovoid fat cells.

Typically, the nerve fibres ran parallel to one another, although at points of nerve branching a certain amount of oblique crossing was seen. The majority of internodes (Figs. 3, 4) appeared to be substantially smooth and cylindrical, although a small proportion deviated from this and showed a regular undulation of fibre contour with constrictions occurring at intervals of approximately 5 μm (Figs. 5, 6).

In the paranodal region (Figs. 5, 6) dilatations of fibre contour, i.e. paranodal bulbs, were observed and were asymmetrical in their principal dimensions. Elongated

profiles of longitudinal shelf-like invaginations and circular profiles of tubular invaginations were constant features of the myelin sheath of the paranodal bulbs. The convex terminations of adjacent bulbs curved inwards, strongly suggesting axonal constriction at these points.



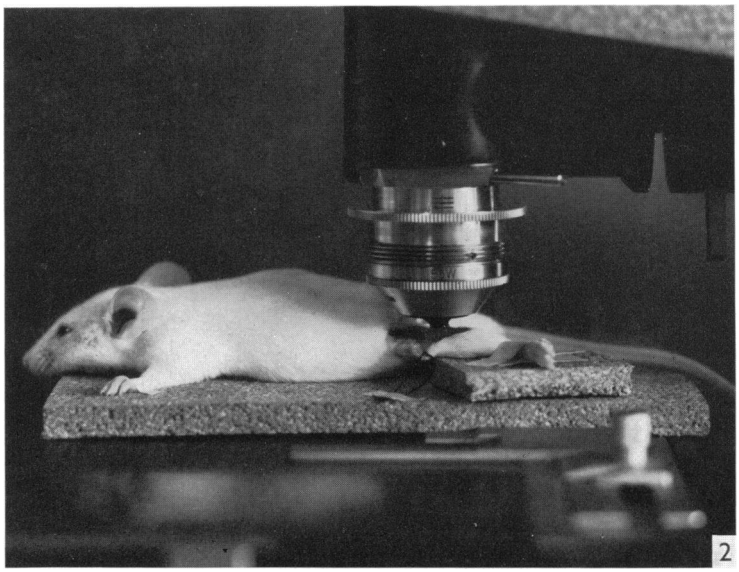


Fig. 1. General view of experimental set-up. A Leitz Orthoplan microscope carries an Ultropak incident light attachment and is fitted with an automatic camera and a high-intensity xenon discharge lamp.

Fig. 2. Close-up of experimental field. An anaesthetized mouse with a thigh nerve exposed is examined using a × 55 water immersion objective using oblique incident illumination.

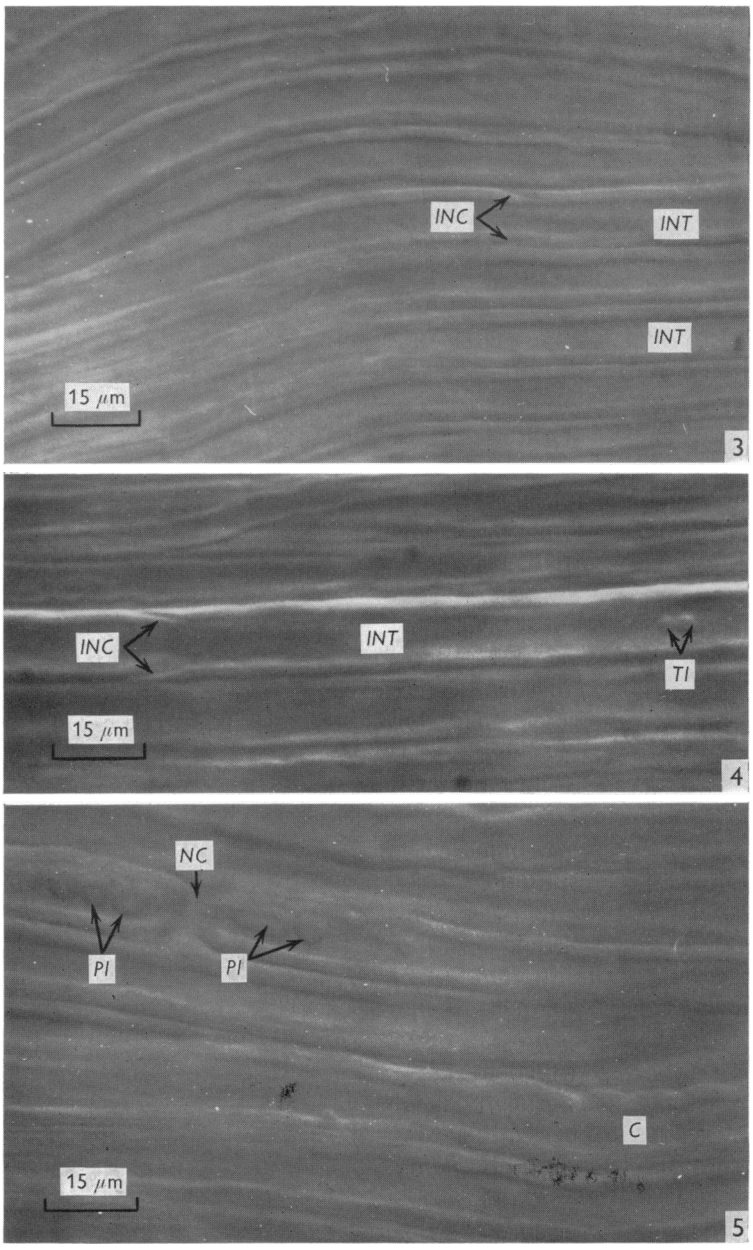


Fig. 3. Internodes seen *in vivo* using oblique incident illumination. The nerve fibres are largely parallel and the majority of internodal sheaths (*INT*) are smooth and cylindrical. A 'closed' incisure of Schmidt–Lanterman is indicated (*INC*).

Fig. 4. A length of cylindrical, uncomplicated internode (*INT*) includes two small tubular invaginations of the myelin sheath (*TI*) and a 'closed' incisure (*INC*).

Fig. 5. An *in vivo* field showing a typical node of Ranvier with its nodal constriction at *NC*. Each paranodal bulb shows profiles of tubular or shelf-like invaginations of the myelin sheath (*PI*). At *C* a regularly constricted internodal myelin sheath is seen.

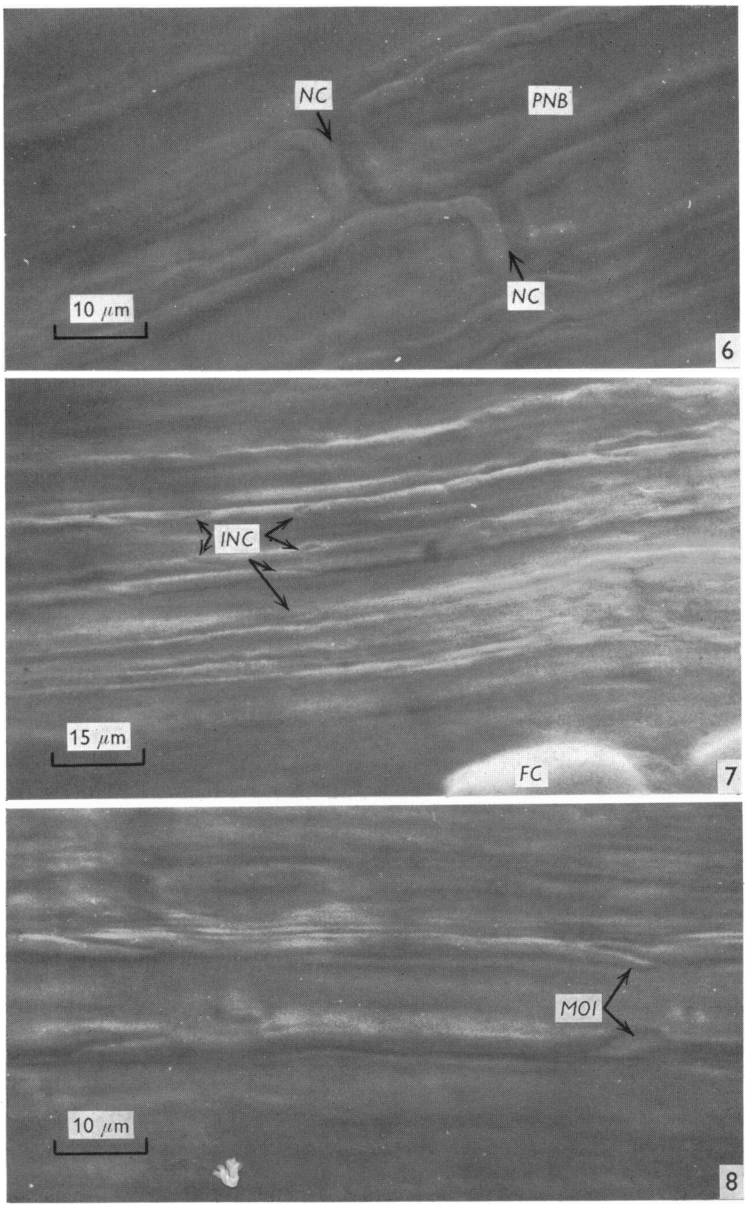


Fig. 6. Two nodes of Ranvier with nodal constrictions at *NC*. The paranodal bulb (*PNB*) shows profiles of shelf-like invaginations and a regular constriction of its myelin sheath.

Fig. 7. Varieties of 'closed' incisures of Schmidt–Lanterman seen *in vivo* are indicated (*INC*), with a fat cell profile at *FC*.

Fig. 8. An internodal myelin sheath showing a 'moderately open' incisure at *MOI*.

Schmidt–Lanterman incisures (Figs. 4, 7, 8) were observed as bilateral, diagonal dark lines crossing the myelin sheath, dividing the internode into cylindrico-conical segments. They exhibited a narrow range of states, as judged from the width of the oblique dark line, usually appearing 'closed' although a minority were 'moderately open'.

After observation, the wound was closed and the animal allowed to recover to permit subsequent assessment of possible trauma to the nerve during the experiment. No motor disturbances were noted over a period of 3 weeks and there was no evidence of degenerative change in fibres prepared by the method of Swank & Davenport (1935) for light microscopy. Electron microscopy showed no evidence of focal demyelination or Wallerian degeneration—in each case the nerve from the unoperated side was used as a control.

DISCUSSION

For the reasons stated previously, acceptable transillumination techniques were found impossible to apply to mature nerves. Further, the significance of features seen in tissue culture remains complex. In the case of nerve culture the cells are removed from their natural vascular and metabolic environment and deprived of contact with both higher centres and end organs.

In the method described above, however, there is minimal disturbance to the fibrous tissues and vascular network and no separation from influential 'input' and 'output' systems. The disadvantages are that the animal is anaesthetized and an incision is made but these are unavoidable except in superficial translucent tissues.

The resolvable detail differs qualitatively from that seen in isolated teased fibres when transilluminated, but it must be remembered that the fibres are deep inside an intact nerve bundle, and are being viewed through a relatively large mass of collagen and the attenuated cytoplasmic processes of a series of perineurial cells (Cravioto, 1966). Moreover, the entire field is subject to transmitted respiratory movements and oscillates with vascular pulsation. For these reasons direct observation through the microscope is often more satisfying than attempts to record the field by 'single-shot' microphotography and in our current series of experiments we are incorporating more continuous recording using cine photographic and television techniques. However, the detail obtained is sufficient to enable appreciation of the *in vivo* contours of the myelin sheath throughout the internode.

With the exception of zones of Schwann cell specialization, i.e. paranodal bulbs, perinuclear regions and Schmidt–Lanterman incisures, it is often assumed that the tranverse-sectional profile of the remaining uncomplicated internode is approximately circular. However, the majority of profiles seen after routine processing for electron microscopy are far from circular and recently further doubts have been expressed by Berthold (1968), who postulated that a cog-wheel-like shape is closer to the *in vivo* shape of the internode, at least in feline fibres, and assumed intuitively that fixation, dehydration and embedding would simply exaggerate an age-dependent *in vivo* pattern. Possible species differences concerning the distribution and amount of internodal Schwann cell cytoplasm may be relevant, since irregularities of myelin profile have been interpreted as indicating reciprocal accumulations of cytoplasm.

Opinions have long been divided between a reticulum-like network of longitudinal cords and connecting 'collars' radiating from the nuclear region (Cajal, 1928) and a thick layer surrounding the internode uniformly (Stockenius & Zeiger, 1956). Certainly, in the mouse and rat, electron micrographs give little indication of any substantial accumulations of Schwann cell cytoplasm in the unspecialized internode, although in the cat Berthold (1968) has described distinct notches filled with cytoplasm along the contour of the fibre.

The present studies show that at least in the mouse the assumed circular profile is largely correct for the majority of internodes, but that there exists in addition a small population of fibres showing regular, almost periodic internodal constrictions. The latter appearance may well reflect a dynamic process involving asynchronously all fibres in the bundle, as for example during the peristaltic movements seen in culture (Murray & Herrmann, 1968).

In vivo observations have confirmed the presence of asymmetric paranodal dilatations (Williams & Kashef, 1968). The indentations of the paranodal bulbs, present both proximally and distally, presumably correspond to the organelle-bearing columns of Schwann cell cytoplasm described by Williams & Landon (1963).

The observation of bilateral oblique dark lines crossing the myelin sheath and dividing the internode into cylindrico-conical segments has confirmed the previously debated existence of the incisures of Schmidt-Lanterman and their variation from a 'closed' to 'moderately open' condition, again possibly reflecting dynamic changes of state in the fibre. However, observation of fields *in vivo* for periods of 1–2 h shows that there are no gross and relatively rapid changes in either internodal profile or state of dilatation of the incisures and that demonstration of such possible changes must await more prolonged observation with continuous recording. These findings contrast sharply with our observations on the obvious and quite rapid changes which occur following a proximally sited nerve crush. Further details of these changes and an analysis of the structure and reactions of the incisures are soon to be published.

It would seem clear that the technique described provides useful and hitherto inaccessible information not only concerning nerve fibre morphology and dynamics, but promises a wide application in other tissues.

SUMMARY

Using oblique incident illumination, small nerve bundles have been observed in the thigh of the adult mouse, with minimal surrounding tissue damage, maintenance of a functioning vascular plexus and with undisturbed central sources and end organs. Typically, the unspecialized regions of the internode appear smooth-walled, approaching the cylindrical form, although a small population of fibres was found showing a regularly constricted contour. Asymmetrical paranodal bulbs and irregular surface indentations of the paranodal region, presumably related to the paranodal columns of Schwann cytoplasm, were constant features of each fibre. The *in vivo* existence of incisures of Schmidt–Lanterman was established and they appeared as bilateral oblique dark lines of varying width—from 'closed' to 'moderately open'—which crossed the compact myelin of every internode.

Suggestions are made concerning the use of this technique in conjunction with time-lapse cinematography for further studies of nerve dynamics, and as a suitable method for application to other tissues.

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