THE DISTRIBUTION OF ELECTRON-DENSE TRACERS IN PERIPHERAL NERVE FIBRES

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SUMMARY

Two electron-dense tracers, ferritin and lanthanum, have been administered to peripheral nerve fibres, and their uptake has been studied ultrastructurally. It was found that the perineurium was an effective barrier to ferritin in vivo, and the tracer was subsequently injected sub-perineurally. Ferritin uptake over a 120-min period was confined to occasional phagocytic vesicles in perineurial and Schwann cells, and to the nodal gap substance and paranodal periaxonal space. No uptake was observed in the myelin sheath, incisural intraperiod line gap, or in the axoplasm. Soaking fibres in ferritin in vitro resulted in a more generalized cytoplasmic and axoplasmic uptake, although the myelin sheath and Schmidt-Lanterman incisures remained devoid of the tracer.

Lanthanum nitrate, included in the fixative solution, delineated the patent incisural intraperiod line gap, and outlined the external surface of the terminal loops of nodal Schwann cell cytoplasm, and the paranodal Schwann cell-axolemmal junction. Unlike ferritin, La\(^{3+}\) penetrated the myelin sheath, being usually confined to the intraperiod line region of the outer lamellae, where it was associated with a widening of the lamellar unit, and an apparent splitting of the intraperiod line.

The results are discussed with regard to distribution of extracellular space in peripheral nerve fibres.

INTRODUCTION

In recent years, the information derived from the use of contrast-enhancing stains employed in electron microscopy has been supplemented by electron-dense tracers, which are presumed to delineate extracellular space channels and preferential routes for the cellular uptake of particulate matter in the diameter range 4-32 nm. Lanthanum acetate has been used as a conventional stain; however, when it is added to fixative and washing solutions as the neutralized nitrate, it has been used as a tracer in invertebrate nerve and retina (Doggenweiler & Frenk, 1965), mouse heart, liver (Revel & Karnovsky, 1967), and peripheral nerve (Revel & Hamilton, 1969), and in rat bile canaliculi (Matter, Orci & Rouiller, 1969).

The distribution of the protein ferritin (estimated molecular diameter 9 \(\pm\) 0.5 nm) in aqueous solution has been analysed in nervous tissue, in the spinal ganglia of the toad (Rosenbluth & Wissig, 1964), the cerebral cortex (Bondareff, 1964), and the perineural sheath of peripheral fibres (Waggner, Bunn & Beggs, 1965). Ferritin has also been employed as an indicator of the so-called 'large pore' (about 3.5 nm diameter) system of cellular particulate transport in capillary endothelium (Palade, 1968).

In view of autoradiographic studies (e.g. Singer, 1968), which have emphasized the...
existence of axon/satellite cell/extracellular space transport systems in peripheral nerve fibres, both the electron-dense tracers, lanthanum and ferritin, have been administered to peripheral nerves in an attempt to determine possible routes for the passage of substances into fibres.

MATERIALS AND METHODS

Lanthanum-containing preparations

Fifteen adult mice were anaesthetized using intraperitoneal Nembutal and the sciatic nerve exposed in the thigh. Small pieces of the nerve were excised; some were immediately immersed in Karnovsky's formaldehyde-glutaraldehyde fixative in 0.2 M cacodylate buffer containing La(NO₃)₃ at a final concentration of 1%, pH 7.3, for 2 h (Revel & Karnovsky, 1967). Other pieces were pre-soaked in anisotonic saline solutions (0.03 M, 0.07 M, 0.3 M and 0.6 M NaCl) for 15 min prior to fixation. Control specimens of both fixed and pre-soaked nerves were processed without exposure to La³⁺. The tissues were washed overnight in 0.2 M cacodylate buffer containing La(NO₃)₃ at a final concentration of 1%, and post-fixed in OsO₄-collidine, pH 7.3, for 2 h. All fixation and washing procedures were carried out at 4 °C. To avoid unnecessary exposure to aqueous solutions, which have been shown to wash La³⁺ out from tissue blocks, dehydration was begun in 75% ethanol for 10 min, followed by 3 15-min changes in 100% ethanol, and propylene oxide, 15 min. The tissue was embedded in TAAB resin, and sectioned on an LKB ultramicrotome. Silver-grey sections were collected on copper grids and some were further stained with uranyl acetate and lead citrate.

Ferritin preparations

An aqueous 10% solution of ferritin (Calbiochem Ltd) was used for both in vitro and in vivo soaking of fibre bundles, and for sub-perineural injection into the sciatic nerves of living adult mice. Times of administration of the solution, both in vivo, to mice anaesthetized with Nembutal, and in vitro, to fibres excised from the body, were 5, 10, 50, 60, 90 and 120 min. On occasions, trypan blue was added to the ferritin solution to facilitate location of the injected material, but was later discarded, since it disappeared within the 120-min period. In all cases, at the end of the period of ferritin-immersion, specimens of excised, unteased, nerve were fixed in ice-cold 3% glutaraldehyde in phosphate buffer, pH 7.3, for 2 h. They were then washed overnight in buffer, post-fixed in 1% OsO₄ in Millonig's phosphate buffer, pH 7.3, for 1 h, dehydrated in an ascending series of ethanols, and embedded as before in TAAB resin, and sectioned on an LKB ultramicrotome.

All sections of both La³⁺- and ferritin-containing material were examined in RCA EMU 3 and 4 electron microscopes, using a 30-μm heated objective aperture, and accelerating voltages of 50 and 100 kV.

RESULTS

Highly electron-dense deposits of La³⁺ were observed approximately corresponding to the position of the intraperiod lines of the compact myelin sheath, confined typically to the outer 2 or 3 lamellae (Fig. 1), although staining of adaxonal lamellae was seen occasionally, particularly near nodes of Ranvier. While the uptake of the ‘stain’ was generally uniform along these intraperiod lines, in some instances it was intermittent, producing a localized ‘beading’ appearance in the sheath (Fig. 2). Closer analysis of the stained regions of myelin sheath, however, revealed a marked increase in the repeat distance of the lamellar unit (i.e. the distance between the midpoint of two adjacent major dense lines), which was about 12 nm in stained myelin and only
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9.5 nm in adjacent unstained sheath. In the stained regions, both the major dense lines, and the pale interzone between the major dense and intraperiod lines, appeared normal. In the region of the intraperiod line, a triple-layered zone was present, comprising the densely stained, separated components of the intraperiod line and an intervening pale stratum. In the neighbouring compact myelin sheath, the intraperiod line was single.

At the nodes of Ranvier (Figs. 5, 6) and incisures of Schmidt–Lanterman (Figs. 3, 8, 9), a more widespread distribution of stain was apparent, with a deeper penetration of paranodal and peri-incisural myelin by the La³⁺ than throughout the surrounding uncomplicated internodal myelin.

At the node, the gap-substance and Schwann cell basement membrane showed enhanced granularity and contrast, and large irregular deposits of tracer were seen outlining the external surfaces of the terminal loops of nodal Schwann cytoplasm, and the paranodal Schwann cell-axolemmal junction (Figs. 5, 6). In the latter situation the stain frequently appeared to have a regular periodicity of 30 nm, resembling the deposition of La³⁺ at central nodes of Ranvier described recently by Hirano & Dembitzer (1969). Electron-dense thickenings of the outer leaflet of the axolemma have also been described in unstained central nodes by Peters (1966).

At the incisures, La³⁺ was present throughout the patent intraperiod line gap of the incisural spiral (Fig. 8; see Hall & Williams, 1970).

In the extracellular space La³⁺ outlined collagen fibres, but was not otherwise evident, reflecting the ease with which the heavy metal can be washed out of the tissue during processing (Revel & Karnovsky, 1967).

In fibres which had been pre-soaked in hypotonic saline solutions (0.03 M or 0.07 M NaCl) prior to fixation in the La³⁺-paraformaldehyde-glutaraldehyde solution, La³⁺ uptake was greatly increased throughout the myelin sheath, along intraperiod lines which had been split by the hypotonic soaking (Fig. 4). La³⁺ was present as before at the node and the incisure; although dilated, the peri-incisural space was devoid of tracer. Pre-soaking fibres in hypertonic saline solutions (0.3 M or 0.6 M NaCl) prior to fixation resulted in a minimal uptake of tracer which, in the uncomplicated internode, was confined to the intraperiod lines of the outer lamellae of only a few fibres. Staining of nodal and incisural regions was unchanged by the pre-soaking procedure.

After soaking excised, unteased fibres with an intact perineurium in ferritin solutions in vitro, no ferritin was observed over the myelin sheath, or within the incisural compartments. However, an almost uniform distribution of ferritin was seen both free, and in vesicles, in perineurial cells, Schwann cells, and throughout the axoplasm and gap substance (Fig. 11). In vitro soaking by surrounding the whole fibre bundle with ferritin solution, was unsuccessful as a means of presenting the ferritin to individual fibres, even after prolonged time intervals. The perineurium was an effective barrier to ferritin passage into the endoneurial space, with minimal uptake into phagocytic vesicles in perineurial cells after 60 min.

Following in vivo sub-perineurial injection, however, ferritin filled the extracellular space, being taken up into occasional phagocytic vesicles in endothelial cells, Schwann
cells and perineurial cells. It would seem unlikely that ferritin uptake was the result of stimulated endocytic activity in these cells, in that few of the vesicles contained the electron-dense micelles. Passage of ferritin outwards across the perineurium was observed within the 120-min period; decreasing concentrations of tracer were present in the spaces between successive perineurial cell layers, and there was some indication of phagocytic uptake of ferritin by these cells.

Ferritin was present throughout the nodal gap substance, between the nodal fingers, and along the nodal and paranodal axolemma, as far as the last paranodal terminal loop of Schwann cytoplasm (i.e. that most distant from the mid-nodal point), within 5 min of injection. After 120 min, there was little alteration in this pattern of distribution, and no indication of any trans-axolemmal movement (Figs. 7, 12).

Ferritin was never observed in either the cytoplasmic spiral or the intraperiod line gap (which was delineated by La\(^{3+}\)) at the incisure of Schmidt–Lanterman (Fig. 10). Occasional ferritin micelles were taken up in vesicles in epi-incisural Schwann cytoplasm, but such uptake was infrequent.

Within the 2-h period of observation, ferritin was not seen in the axons of either myelinated or non-myelinated fibres, although Kasa (1968) has described ferritin distributed both free and in vesicles, in the axoplasm after local application of ferritin. It is interesting to note that Waggener et al. (1965) could find no axonal uptake in epineural non-myelinated nerve fibres following *in vivo* soaking with ferritin over a similar 120-min period. In the present study, ferritin has been observed, never in any great concentration, in the mesaxon gap of non-myelinated fibres, appearing within 30 min of administration, suggesting either that the ferritin micelle is too large, or that these channels offer some resistance to the passage of ferritin.

**DISCUSSION**

It has been demonstrated recently that La\(^{3+}\) can penetrate the myelin sheath of peripheral nerve fibres in the region of the intraperiod line (Revel & Hamilton, 1969). The extent to which the appearance of the La\(^{3+}\)-stained sheath reflects the *in vivo* unfixed state of myelin is uncertain; it has been proposed on the basis of such treatment that the intraperiod line is double in normal myelin (Revel & Hamilton, 1969), although previously such a condition has been reported only after manipulative or osmotic traumatization (Robertson, 1958), and consequently considered artifactual. In this laboratory, using conventional electron-microscope preparative techniques, the intraperiod line of uncomplicated internodal, and of paranodal myelin, characteristically appears single, while throughout the incisural spiral the intraperiod line is split to enclose an experimentally variable volume of extracellular space (Hall & Williams, 1970).

In considering the results of La\(^{3+}\) treatment certain points are relevant. Does La\(^{3+}\) act (i) by penetration of existing, i.e. *in vivo*, spaces; or (ii) by penetration of previously closed regions, by virtue either of the altered osmolarity of the solution or of some specific property of La\(^{3+}\); or (iii) by penetration of labile regions, i.e. regions which may be open or closed *in vivo*, depending upon the physicochemical state of the tissue at any time.
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In terms of ultrastructural interpretation, such points are dependent upon La\(^{3+}\) acting as (i) a true tracer, delineating extracellular space, (ii) a toxic agent staining artifactually dilated false space, or (iii) as an active agent capable of demonstrating physiologically labile in vivo states, possibly by the disruption of weak bonds. Previous studies have tended to assume that La\(^{3+}\) delineates true space (Doggenweiler & Frenk, 1965; Revel & Karnovsky, 1967; Revel & Hamilton, 1969; Hirano & Dembitzer, 1969). To satisfy the conditions for consideration as a true diffusion tracer, however, it is necessary that preferential adsorption, lipid solubility, alteration of the tracer molecule, or chemical binding, do not affect La\(^{3+}\) distribution, and further, that tissue damage does not occur. From work with La\(^{3+}\) on liver specimens, Matter et al. (1969) concluded that such conditions were not fulfilled.

Fixation in La\(^{3+}\)-containing fixatives does not produce a uniformly stained sheath; typically only the outer lamellae contain electron-dense deposits along the intraperiod lines. Such a pattern may be indicative of a progressively diminishing diffusion gradient for La\(^{3+}\), as described by Matter et al. (1969), in the bile canaliculi, or may reflect a limited penetration prior to fixation proper, bearing in mind the high molecular weight of the fixative. Neither of these alternatives is necessarily dependent upon normally patent intraperiod lines. Although most studies have been made on the assumption that La\(^{3+}\) is acting in a colloidal form, a recent investigation has questioned the validity of such an assumption: Matter et al. (1969) found that over the pH range 5–8–5, measurements of osmolarity and conductivity indicated that La\(^{3+}\) is monodisperse.

It has been suggested (Doggenweiler & Frenk, 1965) that La\(^{3+}\) acts as a ‘super Ca\(^{2+}\)’, staining intercellular gaps by differential binding to the outer stratum of the unit membrane, possibly as a result of the asymmetric distribution of membrane phospholipids (Rojas, 1967). La\(^{3+}\) has also been claimed to bind to protein (Srivastava, 1964) and to the COO\(^{-}\) groups of polysaccharides (de Jong, 1949). Membrane binding may explain why the width of the space occupied by La\(^{3+}\) exceeds the 2-nm intraperiod gap described after uranyl acetate treatment (Revel & Hamilton, 1969), although it could be that non-uniformity of La\(^{3+}\) deposition reflects adsorption to specific groups uncovered by altered surface configurations due to the presence of a trivalent ion.

Allowing for a certain amount of washing-out of a labile La\(^{3+}\) complex, during initial dehydration, common to all procedures and to all fibres, the presence of La\(^{3+}\) deposits, albeit intermittent, throughout every layer of the internodal sheath following experimental splitting of the intraperiod lines, contrasts sharply with the pattern of distribution obtained after straight fixation, or hypertonic pre-soaking.

Unlike La\(^{3+}\), ferritin may be administered in vivo and consequently the time available for penetration of the latter into the nerve fibre is considerably increased. Certain difficulties were found, however, in the successful penetration of the connective tissue sheaths, to allow presentation of the ferritin micelles directly to the fibres in vivo. In previous studies on the permeability of the sheaths of nerve fibre bundles, both perineurium (de No', 1950; Nordquist, 1952; Krnjevic, 1954), and epineurium (Causey & Palmer, 1953) have been implicated as the site of a diffusion barrier. More
recently, Shanthaveerappa & Bourne (1962), in a light-microscope analysis, claimed the perineurial 'epithelium' to be a metabolically active, continuous protoplasmic cell barrier. The poor uptake of ferritin into phagocytic vesicles in the outer layer of perineurial cells, and subsequent low concentration of the tracer in succeeding intercellular perineural spaces, described in this study, indicate that, for ferritin at least, the perineurium functions \textit{in vivo} as a diffusion barrier (compare Waggener \textit{et al.} 1965).

Soaking excised segments of nerve bundles in the ferritin solution for periods varying from 5 to 120 min resulted in the rapid non-specific deposition of ferritin throughout the perineurium, Schwann cell cytoplasm, and axoplasm of every fibre. Such a distribution pattern is probably attributable to (i) membrane permeability changes on excision, since intracellular limitation of the tracer to vesicles after subperineurial injection \textit{in vivo} reduces the possibility of toxic damage due to ferritin itself; and (ii) longitudinal diffusion both along the endoneurial spaces (Weiss, Wang, Taylor & Edds, 1945) and the axon (Perdrau, 1937; Ottoson, 1952).

Sub-perineurial injection of ferritin proved effective in surrounding fibres with tracer without causing any observed alterations in fibre morphology. After 120 min there was no generalized cytoplasmic uptake of ferritin, little phagocytic activity on the part of Schwann cells, no trans-myelin sheath passage of the tracer, and no ferritin was observed either free, or in vesicles, in the axoplasm of myelinated and non-myelinated fibres. No ferritin was present in either the cytoplasmic spiral or in the patent intraperiod line gap of the Schmidt–Lanterman incisures. The absence of ferritin from the incisural region may be due to several reasons: (i) the ferritin micelle is too large to enter the intraperiod line gap; (ii) transport of substances at the incisure may be highly selective (...'the localization of a barrier effect for ferritin (by the perineurium) does not indicate that small ions are impeded by the same mechanism...' (Waggener \textit{et al.} 1965)); (iii) incisures are not related to a neuron/satellite cell/extracellular space transport mechanism, and, as yet, autoradiographic studies do not support such a preferential system (Singer & Salpeter, 1966; Friede & Samorajski, 1969).

The distribution pattern of ferritin at the node of Ranvier closely resembled that of La\textsuperscript{3+}, although ferritin was not observed in paranodal myelin. There was presumably some barrier effect to ferritin at the node, in that the concentration of micelles in the gap substance was much less than that in the neighbouring extracellular space. Again, while such a pattern became established within 5 min, and appeared to remain unchanged after 120 min, it is not known whether there was any turnover of micelles between gap substance and extracellular space in that time. The rapid entry of ferritin into the gap substance is interesting in the light of work on Hale's iron staining at the node (Langley & Landon, 1968). These workers described characteristic nodal staining after 5 min, remaining unchanged after longer periods of incubation. Uptake of the ferric hydroxide-phosphate-containing ferritin micelle (FeOH)\textsubscript{8} (FeOPO\textsubscript{4}H\textsubscript{2}), (Farrant, 1954), at the node within a similar time interval, may be indicative of a specific reaction between the ferritin and some component of the gap substance. Saturation of this component could explain the failure to accumulate further ferritin.
within the gap substance with time. In one of the few light-microscope studies investigating the passage of particulates into nerve bundles and individual fibres, Ottoson (1952) observed a slow diffusion of methylene blue out of the node after intra-axonal injection, and no trans-myelin or trans-incisural passage after intra-axonal or sub-neurilemmal injection.

Recent work has indicated the presence of as yet unspecified routes for auxiliary metabolic support of the fibre, via extracellular space/satellite cell/neuron transport systems (Singer & Salpeter, 1966).

The present study has indicated striking differences in La$^{3+}$ 'uptake' by the intraperiod line of the normal compact myelin sheath and the intraperiod line of the incisure of Schmidt–Lanterman, these differences reflecting the single nature of the former, and the double nature of the latter, in conventionally processed tissue. It would therefore appear probable that La$^{3+}$ is delineating a true anatomical channel at the incisure. Failure of ferritin to penetrate this space may be mechanical or selective. However, it would seem from the experimentally induced shape changes in this region (Hall & Williams, 1970) that the incisural intraperiod line gap is open to the passage of water.

The apparent ease and rapidity with which both La$^{3+}$ and ferritin entered the nodal and paranodal regions may indeed reflect a true communication between the periaxonal, and the general, extracellular spaces. Alternatively, or in addition, it may reflect favourable sites for the specific uptake of these tracers, or artifactual spaces attributable to the experimental procedures, or to the disruptive effects of these substances. Although the existence of a channel open to the rapid flux of ions between the immediate nodal periaxonal space and the general extracellular space has been acknowledged for many years, this study has demonstrated the extension of this channel apparently to include paranodal periaxonal space. It is interesting that Hirano & Dembitzer (1969) describe similar distribution patterns for La$^{3+}$ at central nodes, and suggested that the periaxonal space was continuous with extracellular space, via spaces between several separate parallel bands, analogous to those described by Peters (1966). Additional evidence of communication channels between paranodal and nodal periaxonal spaces and extracellular space, has been provided using horseradish peroxidase (Hirano, Becker & Zimmerman, 1969) and microperoxidase (Feder, Reese & Brightman, 1969).

The results with La$^{3+}$ and ferritin distribution in relation to the uncomplicated internodal myelin sheath are equivocal: ferritin is not taken up, while La$^{3+}$ is, at least to some extent, and particularly after experimental splitting of the intraperiod lines. To what extent this pattern is related to the state of the sheath in vivo is not known. The ease with which the intraperiod line may be experimentally split may indicate a similar structural lability in vivo, although the staining may reflect a disruptive effect of La$^{3+}$, which is not shared by ferritin. If there is a degree of instability at the intraperiod line, La$^{3+}$ may indeed penetrate the potential extracellular space channel of the outer layers of the sheath, within the time limits imposed by the rapidity of onset of the effects of fixation, perhaps by linkage with polysaccharide groupings, or by adsorption to the polar ends of phospholipids. It is interesting that Brightman &
Reese (1969) failed to obtain La$^{3+}$ or peroxidase penetration of the normal myelin sheath in mouse and fish brain. This may reflect the effectiveness of junctional complexes in preventing La$^{3+}$ reaching the fibres in these situations.

With regard to ferritin, an interpretive problem arises. Clearly, the same pictorial evidence would be obtained if ferritin were unable to enter the occluded interspace of in vivo tight junctions—in this case, the myelin sheath—or if ferritin micelles were squeezed out from interspaces during the artifactual formation of a close apposition. From the experimental evidence described above, it would seem that the first of these is the more likely alternative.

In conclusion, it appears that the distribution of extracellular space within conventionally processed individual peripheral nerve fibres is minimal along the internodes, confined to a definite spiral channel at the Schmidt–Lanterman incisure, via the permanently patent incisural intraperiod line gap. A similar distribution of ferritin and La$^{3+}$ at the node of Ranvier has confirmed previous views on the existence of an extracellular space-periaxonal space channel at this point, and tracer distribution patterns have suggested an extension of this channel to include paranodal periaxonal space.

REFERENCES


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Figs. 1, 2. The typical distribution pattern of La^{3+} in uncomplicated internodal compact myelin. Note the single intraperiod line except where La^{3+} is deposited, and the absence of La^{3+} from the outermost lamellae, possibly due to washing-out during processing. In the stained areas there is an increase in the repeat distance of the myelin and the stained 'intraperiod line' appears trilaminar, with a central pale zone. \( \times 250000 \) and \( \times 150000 \), respectively.

Fig. 3. A small area of the incisure of Schmidt–Lanterman. The fibre had been soaked in 0.07 M NaCl prior to fixation. The intraperiod line gap is filled with La^{3+} deposit, yet the peri-incisural dilated space is devoid of tracer. \( \times 250000 \).

Fig. 4. A fibre soaked in 0.07 M NaCl for 15 min prior to fixation. The intraperiod line is double throughout the sheath, and La^{3+} deposition, while intermittent, occurs in every layer. \( \times 200000 \).
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Figs. 5, 6. La$^{3+}$ distribution at nodes of Ranvier in fibres without anisotonic pre-soaking. The paranodal myelin shows a greater deposition of La$^{3+}$ than internodal myelin (compare with Fig. 1). The gap substance has an increased granularity, but the greatest deposition is around the terminal loops of myelin, and along the Schwann cell aspect of the axolemma at the node and paranode. In Fig. 5 the La$^{3+}$ distribution is periodic at the point indicated by the asterisk (*). Fig. 5, × 60,000; Fig. 6, × 21,200.

Fig. 7. Peripheral nerve fibres 10 min after sub-perineurial injection of ferritin. Note the distribution of tracer in relation to the terminal loops of myelin at the node of Ranvier. No tracer is evident in the compact myelin or axoplasm. × 100,000.
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Figs. 8, 9. Incisures of Schmidt-Lanterman in fibres fixed as for Figs. 5, 6, without prior soaking in anisotonic saline. There is La³⁺ deposition throughout the incisural intraperiod line gap. Fig. 8, × 70 000; Fig. 9, × 250 000.

Fig. 10. Incisural region 15 min after sub-perineurial injection of ferritin. Micelles are present in the basement membrane, and in 3 invaginations of the Schwann cell plasma membrane. No free tracer is present either in the cytoplasm or in the incisure. Unstained, × 18 000.

Fig. 11. This shows the uniform distribution of ferritin within axoplasm and gap substance after 60 min soaking in vitro. Unstained, × 21 200.

Fig. 12. This is of a similar field to that shown in Fig. 11, 120 min after sub-perineurial injection of ferritin. No axoplasmic uptake is evident. Unstained, × 32 000.