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# The role of non-resident cells in Wallerian degeneration

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## Summary

Wallerian degeneration was studied in the phrenic or sciatic nerves of mice following transplantation into Millipore diffusion chambers of 0.22 μm pore size which were implanted in the peritoneal cavity and kept for up to eight weeks. This method positively eliminates the access of nonresident cells to the tissue, at the same time providing proper conditions for tissue survival. Such nerves showed no proliferation of Schwann cells and no evidence for their active role in the removal or digestion of myelin. Schwann cells rejected their sheaths and the latter persisted for weeks, leading either to sheath distension (the sheath becoming wider and thinner) or to collapse (the sheath becoming thicker, collapsing upon the empty axis cylinder). The outer envelope of Schwann cytoplasm separated into pseudopodia rich in microtubules. Sheath rejection led to a slow decay of the myelin in the absence of active phagocytosis. There was profuse fibroblastic proliferation from the epineurium and perineurium, from which cells migrated into the chambers developing fatty change. No evidence was found to link the fatty change in fibroblasts to sheath decay.

Diffusion chambers of 5.0 μm pore size were invaded by leukocytes and monocytes. Nerves kept in such chambers showed active phagocytosis of myelin leading to its removal, similar to Wallerian degeneration *in situ*. Phagocytes were shown to attack selectively the rejected myelin sheaths, distinguishing the latter from the surviving Schwann cells, even though both structures derive from the same cell.

The activity of phagocytes in digesting myelin was mediated by a signal which diminished in intensity with time; there was very little active phagocytosis of myelin in nerves that had been predegenerated in 0.22 μm pore chambers. Various modifications of the experiment, including studies with co-cultured peritoneal macrophages or bone marrow, indicate a need for additional activating factors to induce myelin phagocytosis.

## Introduction

There is still controversy as to the type of cells involved in the removal of lipids during Wallerian degeneration. One widely held opinion is that the activities of the Schwann cells in producing and maintaining the myelin sheath are directly controlled by the axon. The absence of a corresponding signal from the axon is thought to trigger off a process of

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involution, whereby the Schwann cell digests and eventually removes its myelin sheath (Weiss & Wang, 1945; Guth, 1956; Wechsler & Hager, 1962; Nathaniel & Pease, 1963; Satinsky *et al.*, 1964; Schlaepfer & Hager, 1964; Blümcke & Niedorf, 1966; Luse, 1968; Cravioto, 1969; and many others). Myelin removal is thought to be accompanied by proliferation of Schwann cells, supplying a pool of uncommitted cells available to receive the regenerating nerve fibres (Causey, 1960). An opposing concept can be traced back as far as Ranvier (1878) and Cajal (1928), who succinctly stated that the removal of the myelin sheath during Wallerian degeneration is accomplished by haematogenous cells invading the fibres. This point was accepted by Nageotte (1932), Speidel (1935) and Young (1945), and was taken up again more recently by Olsson & Sjöstrand (1969), Liu (1973, 1974) and Schubert & Friede (1981).

When reviewing the vast literature on this subject, one becomes impressed by the scarcity of reliable markers of cell lineage (Hall, 1978). The basement membrane of the nerve fibre (the 'neural tube') is often used as an index of the origin of cells; an increase in intratubal cells is thought to indicate Schwann cell proliferation, while increase in extratubal cells is attributed to mesenchymal elements. This criterion is quite unreliable. Extratubal migratory cells may be seen entering the basement membrane of damaged fibres (Schubert & Friede, 1981); conversely, proliferation of Schwann cells is claimed as the source of migratory Schwann cells leaving the neural tube (Abercrombie & Johnson, 1942). The basement membrane itself is also an inconsistent marker, as Schwann cells acquire basement membranes only upon contact with axons (Bunge *et al.*, 1982). There are few experiments for which the claim can be made that specific markers of cell lineage were used. Prelabelling of Schwann cell populations in developing nerves (Asbury, 1970, 1975) showed that the pre-existing cell population does not contribute substantially to the increase in cells during Wallerian degeneration. However, a moot point in using tritiated thymidine for assessing Schwann cell proliferation during development or during Wallerian degeneration (Leblond *et al.*, 1959; Asbury, 1967; Friede & Samorajski, 1968; Olsson & Sjöstrand, 1969) is the relatively late developmental increase in endoneurial fibroblasts (Mustafa & Gamble, 1978) and the unresolved question of concurrent proliferation of fibroblasts and Schwann cells.

In the present paper, we report a new approach for studying Wallerian degeneration using nerves enclosed in diffusion chambers. Chambers with membranes of 0.22 $\mu$m pore size permit study of the course of Wallerian degeneration under conditions which rigorously exclude the entrance of nonresident cells into the tissue. Chambers with membranes of 5.0 $\mu$m pore size, however, admit cells.

## Materials and methods

Phrenic or sciatic nerves and/or fine strips of muscles were obtained from mice of either sex from different strains, including C57BL, NMRI, Swiss and Beige C57BL bg/bg (Chediak-Higashi). The latter was used in some experiments to obtain additional information using the giant lysosomes as strain-specific markers; however, these markers were less specific than anticipated. Animals were

decapitated under deep ether anaesthesia. Skin and abdominal contents were removed. In order to obtain sciatic nerves, the pelvis and lower extremities were severed from the rest of the body. The specimen was washed for 3 min in 80% alcohol; nerve samples, 3–5 mm in length, were dissected and washed in phosphate-buffered saline (PBS), pH 7.4, containing 200 $\mu$g ml$^{-1}$ Gentamicin.

*Preparation of Millipore diffusion chambers*

Millipore diffusion chambers of 0.22 ± 0.02 $\mu$m or 5.0 ± 1.2 $\mu$m pore size were sterilized, with their bottom membrane pasted, by overnight exposure at 80° C dry heat. Sterile tissue specimens were placed into the chambers and excess fluid was drained through the bottom membranes. The top membranes of the chambers were then pasted and the chamber was implanted into the peritoneal cavity of the host animal. One or two chambers were implanted.

*Dialysis tubes*

Dialysis tubes of 2 nm pore size were obtained from Serva (Feinbiochemische GmbH, D-6900 Heidelberg 1, FRG). The tubes were stored in 0.1% sodium azide and were sterilized in PBS in the autoclave. Segments of nerves or small strips of muscle fibres were placed into the tubes, and the latter were closed by three tight ligatures at each end. Three tubes were implanted into the peritoneal cavity of each host mouse.

*Nerves predegenerated* in situ

Sciatic nerves were cut in mice deeply anaesthetized with Rompun-Ketanest, and the animals were killed 50 h later. Predegenerated or contralateral control nerves were placed into 0.22 $\mu$m pore chambers and implanted into the peritoneal cavity, where they remained for four weeks.

*Injection of myelin preparation*

Peripheral nerve tissue of 200 mg wet weight was obtained from 15 C57BL mice and was frozen in liquid nitrogen. Preparation of purified myelin was obtained with the courtesy of Dr Waehneldt, Max-Planck-Institut für experimentelle Medizin, Göttingen. The myelin fraction contained 13.75 $\mu$mol $\mu$l$^{-1}$ myelin in 0.9% saline, and was stored at −70° C. For injections, solutions were brought to room temperature and were diluted 1:10 in PBS containing Gentamicin. Three injections of 23 $\mu$l of the diluted myelin preparation, giving a total of 103.1 $\mu$g myelin, were injected into the nerves before they were placed into 0.22 $\mu$m pore chambers. The purpose of this experiment was that of 'loading' the nerve with substrate for phagocytosis. The *in situ* effect of such injections was studied by Schubert & Friede (1981).

*Co-culture with peritoneal macrophages or serum monocytes*

Peritoneal macrophages were obtained by peritoneal lavage from Chediak-Higashi mice after the method of Cohn & Benson (1965) and Parwaresch *et al.* (1981), using PBS as lavage fluid. The suspensions were centrifuged at 600 rpm in glass tubes, supernatants were decanted and the cells were washed once more for 3 min in PBS. The final fluid volume of 1 ml was gently shaken, and a 100 $\mu$l aliquot was placed in each 0.22 $\mu$m pore chamber along with peripheral nerve tissue and/or fine strips of muscles.

Blood monocytes were obtained from decapitated Chediak-Higashi mice. Blood was collected in heparinized glass tubes. After centrifugation at 600 rpm for 10 min, the thin layer of leukocytes with some admixed serum and erythrocytes was aspirated and diluted 1:5 in PBS containing Gentamicin. Following centrifugation for 3 min, the supernatant was decanted; 100 $\mu$l aliquots of

the remaining fluid of 1 ml were added to peripheral nerves or to fine strips of muscle in the 0.22 μm pore chambers. Cell counts were done using a Fuchs–Rosenthal chamber.

In another series of experiments, small sterile specimens of bone marrow, splenic, lung or lymphatic tissue were obtained from Chediak-Higashi donors and were placed in close proximity to the nerves in 0.22 μm pore chambers.

*Opening of diffusion chambers of 0.22 μm pore size after four weeks*

Sciatic nerves from Swiss mice were implanted for 28 days into autologous donor mice using 0.22 μm pore chambers. After 28 days, the chambers were removed under deep anaesthesia, the top membranes were cut and the contents of the chambers were transferred into 5.0 μm pore chambers, which were reimplanted into the same donor mice. The latter were killed after 3, 14 and 28 days, and the chambers were removed.

*Specimen preparation*

The top membranes of the diffusion chambers were removed and tissue adhering to the bottom membrane was fixed in 3% glutaraldehyde in 0.067 M phosphate buffer, pH 7.4. Tissues were postfixed in 1% osmium tetroxide in the same buffer for 2 h and embedded in Araldite after dehydration in graded concentrations of alcohol passing through 2,4-epoxypropane. Semithin sections (1 μm) were stained with toluidine blue 0. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined in a Zeiss EM 10B electron microscope.

Computer-assisted measurements of fibres were made with a Kontron Videoplan at a final magnification of × 3400. Inner and outer surfaces of the sheaths were traced with a cursor. Only solid sheaths were measured, thus avoiding error from splitting or separation of myelin lamellae. Special computer software determined inner and outer circumferences and calculated the inner and outer diameters for a fibre with a circular profile. By mathematically 'expanding' the sheaths to a full circle, one eliminated scatter from variation in shrinkage and documented, at the same time, exactly to what degree shrinkage occurred. This method enables detection of changes in the thickness of the sheath compared to the size of the axis cylinder.

For point sampling, a Zeiss Kpl ocular × 8 with 25 points was used, at a final magnification of × 800 oil immersion, and 40 fields were counted per nerve. Three nerves were counted for each experiment. Data are expressed as means ± S.E.M.; to obtain S.D., the S.E.M. should be multiplied by a factor of 1.8.

## Results

### CHANGES IN NERVES DEGENERATING IN DIFFUSION CHAMBERS OF 0.22 μm PORE SIZE

The mouse phrenic nerve was used in initial experiments because of its small calibre; it contains approximately 250 myelinated fibres, most of which are thick and of uniform size. A sample of each nerve was taken before placing the tissue in the diffusion chamber, permitting direct assessment of changes in the number of nerve fibres and also of changes in fibre calibre. Sciatic nerves were used in later experiments.

Nerves were kept in Millipore diffusion chambers of 0.22 μm pore size for 1, 2, 4, 6 and 8 weeks, with at least four samples for each period. Most specimens survived without difficulty up to the end of the first month. Afterwards, necrosis was found occasionally in a chamber; however, this mainly occurred when we initially transplanted

more than two chambers into one host animal, where they tended to form stacks with necrosis developing in the chamber sandwiched in the middle. The development of dense peritoneal fibrosis was also an adverse factor to survival. Generally, chambers contained viable nerve tissue, as verified by the intact fine structure of the Schwann cells, the profuse proliferation of fibroblasts and the active regeneration of muscle tissues described below.

*Absence of Schwann cell proliferation*

There was no noticeable increase in the density of nuclei in nerves kept in diffusion chambers for up to 8 weeks. This was much in contrast to the marked increase in cell density, which normally accompanies Wallerian degeneration from about the third day onwards (Abercrombie & Johnson, 1946).

Using a point sampling method, there was no change in nuclear density between normal nerves ($0.58 \pm 0.07$ nuclei per field) and nerves kept in chambers for 7 days ($0.55 \pm 0.04$) or 28 days ($0.47 \pm 0.1$), compared to a ten-fold increase ($5.0 \pm 0.07$) within 7 days for nerves undergoing Wallerian degeneration *in situ*. Proliferative changes were confined to fibroblasts, which migrated into the chamber as described below.

*Collapse and inflation of myelin sheaths*

Semithin sections of transplanted nerves showed unaltered or slightly shrunken profiles of myelinated fibres during the first few weeks after transplantation (Fig. 1). Concurrently, there was an increasing number of distorted sheaths, showing either collapse or distension of the profile of the fibre. Collapsed profiles were always in the majority; they had a smaller diameter than the fibres in the tissue sample taken from the nerve before transplantation, and their sheaths were thicker than normal. Such sheaths were often flattened, or showed irregular invaginations. An extreme degree of collapse consisted of concentric myelin figures with little or no central lumen. Distended fibres had a larger diameter than the largest fibres found in the nerve before transplantation, and their sheaths were correspondingly thinner than normal. These changes were evident from computer-assisted measurements of fibre calibre and the thickness of the sheath as expressed by the quotient axon diameter/fibre diameter ($g$-ratio). The data shown in Fig. 2 are recalculated for circular fibre profiles, thus showing changes in sheath thickness independently of the degree of collapse of the axis cylinder.

*Decay of sheaths*

The number of profiles of myelinated fibres did not change during the first 2 weeks after transplantation. With longer survival, there were fewer myelin profiles than seen in the samples taken before transplantation, indicating a slow, progressive loss of myelin sheaths. Such decay, however, occurred in the absence of phagocytosis and it appeared to involve a process akin to autolysis. We did not observe focal clusters of cellular debris in the distribution pattern previously occupied by fibre profiles.

*Fine structure of the axon*

Axoplasm had disappeared 1 week after transplantation; residues of axoplasmic organelles were identifiable. The optically empty space inside the sheath was occupied, in part, by enormous tongues of Schwann cell cytoplasm, projecting from the inner mesaxonal lip and containing mitochondria and electron-dense, floccular material (Fig. 3). Similar mesaxonal tongues may be found during Wallerian degeneration *in situ*, although never to the extent seen here. These tongues of Schwann cell cytoplasm were conspicuous after 7 days. Degenerative changes developed after 2 weeks with disorganization of mitochondria and occasional cell membrane defects. The tongues had for the most part disappeared by 6 to 8 weeks. All nonmyelinated axons had disappeared 1 week after transplantation.

*Fine structure of the myelin sheath*

Myelin sheaths retained their shape and fine structure during the first week after transplantation; the shape of the nodes of Ranvier (Fig. 4) and clefts of Schmidt-Lanterman were still present. Longitudinal sections showed beginning fragmentation of the internodes into shorter segments of sheath, corresponding to the formation of ovoids during Wallerian degeneration *in situ*. The presence of this fragmentation, however, did not manifest itself in the sheath profiles seen on cross-sections. Empty sheaths filled their envelope of basement membrane without evidence of retraction (Fig. 5). After the first week, there was some splitting of myelin lamellae at the minor dense lines. The major dense line remained intact, even when

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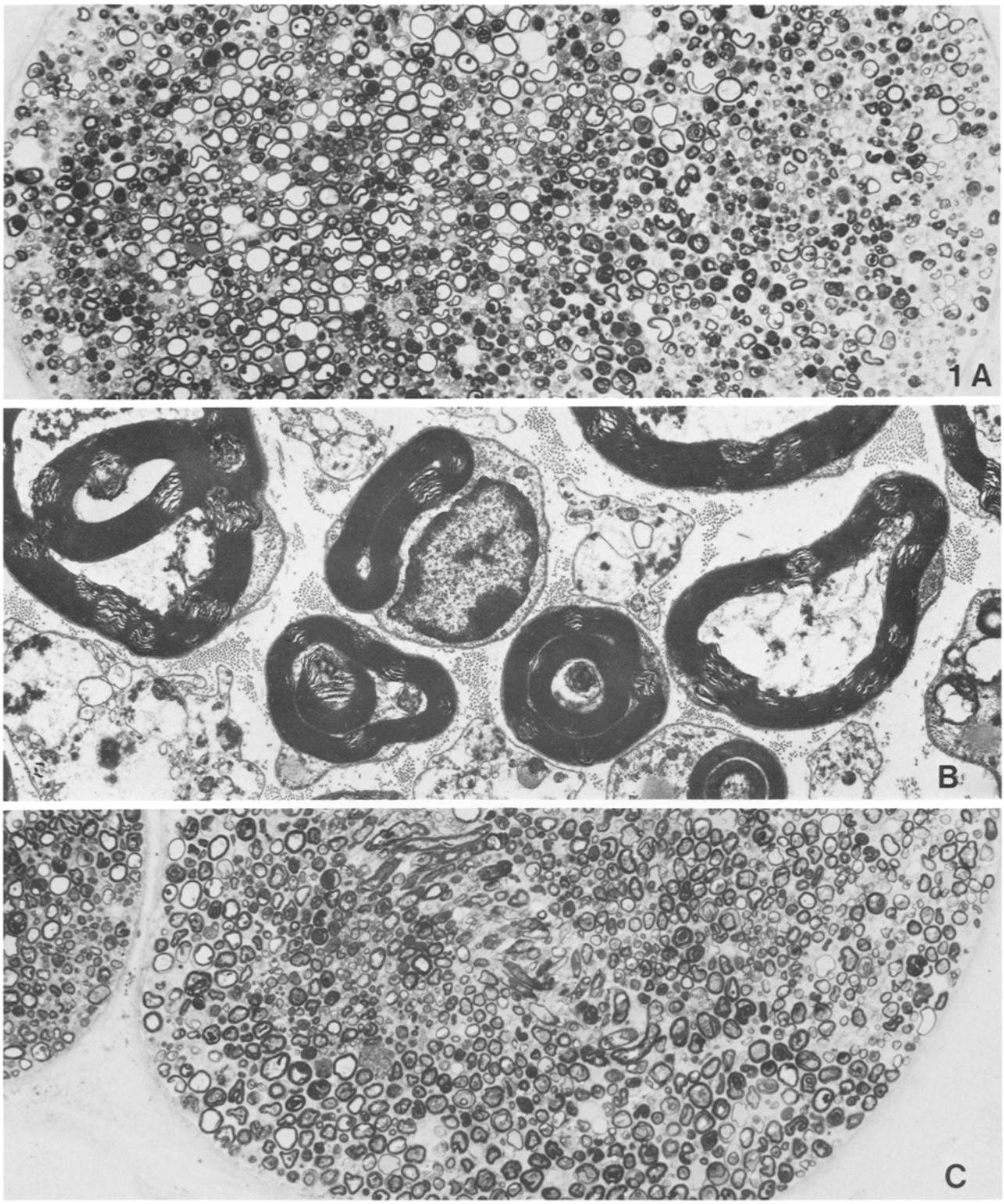
**Fig. 1.** (A) This sciatic nerve was kept in a 0.22 μm pore chamber for 1 week. There is no breakdown of myelin sheaths, and no increase in cell density. Collapsed thickened sheaths and distended thin sheaths are visible. × 425. (B) Axoplasm has largely disappeared after 1 week. Most of the sheaths shown are collapsed. The perikaryon of normal Schwann cell is visible in the centre. Nonmyelinated fibres have disappeared (left lower corner). × 7700. (C) After 4 weeks in the chamber, there is still hardly any loss of myelin sheaths. Cell proliferation is absent, and there are no signs of myelin phagocytosis. × 425.

**Fig. 2.** Computer prints of axon calibre and sheath thickness for normal phrenic nerves (A) in comparison to those for phrenic nerves kept in a 0.22 μm pore chamber for (B) 1, (C) 2 and (D) 4 weeks. Sheath thickness is shown by the quotient axon diameter/fibre diameter ($g$-ratio). Calibres refer to the diameter of a circle having the same circumference as the inner perimeter of the sheath. Fibres kept in the chambers show a shift to the right and up, indicating larger than normal axis cylinders with thinner sheaths, and also a shift to the left and down, indicating shrunken axis cylinders with thickened sheaths. These changes, developing in the absence of axons, indicate passive adjustment of the rejected myelin sheaths.

**Fig. 3.** Sciatic nerve kept for 1 week in a 0.22 μm pore chamber. A distended tongue of Schwann cell cytoplasm protrudes from the inner surface of the sheath into the optically empty axon space. There are no residua of axoplasm. × 25 600.

**Fig. 4.** Longitudinal section of a nerve fibre kept for 1 week in a 2 nm dialysis tube. Axoplasm has disappeared, but the sheath structure of the node of Ranvier is still preserved. × 13 700.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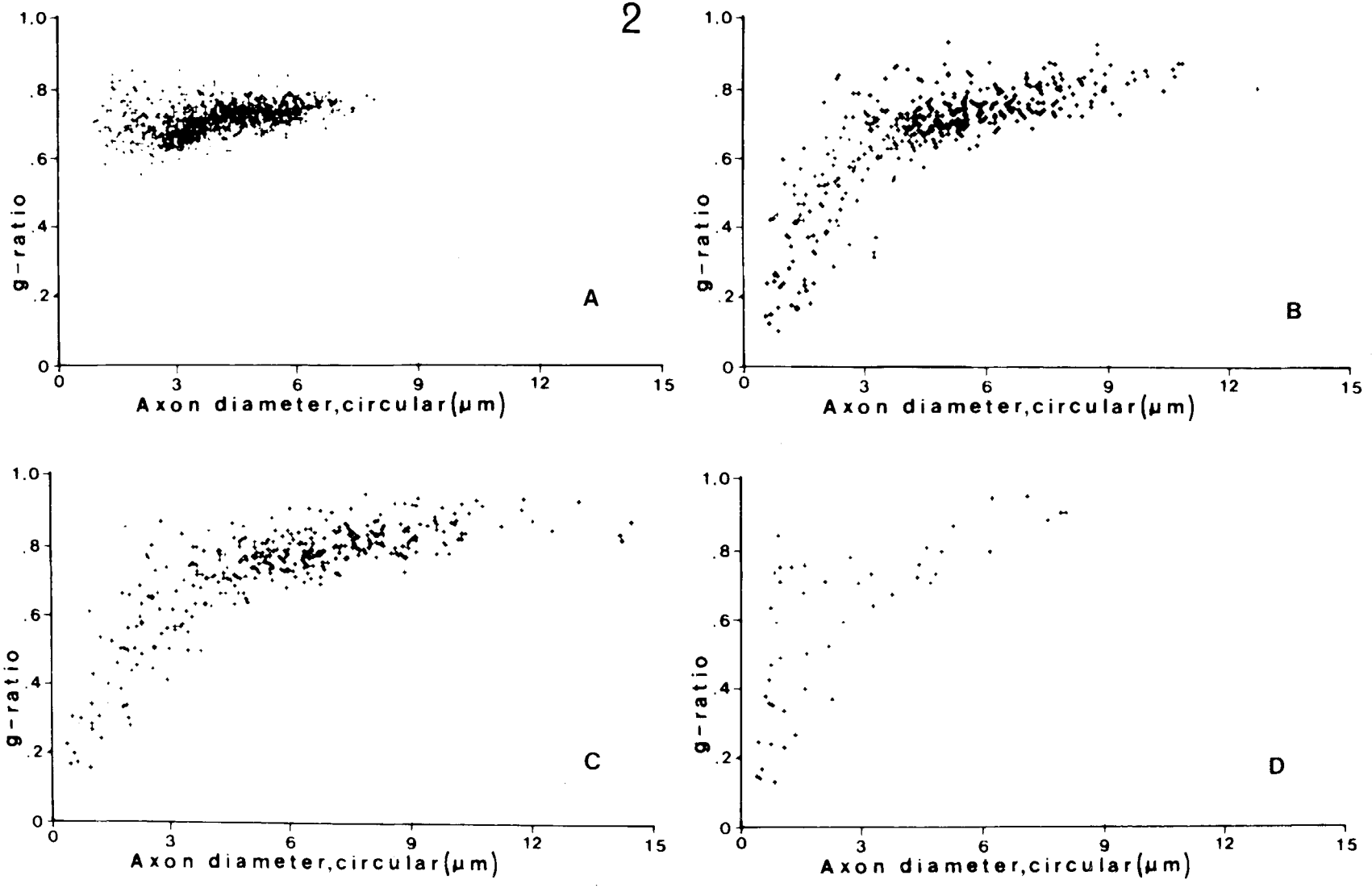
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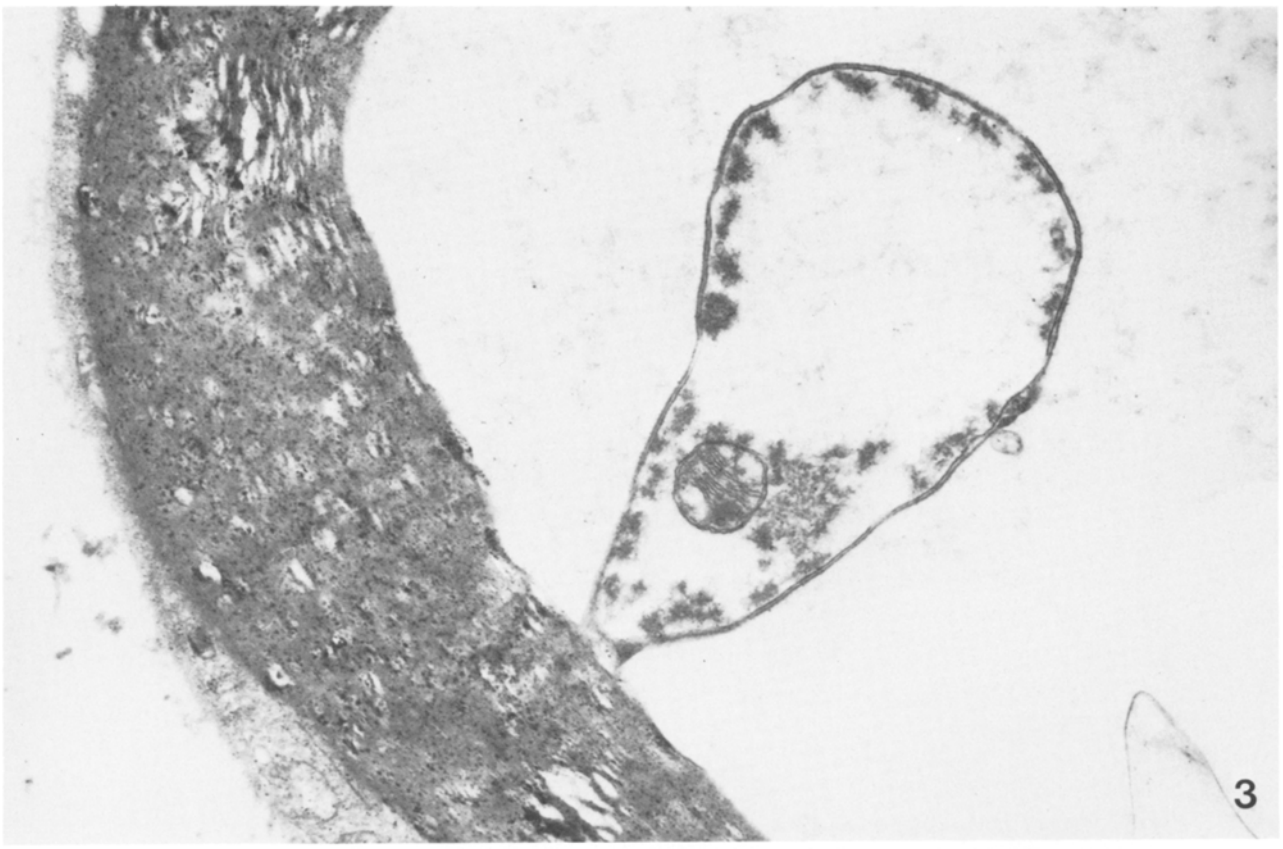
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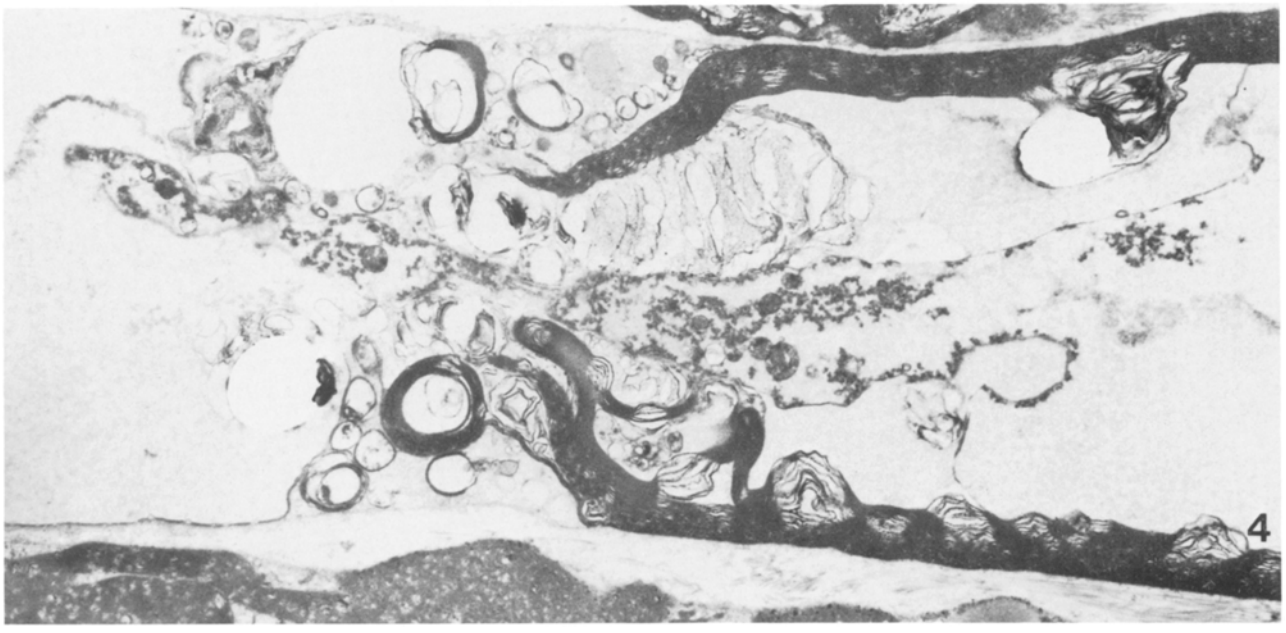
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subsequent fragmentation of the sheaths developed. Collapse of fibres caused myelin profiles to become less regular. Some sheaths separated into multiple concentric rings (Fig. 6). A thin outer shell of myelin persisted occasionally at the basement membrane, while the inner portions of the sheaths loosened up, collapsing upon the centre of the fibre. Isolated fragments of myelin were rarely seen after the first week. Their number increased thereafter, forming deposits between the main bulk of the sheath and its enveloping basal membrane (Fig. 5). Myelin did not spill beyond the latter. Collapse and fragmentation of sheaths were usually accompanied by sheath rejection, as described next.

*Rejection of sheaths*

A slow progress of sheath rejection was evident from the first week onwards. Initially, most fibres still showed an outer layer of Schwann cell cytoplasm encompassing the sheath. Rejection led to the separation of this outer cylinder of Schwann cell cytoplasm from the sheaths, with no discernible mesaxons (Fig. 7); this occurred approximately concurrent with the necrosis of the inner tongues of cytoplasm. The outer cylinder of cytoplasm subsequently broke into single or multiple profiles of processes or thin pseudopodia projecting between the decaying sheaths and the basement membrane (Figs. 5, 6).

*Changes in the Schwann cells*

Nuclei of the Schwann cells had marginal heterochromatin, abundant nuclear pores and, occasionally, prominent nucleoli (Fig. 7). After more than 4 weeks, nuclear profiles tended to become folded, with deep invaginations of the nuclear membrane. Relatively few mitochondria were seen in the perikarya, and there was sparse rough endoplasmic reticulum and a few fat droplets. There were also mitochondria and branching tubules of smooth endoplasmic reticulum in the Schwann cell processes (Fig. 6). The most striking difference between Schwann cell perikarya and processes was a profusion of microtubules in the latter, nearly all parallel to the long axis of the fibre (Fig. 5). The membranes of the processes showed subplasmalemmal density; pinocytosis was rare, and junctions between processes were seldom. Some processes tended to form new (Figs 5, 6).

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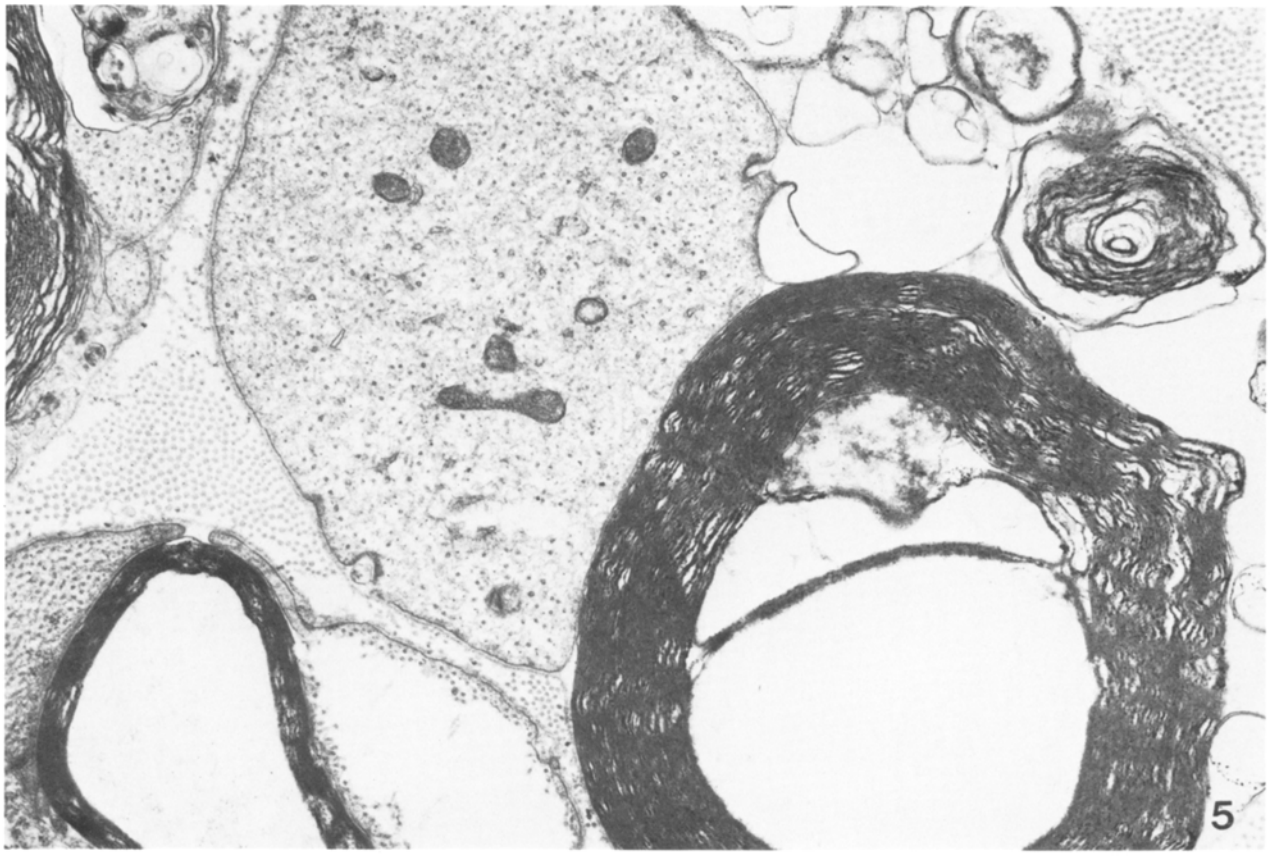
**Fig. 5.** Sciatic nerve kept for 4 weeks in a 0.22 $\mu$m pore chamber. An empty myelin sheath is rejected from its Schwann cell. The sheath has shrunken upon the empty axon space. The Schwann cell process contains mitochondria and many microtubules running parallel to the axis of the fibre. Some myelin debris is visible within the basement membrane next to the empty sheath and the Schwann cell process. × 19 400.

**Fig. 6.** Sciatic nerve kept for 4 weeks in a 0.22 $\mu$m pore chamber. This rejected sheath has collapsed to form concentric rings. Sheath and Schwann cell processes are enveloped by their common basement membrane. No mesaxon was found between the sheath and the Schwann cell cytoplasm. The latter contains many mitochondria and much rough endoplasmic reticulum as well as a Golgi apparatus. There is no evidence of myelin phagocytosis. × 25 600.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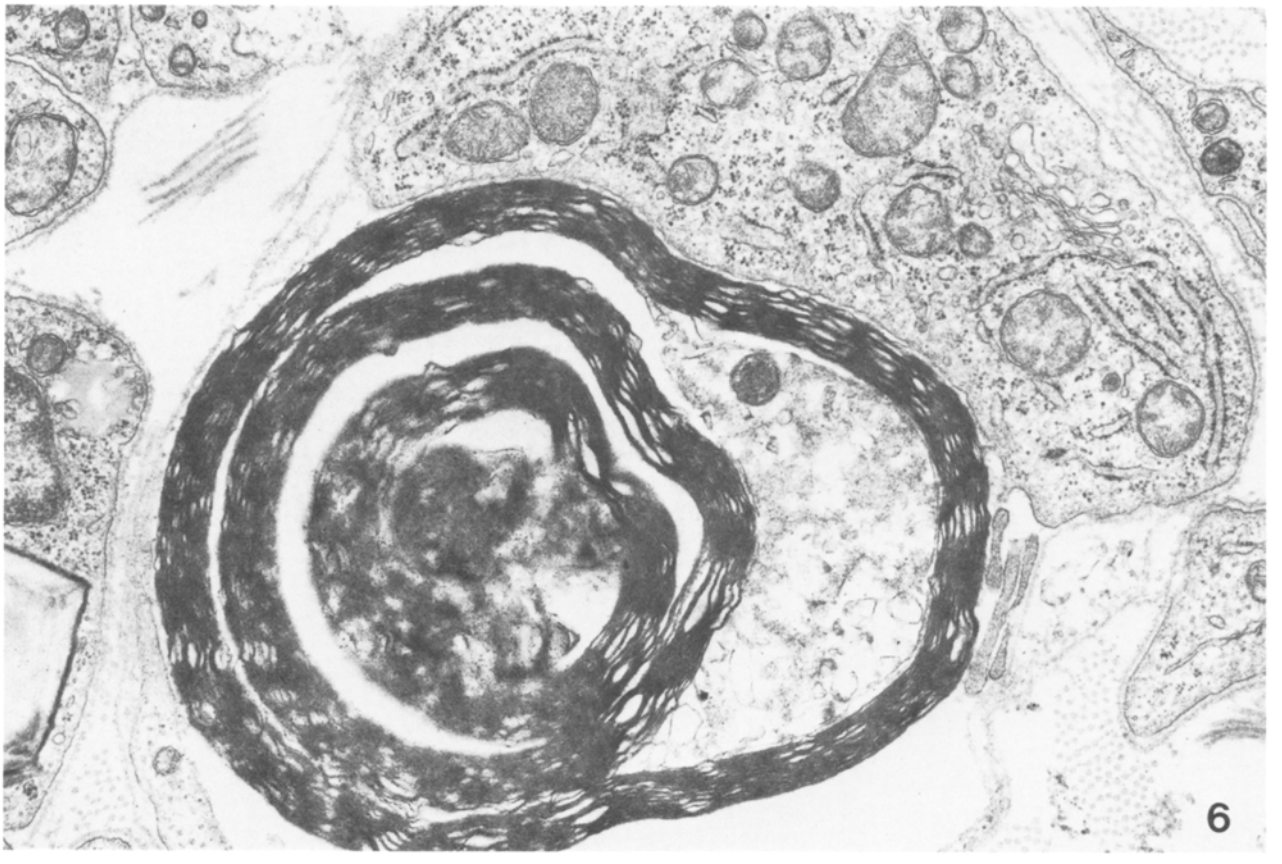
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basement membranes at sites where they had neither contact with the original basement membrane of the fibre nor with the rejected myelin sheath.

No evidence of proliferation of Schwann cells was found. Also, neither perikarya nor processes of Schwann cells were enriched with lysosomes, nor did they show any evidence of myelin phagocytosis. Fragments of myelin were situated occasionally in niches of Schwann cell cytoplasm underneath the basement membrane, though without evidence of incorporation by the Schwann cell (Fig. 5). The occasional occurrence of a few cytoplasmic fat droplets was not apparently related to changes in the myelin sheaths.

*Endoneurial fibroblasts*

The endoneurial connective tissue remained passive throughout the period of observation, although one had the impression of some increase in endoneurial collagen. Endoneurial fibroblasts showed a progressive accumulation of fat droplets. The number of cells did not increase appreciably, except in the superficial portion of the nerve where the arrangement of cells suggested inward migration of perineurial cells.

*Changes in the perineurium*

There was a progressive outgrowth of fibroblasts from the epineurium and perineurium, similar to that seen in cultured nerves (Abercrombie & Johnson, 1942). The entire diffusion chambers were eventually filled with a fine gel of tissue. This outgrowth developed at the expense of the perineurium, which was gradually reduced from its normal thickness of 10 to 15 layers of cells to about 6 to 8 layers after 4 weeks and 2 to 4 layers (or only a single one) after 8 weeks. There was increasing fragmentation of the perineurial basement membrane. Emigrating perineurial cells were initially identified by their abundant pinocytosis, which could be used as a marker. Pinocytosis was diminished in cells found at a greater distance from the transplant, and these cells had the features of fibroblasts. After 2 to 4 weeks, the cells contained increasing amounts of glycogen and fat droplets in their cytoplasm; the latter, in particular, were so prominent that they suggested themselves to be fibroblast markers (Fig. 8). None of these cells showed an increase in lysosomes. There was no phagocytosis of myelin debris nor were there transitional stages of the transformation of myelin debris into fat. Newly formed capillary-like structures were also found in this tissue (Fig. 15). These consisted of tubes formed by endothelial cells linked by a tight junction and encompassed by a thin

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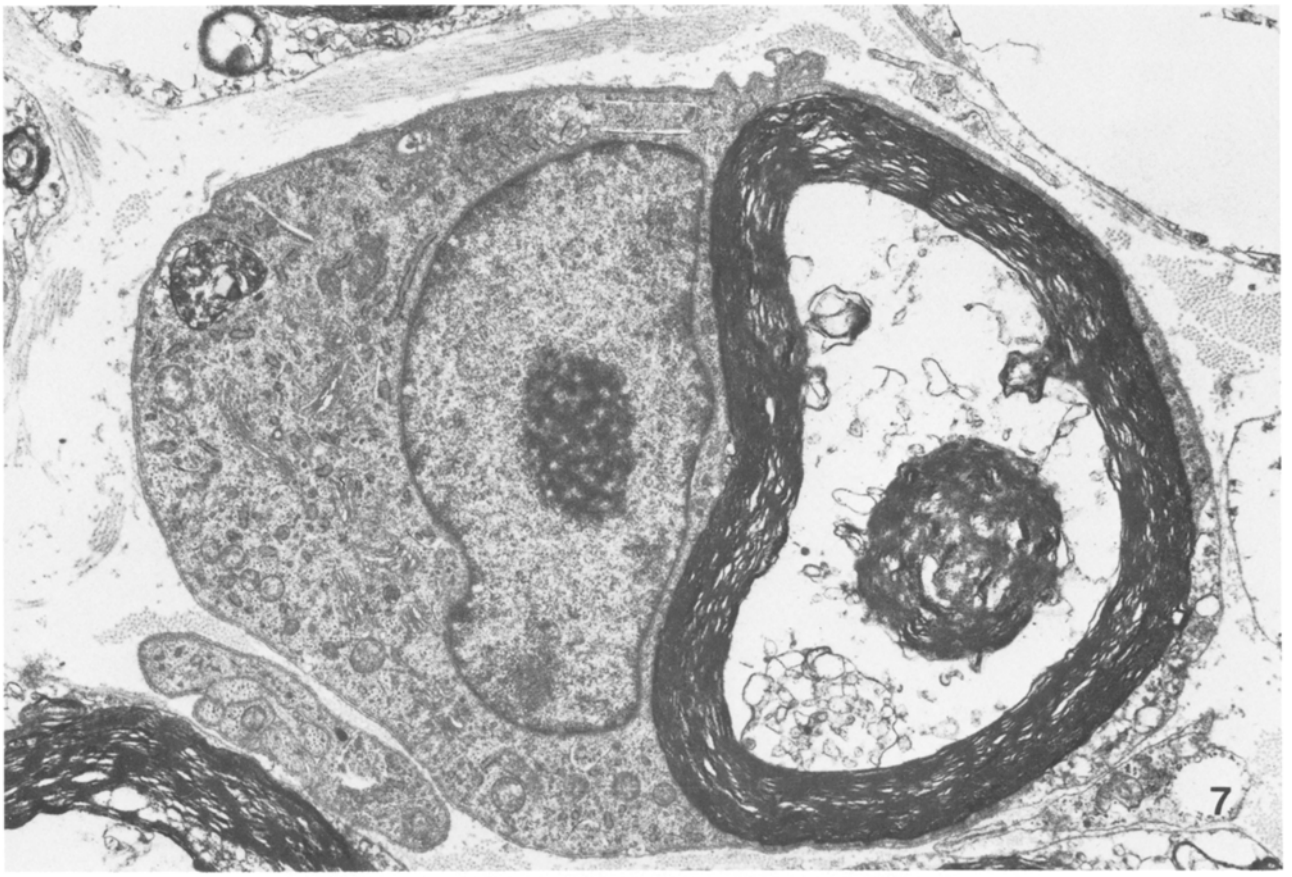
**Fig. 7.** Sciatic nerve kept for 5 weeks in a 0.22 μm pore chamber. The perikaryon of the Schwann cell shows well-preserved nuclear and cytoplasmic structures. The axon space contains myelin debris. The sheath is rejected, but the Schwann cell still embraces four-fifths of the sheath with a thin layer of cytoplasm. × 9900.

**Fig. 8.** Tissue adjacent to a sciatic nerve kept for 4 weeks in a 0.22 μm pore chamber. A profuse proliferation of fibroblasts, originating from the epineurium and perineurium, had filled the entire chamber. Accumulation of fat droplets is common in these cells, but it is not associated with evidence of uptake or digestion of myelin. × 9900.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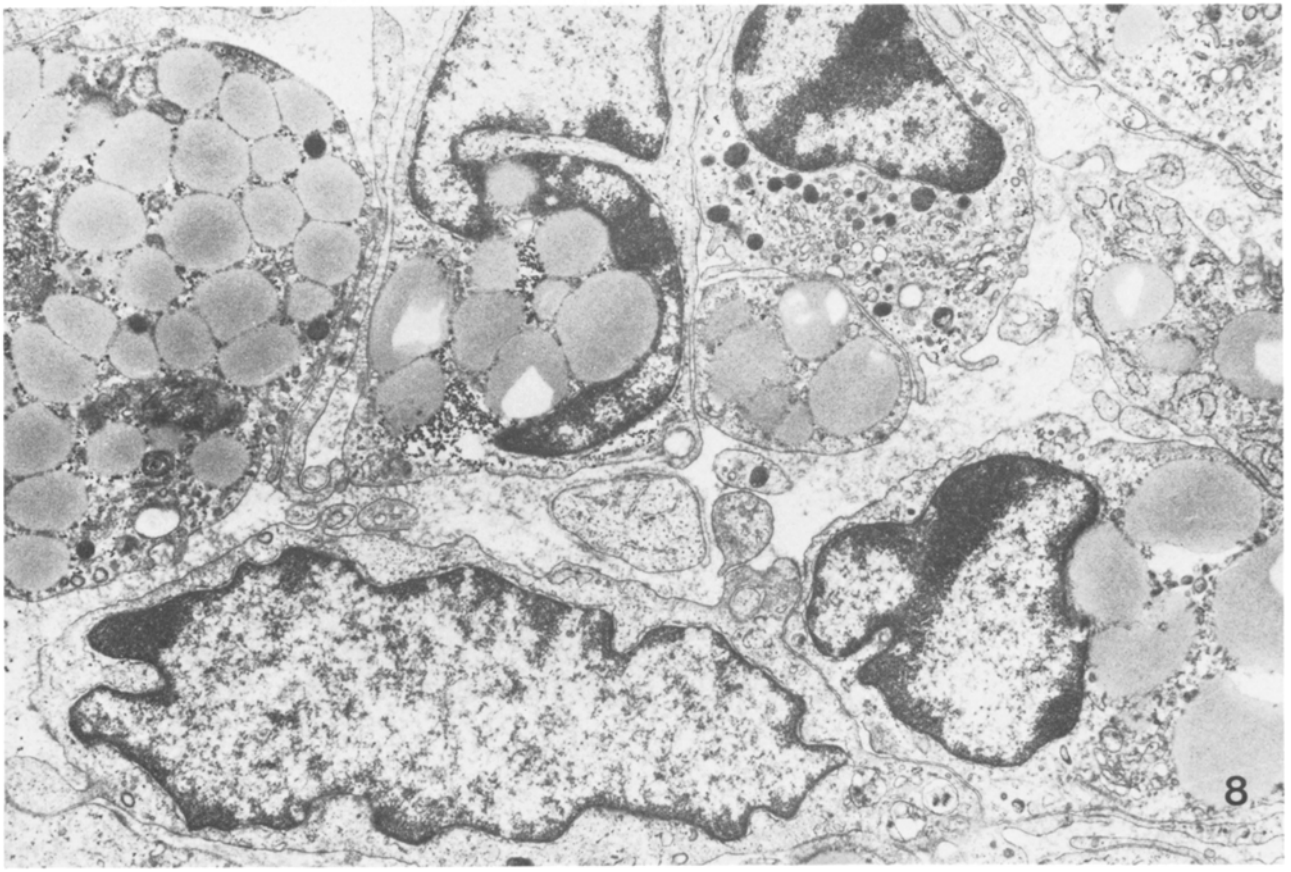
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basement membrane. They were embedded into a matrix of collagen fibres. We attributed these to regenerative changes in fragments of capillaries transplanted with the epineurium.

*Changes in muscle tissue*

Small fragments of skeletal muscle transplanted alone or together with some of the nerves furnished additional evidence of the conditions for survival. The presence of muscle tissue in chambers containing nerves had no effect whatsoever on the changes in the nerves. Cross-striated muscle fibres had undergone necrosis after 1 week, but their clumped mitochondria were still discernible for up to 4 weeks; this delayed resorption differed from the *in vivo* removal of sarcoplasmic debris within 48 h. Proliferation of satellite cells ensued. After 7 days, these cells already showed minute bundles of cytoplasmic filaments in their small perikarya. After 4 weeks, there were much larger bundles of myofilaments with distinct Z-discs and mitochondria, and even an occasional 'mature' muscle cell with marginal nuclei and normal sarcoplasmic structures was seen. In a separate series of experiments, we could show the proliferation and growth of transplanted embryonal spinal cord and ganglia, including the formation of synapses and the myelination of axons. All of these observations were considered an index of the quality of tissue survival in the diffusion chamber.

## CELL INVASION IN MILLIPORE DIFFUSION CHAMBERS OF 5.0 μm PORE SIZE

Diffusion chambers of 5.0 μm pore size containing autologous peripheral nerves were invaded by cells from the earliest observation at 3 days onwards. Many polymorphonuclear leukocytes, eosinophils and mast cells were found after 1 week within the pores of the membrane (Fig. 10), at its inner surface, and within the nerve tissue. Along with these readily identifiable cells were many others, having the features of monocytes. These had large oval nuclei with nucleoli (Figs. 12, 13). Their nuclear membrane was folded, with a broad rim of heterochromatin. The cytoplasm was

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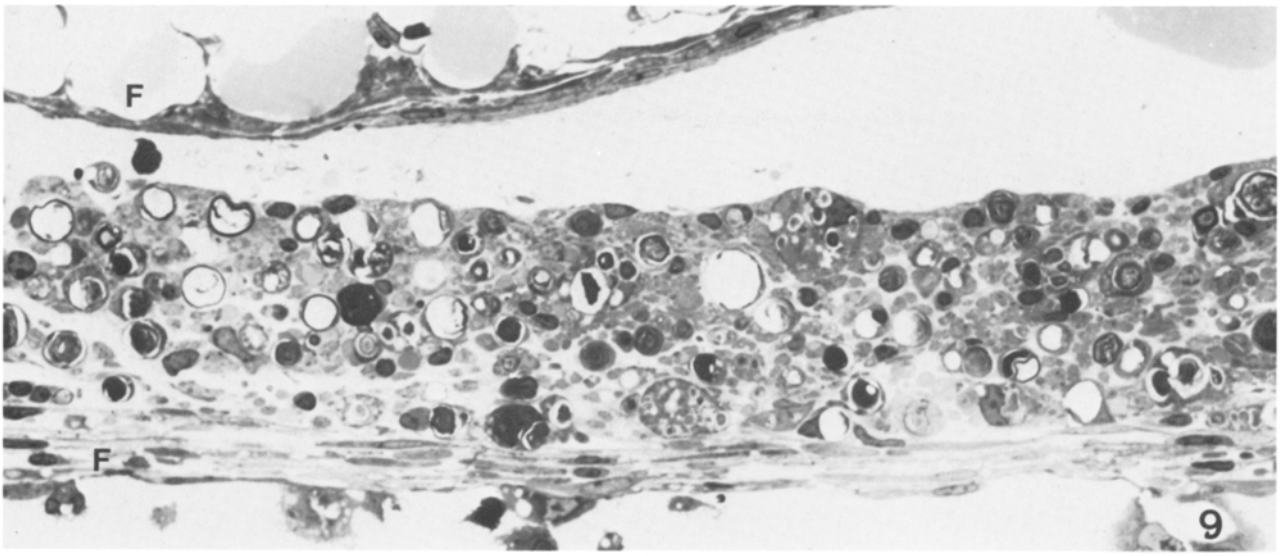
**Fig. 9.** Nerve tissue kept for 2 weeks in a 5.0 μm pore chamber is shrunken with advanced disintegration of sheaths and invasion by myelinophages. In this preparation, the nerve tissue had separated from membrane; it is sandwiched between sheets of flattened fibroblasts (F). × 696.

**Fig. 10.** Nerve tissue kept for 4 weeks in a 5.0 μm pore chamber is crowded with myelinophagic cells; no sheaths remain. The nerve tissue adheres to the Millipore membrane (M) which contains scattered migratory cells. The outer surface of the membrane is coated by a thin sheet of peritoneal cells (P). A sheet of fibroblasts (F) separates the nerve tissue from the fluid-filled lumen of the diffusion chamber. × 695.

**Fig. 11.** Phagocytosis of myelin debris within the basement membrane of a nerve fibre after 1 week in a 5.0 μm pore chamber. The electron-lucent cytoplasm of the Schwann cell (S) forms a thin rim next to the myelinophage. × 8029. The inset shows the close contact and interdigitation between the electron-dense myelinophage and the electron-lucent Schwann cell. × 37 800.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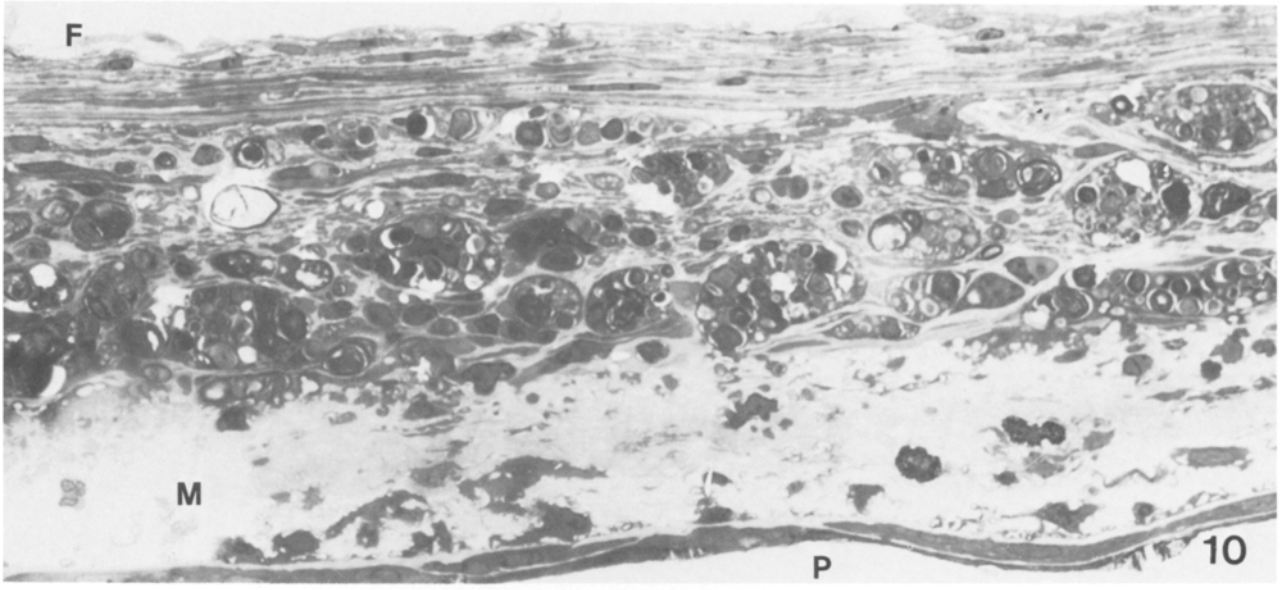
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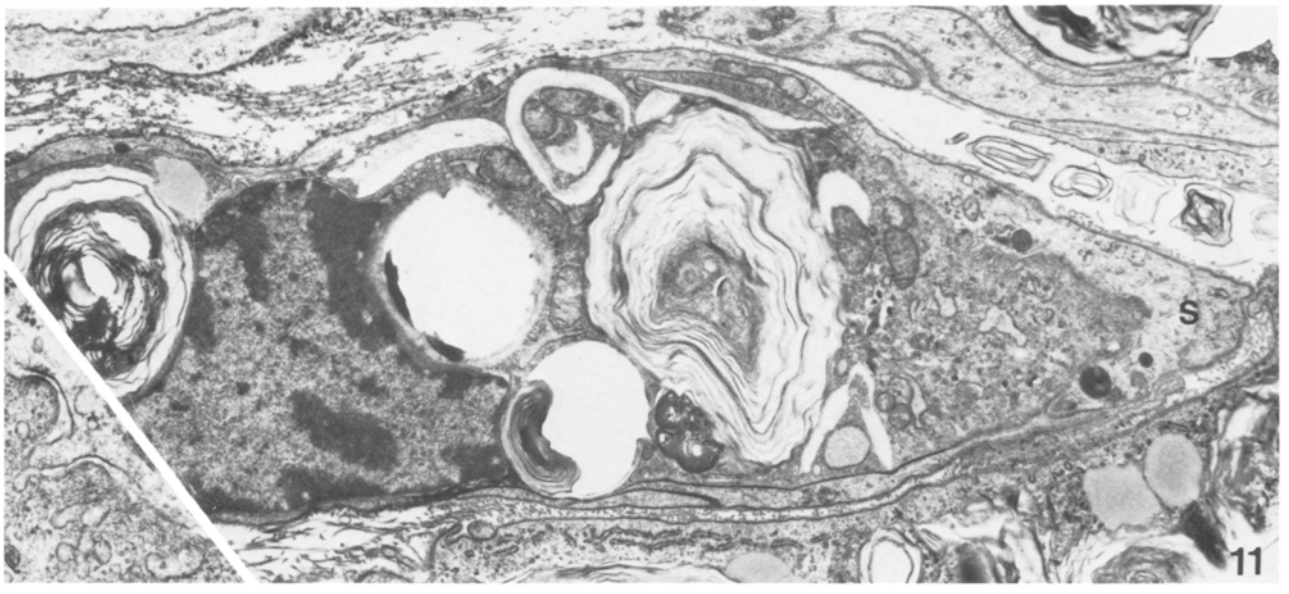
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electron dense, containing abundant small vesicles, polyribosomes and stacks of smooth endoplasmic reticulum; rough endoplasmic reticulum was less common. The Golgi apparatus was prominent. There were few mitochondria or lysosomes, but occasional fat droplets. An abundance of pseudopodia indicated active migration. Pinocytosis was not remarkable in cells situated in the outer parts of the pore membrane, but it increased if cells were near the nerve tissue. Such cells also had coated pits and coated vesicles.

*Myelin phagocytosis*

All the cell types that were observed traversing the membrane (Fig. 10) were also found within the nerve tissue after 1 week's exposure, increasing in number with time. Leukocytes and mast cells were readily recognized, but the distinction between invading myelinophagic cells and Schwann cells was only tentative, particularly for those cells found within the envelope of basement membrane. We based identification on the comparison of 0.22 and 5.0 $\mu$m pore chambers. Schwann cells had a relatively electron-lucent cytoplasm, both in their processes and in the perikarya. Schwann cell proceses contained moderate numbers of organelles, but there were many microtubules. Myelinophagic cells were found only in 5.0 $\mu$m pore chambers and had a relatively electron-dense cytoplasm (Fig. 11). Their nuclei were larger than those of Schwann cells, and they had more organelles in their cytoplasm, often along with ingested myelin fragments. Both cell types were found in close contact, enveloped by the same basement membrane. All features of myelin uptake in 5.0 $\mu$m pore chambers (Figs 9–11) were similar to those seen during Wallerian degeneration *in situ*, but the total increase in cell numbers was much smaller in the chambers (Fig. 14). Myelin particles were initially embraced by thin processes, followed by intake of myelin into the cytoplasm. Myelin particles embraced by phagocytic cells showed alterations of fine structure; the latter were more advanced when myelin fragments were seen within the cytoplasm, the myelin being degraded into whirls of membranes, often lying free in the cytoplasm without surrounding lysosomal membrane. Diminution of such fragments was seen in membrane-bound vacuoles filled with amorphous material. Some fat droplets were seen in myelin-ingesting phagocytes, but they were not a prominent feature.

Myelin phagocytic cells made contact with Schwann cells by forming small pseudopodia directed towards the latter. Such processes showed occasionally coated pits and increased pinocytosis. Coated pits and coated vesicles were not seen where the phagocytes were engaged in myelin uptake, nor were myelin-like structures associated with coated pits. Mitotic figures were never seen in the degenerating nerve up to 4 weeks of observation. There was no evidence for a reverse migration of myelinophagic cells out of the neural tubes.

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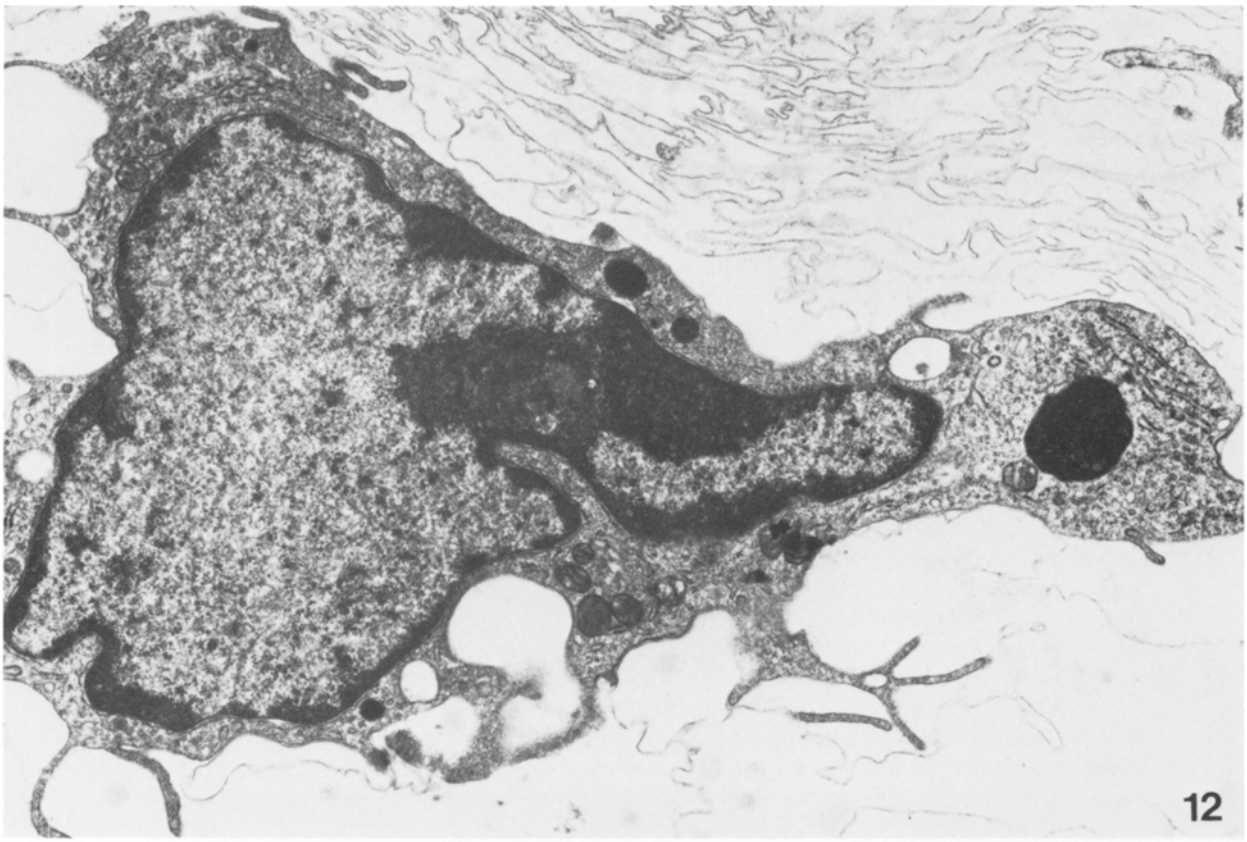
**Fig. 12.** A migratory cell, presumably monocyte, traverses the meshwork of a 5.0 $\mu$m pore membrane at 4 weeks. × 12 600.

**Fig. 13.** Free monocytes are found within a 5.0 $\mu$m pore chamber at 2 weeks. These cells are representative of the single cells seen at the bottom of Fig. 9. × 9900.



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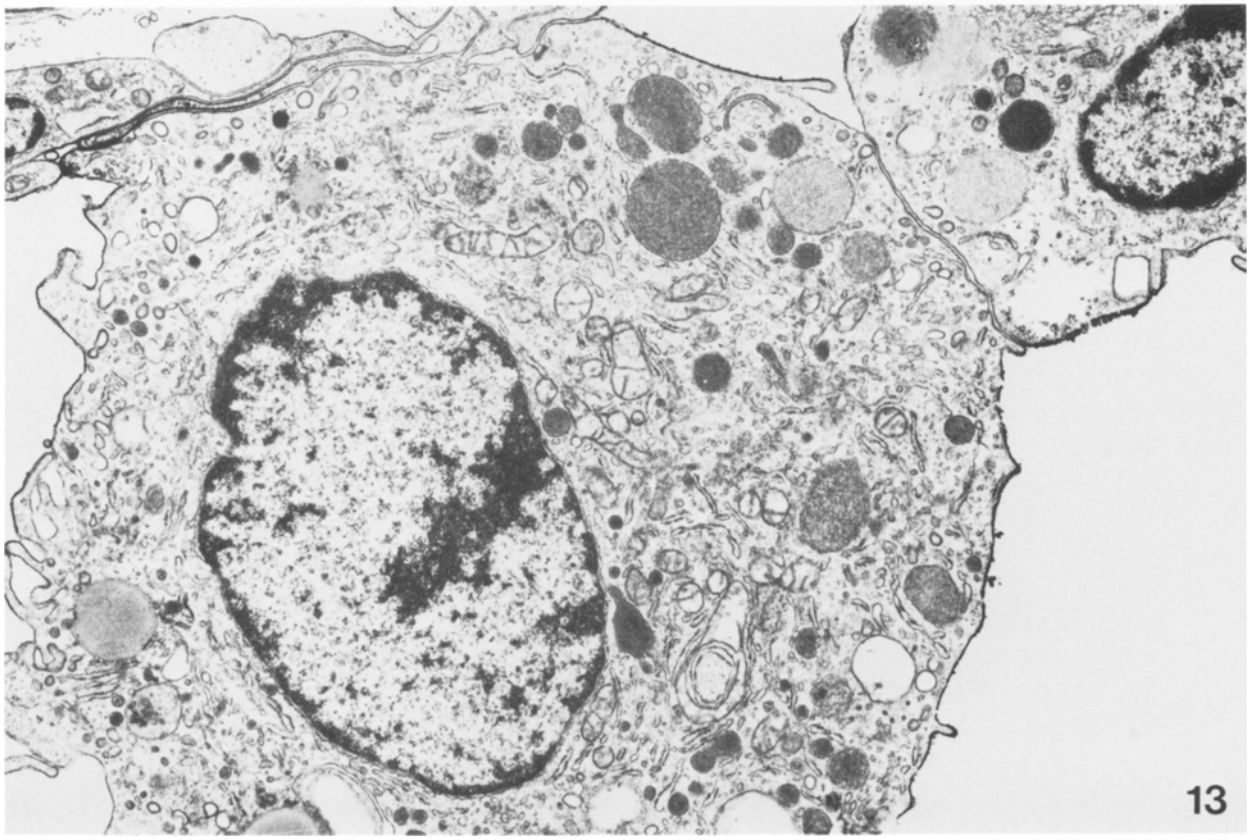

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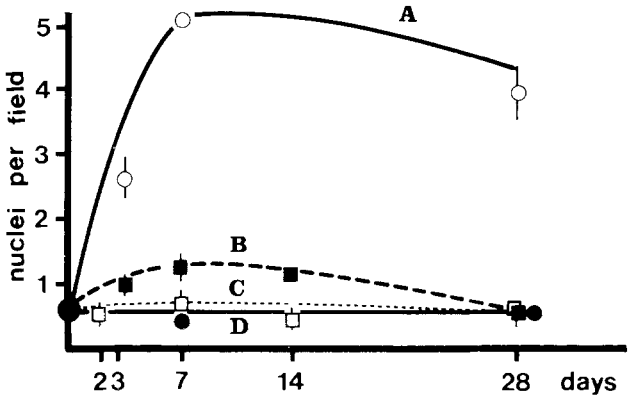



**Fig. 14.** A comparison of nuclear densities in sciatic nerves under different experimental conditions. The curves illustrate from top to bottom: (A) Wallerian degeneration of nerves left *in situ* showing marked increase in cell density (○—); (B) nerves kept in diffusion chambers of 5.0 μm pore size (■- - - -) show cellular invasion, though distinctly less than for nerves left *in situ*; (C) predegenerated nerves that had been kept for 4 weeks in a 0.22 μm pore chamber (not shown) and subsequently transferred into a 5.0 μm pore chamber (□- - - -) show greatly diminished cellular invasion; (D) nerves kept in 0.22 μm pore chambers (●—) show no changes in cell density. Points indicate means and S.E.M. for three samples each. If no bars are shown, the S.E.M. was smaller than the symbol. To obtain S.D., the bar length for the S.E.M. should be multiplied by 1.8.

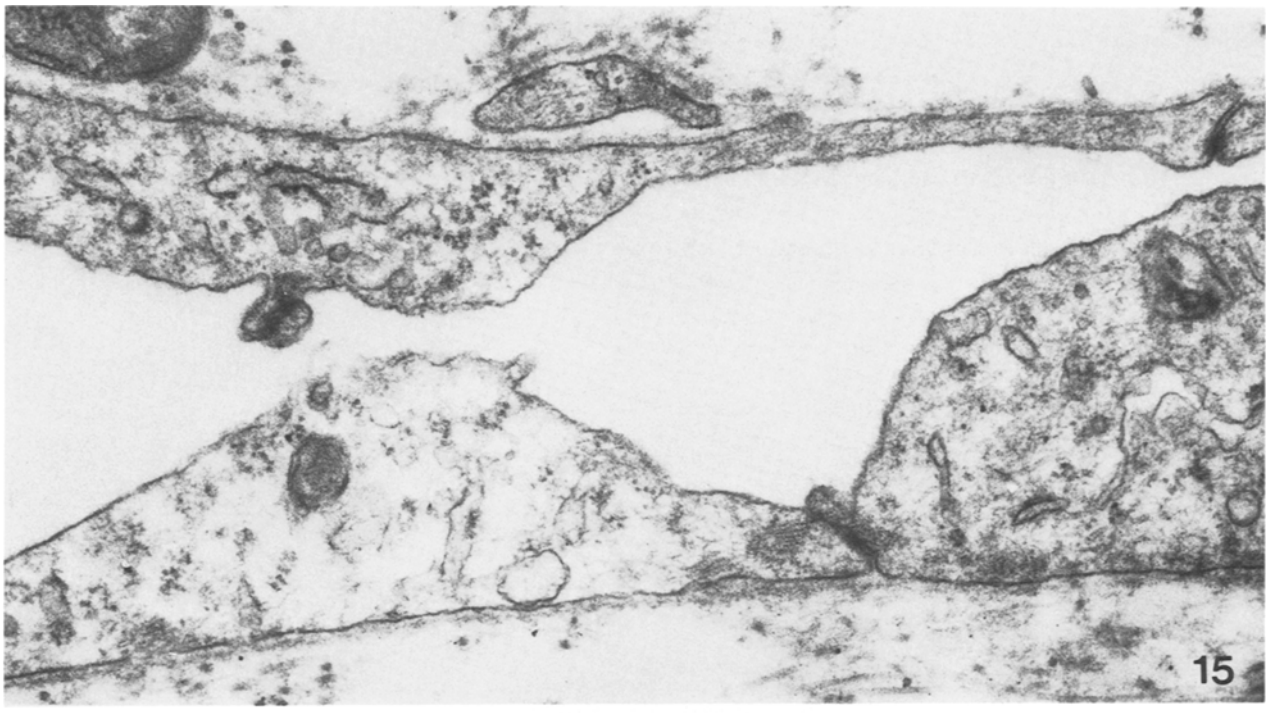
After 4 weeks, much myelin had disappeared from the nerves. Profiles of cell processes were packed within the basement membrane (Fig. 16), similar to the bands of Büngner, seen after Wallerian degeneration *in situ*. There was no fat in these cells. Some of the cells seen in these bands had rather dense cytoplasm, suggesting that they were resting macrophages. A few residual myelinophagic cells had giant nuclei and huge cytoplasmic bodies containing whorls of myelin and many fat droplets. Aside from the myelinophages, there were always leukocytes present in 5.0 μm pore chambers, many of them being eosinophils. These cells showed neither myelin uptake nor other evident interaction with other cells.

*Reduced fibroblast proliferation in chambers of 5.0 μm pore size*

Chambers of 5.0 μm pore size contained a liquid and they failed to show the gel marking the profuse outgrowth of fibroblasts seen in the 0.2 μm pore chambers. Spindle-shaped

**Fig. 15.** Fine structure of a newly formed capillary among the fibroblasts filling a 0.22 μm pore chamber after 7 days. Endothelial cells joined by tight junctions are encompassed by a basal lamina. × 36 300.

**Fig. 16.** This nerve was kept for 4 weeks in a 5.0 μm pore chamber. There are no residua of myelin in this field. The Schwann cell processes are arranged in register within their basement membranes, forming the bands of Büngner. Rounded cells occupy some of the basement membrane tubes; some of these may be resting migratory cells, based on cytoplasmic density. × 1000.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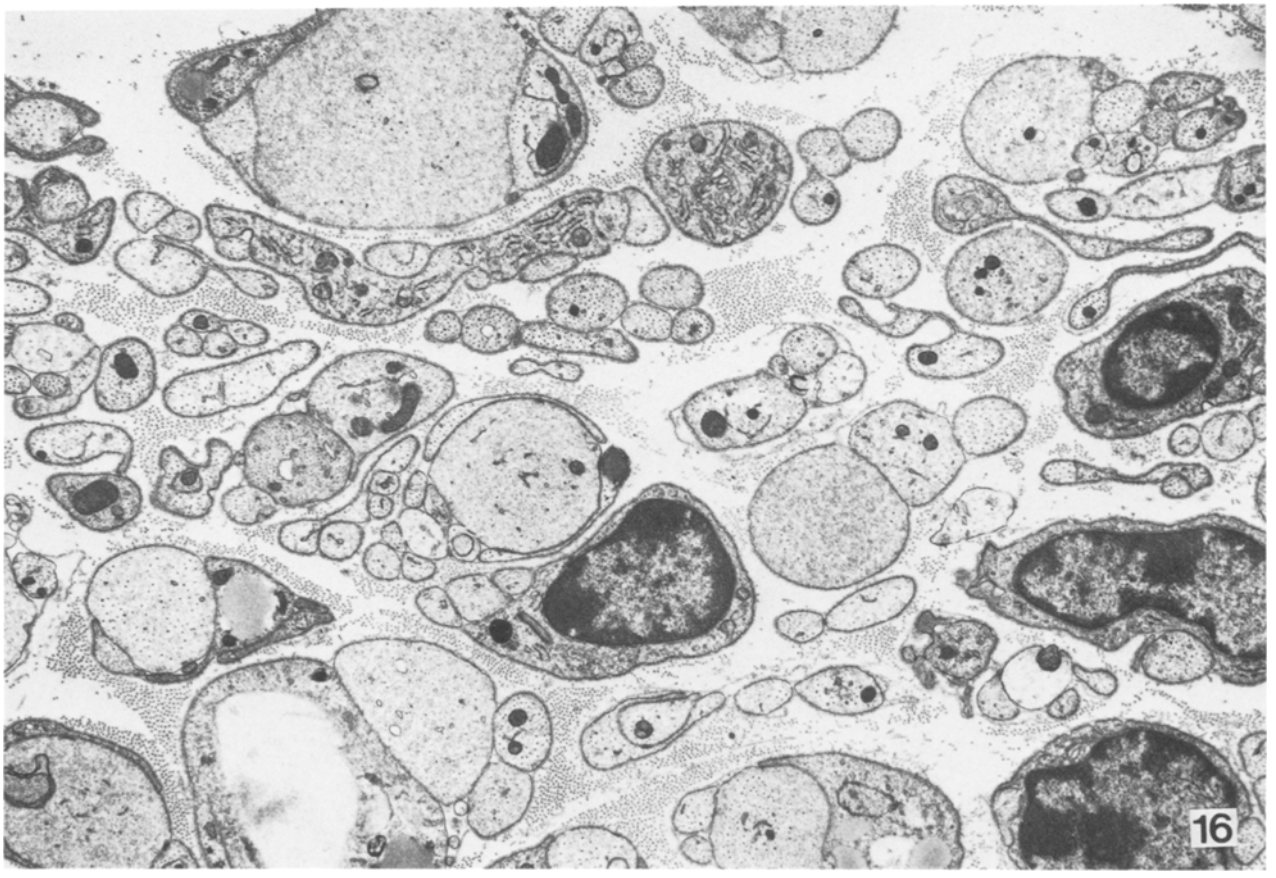
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fibroblasts had aggregated to form a thin, multilayered sheath adhering to the chamber's bottom membrane and forming abundant collagen (Figs 9, 10). Fat droplets were absent from the cytoplasm. Where the nerve touched the membrane of the chamber, there was loosening of the perineurium, and fibroblasts had nearly disappeared. On the opposite surface, the perineurium had fused with the sheaths of fibroblasts, which covered the degenerating nerve tissue.

### EFFECT OF TRANSPLANTING NERVES FROM CHAMBERS OF 0.22 μm PORE SIZE INTO CHAMBERS OF 5.0 μm PORE SIZE

There was a remarkable difference in the intensity of cellular reactions if nerves were kept in 0.22 μm pore chambers for 1 to 4 weeks, followed by transfer into 5.0 μm pore chambers (Figs 17–19). In such nerves, myelin phagocytes were greatly reduced compared with nerves placed immediately into 5.0 μm pore chambers, and the macrophages were mostly found in the outer parts of the profile of the nerve. Only a few typical myelin phagocytes were seen with the electron microscope, and those present were often located outside of the fibres. Very little degeneration of myelin had occurred after 8 weeks, even though the nerve had been in the 5.0 μm pore chambers for 4 weeks. Such nerves, indeed, were more similar to nerves kept for 8 weeks in 0.22 μm pore chambers. Delayed admittance of phagocytic cells had greatly diminished the intensity of their action. Other tissue changes were not similarly effected; the outgrowth of fibroblasts was diminished after transplantation into 5.0 μm pore chambers, and there was also invasion by leukocytes. Most of these were neutrophilic granulocytes, often showing uptake of small amounts of myelin. There were few eosinophils.

### MODIFICATIONS OF THE EXPERIMENTS

*Absence of myelin phagocytosis in dialysis tubes*

If the pore size of the chamber was decreased to 2 nm by using dialysis tubes, there was no significant change in myelin degradation, in the reaction of Schwann cells, in fibroblast growth or in formation of myoblasts. Newly formed fibroblastic tissue filled the lumen of the closed dialysis tube in a fashion similar to that seen in 0.22 μm pore chambers.

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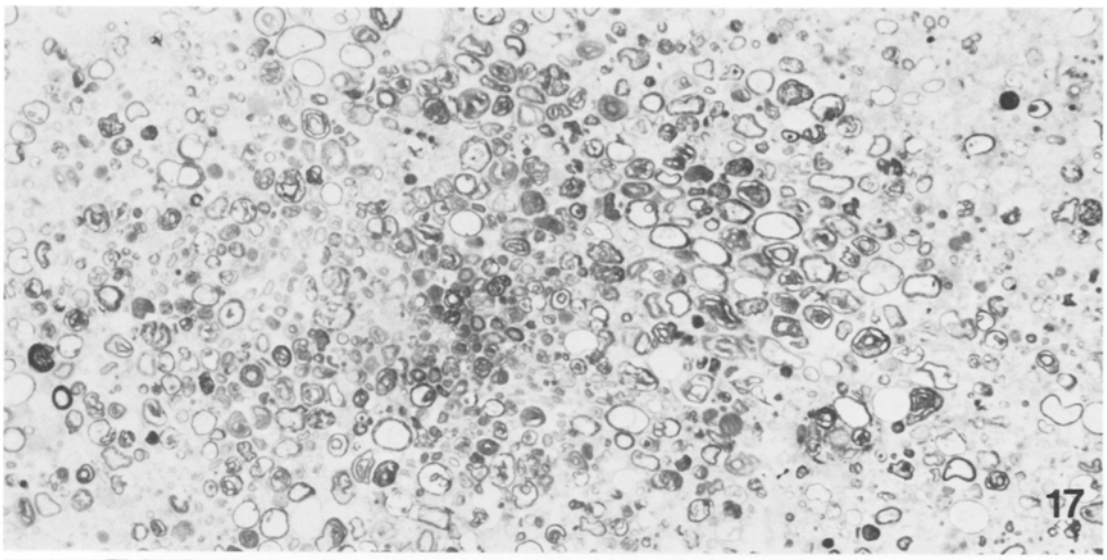
**Fig. 17.** This sciatic nerve was kept for 8 weeks in a 0.22 μm pore chamber; there is decay of sheaths but no active phagocytosis of myelin. × 435.

**Fig. 18.** This nerve was kept for 4 weeks in a 0.22 μm pore chamber and was then transferred into a chamber of 5.0 μm for another 2 days. There is no evidence of cellular invasion. × 435.

**Fig. 19.** This nerve was kept for 4 weeks in a 0.22 μm pore chamber and then for another 4 weeks in a 5.0 μm pore chamber. Even though the chamber was accessible to cells for 4 weeks (compare Fig. 10), there is no intense myelin phagocytosis, and the decay of the sheaths is about the same as in the nerve kept in a 0.22 μm pore chamber for the entire 8 weeks (Fig. 17). × 435.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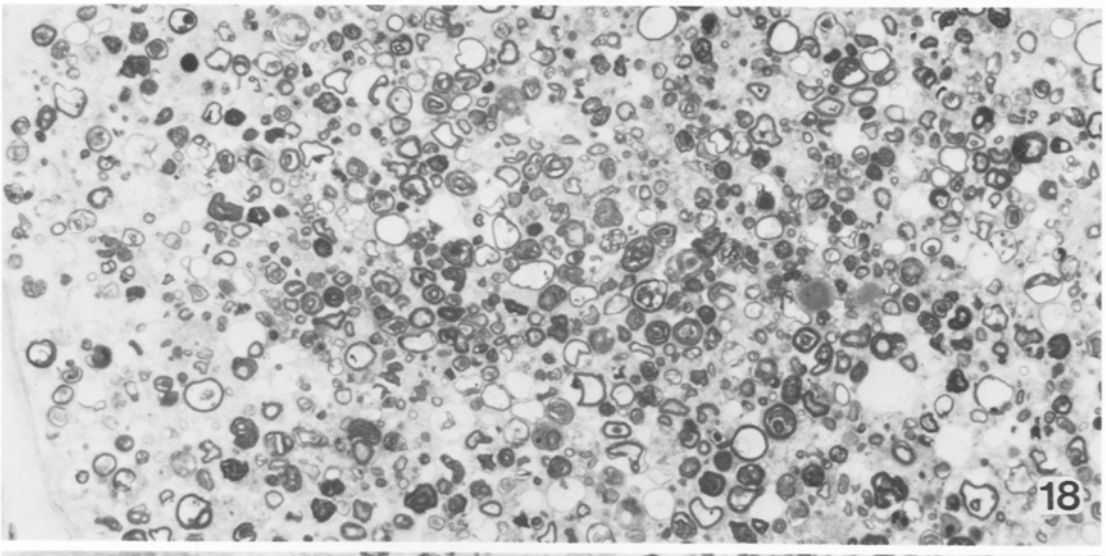
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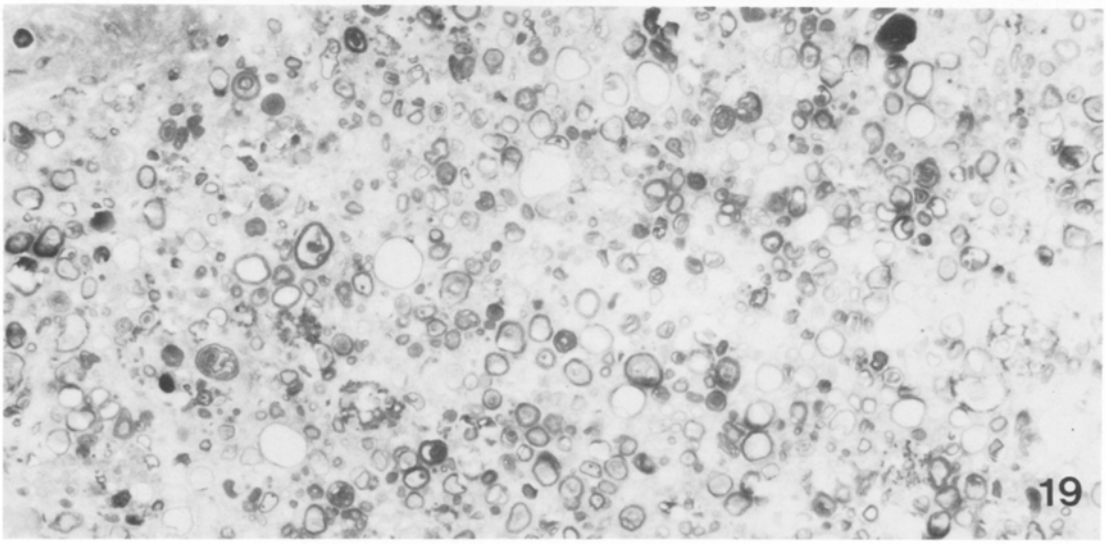
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*Changes in nerves predegenerated* in situ *for 50 h before being placed for 28 days in a Millipore diffusion chamber of 0.22 μm pore size*

Examination with the light microscope showed no differences compared with nerves placed into chambers immediately. Using the electron microscope, there were occasional leukocytes and a few heteromorphic cells within the neural tubes, indicating some cellular invasion during the 50 h before the nerves were transferred into chambers.

*Nerves injected with purified myelin*

Nerves loaded with injections of purified myelin before being placed in 0.22 μm pore chambers showed no phagocytosis of myelin by Schwann cells. Perineurial or endoneurial fibroblasts showed neither proliferative changes nor myelin phagocytosis. Some of the injected myelin debris had spilled outside the nerve. Such myelin was ingested by migratory fibroblasts presumbly of perineurial origin.

*Co-culture of peritoneal macrophages or serum monocytes in Millipore diffusion chambers of 0.22 μm pore size*

There was no myelin phagocytosis, even though $5.7 \times 10^6$ peritoneal cells or $2.5 \times 10^6$ serum cells had been placed into the chambers.

*Co-culture of bone marrow, splenic, lymphatic or lung tissue in Millipore diffusion chambers of 0.22 μm pore size*

There was no significant removal of myelin, even though the marrow cells proliferated and persisted for at least 4 weeks (Berman & Kaplan, 1959; Benestad, 1970). After 7 days, there were many well-preserved marrow cells and mitoses, but the cells did not aggregate toward the nerve.

Immature narrow cells had sparse lyosomes, or absent specific granules in the leukopoetic line. After 4 weeks the cells were arranged in small clusters or bands. Lyosomes had increased in number and size, and the specific lysosomes of the eosinophilic leukocytes were present. Occasionally some showed phagocytosis of myelin figures or of other electron-dense material, but there was no significant degradation of myelin or invasion of the nerves by marrow cells.

Splenic and lymphatic tissue was necrotic after 4 weeks without changes in the co-cultured nerves. Nerves co-cultured with lung tissue showed less outgrowth of fibroblasts than controls.

## Discussion

Three types of 'cages' were used to manipulate the access of cells and macromolecules to the nerve tissue: (1) dialysis tubes of 2.0 nm pore size which are impermeable to cells and to macromolecules; (2) Millipore diffusion chambers of $0.22 \pm 0.02$ μm pore size; the pores are about 100 times larger than in dialysis tubes and impermeable to cells, but they

admit any soluble macromolecular material; (3) Millipore diffusion chambers of 5.0 ± 1.2 μm pore size which admit migratory cells. This permits a crude classification of factors involved in Wallerian degeneration.

The opportunities of using Millipore diffusion chambers for organ culture reach far beyond the present experiments. The rigorous exclusion of cells eliminates mechanisms of cell-mediated immunity. Heterologous tissue can be grown in such chambers similar to transplants into athymic nude mice. In the present experiments, we had an effort to avoid these aspects as we wanted to understand the basic cellular interactions of Wallerian degeneration. Accordingly, we transplanted only autologous or homologous nerves into animals of the same strain; care was taken to use only autologous nerves in all experiments which involved 5.0 μm pore chambers or the reopening of the chambers (see Material and methods). The interaction between cell-mediated immunity and myelin phagocytosis in heterologous tissue is subject to a subsequent study.

The nerves transplanted into the 0.22 μm pore chambers contained essentially the decaying axons, the Schwann cells and the endoneurial and perineurial connective tissue. A few haematogenous cells within the capillaries and a few resident monocytes also had to be transplanted. Resident monocytes, however, constitute approximately 2–4% (Oldfors, 1980) or 1% (Schubert & Friede, 1981) of the total cell population of a nerve. It is now widely accepted that these cells are no longer capable of sustained division once they have left the blood-stream (Adams *et al.*, 1981). At most, there could be one cycle of cell division of resident monocytes which could render their contribution insignificant.

The excellent survival of tissue in diffusion chambers showed that the changes observed in 0.22 μm pore chambers were not a result of unfavourable conditions. Muscle tissue, fibroblasts and marrow tissue were capable of proliferation and maturation. Indeed, in other experiments, explanted foetal spinal cord and ganglia showed nerve cell proliferation, axon sprouting and myelination of fibres in 0.22 μm pore chambers The only difference between the two diffusion chambers used was the size of their pores, and this single factor determined whether there was myelin phagocytosis or not. Even if the tissue was overloaded with purified myelin before placement into the chamber, there was still no myelin digestion by Schwann cells or by fibroblasts.

One may still speculate that the absence of myelin phagocytosis in 0.22 μm pore chambers was due to some severe nutritional deprivation or else to the accumulation of an unknown substance within the chambers. Such speculation is without foundation. Dialysis membranes permit the passage of small molecules below a molecular weight of 500, thus admitting freely glucose, oxygen and nutrients to the tissue. Millipore membranes are basically inert sieves, mostly used for the filtration of biological fluids. Their openings were 100 times larger than those of dialysis membranes. Very large molecules pass freely through these membranes, much larger ones than those that pass through a normal capillary wall or through the blood–brain barrier. Conceivably, some macromolecules might adhere to the membranes; this is most unlikely, but even if so, adherence would be the same for 0.22 and 5.0 μm pore chambers which are made of the

same material. We also considered and rejected the possibility that washing tissues with Gentamicin would inhibit cell proliferation. Decrease of concentration to 50 mg $ml^{-1}$ had no different effect and mitoses were evident in bone marrow tissue and spinal cord–root ganglia transplants. The washing was short, and any effect dependent on this factor could not possibly differ between types of chambers.

We must also reject the assumption that the migration of cells through 5.0 $\mu$m pore membranes may represent outward migration of cells from the transplanted nerves. The cells found within the pores of the membrane and next to it were clearly not Schwann cells and they were readily identifiable with leukocytes, eosinophils, mast cells and monocytes. Identification became difficult only for cells that had entered the basement membrane of the nerve fibres or for phagocytic cells.

Wallerian degeneration proceeding in the absence of nonresident cells showed no cell proliferation and no active intracellular digestion of myelin. These observations contradict the widely accepted concept that Schwann cells proliferate and digest their sheaths once they lose contact with the parent axon. A characteristic aspect of sheath decay was the torpidity of all alterations following the loss of the axon. Neurofilament antigen, detected by immunofluorescence, is known to have disappeared by the tenth day in nerves degenerating *in situ* (Bignami *et al.*, 1981). Myelin sheaths persisted as empty tubes for many weeks, similar to the central fibres reported by Lampert & Cressman (1966), having collapsed but intact sheaths as late as 52 days after transection. In such tracts, macrophages were shown to be haematogenous monocytes through carbon labelling (Ling, 1978, 1979).

Rejected sheaths developed passive changes in configuration whereby the space within the sheath became either larger than the normal axon with a corresponding reduction in sheath thickness, or else it became smaller with a corresponding increase in sheath thickness. These changes were quite evident in phrenic fibres because of their uniform calibre range. Similar changes were previously described as the consequence of outward slippage of the sheath in swollen fibres (Friede & Martinez, 1970a; Friede & Miyagishi, 1972) or of inward slippage of the sheaths during early Wallerian degeneration (Friede & Martinez, 1970b). The observations made here were similar, but we used a more sophisticated computer-assisted method than the cumbersome counts of myelin lamellae. The documentation of these changes in diffusion chambers is important; such extreme slippage of the sheath developed after the sheaths were rejected by the Schwann cell. Our data, accordingly, caution against the interpretation of sheath slippage as a physiological nontraumatic type of adjustment of the sheath to rapid changes in axon calibre.

The massive proliferation of cells, which is so characteristic of Wallerian degeneration, is evidently not an immediate and direct response originating in the Schwann cells, as is often taken for granted. Our observations agree with the data of Asbury (1970, 1975), showing no proliferation of prelabelled Schwann cells. These findings are at variance with a recent report of Salzer & Bunge (1980) showing a mitogenic signal to myelinating Schwann cells upon excision of their parent spinal

ganglion in culture; perhaps drastic conditions, such as foetal cells myelinating in culture, may evoke cell activities not normally expressed in adult animals. One must assume that the bulk of increase in cell density is from the invasion of haematogenous cells, or at least, dependent on the presence of these cells. Gibson (1979), likewise, found no evidence to support the notion that Schwann cells transform into macrophages. The increase in thymidine-labelled cells during Wallerian degeneration (Friede & Johnstone, 1967) may also show recent macrophage invasion, as the first peak of labelled macrophages in the blood stream occurs 18 h after labelling (Volkman, 1976). Amoeboid phagocytes are known to enter degenerating nerves in lead poisoning (Lampert & Schochet, 1968) or after crush (Olsson & Sjöstrand, 1969), and similar findings were amply documented for experimental allergic neuritis. This was thought to differ from the assumed role of Schwann cells in Wallerian degeneration (Wiśniewski *et al.*, 1969). If either purified myelin or carbon particles were injected into peripheral nerves (Schubert & Friede, 1981), there was evidence of the invasion of fibres by migratory cells labelled by that material, but definite identification of the origin of the cells from either monocytes or fibroblasts was not possible.

Monocytes and leukocytes may become attracted to the degenerating nerve by chemotactic stimuli, or else may reach the nerve during random migration and recognize the damaged myelin sheaths upon contact. These two modes of action are not mutually exclusive. Phagocytes may conceivably be attracted by the rapid disintegration of the axon rather than by the slow decay of the myelin sheaths. This is unlikely, however, as there is very little proliferation of cells during Wallerian degeneration of nonmyelinated nerves (Joseph, 1950; Peyronnard *et al.*, 1973; Romine *et al.*, 1976).

An important aspect of the activation of phagocytes was observed when 0.22 $\mu$m pore chambers were opened after an interval. Such predegenerated nerves were no longer capable of eliciting a strong phagocytic invasion. Indeed, the intensity of the phagocytic response diminished with the length of the decay in the absence of phagocytes. This indicates the existence of a specific and transient signal attracting phagocytes to the rejected sheaths.

Another line of thought implies a rather specific interaction between phagocytes and rejected myelin. Myelin membranes are modified surface membranes of Schwann cells, yet the macrophages were capable of distinguishing the rejected sheaths, which they ingested, from the Schwann cells, which they did not attack. This distinction took place at a very early phase of Wallerian degeneration, when the rejected sheath was still close to its cell. Sheath rejection may modify the myelin in such a way that it can be recognized by the phagocyte as being 'damaged'. Also, sheath rejection becomes evident only if phagocytes are kept out of the tissue. Otherwise, sheath rejection and sheath phagocytosis interdigitate so intimately that the two phenomena cannot be recognized as being different.

The nature of target recognition by phagocytes is still obscure. Myelin could be recognized by its content of basic protein or Wolfgram protein, or by myelin-associated glycoproteins which are found in the sheath but not in the Schwann cell, e.g. the 170 000

molecular weight glycoprotein (Shuman *et al.*, 1983), but this alone would not help the distinction between normal and rejected myelin. Antibodies to myelin autoantigens occur following sciatic transection (Schwartz *et al.*, 1982) and immunologic mechanisms of target recognition may be involved in sheath digestion. Other mechanisms seem possible as well. We found no evident relationship between coated pits and myelin uptake into macrophages as claimed by Epstein *et al.* (1983), as one would expect from the known role of coated pits in uptake at a molecular level (Goldstein *et al.*, 1979).

The possibility needs to be considered that fibroblasts may transform into macrophages if stimulated by haematogenous cells; macrophages may stimulate fibroblast proliferation (Leibovich & Ross, 1976; Leibovich, 1978). This interpretation was unlikely since the profuse proliferation of perineurial fibroblasts filling the 0.22 $\mu$m pore chambers was inhibited in 5.0 $\mu$m pore chambers. This restriction may be attributed to the capacity of macrophages to remove a collagen matrix upon contact (Werb, 1983).

Monocytes are most probably responsible for myelin phagocytosis. Polymorphonuclear leukocytes, plasma cells or mast cells are readily identified with the electron microscope, and these cells did not play a significant role in the digestion of myelin. We do not have any conclusive data at this point as to whether lymphocytes are admitted into 5.0 $\mu$m pore chambers. However, the simple absence or presence of monocytes was not the only factor causing myelin digestion. Co-cultures of peritoneal or blood monocytes brought negative results. The meaning of these observations is not clear. Peritoneal lavage may damage cells; furthermore, peritoneal macrophages are heterogeneous, including resident and immigrated cells having different functional properties (Daems & Brederoo, 1973; Daems *et al.*, 1973; Daems & Koerten, 1978; Blok *et al.*, 1982; Volkman *et al.*, 1983). The contribution of these populations to myelin phagocytosis is presently undefined. These arguments do not apply to co-cultured bone marrow, but marrow cells had to undergo proliferation and maturation within the diffusion chambers. Further studies are necessary to show whether the absence of myelin phagocytosis with co-cultured tissues indicates a need for T-lymphocytes, lymphokines or other activating factors, or for the maturation or programming of macrophages.

Very little is known about the role of polymorphonuclear leukocytes in Wallerian degeneration, even though these cells enter nerves in large numbers during a transient initial phase. This may simply be caused by the co-activation of all haematogenous phagocytes. Leukocytes possess soluble enzymes for degrading myelin basic protein and Wolfgram protein (Rastogi & Clausen, 1981), but the electron microscope did not show their participation in myelin degradation. On the other hand, leukocytes may play an unknown role in presenting tissue components for phagocytosis. The latter assumption was tested by the two days' predegeneration of transected nerves *in situ* before transfer into a 0.22 $\mu$m pore chamber. Such nerves were exposed to the initial burst of leukocyte invasion, but this alone did not suffice to modify the events later on, if no macrophages were admitted.

A last feature requiring consideration is the appearance of intracellular neutral fat in

Wallerian degeneration. Traditionally, neutral fat is thought to be an end-product of myelin digestion which appears in the Schwann cell during the final phase. In the present series, droplets of neutral fat were abundant in the fibroblasts that had migrated into the diffusion chamber. This fatty change in fibroblasts was rather similar to that seen in tissue cultures, and no evidence was found to link this change to myelin degradation. Cells containing fat were not seen to be involved in the digestion of myelin and vice versa, and there were no transitional stages; indeed, in terms of distance, the cells containing fat were located the farthest away from the sites of the sheath decay. One must conclude, therefore, that what was thought to be a continuous process of degradation of myelin lipids to neutral fat within one and the same cell population may well be independent, overlapping phenomena involving populations of haematogenous and pre-existing connective tissue cells.

Wallerian degeneration, in conclusion, emerges as a phenomenon far more intricate than the previously formulated concepts allow. A better understanding of the process of target recognition between rejected sheaths and phagocytes may not only be of cell-biological interest; it may also open new bases for understanding demyelinating diseases.

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