Myelin Sheath Survival after Guanethidine-induced Axonal Degeneration

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Abstract. Membrane–membrane interactions between axons and Schwann cells are required for initial myelin formation in the peripheral nervous system. However, recent studies of double myelination in sympathetic nerve have indicated that myelin sheaths continue to exist after complete loss of axonal contact (Kidd, G. J., and J. W. Heath. 1988. *J. Neurocytol.* 17:245–261). This suggests that myelin maintenance may be regulated either by diffusible axonal factors or by nonaxonal mechanisms. To test these hypotheses, axons involved in double myelination in the rat superior cervical ganglion were destroyed by chronic guanethidine treatment. Guanethidine-induced sympathectomy resulted in a Wallerian-like pattern of myelin degeneration within 10 d. In doubly myelinated configurations the axon, inner myelin sheath (which lies in contact with the axon), and ~75% of outer myelin sheaths broke down by this time. Degenerating outer sheaths were not found at later periods. It is probably that outer sheaths that degenerated were only partially displaced from the axon at the commencement of guanethidine treatment. In contrast, analysis of serial sections showed that completely displaced outer internodes remained ultrastructurally intact. These internodes survived degeneration of the axon and inner sheath, and during the later time points (2–6 wk) they enclosed only connective tissue elements and reorganized Schwann cells/processes. Axonal regeneration was not observed within surviving outer internodes. We therefore conclude that myelin maintenance in the superior cervical ganglion is not dependent on direct axonal contact or diffusible axonal factors. In addition, physical association of Schwann cells with the degenerating axon may be an important factor in precipitating myelin breakdown during Wallerian degeneration.

A primary role of the axon is to instruct Schwann cells to commence myelin formation (Simpson and Young, 1945; Aguayo et al., 1976; Weinberg and Spencer, 1976). This communication is thought to be mediated by molecular interactions between the plasma membranes of these two cells. Continued axon–Schwann cell contact has also been considered essential for subsequent maintenance of the compact sheath, since myelin breakdown and the down regulation of myelin component production occur rapidly after axonal degeneration or when Schwann cells are cultured without axons (Raff et al., 1979; Brockes et al., 1980; Mirsky et al., 1980; Politis et al., 1982; Winter et al., 1982; Poduslo et al., 1984, 1985; Trapp et al., 1988; Le Blanc and Poduslo, 1990; Rutkowski et al., 1990; Yao et al., 1990). However, recent studies of double myelination (Heath, 1982, 1983; Kidd and Heath, 1988*a*,*b*) have demonstrated that myelin sheath integrity can be retained in vivo in Schwann cells that have lost physical contact with an axon.

Double myelination is found in adult sympathetic nerve and consists of a myelinated fiber that is concentrically surrounded by a second myelin sheath and Schwann cell (Heath, 1983; Kidd and Heath, 1988*a*). This arrangement results from the progressive displacement of a myelinating Schwann cell from axonal contact through invasion of another Schwann cell at a node or heminode (Kidd and Heath, 1988*b*). Subsequent myelin formation by this interposed (now "inner") Schwann cell results in a region of axon surrounded by two myelin sheaths, each produced by an individual Schwann cell (Heath, 1982; Kidd and Heath, 1988*a*). In ~25% of configurations, the original (now "outer") Schwann cell becomes completely displaced from axonal contact, yet the lamellar organization of these outer myelin sheaths is invariably retained and they often undergo further conformational reorganization ("infolding") which requires the supply and ordered intercalation of myelin-specific components (Kidd and Heath, 1988*a*; Heath, J. W., B. D. Trapp, and G. J. Kidd, manuscript in preparation).

These observations raise two novel hypotheses. First, myelin maintenance may be regulated by diffusible axonal factors operating over micron distances, in contrast to membrane–membrane contact. Alternatively, axonal influences are no longer required by the outer, fully displaced Schwann cells. To test these hypotheses, the response of outer Schwann cells to axonal degeneration was investigated. Chronic treatment of rats with the neurotoxic drug guanethidine was used to produce a selective degeneration of post-

ganglionic sympathetic neurons (Burnstock et al., 1971; Eränkö and Eränkö, 1971; Jensen-Holm and Juul, 1971; Heath and Burnstock, 1977; Johnson and Manning, 1984), which are the source of doubly-myelinated axons in the superior cervical ganglion (SCG)[1] (Heath, 1983; Kidd and Heath, 1988*a*). We have previously established that the primary action of guanethidine is on the neuron, and that myelin breakdown in nondisplaced internodes occurs only as a secondary, Wallerian-like response to the axonal degeneration produced by this drug (Kidd et al., 1986). Based on EM analysis of serial sections, we now provide evidence that completely displaced outer internodes survive axonal degeneration. A preliminary account of this work has been presented (Kidd et al., 1990).

Materials and Methods

Animal Groups, Treatment, and Tissue Preparation

Guanethidine treatment and tissue preparation closely followed the method of Kidd et al. (1986). An initial group of 32 male Sprague-Dawley rats aged 43 wk was used in this study. Considerable variation exists among individual animals in the population of myelinated fibers in the SCG (Heath, 1983), although numbers are more uniform between left and right sides of the same animal (Kidd et al., 1986). To ensure that animals chosen for guanethidine administration contained sufficient numbers of doubly myelinated fibers to make subsequent EM analysis practicable, the right SCG was removed surgically under chloral hydrate anesthesia, fixed, and resin-embedded, and the number of doubly myelinated profiles was assessed by light microscopy. 23 rats from the original group had one or more doubly myelinated fibers (usually two or three) in a sample transverse section and on this basis were selected for subsequent treatment.

After recovery for at least 2 wk, rats were injected chronically with guanethidine sulphate (Ismelin, a gift from Ciba-Geigy, Sydney, Australia; 50 mg/kg per day, i.p.) dissolved in 0.9% saline. Groups of animals were sacrificed after treatment for 5 d (n = 2), 7 d (n = 3), 10 d (n = 3), 2 wk (n = 2), 3 wk (n = 3), 4 wk (n = 6), and 6 wk (n = 4). Under deep Nembutal anesthesia, animals were systemically perfused with 1% sodium nitrite, then 2.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) and 1% formaldehyde in 0.1 M sodium cacodylate buffer (Kidd et al., 1986). The remaining left SCG, complete with internal and external carotid nerves and cervical sympathetic trunk, was dissected out and desheathed, osmicated, and embedded for LM and EM analysis (see Kidd et al., 1986).

Sections were generated at a standard level immediately inferior to the site where the external carotid nerve exits the ganglion (Heath, 1983; Kidd and Heath, 1988*a*). This region includes the postganglionic nerve fiber tracts projecting into the external carotid nerve, where the majority of doubly myelinated fibers are located (Heath, 1982, 1983). Serial sections for LM and EM were generated as repeating sequences consisting of one thick section (~0.5 μm) followed immediately by six to eight thin sections, using a Reichart Ultracut E ultramicrotome (Reichart Jung, Vienna). A typical sequence of sections consisted of ~50 thick–thin sequences, representing 50 μm in tissue depth. Internodes in the normal SCG range from 15 to 50 μm in length (Heath, 1982). Individual thin sections were examined for doubly myelinated fibers and for any intact myelin sheaths lacking an axon. These profiles were then followed through serial sections. Thick sections for LM were stained with toluidine blue. Thin sections were mounted on uncoated 300-mesh high transmission hexagonal grids, double stained with uranyl acetate and lead citrate, carbon coated, and examined in a JEOL 100CX transmission electron microscope (JEOL Scientific Instruments, Sydney, Australia).

Results

In control ganglia two or three doubly myelinated profiles were typically observed in single thick or thin sections at the standard level of analysis (see Heath, 1982, 1983; Kidd and Heath, 1988*a*,*b*), although considerable variation existed. In approximately a quarter of these configurations the outer internode was completely displaced from the axon and surrounded an inner myelin sheath and Schwann cell (Fig. 1). The remaining three quarters represented formative stages of double myelination, in which either the outer sheath still retained some region of axonal contact, or the invading inner Schwann cell had not yet formed a myelin sheath, or both (Kidd and Heath, 1988*b*).

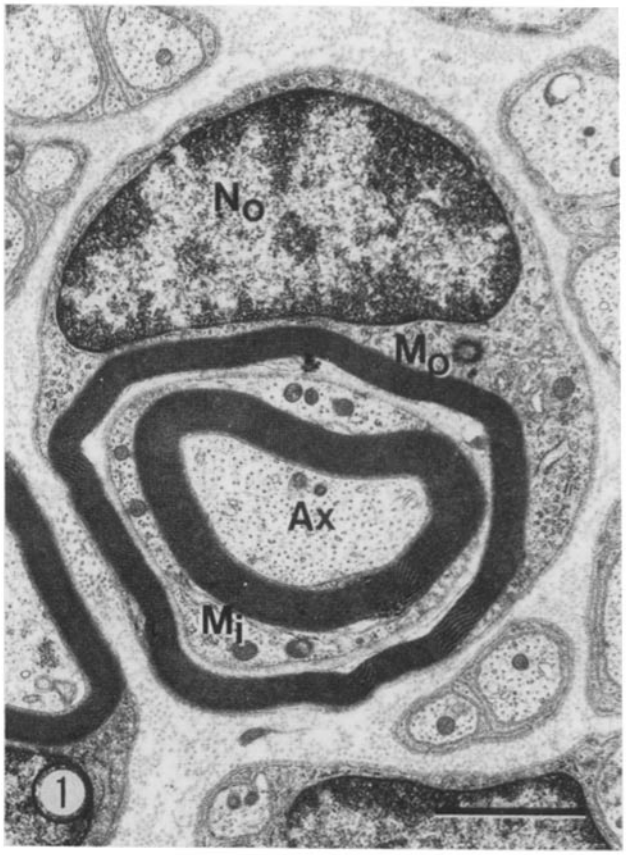


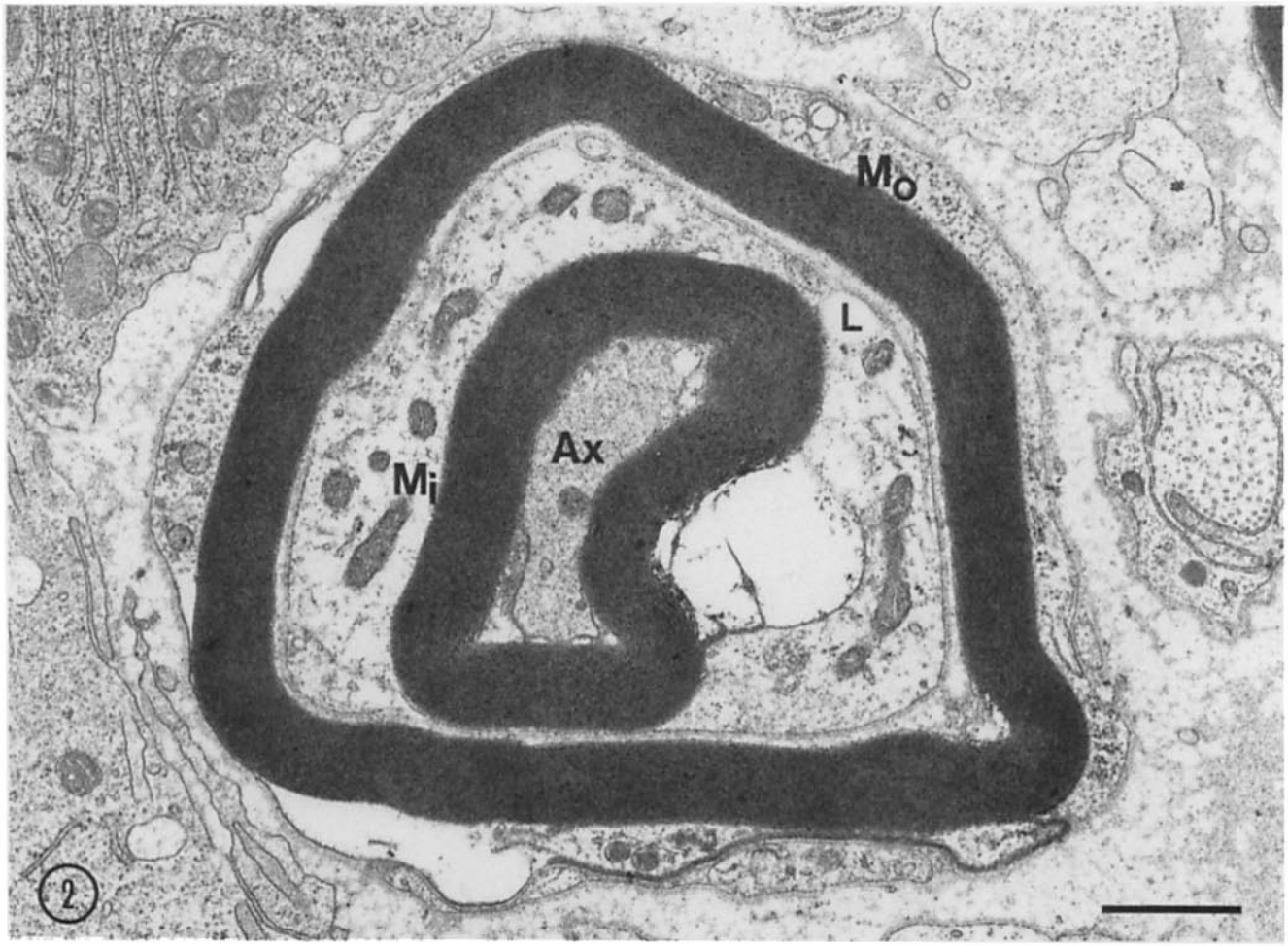
Figure 1. Doubly myelinated axon from control SCG in TS (immersion fixation). The central axon (*Ax*) is ensheathed in the usual intimate fashion by an inner Schwann cell and myelin sheath (M_i). This region of the fiber is further ensheathed by a second or outer myelin sheath (M_o) produced by an individual Schwann cell (nucleus N_o). Bar, 1.5 μm.

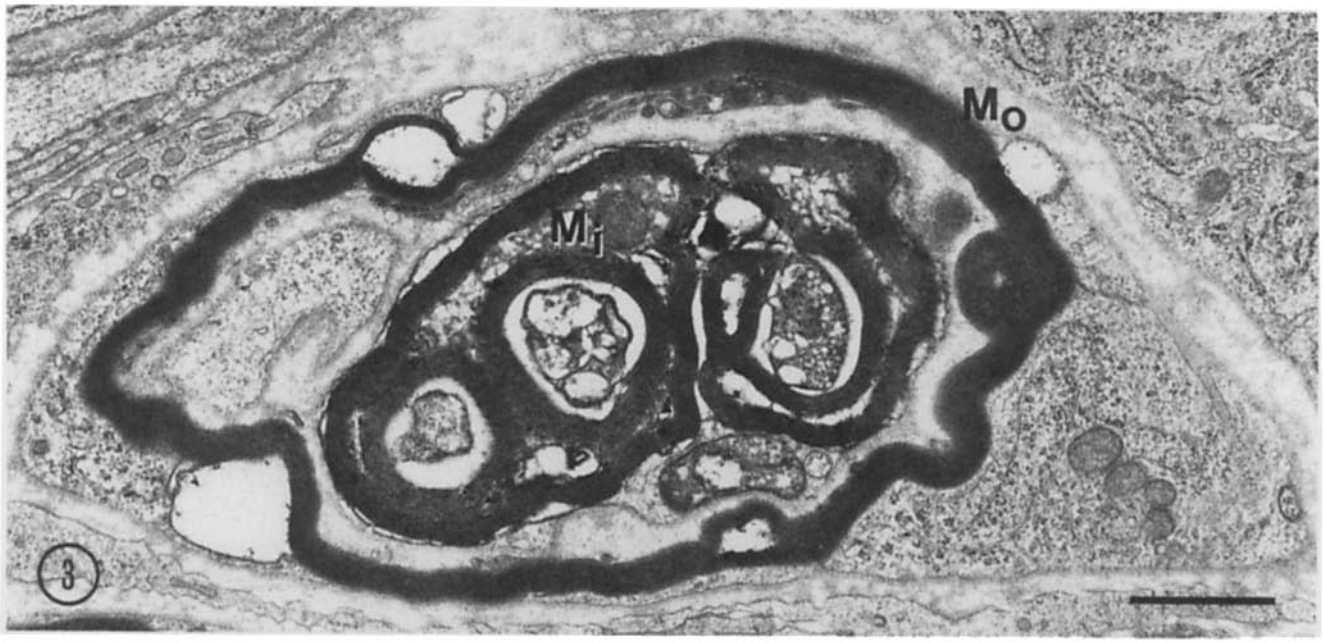
Guanethidine treatment was effective in producing selective degeneration of postganglionic myelinated fibers (1–3 μm diam), as reported previously (Kidd et al., 1986). The number of myelinated fibers declined rapidly at 5 and 7 d, falling to 15% of those in control ganglia at 10 d and declining only slightly thereafter. The remaining intact fibers are likely to represent preganglionic (cholinergic) fibers which are unaffected by guanethidine. Myelin degeneration followed a Wallerian-like pattern in which myelin breakdown was secondary to axonal degeneration.

Response of Doubly Myelinated Fibers to Guanethidine

The degenerative responses of the axon and inner myelin internodes were uniform and we have described them first. In contrast, there were two distinct patterns of response among outer internodes, which are described subsequently.

1. *Abbreviation used in this paper*: SCG, superior cervical ganglion.





Figures 2 and 3. (*Fig. 2*) Guanethidine sulphate (50 mg/kg per day) for 7 d. Early degeneration. The central axon (*Ax*) appears flocculent and lacks organelles. Focal splitting of lamellae is evident but restricted to the outer margins both of inner (M_i) and outer (M_o) myelin sheaths, and a small lipid droplet (*L*) is present in the inner Schwann cell. Bar, 1 μm. (*Fig. 3*) Guanethidine sulphate (50 mg/kg per day) for 7 d. More advanced disruption of a displaced outer sheath (M_o), including splitting of lamellae within the sheath. Extensive disruption of the inner sheath (M_i) has obscured remnants of the axon. Bar, 1.25 μm.

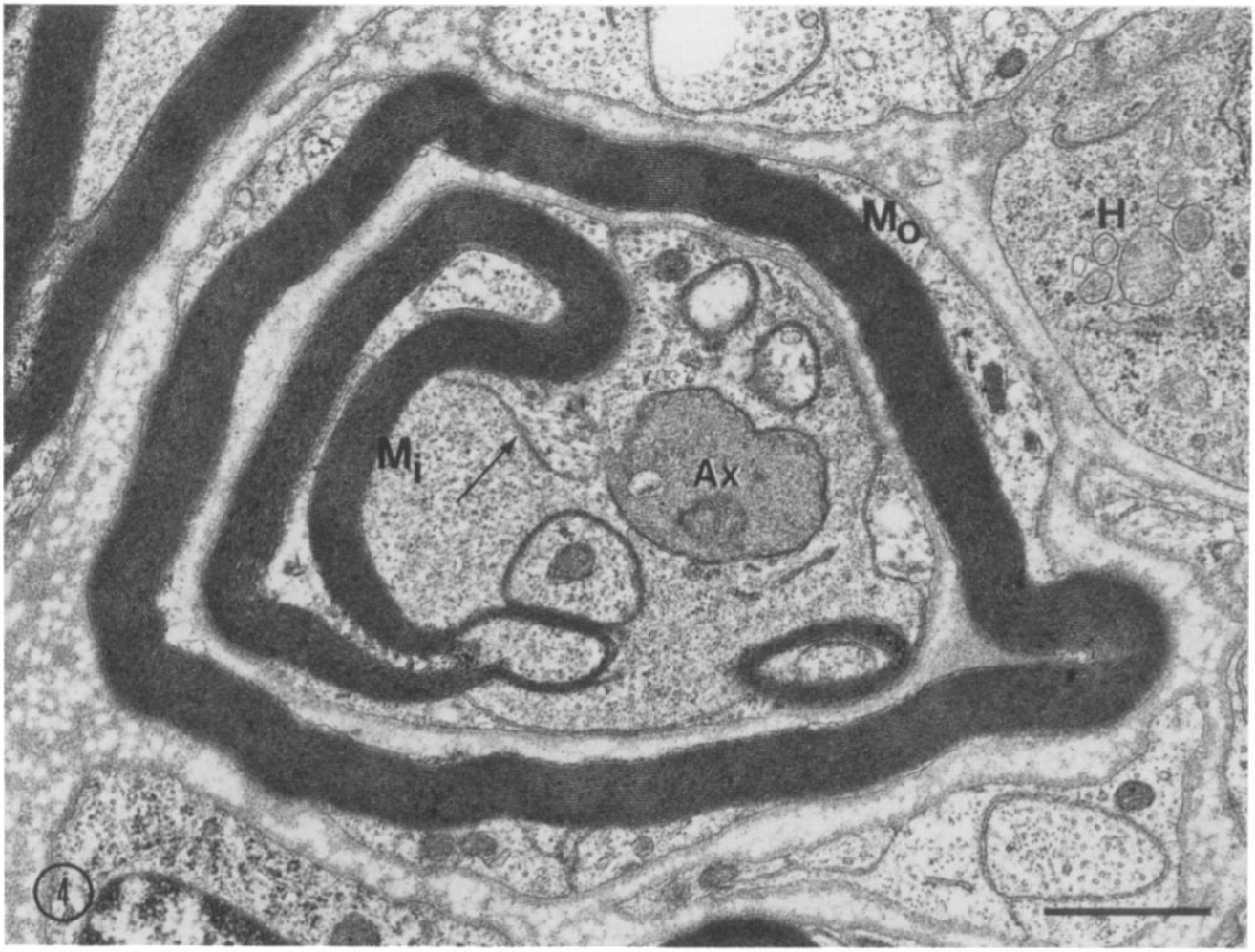


Figure 4. Guanethidine sulphate (50 mg/kg per day) for 5 d. A surviving outer sheath and Schwann cell in TS. The degenerating axon (*Ax*) appears flocculent, and retains contact with the inner sheath (M_i) via an extended mesaxon (*arrow*). The inner sheath has collapsed, and loosened lamellar membranes and myelin debris are located within the cytoplasm. However, the outer myelin sheath (M_o) remains free from focal disruption and the orderly lamellar structure is retained. *H*, haematogenous cell. Bar, 1 μm.

The earliest detectable change in doubly myelinated fibers involved disruption of the axon, characterized by the accumulation of organelles, membranous debris and dense bodies, disruption of the cytoskeleton, and widening of the periaxonal space. At more advanced stages, breakdown of the inner sheath also occurred (Figs. 2 and 3). This involved splitting of individual myelin lamellae, initially at the margins of the sheath, ultimately resulting in the collapse of the inner sheath and in the formation of myelin ovoids within the inner Schwann cell cytoplasm. Lipid droplets and myelin debris accumulated within the Schwann cell cytoplasm (Figs. 2 and 3). In other profiles, reorganization of cytoplasmic processes of the inner Schwann cell was evident but myelin debris was absent, even at early time points (see Fig. 5), suggesting that the inner Schwann cell had not yet produced a myelin sheath at the time of axonal degeneration. In particular, intact doubly myelinated fibers in which the axon and inner sheath remained unaffected by guanethidine (i.e., resembling Fig. 1) were not observed after 10 d in this study.

Based on the numbers of doubly myelinated profiles in the control and treated SCG of each animal, ~75% of the outer myelin internodes degenerated within the first 10 d of treatment. In these profiles, the outer internodes degenerated at the same time as the inner sheath and axon (Figs. 2 and 3). We did not observe degeneration of outer internodes in the absence of degeneration of the centrally enclosed axon.

In contrast, in the remaining 25% of profiles the outer internode remained intact, irrespective of the state of degeneration and phagocytosis of the central axon and inner myelin sheath or the duration of guanethidine treatment (Figs. 4–6). 12 surviving outer sheaths were observed in this study. In these profiles, the ordered lamellar structure of the outer sheath was well maintained, and lipid droplets or whorls of degenerating myelin were not present within the cytoplasm of the outer Schwann cell. Serial reconstruction of profiles in which the degenerating axon and inner sheath were still identifiable (Fig. 4) indicated that these outer Schwann cells were completely displaced from the axon. Additional features characteristic of fully displaced outer myelin sheaths were retained, such as the infolding of both sets of "paranodes" (Fig. 5, *a*, *c*, and *e*) and the formation of new lamellae in some infolded regions by compaction of juxtaposed "adaxonal" plasma membranes (Fig. 5, *a* and *c*; and Fig. 6, *a* and *b*; see also Kidd and Heath, 1988*a*,*b* for details of these

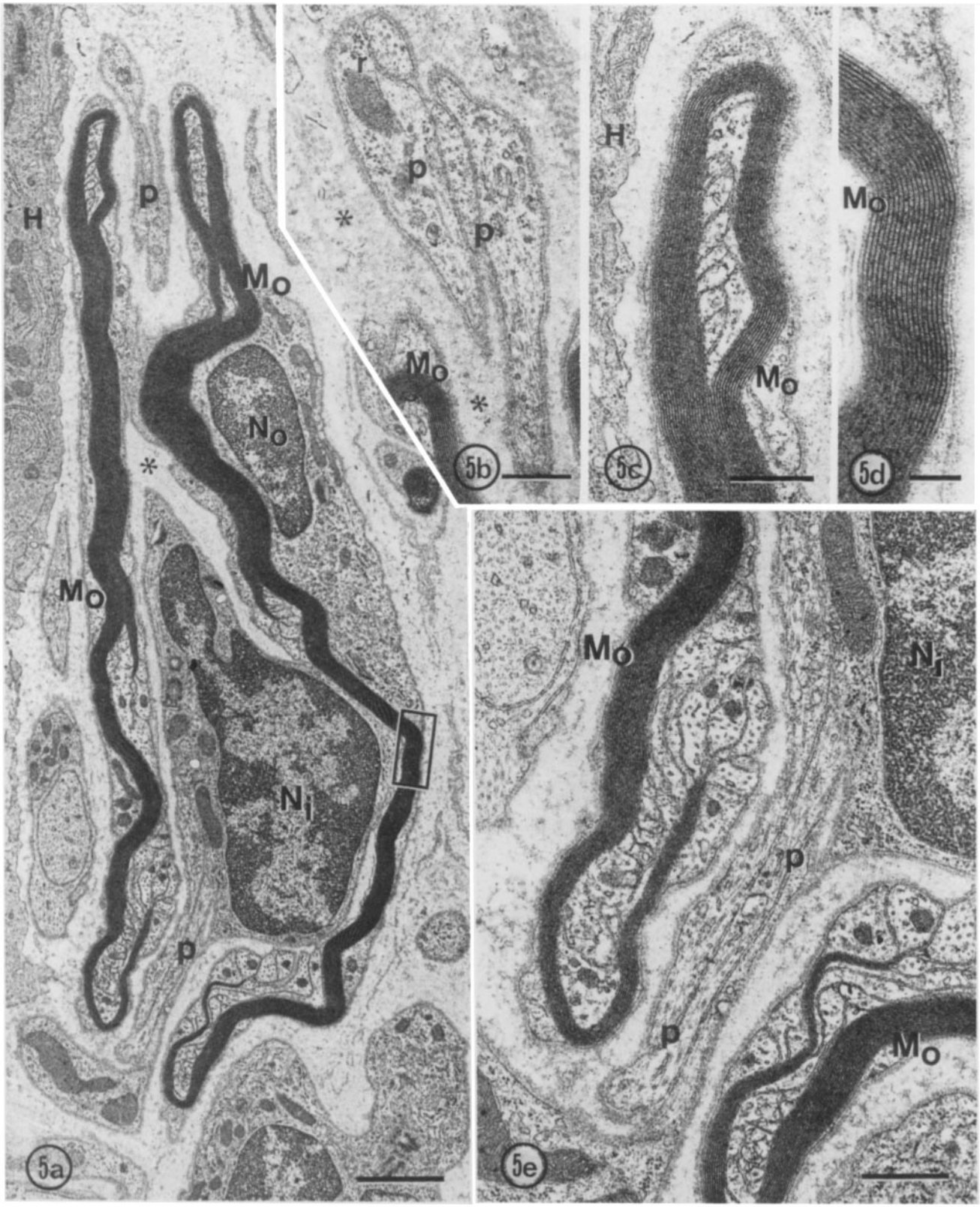


Figure 5. Guanethidine sulphate (50 mg/kg per day) for 7 d. Profile in longitudinal section. (*a*) A surviving outer sheath (M_o) surrounds an inner Schwann cell (nucleus N_i) and numerous cytoplasmic processes (*p*), but axonal and myelin debris are not evident. At higher magnification a group of Schwann cell processes containing ribosomes (*r*) extend from the upper end (*b*, from deeper section) and lower end (*e*) of the sheath. The lamellar structure of the outer sheath remains intact (*c* from upper left in *a*; *d* from box), and characteristic infolding with compaction of juxtaposed "adaxonal" plasma membranes is retained (pivotal regions illustrated in *c* and *e*). *, collagen fibrils; N_o, outer Schwann cell nucleus; and *H*, haematogenous cell. Bars: (*a*) 1.0 μm; (*b*) 0.5 μm; (*c* and *d*) 0.6 μm; (*e*) 0.2 μm.

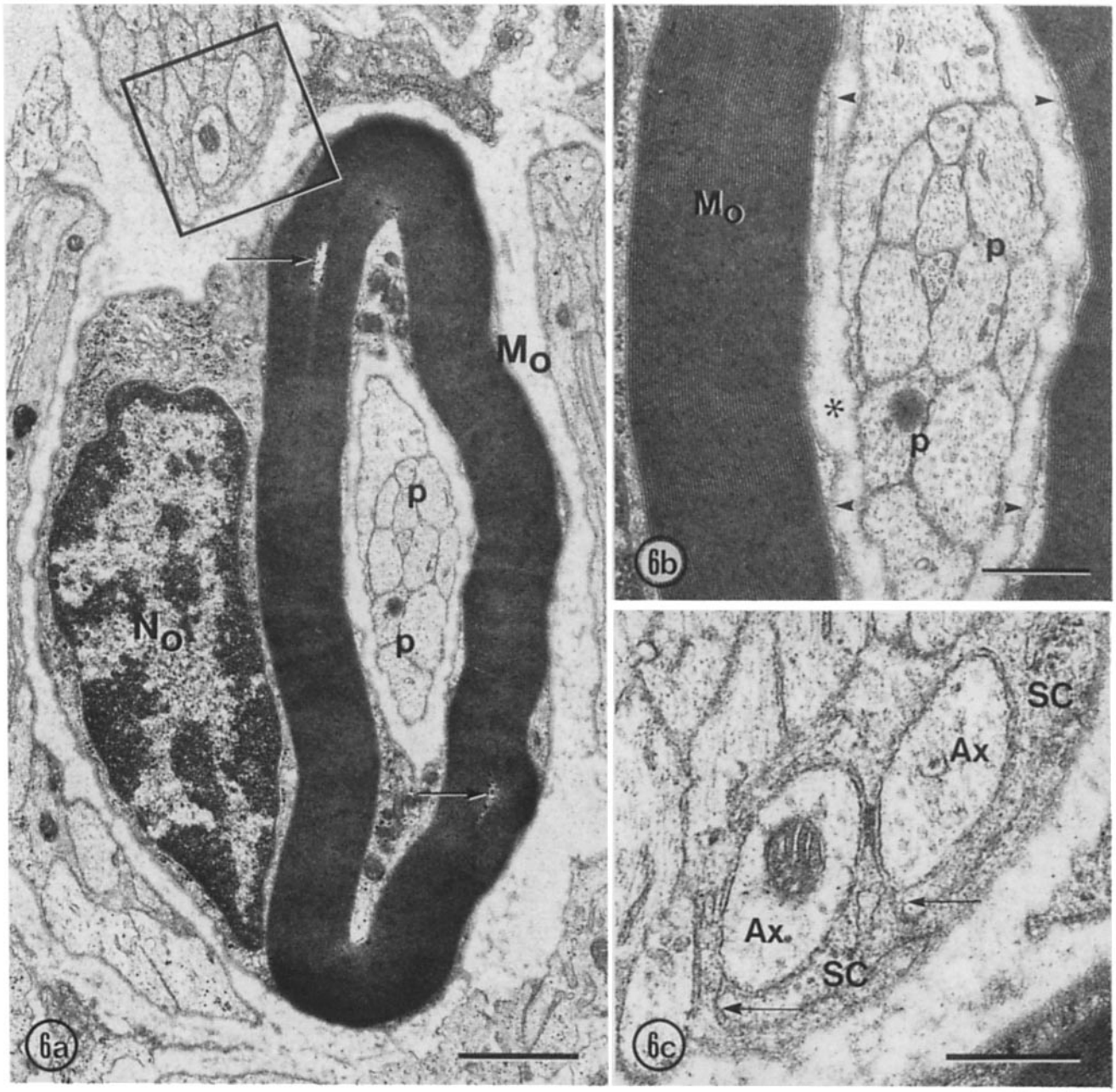


Figure 6. (*g*) Guanethidine sulphate (50 mg/kg per day) for 6 wk. A surviving outer myelin sheath (M_o) and Schwann cell (nucleus N_o) surround numerous cytoplasmic processes (*p*, higher magnification in *b*), and collagen fibrils (*asterisk*). The lamellar structure of the outer sheath is retained (*b*) and the perinuclear Schwann cell cytoplasm is free from myelin debris and lipid droplets. A basal lamina is present on the "inner" aspect of the outer Schwann cell (*arrowheads* in *b*), indicating that the sheath is infolded (see Kidd and Heath, 1988*a*). As a result, the sheath has compacted against itself around almost its whole circumference (*a* and *b*). Thus, focal regions of noncompaction (*arrows* in *a*) within the surviving sheath represent the residuum of the formerly adaxonal cytoplasmic collar and not pathological splitting of lamellae. In contrast to the stacking of the inner processes (*p*), adjacent unmyelinated axons (*Ax*; from box in *a*) are ensheathed in typical manner by Schwann cell processes (*SC*), forming mesaxons (*arrows*). Bars: (*a*) 1.25 μm; (*b*) 0.5 μm; (*c*) 0.2 μm.

events in control tissue). Mitosis and reorganization of the inner Schwann cells resulted in the formation of bands of Büngner enclosed by the surviving outer internodes (Figs. 5 and 6). These Schwann cells and their processes were bounded by the residual inner basal lamina, and by a matrix of small-diameter (<20 nm) collagen fibrils. Degeneration of outer sheaths at later time points (2–6 wk), i.e., after they had survived loss of the axon and inner sheath, was not observed. Haematogenous cells were found in close proximity to surviving outer internodes but did not engage in myelin stripping (Figs. 4 and 5, *a* and *c*). In contrast, haematogenous cells were active in phagocytosis of myelin both from adjacent "singly" myelinated fibers (see also Kidd et al., 1986) and from inner sheaths. Recruitment of hematogenous cells is a hallmark of guanethidine-induced sympathectomy (see Johnson and Manning, 1984).

In no instance was evidence of axonal regeneration found within a surviving outer sheath, even at 6 wk. Typically, the

Schwann cell processes of the inner bands of Büngner lay closely stacked against one another, with no evidence of mesaxon formation (Fig. 6, *a* and *b*). In contrast, surviving unmyelinated axons in the adjacent tissue were ensheathed by Schwann cells in typical Remak manner, including prominent mesaxons (Fig. 6, *a* and *c*). In addition, Schwann cell processes often contained free ribosomes, RER, and numerous intermediate filaments (Fig. 5, *a* and *b*; and Fig. 6 *b*). Axons lacked ribosomes and RER and were usually more electron lucent than their Schwann cell investment (Fig. 6, *a* and *c*).

Discussion

Previous studies of double myelination in the normal SCG (Heath, 1982, 1983; Kidd and Heath, 1988*a*,*b*) have provided evidence that the outer myelin sheath is maintained and indeed reorganized by the outer Schwann cell, which no longer contacts an axon directly. Extending these observations, the principal finding of this study is that completely displaced outer internodes survive guanethidine-induced degeneration of the centrally enclosed axon. We conclude that neither surface-bound nor diffusible axonal factors are required for myelin maintenance after the axon-dependent induction of myelination, at least in displaced Schwann cells in the SCG.

In this study two distinct responses were observed among outer sheaths after degeneration of the axon. In approximately three quarters of profiles, the outer sheath degenerated at the same time as the inner myelin sheath and axon. However, in the remaining group of profiles, the outer sheath survived. Where the outer sheath degenerated, the pattern and time course of myelin disruption was similar to that of the inner sheath of the same configuration, and within the general time frame of degeneration of "singly" myelinated fibers in the surrounding tissue, indicating a Wallerian-like response secondary to axonal degeneration. Surviving outer internodes that were serially sectioned during the progress of axonal and inner sheath degeneration were found to be completely displaced from the axon. Consistent with this observation, surviving outer sheaths often exhibited an advanced degree of conformational reorganization in which both sets of paranodes were infolded; this is unlikely to occur unless the entire outer Schwann cell including both paranodes has been displaced from the axon (Kidd and Heath, 1988*a*,*b*). The ratio of degenerating to surviving outer sheaths ($\sim$3:1) is consistent with previous estimates of partially versus completely displaced outer sheaths in the normal SCG (Kidd and Heath, 1988*b*). Taken together, these observations suggest that contact with a degenerating axon was necessary for myelin breakdown. The alternative possibility that guanethidine itself might exert a cytoxic effect directly on myelin or Schwann cells has been discounted previously (Kidd et al., 1986). Degeneration of partially displaced outer sheaths was also noted after axotomy produced by a transganglionic surgical lesion (Kidd and Heath, 1991).

As expected, a small number of axons persisted in the SCG even after prolonged guanethidine treatment (Kidd et al., 1986) and represented a potential source of diffusible trophic factors which may have mediated outer sheath survival. However, it is unlikely that outer sheath survival in the SCG resulted directly from supply of trophic factors by nearby nondegenerating axons, since their presence did not prevent degeneration of singly myelinated internodes or partially displaced sheaths. In addition, most of the surviving axons were unmyelinated, and so, unlikely to contribute to myelin maintenance by other Schwann cells.

In surviving outer internodes the myelin sheath remained intact at all stages, even though the inner sheath followed the expected course of focal splitting, ovoid formation, and finally reorganization of Schwann cells and their processes into bands of Büngner. In some instances, a myelin sheath had not been formed by the inner Schwann cell before guanethidine treatment, and in the absence of myelin debris the events of phagocytosis were complete at earlier time courses (5–7 d; e.g., Fig. 5 probably represents such a profile). Degeneration of susceptible myelinated fibers was largely complete after treatment for 10 d (see also Kidd et al., 1986). In particular, no doubly myelinated fibers unaffected by guanethidine were found beyond this time point. This indicates that surviving outer internodes in animals treated for 6 wk (the longest period studied) have persisted in the absence of the axon for at least 4 wk. Similarly, outer internodes survive for at least 16 wk after axonal degeneration produced by surgical transection of the SCG (Kidd and Heath, 1991).

The sympathectomy produced by guanethidine involves destruction of the entire neuron (Burnstock et al., 1971; Heath et al., 1972; see Johnson and Manning, 1984). Thus, in this study it would be expected that the potential for axonal regeneration was minimized, particularly since daily guanethidine administration was continued throughout the course of the experiment. Regenerating axons ensheathed by membranes of inner Schwann cells were not found within any surviving outer internodes. Rather, the reorganized cytoplasmic processes of the inner cells merely abutted against one another. For these reasons, we conclude that outer sheath survival was not influenced by possible trophic factors produced by axons regenerating within the confines of the outer Schwann cell. In a complementary surgical study (Kidd and Heath, 1991), axonal regeneration was either prevented or facilitated by variation of the experimental design. Where prevented, the results were similar to the present study. Where facilitated, axonal sprouts were readily identified as they grew within surviving outer internodes and became invested by inner Schwann cells in typical Remak fashion, including the development of prominent mesaxons.

Outer sheath survival is unlikely to reflect diminished Schwann cell myelinolytic competence because of toxic effects of guanethidine, since daily guanethidine treatment did not interfere with myelin breakdown in inner Schwann cells, those providing "single" myelin ensheathment or those only partly displaced from the axon (see also Kidd et al., 1986). All surviving internodes observed in this study surrounded collagen, basal lamina, and residual inner Schwann cells/processes, indicating the former existence of a region of double myelination rather than the continued survival of a "single" sheath.

It is apparent that conditions in the SCG promote formation of double ensheathment. The results of this study indicate that most if not all displacement leading to double myelination in the SCG involves postganglionic adrenergic axons rather than preganglionic or nonsympathetic axons. However, observations of similar profiles (see Kidd and

Heath, 1988*a*) and those resembling formative stages (Bischoff and Thomas, 1975; Weller and Cervós-Navarro, 1977; Smith et al., 1985; Vital and Vallat, 1987) in other nerve tissues suggest that Schwann cell behavior in the SCG is not intrinsically different from that in other tissues (see Kidd and Heath, 1988*b*). The results of the current study and previous work on double myelination therefore suggest that Schwann cells may continue to synthesize myelin components and intercalate them into an existing myelin sheath in the absence of membrane level interaction with the axon or diffusible axonal trophic factors. One explanation for these data may be that the axonal signal is mimicked by some factor in the endoneurium, although there is no evidence to support this hypothesis at present. Alternatively, the gradual process of displacement of the outer Schwann cell from the axon may be accompanied by loss of sensitivity to axonally derived regulators. It was not possible in this study to statistically assess the degree of displacement required to provide a basis for such loss of sensitivity. However, as far as could be determined, no surviving outer sheath retained any contact with the axon, suggesting that even minor contact with a degenerating axon may be sufficient to precipitate myelin breakdown in the outer cell. The most likely explanation is that the maintenance phase of myelination, which requires synthesis of relatively low levels of components to match to their slow turnover rates in the sheath (Benjamins and Smith, 1984), may not be directly regulated by the axon, unlike initial myelin formation which requires much higher synthetic rates.

In Wallerian degeneration, the mechanism that initiates myelin breakdown after axonal degeneration remains unknown (Bigbee et al., 1987; Oaklander et al., 1987; Oaklander and Spencer, 1988; White et al., 1989; LoPachin et al., 1990), though it has been suggested that the ensheathing Schwann cells are responding to lack of a signal factor normally supplied by the axon (Joseph, 1973; Malbouisson et al., 1984; Oaklander and Spencer, 1988). However, the continued maintenance of the outer sheath in regions of double myelination after complete displacement from the axon (Kidd and Heath, 1988*a*,*b*), and following degeneration induced by guanethidine (this study) or surgery (Kidd and Heath, 1991), raise the alternative possibility that secondary myelin breakdown is precipitated by a positive signal, probably originating from the degenerating axon (Joseph, 1973; Lubinska and Jastreboff, 1977). According to this hypothesis, a completely displaced outer internode would not receive this degenerative stimulus because of its remote position and the intervening basal lamina of the inner cells, and would continue to maintain its sheath. Potential stimuli might include molecules formed during axonal proteolysis, or lytic enzymes involved in degrading the axon may also damage the sheath and initiate myelin breakdown. A further possibility is that damage to the periaxonal membrane during early Wallerian degeneration may result in raised calcium levels within the Schwann cell, activating enzymes and sheath damage (Smith and Hall, 1988). Raised calcium levels have also been implicated in initiation of axonal degeneration (Schlaepfer and Bunge, 1973; Schlaepfer, 1974). Complementary evidence, from studies of C57BL/6/Ola mice, indicates that Schwann cells in this mutant continue to maintain a morphologically intact myelin sheath for a prolonged period after loss of axonal contact through axonal degeneration (Glass J., Johns Hopkins University, Baltimore, MD, personal communication). Wallerian degeneration is retarded in this strain, probably through inadequate recruitment of macrophages (Lunn et al., 1989), suggesting that nonneuronal factors may also be required for a Schwann cell response. These results suggest that Schwann cells continue to maintain a myelin sheath until appropriate axonal and nonneuronal signals initiate myelin degeneration and dedifferentiation.

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