CHEMICAL AND STRUCTURAL CHANGES

OF NEUROFILAMENTS

IN TRANSECTED RAT SCIATIC NERVE

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ABSTRACT

The sequence of changes occurring in transected rat sciatic nerve was examined by electron microscopy and by sodium dodecyl sulfate (SDS) polyacrylamide disc gel electrophoresis. Representative segments of transected nerves were processed for ultrastructural examinations between 0 and 34 days after the transection of sciatic nerves immediately below the sacro-sciatic notch. The remainder of the transected nerves and the intact portions of sciatic nerves were desheathed and immediately homogenized in 1% SDS containing 8 M urea and 50 mM dithioerythritol. Solubilized proteins were analyzed on 12% gels at pH 8.3 in a discontinuous electrophoretic system.

Initial changes were limited to the axons of transected nerve fibers and were characterized by the loss of microtubules and neurofilaments and their replacement by an amorphous floccular material. These changes became widespread between 24 and 48 h after transection. The disruption of neurofilaments during this interval occurred in parallel with a selective loss of 69,000, 150,000 and 200,000 mol wt proteins from nerve homogenates, thus corroborating the view that these proteins represent component subunits of mammalian neurofilaments. Furthermore, the selective changes of neurofilament proteins in transected nerves indicate their inherent lability and suggest their susceptibility to calcium-mediated alterations.

Electrophoretic profiles of nerve proteins during the 4–34-day interval after nerve transection reflected the breakdown and removal of myelin, the proliferation of Schwann cells and the deposition of endoneurial collagen. A marked increase of intermediate-sized filaments within proliferating Schwann cell processes was not accompanied by the appearance of neurofilamentlike proteins in gels of nerve homogenates.

KEY WORDS protein subunits · neurofilaments · intermediate-sized filaments · gel electrophoresis · nerve degeneration

A well-defined sequence of structural changes is known to occur in transected peripheral nerve. These degenerative changes, commonly referred to as Wallerian degeneration after their original description (48), appear initially in the axon (10, 36, 48) and are characterized ultrastructurally by degradative changes of axonal microtubules and neurofilaments (14, 17, 20, 40, 43, 47, 49).

Primary axonal changes are associated with collapse and fragmentation of myelin sheaths, but chemical breakdown of myelin occurs at a later time (5, 6, 9, 22, 28, 29, 35, 38). Progressive degradation of myelin and axonal debris is accompanied by a proliferation of Schwann cells (1, 11, 15, 23, 46) and fibroblasts (1, 15) and deposition of collagen (1, 2, 15).

Changes in transected nerves involve specific axonal and Schwann cell elements at different time periods, thus providing a useful model to correlate chemical and structural changes of these tissue components. Biochemical studies of Wallerian degeneration have largely focused on the breakdown of myelin and the loss of myelin lipids (5, 6, 9, 22, 28, 29, 35, 38). Little attention has been directed to early axonal alterations or to the overall changes in nerve proteins that occur in transected nerves.

Our recent studies have identified the major proteins of peripheral nerve on sodium dodecyl sulfate (SDS) gels of whole nerve homogenates on the basis of their co-migration with proteins of purified axonal and myelin fractions (31). Neurofilament proteins have also been identified in isolates of intact neurofilaments (44). The present study of nerve proteins during Wallerian degeneration has confirmed the identity of different proteins in nerve homogenates. Furthermore, the study demonstrates that the breakdown of neurofilaments is associated with profound changes in their protein subunits, indicating an inherent lability of neurofilament proteins in situ. Since neurofilament disruption in degenerating nerves is believed to be mediated by an influx of calcium (40–43), alterations of neurofilament subunits may be due to a calcium-activated process.

MATERIALS AND METHODS

Experimental Manipulations

Sciatic nerves of 300–350-g male Sprague-Dawley rats under Nembutal (Abbott Laboratories, North Chicago, Ill.) anesthesia were transected bilaterally immediately below the greater sciatic foramen, and the overlying muscle and skin incisions were sutured with 5-0 and 3-0 silk, respectively. After various intervals (0–34 days), the rats were anesthetized with ether and perfused through the heart with isotonic saline to obtain blood-free tissues for chemical studies. The nerves, nerve roots, and sensory ganglia of the sciatic nerve complex were excised, temporarily placed in saline, and divided according to the schema in Fig. 1. Electron microscopic examinations were carried out on 2-mm segments (EM)
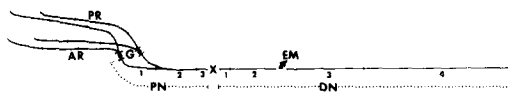

FIGURE 1 Schematic outline of rat sciatic nerve complex indicating sites of tissue samples in relation to nerve transection. For details, see Material and Methods.

excised 15 mm distal to the site of transection (X) after fixation by immersion in 2% glutaraldehyde and 5% p-formaldehyde in 0.1 M phosphate buffer, pH 7.2. Identical segments of transected nerves were also examined ultrastructurally after fixation by perfusion with Ringer's solution containing 1% glutaraldehyde and 2.5% p-formaldehyde. Transected nerves proximal to EM segments were desheathed and divided into 2–3-mm distal stumps (DN1) and 12–13-mm mid-distal segments (DN2). Desheathed distal portions of transected nerves were divided into 20-mm distal segments (DN3) and 35–45-mm distalmost segments (DN4). Intact portions of the sciatic nerve complex were separated just above the junction of the L4 and L5 nerve trunks, desheathed, and divided into 2–3-mm proximal stumps (PN3) and 9–12-mm mid-proximal nerve segments (PN2). After careful exposure of the lumbar cord, the anterior roots (AR) and posterior roots (PR) L4 and L5 nerve trunks were excised and divided into proximal (AR1 and PR1), middle (AR2 and PR2), and distal (AR3 and PR3) segments ~9 mm each, according to their relationship to their respective neuronal soma. The L4 and L5 sensory ganglia (G) were cleanly dissected. Proximal nerve segments (PN1) were obtained by desheathing the remaining portions of L4 and L5 nerve trunks distal to the ganglia. Control roots (CR) and control ganglia (CG) were obtained from the L1, L2, and L3 nerve trunks, and control nerve (CN) was taken from the middle portion of desheathed axillary nerve. Contralateral tissue samples of each animal were combined and their approximate weights (in milligrams) were as follows: DN1, 6; DN2, 21; DN3, 34; DN4, 25; PN1, 22; PN2, 40; PN3, 7; AR1, 6; AR2, 6; AR3, 7; PR1, 10; PR2, 9; PR3, 9; G, 7; CR, 22; CG, 7; CN, 20.

Solubilization of Nerve Proteins

Each weighed nerve specimen was immediately placed into a Duall tissue grinder (Kontes Co., Vineland, N.J.) containing freshly prepared 8 M urea, 1% SDS, and 50 mM dithioerythritol (DTE). The samples were homogenized thoroughly, heated at 100°C for 5 min, rehomogenized and centrifuged for 60 min at 40,000 rpm in a Beckman SW 50.1 rotor (Beckman Spinco, Palo Alto, Calif.). The clear supernate was collected through the side of the tube, avoiding the floating lipid layer. For protein determinations, nerve segments were heated at 70°C for 15 min in 1 N NaOH, the lipid was allowed to float to the top, and the clear bottom layer was assayed by the method of Lowry et al. (27).

Polyacrylamide Gel Electrophoresis

A modification of the SDS gel system of Laemmli (25) was used to analyze the solubilized proteins of each nerve segment. Resolving gels were 8.4 cm long, stacking gels were 1.7 cm, and electrophoresis was performed at 20°C in acid-washed tubes of 0.55-cm inside diameter. Electrode buffer was 0.05 M Tris, 0.38 M glycine, 0.1% SDS, pH 8.3. Samples were prepared in 10 × 74-mm tubes containing 5 × 10⁻⁴% bromphenol blue, 10% sucrose, 1% SDS, 40 mM DTE, 1 mM sodium ethylenedinitrilotetraacetate (EDTA), 40 μg of protein, and 10 mM Tris-HCl, pH 8.9, in a total volume of 75–300 μl. Electrophoresis was carried out at 1.5 mA per gel through the stacking gel and 2.5 mA per gel through the resolving gel. When the tracker dye came to within 0.5 cm of the end of the gel, electrophoresis was stopped, and the gels were removed from the tubes and cut off at the tracker dye. Fixation and staining of gels and estimation of molecular weights were performed as previously reported (31).

Electron Microscopy

Aldehyde-fixed nerve segments (EM) were washed in 0.1 M sodium cacodylate buffer, pH 7.2, and placed in the same buffer containing 1% OsO_4 for ~30 min before and after transversely sectioning the tissues into 0.2–0.4-mm sections. Nerve tissues were stained en bloc in uranyl acetate (24), dehydrated through propylene oxide, and embedded in epoxy 812 resin. Ultrathin sections were stained with lead citrate (37) and examined in a Siemens Elmiskop 1A electron microscope operated at 80 kV and equipped with 100-μm condenser and 15-μm objective apertures.

RESULTS

Ultrastructural Changes in Transected Nerves

The nature and sequence of ultrastructural changes in transected nerves closely resembled the published reports on Wallerian degeneration (14, 17, 20, 40, 43, 47, 49). Alterations were not detected at 0, 6, 12, or 18 h but became prominent between 24 and 48 h after transection. Initial changes appeared in axons and were characterized by the loss of microtubules and neurofilaments and their replacement by an amorphous floccular material (Fig. 2). The loss of axoplasmic microtubules generally preceded the loss of neurofilaments. The longitudinal orientation of both organelles became focally disarrayed during the early phase of axoplasmic disruption. 24 h after transection, axoplasmic degeneration was limited to scattered unmyelinated fibers and <5% of myelinated fibers. By 48 h, all unmyelinated and >90% of myelinated fibers had degenerated axoplasm. In-

termediate degeneration was noted at 30, 36, 39, and 42 h. No intact axons were seen 60 or 72 h after transection.

Axoplasmic degeneration was accompanied and succeeded by collapse of the surrounding myelin sheaths. Schwann cells filled with disorganized myelin debris became increasingly evident 3, 4, 6, and 8 days after transection. After 4 days, fragmentation of myelin debris could be seen associated with reactive changes of Schwann cells. The reactive Schwann cells displayed increased numbers of organelles as well as lipid droplets mixed with myelin debris. Progressive degradation of myelin in 11- and 13-day transected nerves was manifested by decreased amounts and increased fragmentation of myelin debris within Schwann cell cytoplasm. Only a few scattered Schwann cells and macrophages with lipid debris remained in the 34-day transected nerve.

The breakdown of myelin was accompanied by a proliferation of Schwann cells and fibroblasts. After 6 days, longitudinally proliferating Schwann cell processes occurred individually or in clusters, the latter representing bands of Büngner (7, 17, 49). Proliferating Schwann cells comprised the predominant cellular component in the 13-day transected nerve. These Schwann cell processes were distinguished by an abundance of intermediate-sized filaments (8–10 nm) which paralleled the long axes of the cell (Fig. 3). Clustered processes were enclosed by a single basement membrane. Proliferating endoneurial fibroblasts were noted during the 6–13-day interval after transection. By 34 days, markedly increased numbers of collagen fibrils filled the endoneurium amidst a relative paucity of cellular elements.

Loss of 69,000, 150,000, and 200,000 Mol Wt Proteins from Transected Nerves

Initial changes in the electrophoretic profile of nerve proteins were seen in the 24–48-h interval after nerve transection. Neurofilament proteins of 69,000, 150,000, and 200,000 mol wt (identified by arrowheads in Figs. 4 and 5) can be seen in unsectioned control nerve (Figs. 4 and 5). They were also found consistently in the proximal (DN1), middle (not shown), and distal (DN3) portions of 24-h transected nerve (Fig. 4). However, 48 h after transection, the amounts of these proteins were markedly reduced. These effects were noted in proximal (DN1), middle (not shown), and distal (DN3) parts of 48-h transected nerve (Fig. 4). Progressively smaller amounts of
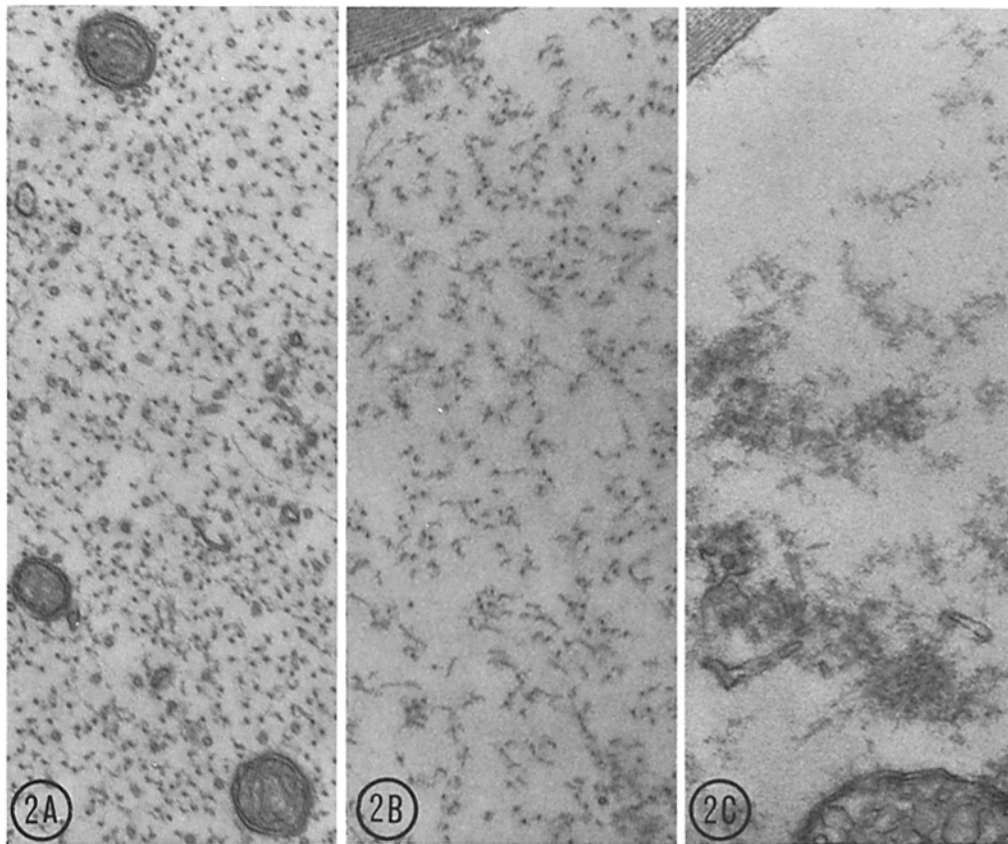
FIGURE 2   Transverse sections of axons showing progressive loss of microtubules and neurofilaments and their replacement by an amorphous granular material after 36 h (*B*) and after 48 h (*C*) compared to 24-h transected nerve (*A*). Sections are representative of large myelinated nerve fibers. × 43,000.

these neurofilament proteins were seen at 30, 36, 39, and 42 h (not shown). Loss of 69,000 and 150,000 mol wt proteins was sometimes more pronounced than the loss of 200,000 mol wt protein during the 30–42-h interval. Loss of the three neurofilament proteins occurred at about the same rate throughout the transected nerve.

Accumulation of 53,000 and 70,000–73,000 Mol Wt Proteins in Transected Nerves

Proteins with the R_f of tubulin (53,000–57,000 mol wt) remained a prominent component in transected nerves. These proteins (Fig. 5, band 4) in unsectioned control nerves (Fig. 5, CN) appeared diffuse compared with the corresponding doublet of purified tubulin on gels of protein standards. In transected nerves, the 53,000–57,000 mol wt protein bands were often asymmetric with a relative increase of faster migrating

proteins of 53,000 mol wt. Increases of 53,000 mol wt protein were most conspicuous in nerves after prolonged posttransectional intervals (Fig. 5, DN3) and represented a major protein band in 34-day transected nerves. Some asymmetry of the 53,000–57,000 mol wt bands with relative increase of 53,000 mol wt protein occurred in transected nerves during the 24–48-h interval. Increases of 53,000 mol wt protein were least apparent in the stumps (DN1) of transected nerves.

A prominent 70,000–73,000 mol wt protein (Fig. 5, band 3) often migrated immediately behind the 69,000 mol wt protein during electrophoresis of nerve proteins, causing an overlap in the densitometric tracings of these protein bands (Fig. 4; CN, DN1–24 h, and DN 3–24 h). Progressively increasing amounts of 70,000–73,000 mol wt protein were observed in nerves during the 24–72-h posttransectional interval. Large amounts of
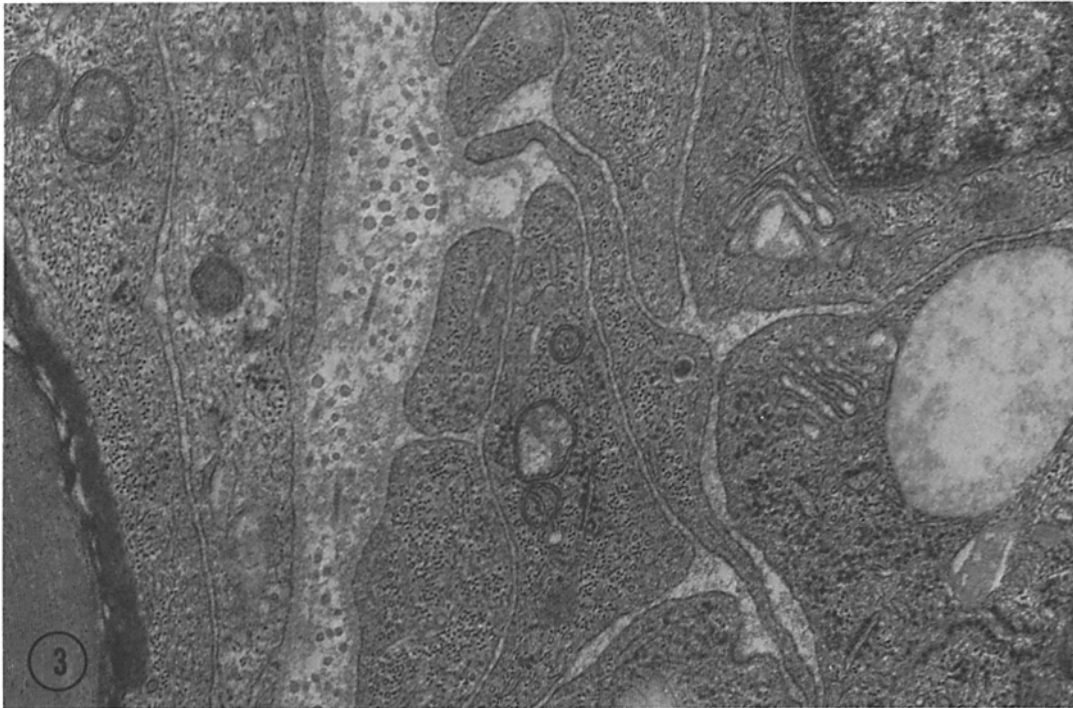
70,000-73,000 mol wt protein remained in transected nerves and became the most conspicuous protein component of the 13-day (Fig. 5, DN3) and the 34-day transected nerves. The accumulation of 70,000-73,000 mol wt protein occurred throughout the transected nerves but was least apparent in the stumps (DN1) of sectioned nerves. An 85,000 mol wt protein band (Fig. 5, band 2) also became accentuated in transected nerves which had large accumulations of 70,000-73,000 mol wt protein.

Loss of Myelin Proteins from Transected Peripheral Nerve

Myelin proteins of peripheral nerve have been isolated and identified (31) and are represented in whole nerve homogenates by a major P_0 glycoprotein of 27,000 mol wt (Fig. 5, band 8), an intermediate P_x glycoprotein of 20,000 mol wt (Fig. 5, band 9) and P1 and P2 basic proteins of 18,000 and 15,000 mol wt (Fig. 5, bands 10 and 11). These proteins remained unaltered during the initial 4 days after nerve transection, were present in diminishing amounts during the 4-13-

day interval (Fig. 5, PN3) and were no longer observed in the 34-day transected nerve. The loss of these proteins occurred at similar rates throughout the transected nerves. The loss of myelin proteins was accompanied by the appearance of a polypeptide band which migrates between bands 10 and 11 (Fig. 5, PN3 and DN3). This abnormal polypeptide remained in 34-day transected nerve.

The 4-13-day posttransectional interval was characterized by the loss of myelin proteins as well as the loss of a diffuse 95,000-100,000 mol wt protein (Fig. 5, band 1). Variable amounts of this protein are present in unsectioned rat peripheral nerve and nerve roots although its origin remains undetermined (31).

Increases of 45,000, 49,000-51,000, and High Mol Wt (HMW) Proteins in Transected Nerves

Some minor protein bands in the electrophoretic profile of whole nerve homogenates became accentuated during the 4-13-day interval after nerve transection. These proteins included a doublet band at 49,000-51,000 mol wt (Fig. 5, band
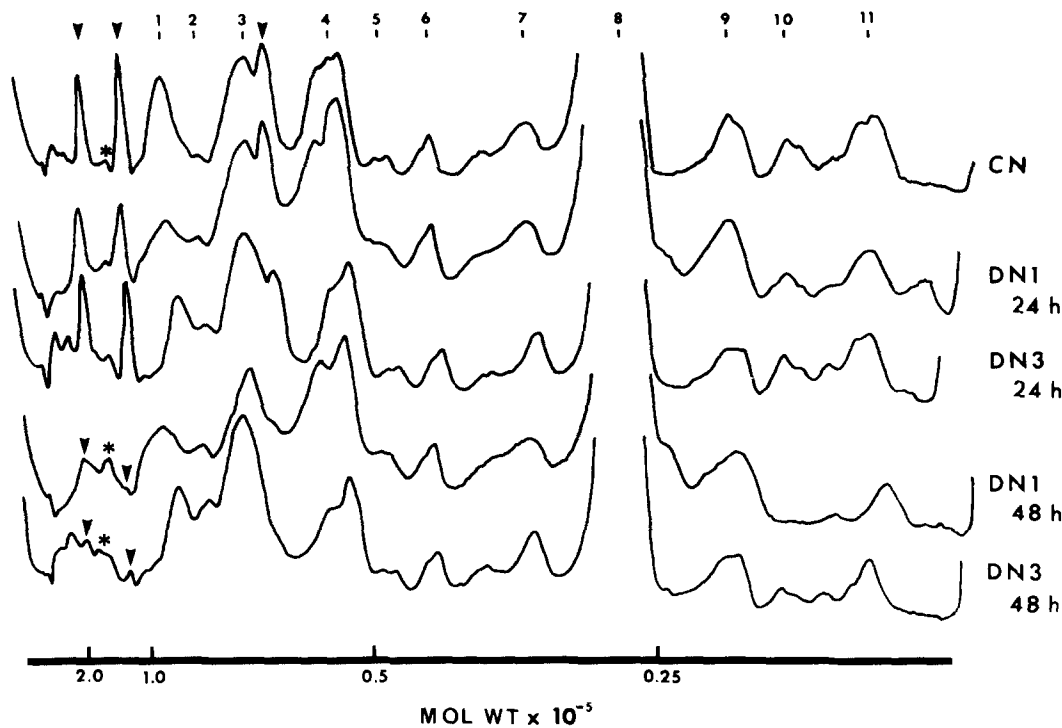
FIGURE 4 Densitometric tracings of protein bands in acrylamide gels of nerve homogenates showing loss of 69,000, 150,000, and 200,000 mol wt proteins (arrowheads) in proximal (*DN1*) and distal (*DN3*) segments of 48-h transected nerve compared with unsectioned control nerve (*CN*) and proximal (*DN1*) and distal (*DN3*) segments of 24-h transected nerve. Asterisk denotes a metachromatic staining collagen protein which served as an internal marker. Mol wt scale constructed from protein standards.

5) and a singlet band at 45,000 mol wt (Fig. 5, band 6). These protein bands became accentuated in severed nerve (DN3) and regenerating stump (PN3) of 13-day transected nerve compared with control nerve (CN) and uncut sciatic nerve (PN1) from the same animal (Fig. 5). In addition, the transected nerve and regenerating stump contained increased amounts of HMW proteins. Several of the HMW proteins stained metachromatically with Coomassie Blue R-250 (ICI United States, Inc., Wilmington, Del.) and co-migrated with commercial calf skin collagen. At least three additional nonmetachromatic protein bands could be recognized among the HMW components, including a single protein of ~175,000 mol wt and at least two other proteins which migrated slower than the myosin standard (210,000 mol wt).

Proteins in the Regenerating Stumps (PN3) of Transected Sciatic Nerves

The proximal stumps (PN3) of cut sciatic nerves

contained the normal nerve proteins of control nerve and the abnormal nerve proteins of transected nerves. Accordingly, the regenerating stump of the 13-day transected nerve (Fig. 5, PN 3) contained increased amounts of 85,000 mol wt protein (band 2), 70,000–73,000 mol wt protein (band 3), 53,000 mol wt protein (band 4), 49,000–51,000 mol wt proteins (band 5), and 45,000 mol wt protein (band 6) as well as the abnormal polypeptide which migrated between bands 10 and 11.

Electrophoretic Analyses of Dorsal Root Ganglia and Nerve Roots, 0–34 Days after Nerve Transection

No consistent alterations of proteins were detected in ganglia or nerve roots during the 0–34-day interval after nerve transection. Electrophoretic profiles of proteins from ganglia were very complex and difficult to analyze.
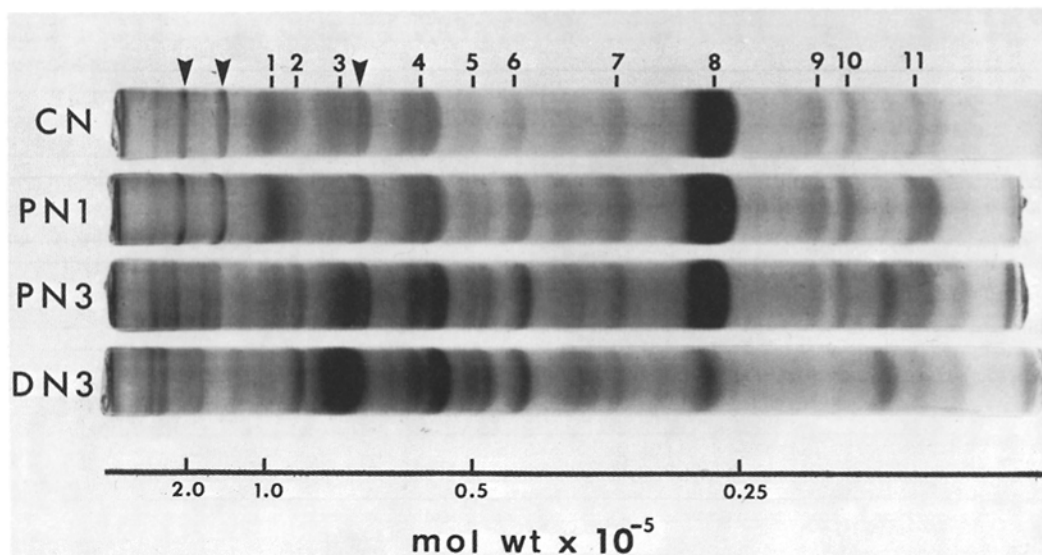
FIGURE 5 Protein bands in acrylamide gels of 13-day transected nerve showing complete loss of 69,000, 150,000, and 200,000 mol wt proteins (arrowheads) in transected nerve (*DN3*) and partial loss in regenerating stump (*PN3*) compared with control nerve (*CN*) and intact sciatic nerve (*PN1*) of the same animal. Myelin proteins (bands 8–11) have been lost from transected nerve. Both transected nerve and regenerating stump show increases of high molecular weight protein bands and nerve protein bands 2–6 as well as accumulation of abnormal protein migrating between bands 10 and 11. Mol wt scale constructed from protein standards.

DISCUSSION

The selective disappearance of 69,000, 150,000, and 200,000 mol wt proteins from polyacrylamide gels of transected peripheral nerve homogenates during the same time interval in which neurofilaments are undergoing granular disintegration corroborates the view that these proteins represent native components of intact neurofilaments. These three proteins were initially suggested to represent neurofilament subunits because of their prominence in the slow component of axonal flow as well as the synchrony of their distad movements (19, 26). Subsequently, the same three proteins were observed to be the major set of proteins in purified axonal fractions which consisted predominantly of neurofilaments by ultrastructural examination (31). The same proteins have recently been identified in isolates of intact neurofilaments from rat peripheral nerve and spinal cord (44).

Chemical changes in neurofilament proteins coincident with their structural disintegration during Wallerian degeneration suggest that this breakdown is more complex than a simple disassembly of polymerized subunits. Axoplasmic degradation in transected nerves is believed to be

associated with an influx of calcium across an altered axolemma (40–43). How an increased calcium concentration causes the disruption of axoplasmic fibrous proteins is presently unclear. Invertebrate neurofilaments are believed to be hydrolyzed by a calcium-activated protease (16). Mediation of neurofilament disruption in transected vertebrate axons by a similar enzyme would require a high degree of enzymatic specificity to account for the lack of parallel breakdown of tubulin during Wallerian degeneration. The disappearance of axonal microtubules during the early phases of Wallerian degeneration could still be due to a calcium-mediated disassembly of these organelles (40, 42, 43).

The nature of the 53,000 and 70,000–73,000 mol wt proteins which accumulate in transected nerves is poorly understood. The variable occurrence of 70,000–73,000 mol wt protein in homogenates of unsectioned nerve (31) and the increased prominence of 53,000 and 70,000–73,000 mol wt proteins in excised nerves after exposure of high calcium levels (W. W. Schlaepfer and S. Micko. Unpublished observations) suggest that these proteins are derived from unstable endogenous nerve proteins. It is noteworthy that a

very prominent 65,000-70,000 mol wt protein appears on acrylamide gels after the calcium-induced disruption of isolated invertebrate neurofilaments (16). Emergence of a prominent neurofilament breakdown product during prolonged isolation procedures could account for the dominant 50,000-54,000 mol wt protein of mammalian brain neurofilament preparations (12, 13, 21, 45, 51), a protein not seen in homogenates of freshly excised, neurofilament-rich mammalian peripheral nerve (19, 26, 31). The continued accumulation of 53,000 and 70,000-73,000 mol wt proteins after the loss of neurofilament proteins remains unexplained but could represent stable products derived from unstable intermediates of neurofilament breakdown.

The biochemical correlates of the marked proliferation of intermediate-sized filaments in Schwann cells in transected nerves cannot be identified with certainty. The increasing prominence of these organelles corresponded with the enhancement of several protein bands in nerve homogenates, including a singlet band with the R_f of actin (45,000 mol wt), a doublet band at 49,000-51,000 mol wt, and several HMW protein bands. Several of these HMW proteins could be identified as collagen proteins on the basis of their metachromatic staining and co-migration with commercial calf skin collagen proteins (32), in accordance with the known increased deposition of endoneurial collagen in transected nerve (1, 2, 15). It is noteworthy that the prominence of intermediate-sized Schwann cell filaments in transected nerve was not associated with the appearance of neurofilamentlike proteins in nerve homogenates although the Schwann cell and neuronal filaments are similar in appearance (7).

The disappearance of myelin proteins between 3 and 13 days after transection generally agrees with previous studies of myelin proteins (4, 30, 50) and myelin lipids (5, 6, 9, 22, 28, 29, 35, 38). Partial loss of P_x and P1 myelin proteins during the initial 24 h after transection (4) was not detected in this study. The breakdown of myelin proteins was associated with the appearance of a small polypeptide which migrated immediately behind the myelin P2 protein, as previously noted (4, 30, 50). The appearance of this abnormal protein in the proximal stump undoubtedly reflects the nerve fiber degeneration known to occur on the proximal edge of nerve transections (8, 10, 18, 33, 34, 36, 39, 52).

The persistence of abnormal proteins in the 34-day transected nerve was unexpected. Most of these proteins were tentatively identified as axonal breakdown products. Yet the presence of intracellular debris had become markedly diminished by the 34-day interval after transection. Additional studies arc needed to determine the ultimate metabolic fate of endogenous nerve proteins released during Wallerian degeneration and their differentiation from exogenous proteins which may accumulate during nerve degeneration.

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