COMPARISON OF NERVE CELL AND NERVE CELL PLUS SCHWANN CELL CULTURES, WITH PARTICULAR EMPHASIS ON BASAL LAMINA AND COLLAGEN FORMATION

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ABSTRACT

The availability of cultures of normal nerve cells (NCs) and Schwann cells (SCs) with and without fibroblasts has allowed us to investigate the sources of endoneurial and perineurial constituents of peripheral nerve. NCs cultured alone, devoid of ensheathment but healthy in appearance, lack basal lamina and extracellular fibrils. In contrast, when SCs accompany NCs, basal lamina and extracellular fibrils are consistently visible around SCs in outgrowth areas formed *de novo* in culture. These fibrils average 18 nm in diameter, exhibit a repeating banding pattern, and are trypsin-resistant and collagenase-sensitive. Collagen synthesis is also indicated by the incorporation of [^{14}C]proline into peptide-bound hydroxyproline in NC + SC or SC cultures. That the [^{14}C]hydroxyproline polypeptides formed in NC + SC cultures are collagenous was determined in part by pepsin digestion-ammonium sulfate precipitation-polyacrylamide gel electrophoresis techniques; the ^{14}C-polypeptides migrate to the positions of $\alpha1$(I), $\alpha2$, $\alpha1$(III), and αB chains of type I, type III, and A-B collagens. Also formed are thin, ruthenium red-preserved strands interconnecting basal laminae. SC ensheathment of axons is similar to that found in the animal; one SC is related to a number of unmyelinated axons or a single myelinated axon. This proclivity to ensheathe and myelinate axons indicates that SC function is not lost during the preparative procedures or after lengthy isolation in culture and provides the most reliable means for SC identification. Perineurial ensheathment and macrophages are lacking in NC + SC culture preparations divested of fibroblasts. We conclude that SCs do not form perineurium or the larger diameter collagen fibrils typical of endoneurium but that in combination with neurons they generate biochemically detectable collagens and morphologically visible basal lamina and thin collagenous fibrils.

KEY WORDS   neuron · Schwann cell · basal lamina formation · collagen synthesis · nerve tissue culture

Within the perineurial compartment of peripheral nerve there are, in addition to nerve fibers and their ensheathing Schwann cells, fibroblasts and endothelial cells, and the extracellular components, basal lamina, reticulin and collagen. The

question of the cellular source of all the extracellular components has not been completely settled: do the Schwann cells or fibroblasts or both synthesize these materials? The source of the perineurial cells is also still debated: do they derive from neural crest (as do Schwann cells) and/or mesenchyme (as do fibroblasts)? Another question involves the nature of interactions between the cell types of peripheral nerve: what interrelationships may be important for the synthesis and organization of the various components?

These questions can now be directly studied by means of newly developed culture systems utilizing normal cells. Fetal rat dorsal root ganglion explants, after treatment with antimitotic agents, will generate a surrounding fibroblast-free outgrowth of either (a) bare axons or (b) axons that are ensheathed by Schwann cells (52). If the explant is excised from cultures in which the outgrowth contains Schwann cell-ensheathed axons, the axons degenerate, leaving a culture containing only Schwann cells. Neurons may then be added back to pure Schwann cell preparations by reintroducing an explant that has been extensively treated to eliminate Schwann cells and fibroblasts. Normal fibroblasts can be added to any of these preparations, thus making possible investigation of the principal cellular elements of peripheral nerve separately or in various combinations with each other.

It is the purpose of this paper, the first in a series, to compare two types of these preparations, those containing only nerve cells (NCs)[1] and those containing nerve cells and Schwann cells (NCs + SCs). In the former, nerve fibers grow radially from the explant to form an outgrowth and remain healthy over periods of weeks or months. When SCs are present, the resulting ensheathment of axons resembles that observed in the animal (as reported in preliminary form, reference 51). This normal relationship between NC and ensheathing cell partners allows identification of the SCs. Moreover, the formation of myelin in culture indicates that the SCs (as well as the axons) retain normal function, including those SCs that form myelin around reintroduced axons (52), even after lengthy spans of weeks or months bereft of NCs. It should be noted that all these culture prepara-

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[1] *Abbreviations used in this paper:* Ara-C, cytosine arabinoside; BSS, balanced salt solution; FdU, fluorodeoxyuridine; MEM, minimal essential medium; NCs, nerve cells; SCs, Schwann cells; wpt, weeks post-transfer.

tions are maintained on a reconstituted rat tail collagen substratum; the importance of this substratum (or an adequate substitute) for the expression of SC function in cultures lacking fibroblasts has been demonstrated recently (11, 13).

MATERIALS AND METHODS

Culture Preparation

Cervical and lumbar dorsal root ganglia were dissected from 17- to 21-d fetal rats, decapsulated as much as possible, and cultured in 25-mm Aclar plastic (Allied Chemical Co., Morristown, N. J.) dishes (12) that had been previously coated with collagen (52). The methods used to eliminate fibroblasts and/or SCs from these cultures have been detailed elsewhere by Wood (52). Briefly, the treatment consists of feeding first with medium containing antimitotic agents (cytosine arabinoside [Ara-C] and fluorodeoxyuridine [FdU]) and then with normal culture medium. This alternating feeding regime is repeated two to four times, after which the ganglia are excised and transplanted to new dishes. To allow the formation of a new NC + SC outgrowth, the cultures are refed three times weekly with normal medium after transplantation. To obtain new NC outgrowth, the cultures are refed three times weekly with medium containing FdU (without Ara-C) after transplantation. Normal medium contained 65% Eagle's minimal essential medium (MEM) with Earle's salts, 25% human placental serum, and 10% 9-d chick embryo extract. Antimitotic media contained 90% MEM, 10% human placental serum, 0.025 M KCl, and each antimitotic agent at 10^{-5} M. All media were supplemented with glucose to ~600 mg% and partially purified nerve growth factor (6) was added at an empirically determined optimum concentration. Cultures were grown at 35° in a humidified atmosphere of 5% CO_2 in air. The transplantation after the antimitotic treatment leaves behind the initial outgrowth which contains considerable debris from dead non-neuronal cells and any surviving fibroblasts and allows regrowth of nerve fibers and formation of a burgeoning population of SCs not exposed to antimitotic agents. In one experiment, this method of preparation of NC + SC cultures was varied by adding clumps of SCs to an NC culture, as depicted in Fig. 3.

Microscopy

Cultures were studied as whole mounts by bright-field or phase microscopy in the living state or after Sudan black staining of fixed cultures (52). To stain for connective tissue components, whole mount cultures were fixed in 10% formalin-Locke's solution overnight and subjected to the Foot-Bielschowsky silver impregnation technique (32, as modified by Mrs. Edith Peterson). At 3, 3.5, 4, 5, 6.5, 7, 12, and 16 weeks post-transfer (wpt), cultures were prepared for electron microscope study. They were rinsed briefly in 35°C Earle's balanced salt solution (BSS) and slowly infused with fixative (at 35°C or room temperature) containing 2% purified glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) in 0.09 M potassium arsenate (0.1 M arsenate buffer, Sigma Chemical Co., St. Louis, Mo.; reference 44) with 0.03 M sucrose (270 mosmol, pH 7.3) (7). Fixation was continued for 1.5 h at room temperature followed by 0.5 h or overnight at 4°C. After rinses in cold BSS, the cultures were postfixed for 1 h at 4°C in 2% OsO_4 in BSS, stained en bloc with buffered uranyl acetate (in some cases), dehydrated through graded ethanols and propylene oxide, and embedded in Epon-Araldite (7).

Some of the cultures were treated with trypsin or collagenase before fixation. For the former, cultures were rinsed three times in Ca-Mg-free Hanks' BSS, subjected to 0.25% trypsin (three times crystallized, Worthington Biochemical Corp., Freehold, N. J.) in the same fluid for 1 h at 35°C, and then rinsed in Earle's BSS before fixation. Other cultures, after rinsing two times with Eagle's MEM, were exposed to 0.05% highly purified collagenase (Type VI, Sigma Chemical Co.) in MEM for 45 min at 35°C. This treatment sometimes partially dissolved the collagen substratum, causing the explant and surrounding outgrowth to float in the medium; the culture chosen for illustration here did not separate from the substratum. The protocol used by Hay (23) was modified to fix and stain some cultures with ruthenium red. The culture was initially fixed as described above and, at room temperature, rinsed in 0.1 M cacodylate buffer, fixed in 2% glutaraldehyde in the same buffer and 0.2% ruthenium red for 2 h, rinsed again in buffer, placed in buffered 1% OsO_4 and 0.05% ruthenium red for 1.5 h, and rinsed further before the en bloc staining procedure.

Areas of interest were selected by microscope examination with a 16 phase objective, circled with a diamond scorer, sawed out, and mounted for sectioning perpendicular to the collagen substratum to obtain transverse sections of the culture. The areas were checked in semithin sections stained with toluidine blue, methylene blue, and borax. Thin sections were cut with a diamond knife on an LKB-Huxley ultramicrotome (LKB Instruments, Inc., Rockville, Md.), picked up on carbon-stabilized Formvar-coated grids, stained with uranyl acetate or tannic acid and lead citrate or lead tartrate (reference 35, communicated to us by Dr. E. Okada), and examined in a Philips 300 electron microscope. The tannic acid method recommended in reference 43 was adapted to sections; best results were obtained by treating the sections for 20 min with fresh, filtered 0.5% tannic acid (Fisher Scientific Co., Pittsburgh, Pa.) before staining with lead citrate. The staining was very effective, although occasionally marred by precipitate.

Collagen Assay

To detect collagen biosynthesis, 5 μCi [^{14}C]proline (30 mCi/mmol) was added in 200 μl of normal culture medium to each culture dish for a period of 48 h at 35°C. Four dishes of each type were pooled for assay. The medium was removed and the cultures were rinsed once with 200 μl of normal medium; this rinse was combined with the medium for assay. The tissue and the pooled fluids were hydrolyzed without prior dialysis in 6 N HCl for 18 h and assayed for [^{14}C]hydroxyproline by the method of Juva and Prockop (26).

To estimate the relative proportions of different types of collagenous ^{14}C-proteins, NC + SC cultures (3.5 wpt) were given 25 μCi/ml [^{14}C]proline (255 mCi/mmol) in culture medium (minus embryo extract) to which 25 μg/ml ascorbic acid and 20 μg/ml β-aminopropionitrile were added and then incubated for 24 h at 35°C. Six culture dishes containing seven explants each were pooled for assay. The medium was removed and added to the two subsequent rinses of 0.5 ml of medium; the tissue was scraped from the dish and kept separate although the data presented in this paper are derived from combined tissue and medium fractions. The samples were dialyzed against 0.15 M NaCl and 0.01 M $CaCl_2$ in 0.05 M Tris-HCl buffer, pH 7.6, at 4°C. Purified bacterial collagenase (Advance Biofactures Corp., Lynbrook, N. Y.), 50 μg/ml, and 2.5 mM N-ethylmaleimide were then added, and the samples were incubated for 15 h at 4°C. The incubation was stopped by adding 1/10 vol of 0.25 M Na_2-EDTA, and the samples were extensively dialyzed against 0.15

M NaCl and 1 mM Na_2-EDTA in 0.05 M Tris-HCl buffer, pH 7.6, at 4°C. The release of radioactive peptides by dialysis was taken as evidence of collagenous ^{14}C-proteins in the sample.

To digest radioactive proteins with pepsin (two times crystallized; Worthington Biochemical Corp.), the samples were dialyzed against 0.5 N acetic acid at 4°C. Pepsin, 100 μg/ml, was added, and the samples were incubated at 4°C for 15 h. After incubation, the pepsin was inactivated by dialyzing the samples against 0.4 M NaCl in 0.1 M Tris-HCl, pH 8.5, at 4°C. The pepsin-resistant ^{14}C-proteins were then precipitated by the addition of 114 mg ammonium sulfate/ml (20% saturation). For electrophoresis on polyacrylamide slab gels in SDS, the ammonium sulfate precipitates of ^{14}C-protein were treated with SDS as described elsewhere (48). The ^{14}C-polypeptides were then electrophoresed on a 6% polyacrylamide gel, as described by Uitto (48), except that the reduction of disulfide bonds by 2-mercaptoethanol was delayed by 60 min to allow separation of disulfide-bonded polypeptides, such as α1(III), from α chains of type 1 collagen. The electrophoresis was run for an additional 180 min. The ^{14}C-polypeptides were detected by radioautographic techniques; the exposed film was scanned at 600 nm using a Zeiss PM 6 spectrophotometer.

RESULTS

After transfer of the explant, neuronal somata remain clustered within the transplant and fascicles of nerve fibers extend radially from the explant to form a new outgrowth. As the light microscope study by Wood (52) has shown, neurites emanating from NC explants are unensheathed, whereas in the case of NC + SC explants, Schwann cells divide, migrate along the newly formed neurites, and provide typical ensheathment, including myelin (by 3 wpt). Electron microscope analysis concentrated an outgrowth areas, but comments and illustrations pertaining to explant regions will also be included. Light micrographs of these preparations appear in Figs. 1–5; salient features are noted in the legends.

NC Cultures

The essential absence of non-neuronal cells precludes ensheathment of both somata and neurites (Fig. 6). Nevertheless, the unensheathed neuronal cell bodies do not manifest a detectable change in survival rate and, in established cultures, appear similar in cytoplasmic content to somata in organotypic sensory ganglion cultures (10). One difference between this preparation and preparations in which ensheathing cells are present is that a small percentage of neurons develops cytoplasmic vacuoles that may be larger than neighboring neurons (Fig. 5). Along with the variation in somal size (18–46 μm diameter) there is variation in cytoplasmic density, depending upon the content of neurofilaments and microtubules. These organelles are most prominent in the perinuclear zone
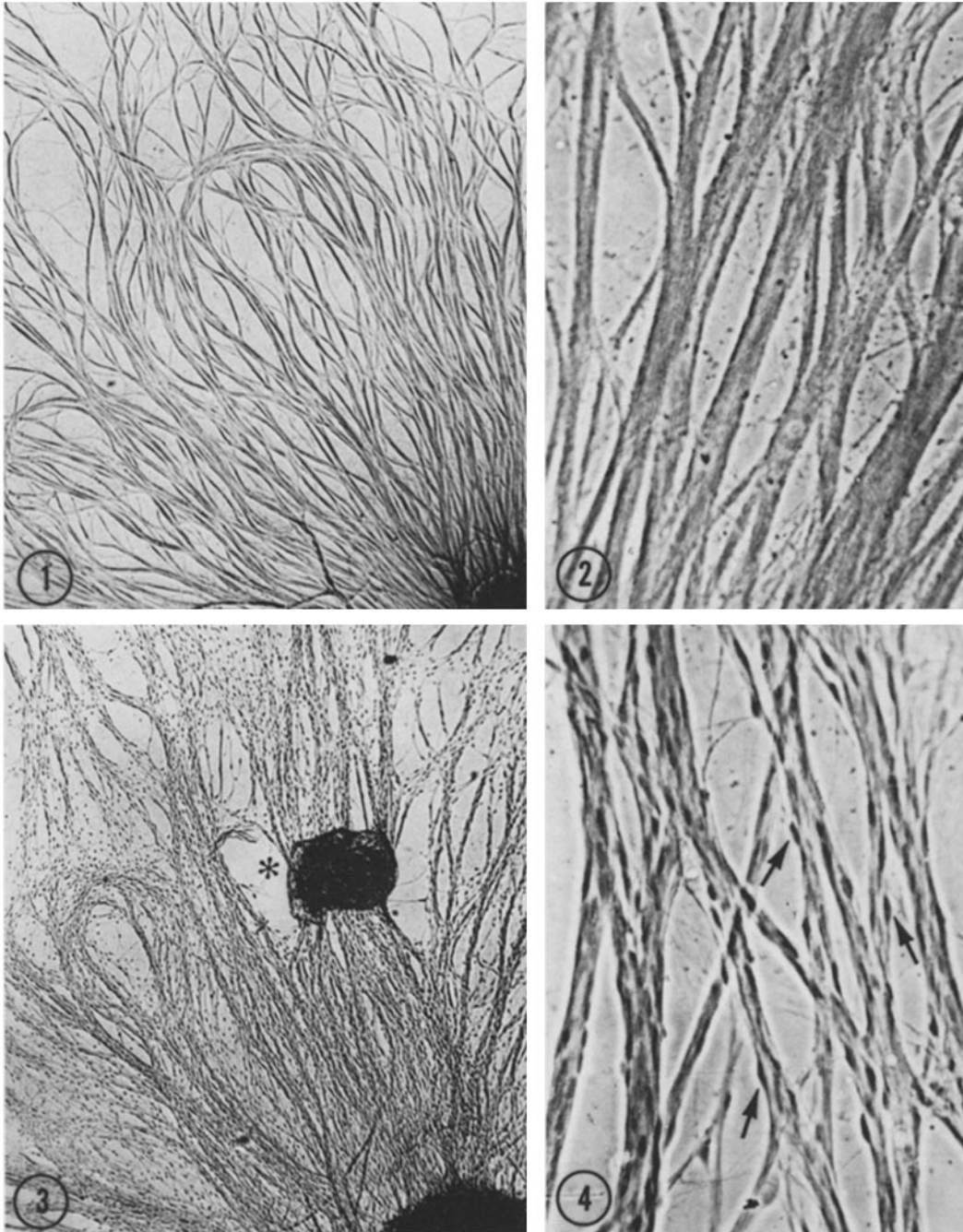
FIGURES 1 and 2   Living NC culture. Outgrowth areas containing fascicles of unensheathed neurites radiating from an explant, the edge of which appears at the lower right in Fig. 1. At higher magnification (Fig. 2), the absence of Schwann (and other non-neuronal) cells from these fascicles is demonstrated. 5 wpt. Fig. 1, × 40; Fig. 2, × 300.

FIGURES 3 and 4   Living NC + SC culture. Outgrowth of neurite fascicles ensheathed with SCs extending from the initial explant (lower right, Fig. 3). This figure illustrates an alternate method of preparation; four clumps of SCs (one shown by an asterisk) were positioned on an outgrowth from NCs only and over the next few weeks these cells divided and migrated to ensheathe the neurites. The minute densities are SC nuclei, better shown at higher magnification (arrows, Fig. 4). Other non-neuronal cells such as fibroblasts are not present. Neurons, 5 wpt; SCs added 8 d after transfer of NC explant. Fig. 3, × 40; Fig. 4, × 300.
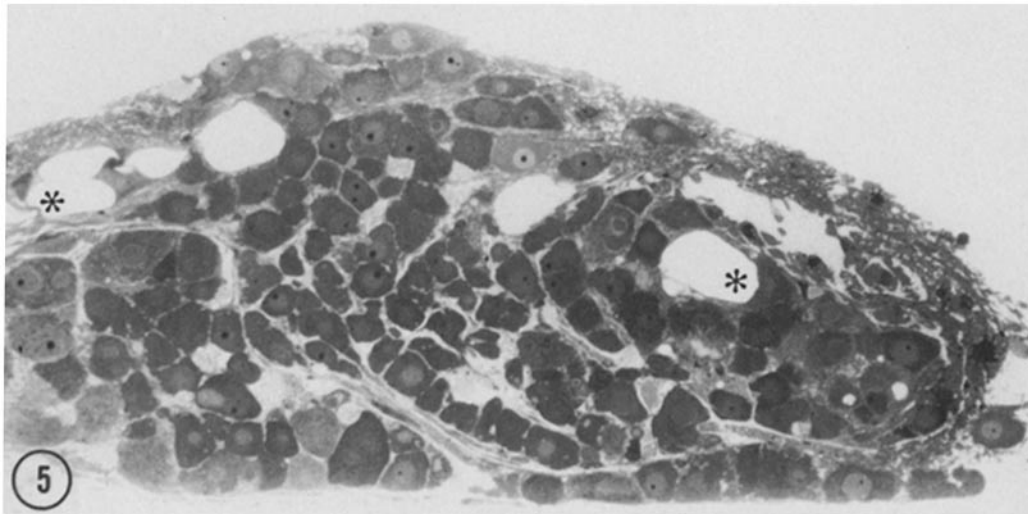
FIGURE 5 Semithin section of NC explant, sectioned in a plane perpendicular to the substratum. This photomicrograph illustrates the general configuration of explant areas and specifically shows the appearance of neuronal somata essentially free of ensheathment. One distinctive feature of an occasional perikaryon is that it contains a very large vacuole, as at the asterisk. The collagen substratum is present though not visible along the lower edge of the figure. 5.5 wk in culture. × 230.

but may extend towards the periphery of the soma either in a dispersed fashion or loosely clustered into curving bundles forming "plasmastrassen" that are more prominent in larger and thus "lighter" somata, as in organotypic cultures (10) and in vivo (1). Somata in dense explant regions may lie in close apposition to one another or be separated by individual or clustered neurites (Fig. 6), a few of which are partially invested by the somal cytoplasm. In spite of the extensive antimitotic treatment, an occasional non-neuronal cell may survive in the explant but does not proliferate or migrate into the outgrowth.

In the outgrowth, neurites are tightly packed into fascicles (Fig. 7). Neurites contain: mitochondria; agranular membrane-composed tubules, vesicles, vacuoles and cup-shaped bodies; dense-cored vesicles; myelin figure-containing bodies, multivesicular bodies and autophagic vacuoles; and neurofilaments and microtubules. Agranular membranous vesicles or tubules may be present throughout the neurite but are most consistently observed around the perimeter beneath the axolemma. The majority of the smaller diameter neurites contain microtubules that are dispersed throughout the fiber, intermixed with a few neurofilaments (Fig. 7). Larger fibers may contain neurofilaments predominantly (as in vivo; reviewed in reference 53), the microtubules being

located peripherally (along with other organelles) or diminished in number (Fig. 7). In their size and predominantly filamentous content, these large neurites correspond to axons that may be myelinated when SCs are present. The nerve fibers in established cultures appear healthy, but occasionally there are neurites that appear to be degenerating because of a predominance of neurofilaments and myelin figure-containing dense bodies. Basal laminae and extracellular fibrils are not observed in the outgrowth regions (Fig. 7).

NC + SC Cultures

With the retention of SCs in the culture, somata and neurites alike become ensheathed. Many neuronal cell bodies are typically encased within a thin layer of satellite or SC cytoplasm (Fig. 8), and this layer often contains a profusion of somal protuberances as has been described in organotypic cultures (10). Other neuronal perikarya are not ensheathed as usual, undoubtedly reflecting the paucity of SCs in some regions of the explant following antimitotic treatment; portions of two neighboring neuronal somata may share a layer of ensheathing cytoplasm, or somata may be devoid of ensheathment. Where ensheathment is lacking, spinelike protuberances from the neuronal perikaryon may envelop adjacent or indenting neuritic processes, as in NC cultures. The range in neuronal
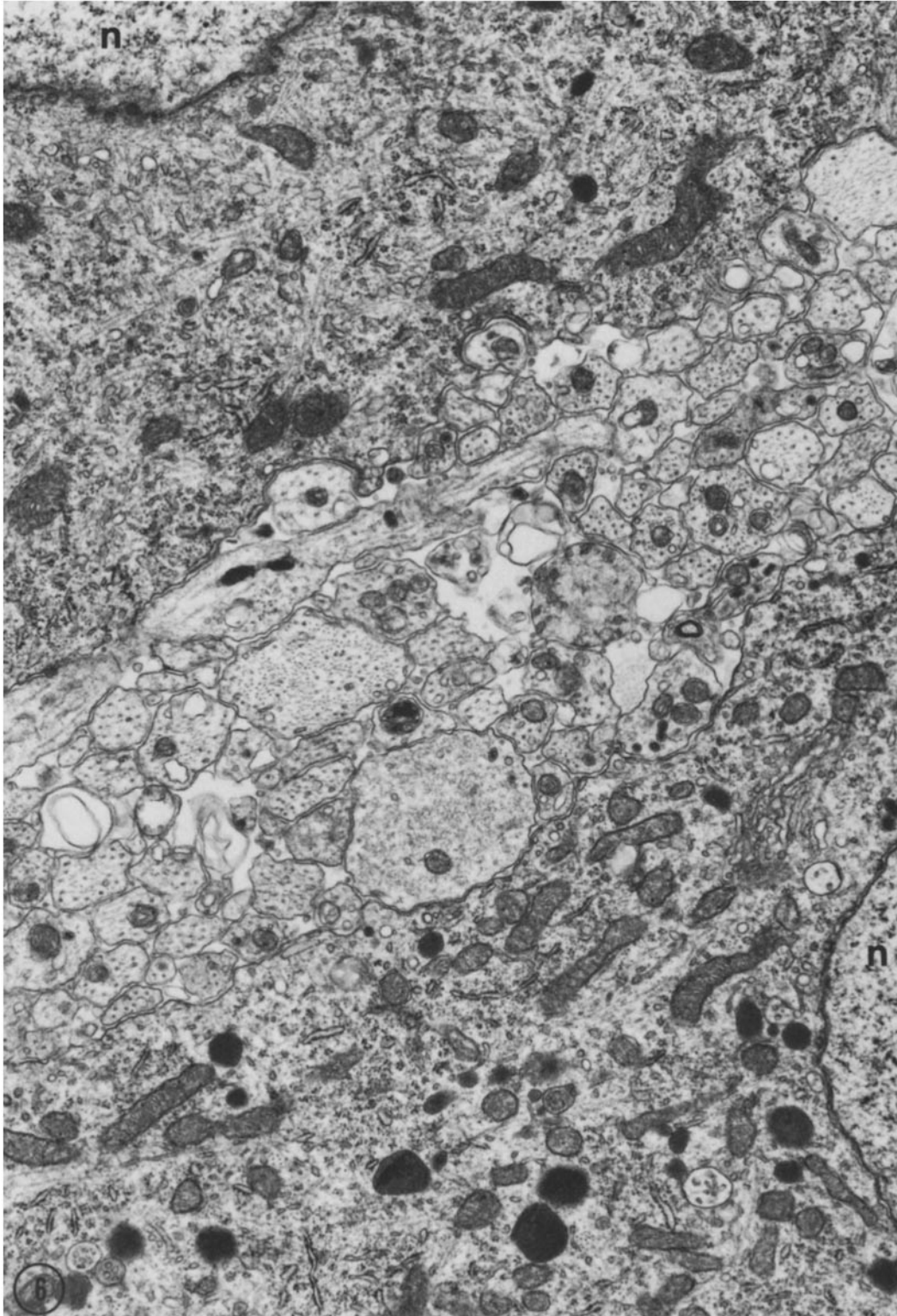
FIGURE 6 Electron micrograph of NC explant region. Portions of two typical-appearing perikarya (and their nuclei, *n*) are shown. Clustered between them are many neurites, some of which indent the somata. Neither the somata nor the nerve fibers are ensheathed. 7 wk in culture. × 21,000.
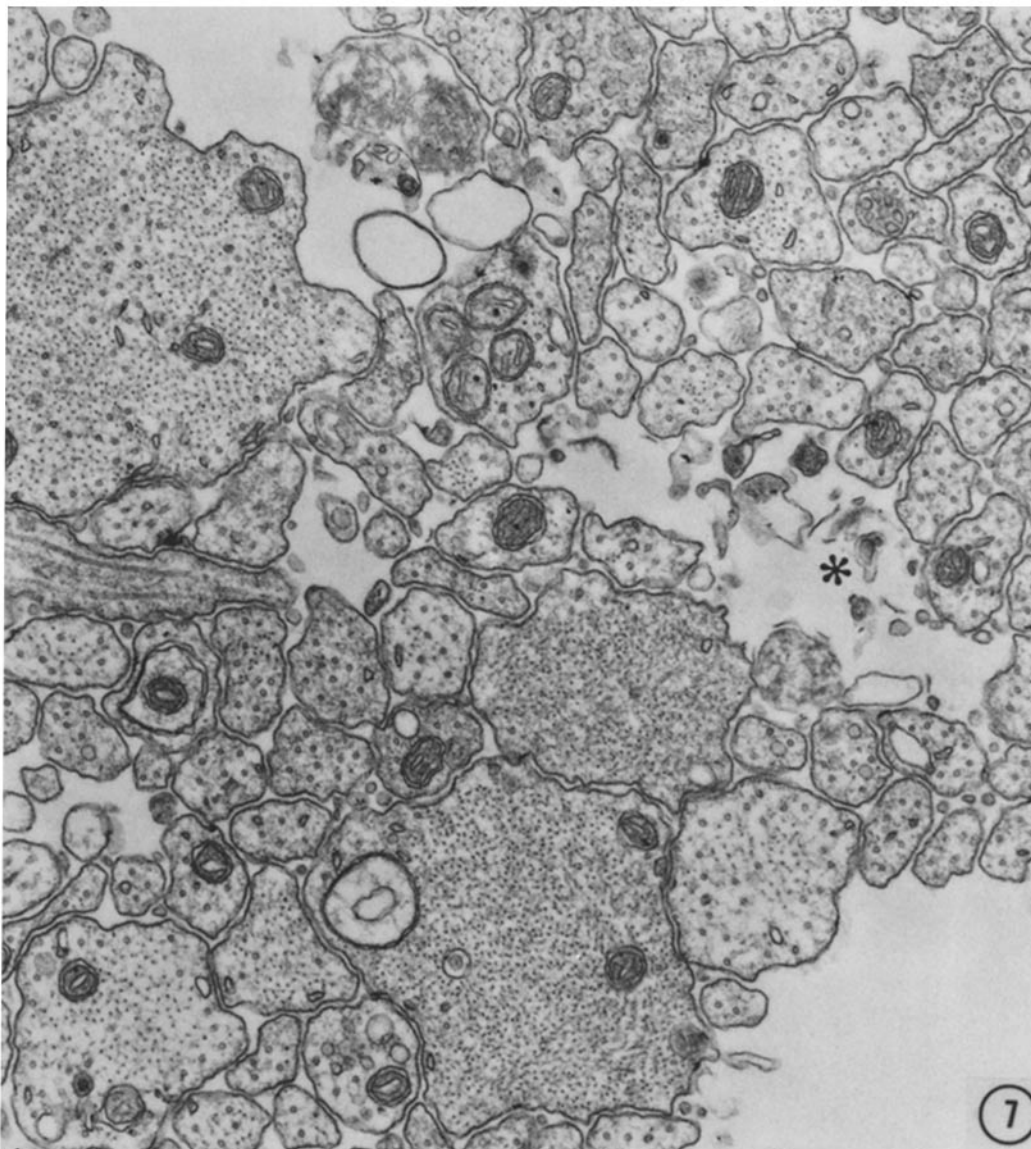
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FIGURE 7   Appearance of outgrowth from the NC explant illustrated in Fig. 6. This fascicle and those in the following figures (except Figs. 13 and 14) have been transversely sectioned. The nerve fibers vary in diameter and also in content (as detailed in the text). The area designated by the asterisk contains debris that probably resulted from gradual neurite or SC demise. × 39,000.

perikaryal diameter (11–54 μm) closely resembles that found earlier in organotypic cultures (11.5-50 μm; reference 10). In the absence of fibroblasts, SCs may spread out to form a thin covering layer over the explant region; these cells are identified as SCs because of their basal lamina coating (vide infra) and associated axons. Macrophages do not appear in cultures with SCs if the former have

been successfully eliminated during antimitotic treatment, confirming Wood's (52) earlier observation.

Outgrowth neurites, including myelinated ones, vary in their proportion and orientation of contained neurofilaments and microtubules (mean diameter, 24 nm), as described above for NC cultures. Healthy-appearing neurites of similar di-
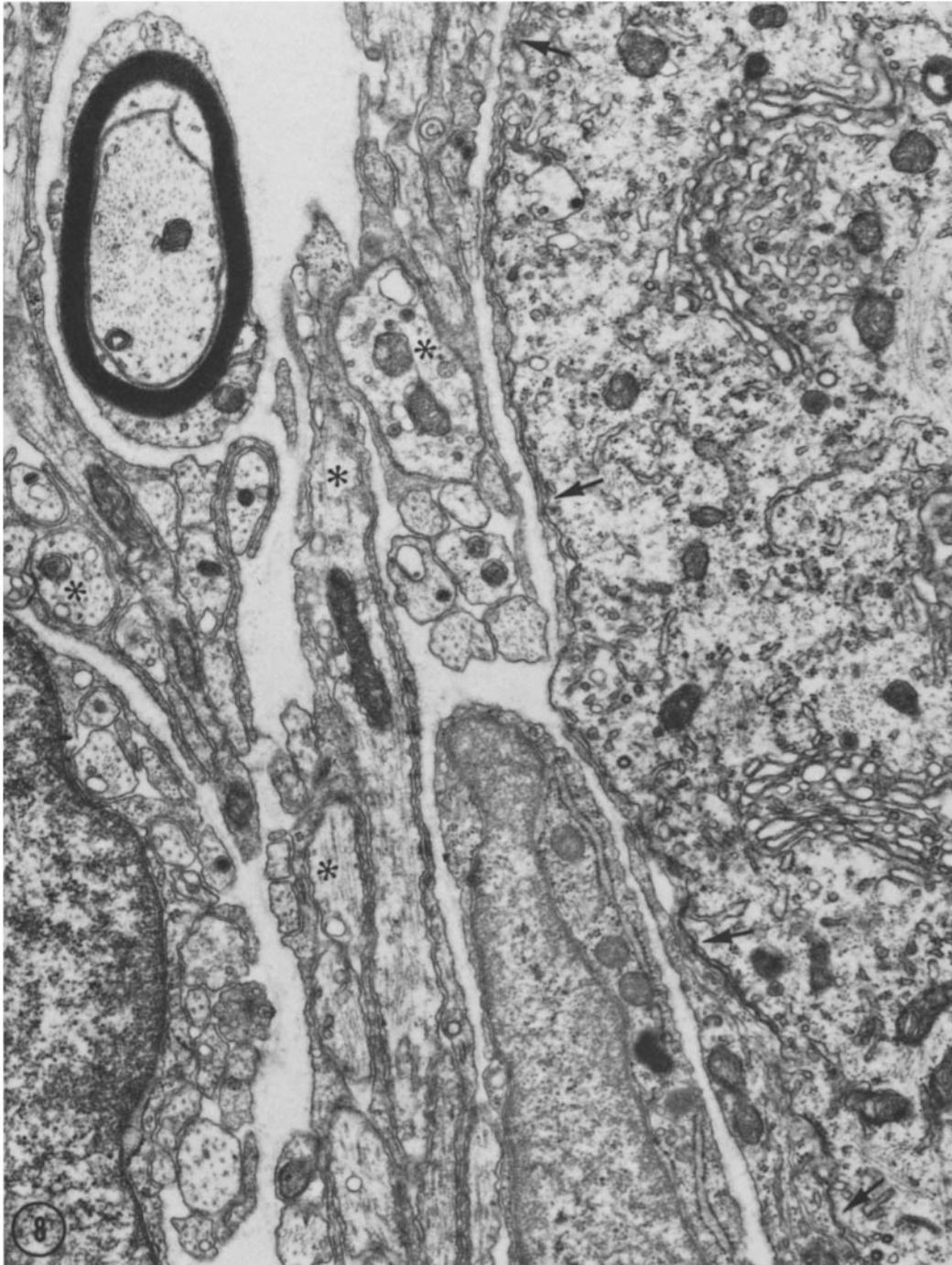
FIGURE 8 NC + SC explant region. This figure, in contrast to the preceding electron micrographs, demonstrates the ensheathment of both somata and axons (as at the asterisks) that develops when SCs are present. The ensheathing cell-neuronal soma interface is indicated by arrows. One axon has been myelinated. 3 wpt. × 23,000.

ameter may contain a mixture of filaments and microtubules or a preponderance of one of these organelle types (Figs. 9 and 11). The prominent neurofilaments range from 6 to 13 nm; finer filamentous material may be discerned beneath the plasmalemma and radiating from neurofilaments and microtubules (as in reference 54). As is typical for peripheral nerve in vivo as well as for organotypic sensory ganglion cultures (10), numerous unmyelinated neurites (0.13–1.03 μm in diameter) are embraced by one SC (Figs. 9–11) whereas a single SC invests only one myelinated fiber (0.94–1.9 μm in diameter) (as in Fig. 8). Many unmyelinated neurites occupy their own cytoplasmic niches, whereas others share a furrow in the SC or, particularly in the younger cultures, occur in clusters nestled between SC processes, as is typical of developing nerve. All SCs in the outgrowth fascicles are associated with neurites. The SC-neurite units are situated singly or in fascicles of varying sizes. Perineurial ensheathment neither covers nor subdivides the fascicles (even after long

FIGURE 9   NC + SC outgrowth. The nucleus belongs to a SC. SC cytoplasm, which may contain prominent rough endoplasmic reticulum (asterisk), invests the neurites. Staining with tannic acid has emphasized the extracellular fibril content; most of these fibrils here, as in the other figures, appear in cross-section because the fascicle has been sectioned transversely. The top surface of the culture (bathed by medium) is at the upper right. 3 wpt. × 20,000.
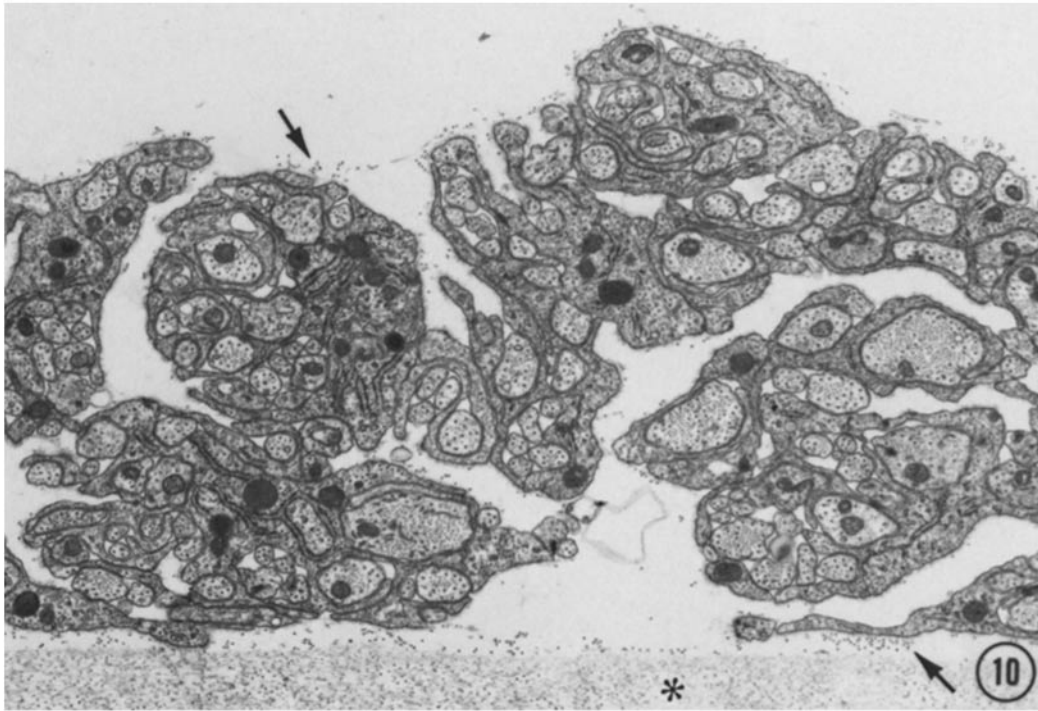
time periods of months), in striking contrast to cultures that also contain fibroblasts, as will be reported subsequently.

The ultrastructure of the SCs resembles that seen in vivo, with prominent signs of synthetic activity. Rough endoplasmic reticulum and Golgi components are prominent. The granular reticulum contains flocculent material and often closely approaches mitochondria or swerves peripherally to occupy a position immediately beneath the plasmalemma (Fig. 12). Subplasmalemmal granular reticulum is sometimes seen to be continuous with the nuclear envelope or related to the Golgi complex. Free polysomes, lysosomal dense bodies and multivesicular bodies, and centrioles (Fig. 12) are also present. In ensheathing but unmyelinating SCs, cilia are frequently found originating from a basal body nestled (along with Golgi elements) near the concavity of a kidney-shaped nucleus and protruding into the extracellular space through a deep invagination of the surface membrane. Neither the shaft nor the invagination cavity is covered with basal lamina. The axoneme often varies

from the 9 + 1 large central vesicle pattern described in references 10 and 20. Intermediate filaments and microtubules, both similar in dimensions to those in neurites, are prominent organelles in the SC cytoplasmic extensions and are oriented parallel to the longitudinal axis of the cell (Figs. 11 and 16) as in vivo (31). Coated caveolae are a feature of the plasmalemma. Small junctions consisting of dense material on the cytoplasmic plasmalemmal surfaces and, to a lesser degree, interposed in the intercellular cleft occasionally link SCs (Fig. 16) or SCs and neurites; small accumulations of dense material may also be found in the SC underneath its basal lamina as in a hemidesmosome configuration.

The SC exterior exhibits basal lamina, and thin extracellular fibrils are present outside this lamina (Figs. 9–11, 13, 14, and 16). The presence of these two types of extracellular components is a highly consistent finding, having been observed in 34 outgrowth areas studied for this investigation and also in numerous comparable preparations obtained by others in our laboratory. Since the out-
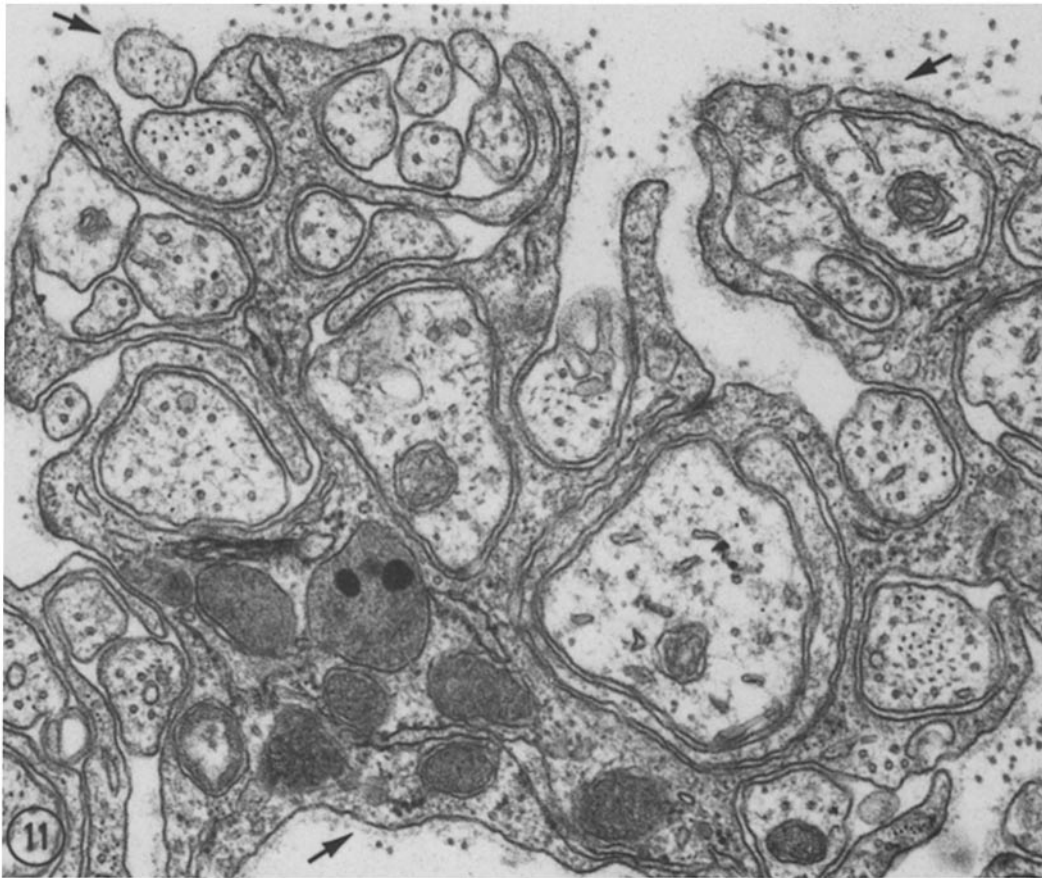
FIGURE 11 Tannic acid-stained section of NC + SC outgrowth, showing transversely sectioned extra-cellular fibrils. Also illustrated is the thin basal lamina (arrows) on the SC exterior. 3 wpt. × 47,000.

growth always forms anew after transfer of the explant, the basal lamina and extracellular fibril components have been formed *de novo*. Most of the cultures were obtained by treating the ganglion adequately to remove fibroblasts before transferring to a new dish, i.e., SCs retained in the explant divided to populate the new outgrowth. In one experiment, however, SCs were added to a pure NC outgrowth (as in Fig. 3); nevertheless, the formation of basal lamina material and fibrils was similar. The thin fibrils are consistently larger and thus distinct from those that populate the reconstituted collagen substratum (Fig. 14).

By 3 wpt, thin basal lamina is apparent on the SC exterior (Fig. 11), but it is not so thick or so continuous as it is at 12 wpt (Figs. 13 and 14). The lamina densa consists of a feltwork of fine filaments in combination with dense material, and some of the filaments appear to span the lamina

rara interna that is usually visible. Preliminary work has shown that ruthenium red stains the basal lamina, partially obscuring the lamina rara interna, and that it makes visible fine strands, often ~25 Å in diameter, that interconnect apposing basal laminae (Fig. 15) or link basal lamina and stained material on the extracellular fibril exterior. Basal lamina is partially disrupted by collagenase (Fig. 17) and completely removed by trypsin (9). Basal lamina is lacking on those portions of the SC plasmalemma apposed to neurites or another SC process facing the interior of the axon-SC complex (Fig. 11); the lamina therefore coats the most exterior regions of the SC.

The extracellular fibrils are of uniformly small diameter (Table I) compared to the fibrils found in the endoneurium in the animal. They are usually oriented parallel to the longitudinal axis of the nearby neurites and SCs (Figs. 9–11, 13, 14,
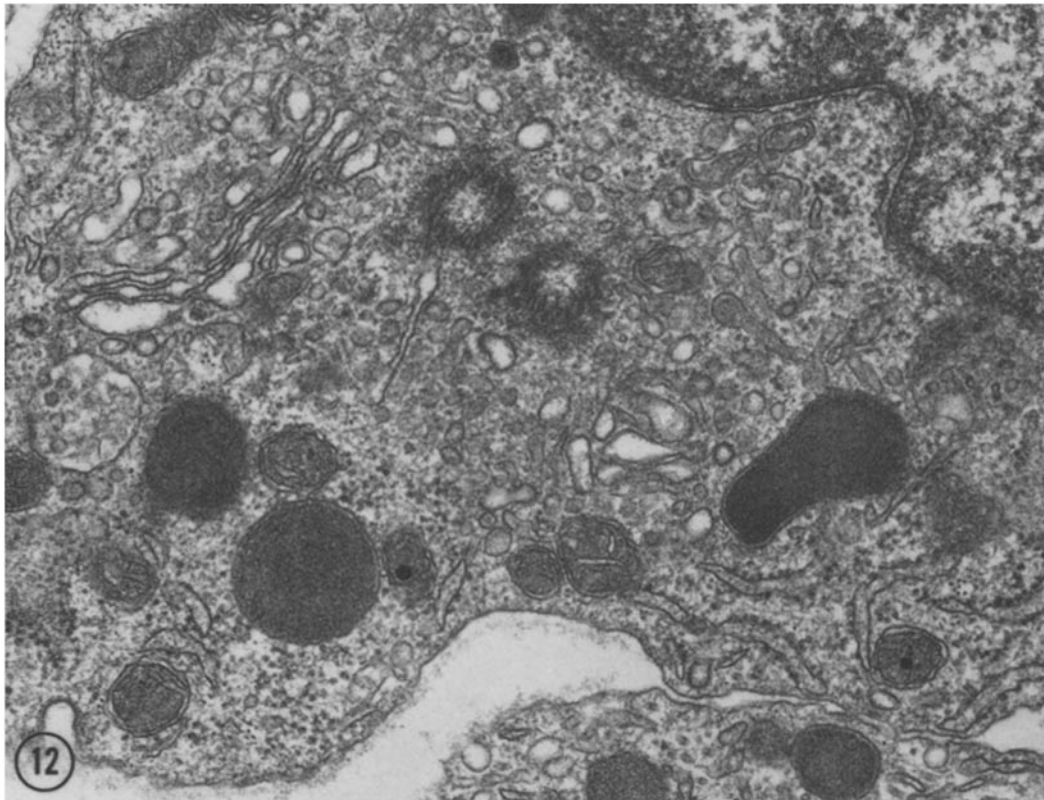
FIGURE 12 Perinuclear region of an SC, typically containing centrioles, Golgi components, lysosomal multivesicular and dense bodies, and granular endoplasmic reticulum components that frequently approach the plasmalemma (as at the lower right). NC + SC outgrowth, 12 wpt. × 47,000.

and 16) and often occur in bundles. Cross-sectioned fibrils appear more heavily stained after tannic acid-lead citrate than after uranyl acetate-lead citrate staining. The major repeating period is difficult to measure; it is not so easily detected in these thin fibrils as it is in the thicker fibrils present in NC + SC cultures to which fibroblasts have been added (8), possibly because of the smaller diameter (16, 41). The cross striations have been visualized best by staining with 2% aqueous uranyl acetate at 64°C for 45 min followed by lead tartrate for 10 min (Figs. 13 and 14), and in these samples they generally exhibit a major repeating period of 50–55 nm. The fibrils are resistant to trypsin treatment but disappear after exposure to collagenase (Figs. 16 and 17). The fibrils neither increase markedly in diameter over a 16-wk culture period (Table I) nor continue to increase in number over a period of months to fill the extracellular expanses. Addition of ascorbic acid to the medium did not enhance fibril formation; un-

doubtedly, the concentration is adequate in the complex culture medium used.

To assess whether these thin fibrils are composed of reticulin, Foot-Bielschowsky impregnations were done. Staining was not evident in the outgrowth as it was in an organotypic ganglion culture prepared at the same time and in earlier organotypic samples (10). Staining varied in the organotypic culture in which some outgrowth fascicles of NCs and SCs were overlain by fibroblasts and others were not; blackened fibrils were not detected in fascicle areas devoid of fibroblasts but were present in areas that did contain fibroblasts. These observations suggest that the fibrils situated close to and along the lengths of the nerve fiber-SC units in NC + SC cultures are not reticulin, at least as far as can be determined by the Foot-Bielschowsky technique we have used. Possibly the fibrils are too thin or inadequately clustered to be detected by this method.

Radioactive proline was added to cultures to
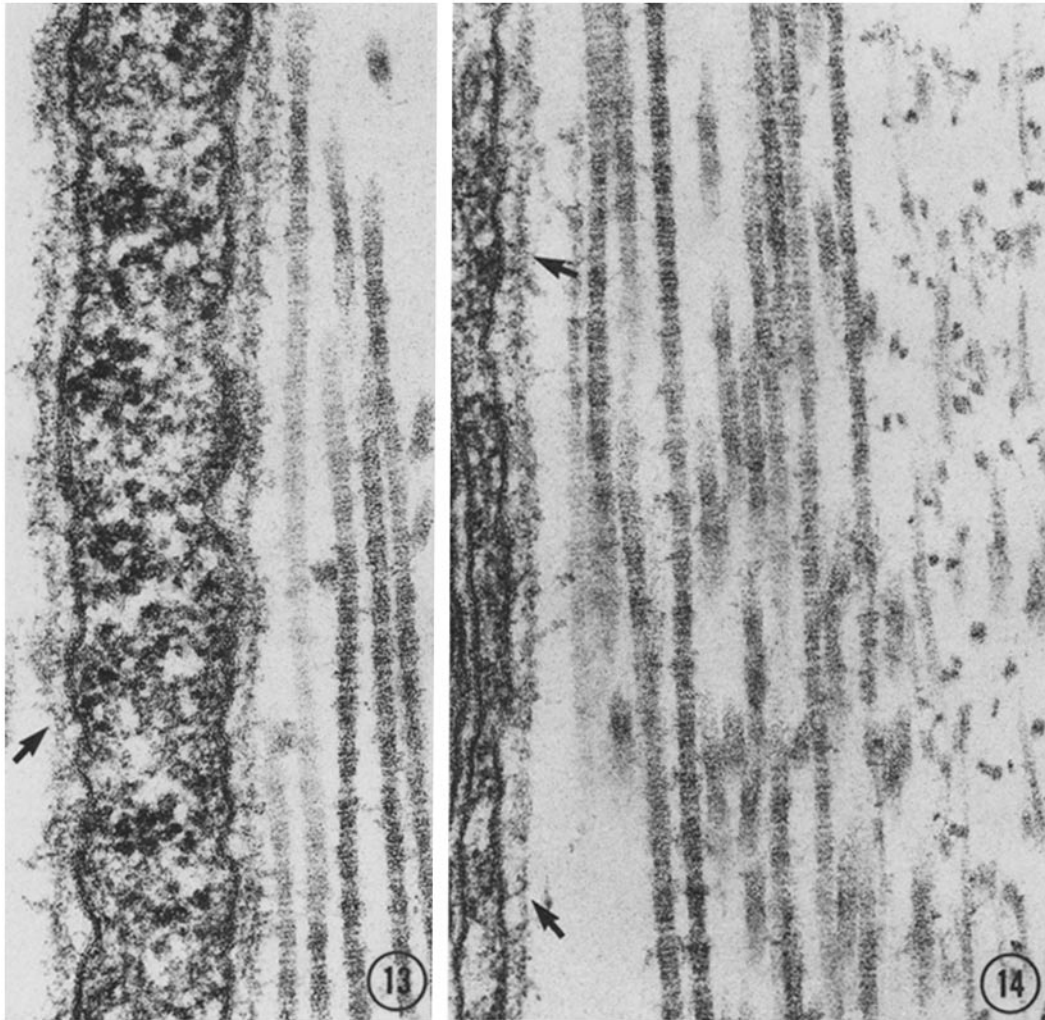
FIGURES 13 and 14 Longitudinally sectioned fascicle regions. The SC cytoplasm is completely covered by basal lamina; fine filaments extend from the plasmalemma to the lamina densa (arrows). Two bundles of striated extracellular fibrils are illustrated. The one in Fig. 14 rests on the reconstituted collagen substratum which contains fibrils of smaller diameter. NC + SC outgrowth, 12 wpt. × 132,000.

assess collagen synthesis by biochemical methods. It was found that SCs alone in the culture dish incorporate [^{14}C]proline into peptide-bound hydroxyproline, suggesting that they have the ability to synthesize collagen (Table II). It appears that more collagen is formed when NCs are also present but this has not yet been studied in a quantitative way.

In NC + SC cultures, the newly synthesized [^{14}C]hydroxyproline-containing macromolecules were identified as collagenous proteins by two independent criteria. First, incubation of the cell

and medium fractions with highly purified bacterial collagenase rendered into dialyzable peptides 82–94% of the [^{14}C]hydroxyproline that was initially nondialyzable. Secondly, limited proteolytic digestion with pepsin, employing conditions under which triple-helical portions of collagen resist proteolysis, indicated that ~70% of the [^{14}C]hydroxy-proline-containing polypeptides were in native triple-helical conformation and could be recovered by subsequent precipitation with ammonium sulfate. Further examination of the ^{14}C-polypeptides by polyacrylamide slab gel electrophoresis, after
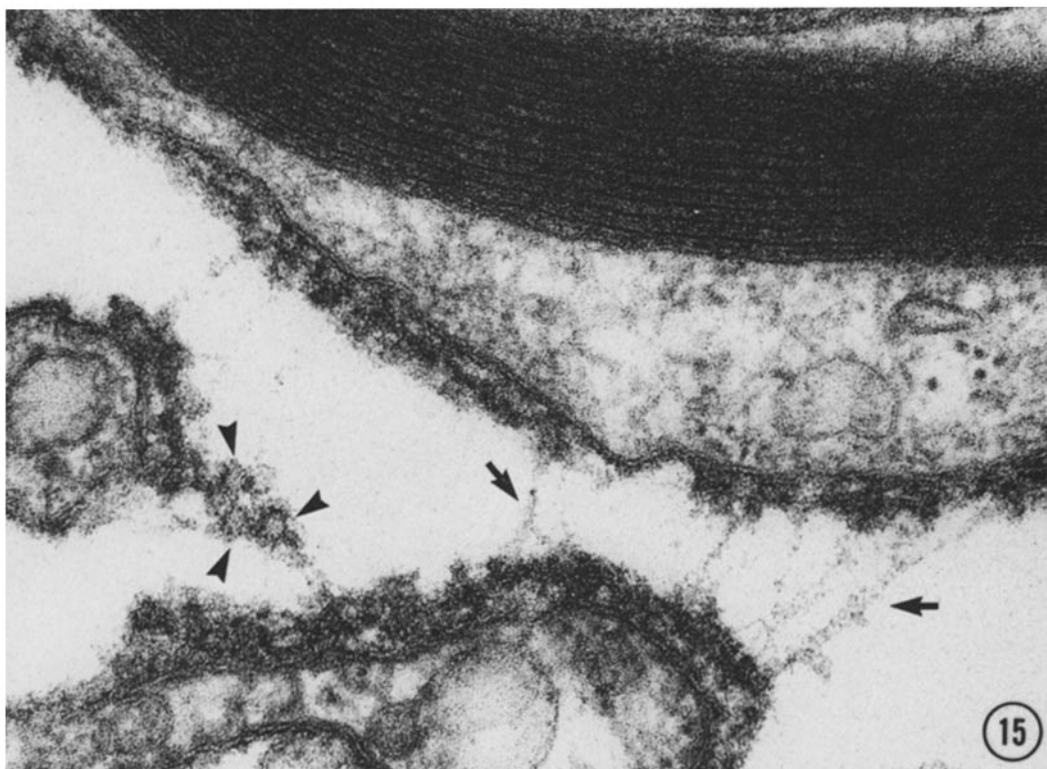
FIGURE 15   After fixation and staining with ruthenium red, the basal lamina is more heavily stained and delicate strands (arrows) are observed to span areas between basal laminae. Ruthenium red-stained material also coats the extracellular fibrils (arrowheads). Part of a myelin sheath is included at the top of the figure. NC + SC outgrowth, 4 wpt. × 150,000.

TABLE I

Measurements of Extracellular Fibril Diameter in Outgrowth Regions of NC + SC Cultures

Culture	Age	En bloc stain	Section stain	Fibril No.	Range	Mean
	wpt				nm	nm
A	3	UA	UA, Pb	50	13–19	15
B*	3	UA	TA, Pb	39	15–25	17
C						
Area 1	12	UA	UA, Pb	37	15–25	22
Area 2*	12	UA	UA, Pb	50	15–21	19
D						
Area 1	16	—	UA, TA, Pb	23	15–21	19
Area 2*	16	—	UA, Pb	50	14–21	18

UA, uranyl acetate; TA, tannic acid; Pb, lead citrate.
* Sample taken from outgrowth farther from the explant than in preceding one for same time period.

denaturation in SDS, indicated the presence of several chains that had an approximate mol wt of 94,000 or larger. Three major peaks of radioactiv-ity migrated in the same positions as $\alpha1(I)$, $\alpha2$, and $\alpha1(III)$ chains of type I and type III collagen, respectively (Fig. 18). Several distinct ^{14}C-poly-
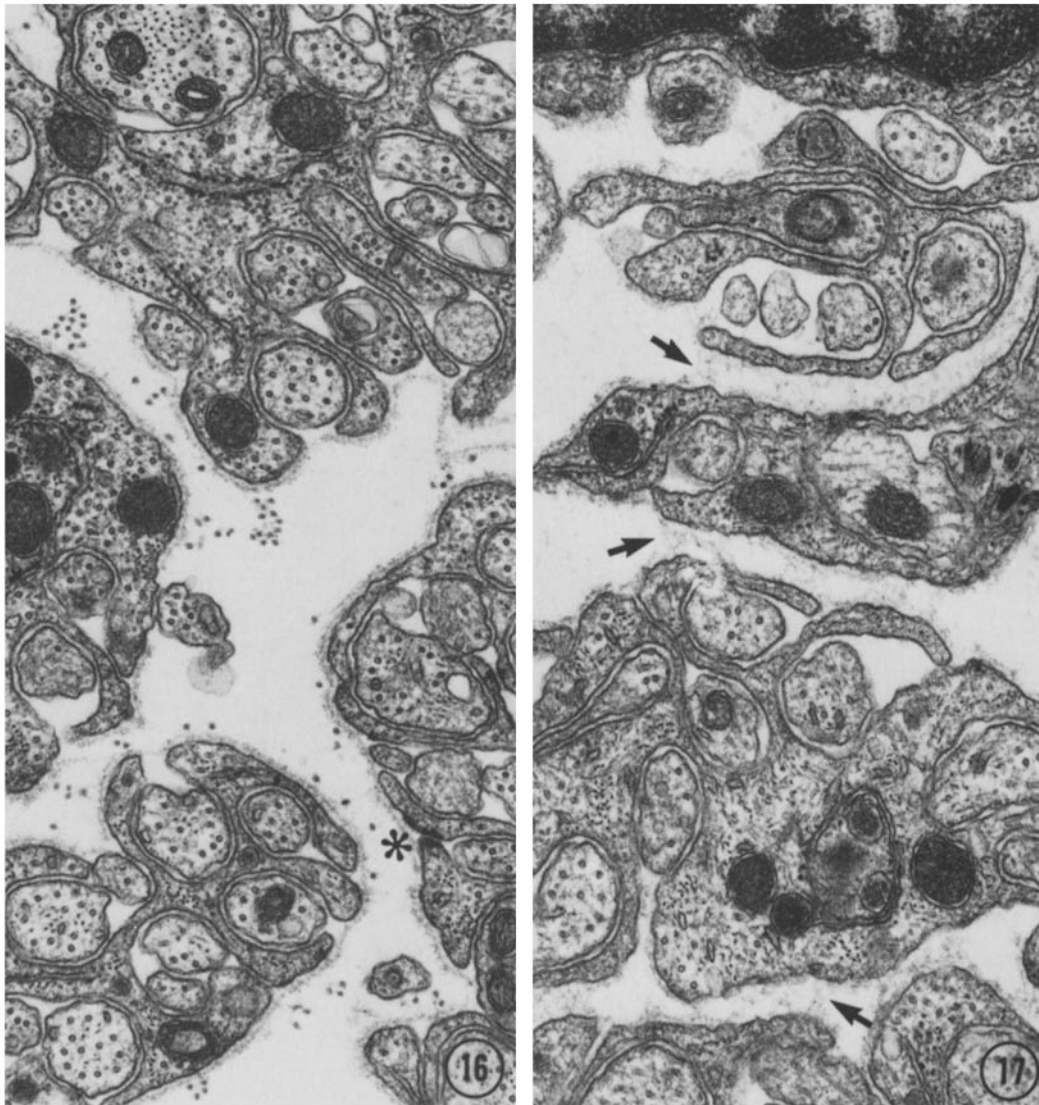
FIGURES 16 and 17   Fig. 16 illustrates a typical outgrowth fascicle region in a control culture of NCs + SCs. Basal laminae and extracellular fibrils are present. Fig. 17 exemplifies the appearance of a comparable area in a sibling culture that has been exposed to collagenase. The extracellular fibrils are no longer present. Also, basal laminae have been partially disrupted (arrows). A junction (asterisk) between SC processes is present in Fig. 16. 4 wpt. × 36,000.

peptides were also noted migrating slightly slower than α1(I); these chains, therefore, have an approximate mol wt of 100,000. Since αB chains of A-B collagens (14, 40) and α1 chains of type IV collagen (17) migrate in a polyacrylamide slab gel electrophoresis to this region, it is conceivable that some of these ¹⁴C-polypeptides represent newly synthesized basement membrane collagen (17).

The isolation and identification of these collagens is currently in progress.

DISCUSSION

The availability of cultures containing either NCs or NCs + SCs allows a new approach to the study of SC function. It has been found that cultured SCs, in the presence of NCs and absence of fibro-

Table II

Incorporation of [^{14}C]Proline into [^{14}C]-Hydroxyproline

	[^{14}C]Hydroxyproline		
Cultures	Tissue	Medium	Total
	dpm × 10^{-3}/culture		
SCs	8.25	72.5	80.75
NCs + SCs	19.0	99.25	118.25

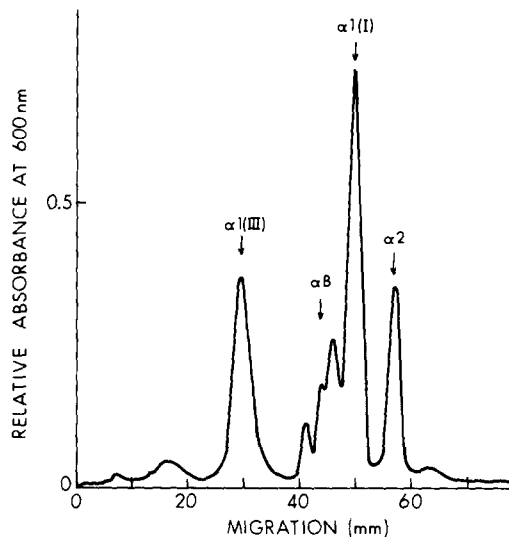SC cultures prepared by removing NCs 2 wk earlier; NC + SC cultures, 2 wpt.

FIGURE 18 Densitometric tracing of fluorogram. Collagenous ^{14}C-polypeptides synthesized in NC + SC cultures, after the administration of [^{14}C]proline. The positions of the ^{14}C-polypeptides correspond to αl(I), α2, αl(III), and αB chains of type I, type III, and A-B collagens.

blasts, ensheathe axons in the same manner as they do in vivo, indicative of their normal functioning in vitro. SCs cultured with NCs do not provide the perineurial ensheathment or the typical population of macrophages commonly observed when fibroblasts have been added to the culture. SCs related to neurons form morphologically detectable basal lamina and thin extracellular fibrils but not the larger, more strikingly cross-striated fibrils characteristic of the endoneurium in vivo. Delicate filamentous strands interconnecting basal laminae are also observed in NC + SC cultures when ruthenium red is employed. These strands resemble ruthenium red-preserved filaments (or "microfibrils") found by others (23, 30) to contribute to a meshwork of embryonic extracellular matrix components. The cross-banded fibrils of NC + SC cultures exhibit a major repeating period (50–55 nm), and are trypsin-resistant and collagenase-sensitive, pointing to their collagenous nature. In their relatively thin diameter the collagen fibrils formed resemble those observed in other fibroblast-free in vitro systems (e.g., references 16 and 24) or at embryonic stages preceding the appearance of fibroblasts (e.g., references 23 and 30). Experimentation utilizing radioactive proline has confirmed that collagens are formed in NC + SC or SC cultures. The labeled proline administered to NC + SC cultures is incorporated into hydroxyproline-containing polypeptides that, by means of collagenase treatment and limited pepsin proteolysis, are found to be collagenous in nature. Furthermore, the newly synthesized radioactive polypeptides that are pepsin resistant, precipitated with ammonium sulfate and separated in an SDS polyacrylamide electrophoresis system are similar in electrophoretic mobility to the αl(I), α2, αl(III) and αB chains of type I, type III, and A-B collagens. There is the possibility that α chains of

type IV collagen have been formed as well.

The finding that SCs are involved in collagen synthesis supports earlier work, much of which utilized tumor cells (see references in 15). The first pertinent study was done by Murray and Stout (36) who demonstrated that reticulin, visualized by the Foot-Bielschowsky method adapted to tissue culture (32), was formed de novo by SCs that grew into fibroblast-free regions from an explant of a human benign nerve sheath tumor (neurilemoma, now called schwannoma). These authors (37) had shown earlier that the cell type populating most areas of outgrowth from this type of tumor was the SC rather than the fibroblast. In fact, because collagen and reticulin were present in large amounts in schwannomas, the identity of the constitutive cells continued to be questioned by others. Electron micrographs have supported the Murray and Stout conclusion that the principal tumor cells are surrounded by basement membrane, which would be true of SCs but not fibroblasts, and collagen fibrils are found between them (49; reviewed in reference 47).

In 1963, Nathaniel and Pease (38) reported that SCs are the probable source and responsible for the polymerization of endoneurial collagen. Their conclusion was based on observations that, in rat

dorsal roots recovering from crush lesions, new collagen fibrils appeared adjacent to SCs but at some distance from the infrequent fibroblasts and, moreover, were interspersed with newly formed basal laminae inside the surviving original basal lamina envelope (the "Schwann tube" or band of Büngner). That new basal lamina and collagen fibrils appeared together in relation to the SC surface inside the original basement membrane was confirmed by Thomas (45) who studied regenerating rabbit peripheral nerve. But the presence of fibroblasts just outside the Schwann tubes complicated the assignment of the initial synthesis of the procollagen polypeptides to one cell type. Was it possible that fibroblast-formed procollagen diffused through the Schwann tube lamina to be cross-linked into fibrils at the SC surface (45)?

Church et al. (15) studied labeled proline incorporation into hydroxyproline in vitro in a clonal rat cell line derived from a chemically induced schwannoma. These schwannoma cells synthesized five different collagenous polypeptides, including $\alpha 1$ and $\alpha 2$ chains that did not become organized into typical fibrils as readily as would have been expected, and two larger chains, one of which was not in the typical collagen triple-helical molecular form. The latter polypeptide may be unique to schwannoma cells (15). Fibril formation may have been low because required cofactors were lacking in the cultures (15). Our results are similar to theirs in that in both investigations there was more incorporated label in the medium than in the cell layer in contrast to fibroblast cultures in which the opposite was observed (Table II and unpublished data).

Our data on normal, neuron-related SCs and those obtained by Church et al. (15) on tumor-derived SCs indicate the simultaneous synthesis of different genetic types of collagen. On the basis of our morphological findings (basal lamina and thin striated fibrils), we might have anticipated the formation of two types, III and IV. If the basal laminae of SCs resemble those in many other tissues, they would be expected to contain type IV collagen (27, 34). That there is collagen of some type present is suggested by the partial sensitivity of the basal lamina to collagenase. The cross-banded fibrils would be expected to be composed of type I or III (reticulin), possibly the latter because the fibrils are thin (33), although we did not demonstrate the presence of reticulin by the Foot-Bielschowsky method. Since both types I and III appear to be synthesized, are there two populations of fibrils among those we observe, or is each fibril a mixture of both collagen types as the uniform diameter population might suggest? Recent work has raised the possibility that fibrils of mixed composition exist (25). Data in Fig. 18 suggest that αB chains may also be present; these may be destined for the basal lamina (reviewed in references 3 and 40).

Adult mammalian peripheral nerve contains types I and III collagens (42) and probably type IV collagen as well. If the tentative identification of the collagenous polypeptides in our cultures is confirmed, it would appear that the SCs with neurons are capable of synthesizing all the endoneurial collagen types. It should be noted that in the animal the population of fibroblasts is low; 5% or less of the resident cells in endoneurium are fibroblasts (2). Are the SCs, then, responsible for the formation of endoneurial collagen? Our culture work suggests that they are in part. Animal endoneurial collagen fibrils range in diameter from 25-55 nm (1, 19, 46). The collagen fibrils in NC + SC preparations do not reach the larger diameters. Furthermore, when fibroblasts are added to these cultures, larger diameter (and far greater numbers of) banded fibrils are seen (8). Thus, in spite of the finding that SCs with neurons may be able to synthesize all the types of α chains required, fibroblasts play a role in the development of the typical array of endoneurial collagen fibrils. It may be of interest in this regard that in spinal root regions in the animal, where SC ensheathment of nerve fibers is comparable to that in the more distal nerve, there are fewer endoneurial collagen fibrils and also a diminution in the number of fibroblasts (50). Another point of interest is that new data of co-authors J. Uitto and P. Wood indicate that, unlike the type III and the presumed basement membrane collagens present, type I collagen is more abundant in the medium than in the tissue layer, suggesting that this type of collagen is assembled into fibrils to a lesser degree in NC + SC cultures. This raises the question whether the presence of fibroblasts is required for polymerization of some of the collagen; fibroblasts could possibly contribute enzymes needed for the conversion of procollagen to tropocollagen or for fibril assembly. Our finding that collagens are synthesized in NC + SC cultures also raises the question why SCs (in cultures with NCs but not fibroblasts) appear to require contact with a collagenous substratum for normal functional development (11). In this respect the SCs resemble

a number of cell types (e.g., corneal epithelium) that require contact with extracellular matrix materials for full expression of their functional capabilities (see reviews 21, 22, 29, and 39).

The presence of fibroblasts does not appear, however, to be a requirement for basal lamina formation on SCs. With increasing time in culture, the basal lamina coating the SCs related to neurons becomes more prominent. The fibroblast may secrete substances that contribute to basal laminae, but the morphologically visible framework of lamina material appears in the absence of fibroblasts. Our finding is consistent with an increasing body of knowledge indicating that basal laminae are not produced by connective tissue fibroblasts but instead are synthesized by the cells in contact with the laminae, namely, a variety of epithelial (e.g., references 4 and 24), endothelial, fat, and muscle cells (see references in 28). There is, in fact, no evidence to suggest that fibroblasts produce basal laminae (18). In our culture system, SCs form morphologically detectable basal lamina only when they are related to nerve cells (9, 51). This is in agreement with a study by Billings-Gagliardi et al. (5) who discovered that migrating SCs have no basal lamina, whereas, after they have stopped migrating and assumed a spindle-shaped contour around an axon, an initially patchy basal lamina makes it appearance on the SC surface. Interestingly, these authors mention that "randomly spaced near the outer aspect of the basal lamina are whisker-like clusters of fibrils;" perhaps these fibrils correspond to those accompanying basal lamina formation in the NC + SC cultures.

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