STUDIES OF SCHWANN CELL PROLIFERATION

I. An Analysis in Tissue Culture of Proliferation during Development, Wallerian Degeneration, and Direct Injury

JAMES L. SALZER and RICHARD P. BUNGE

From the Departments of Biological Chemistry and Anatomy and Neurobiology, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT

In this paper the stimuli for and pattern of Schwann cell proliferation are defined under various experimental conditions. We used a tissue culture system in which fetal rat dorsal root ganglia, treated to eliminate contaminating fibroblasts (Wood, P., 1976, *Brain Res.* **115**:361–375), appear to recapitulate many aspects of the developing peripheral nervous system. We observed that: (*a*) proliferation of Schwann cells on neurites is initially rapid, but, as each neurite becomes fully ensheathed, division slows considerably and is confined to the periphery of the outgrowth; (*b*) during the period of rapid proliferation, excision of the ganglion causes a rapid decay in the number of dividing cells; (*c*) excision of the ganglion from more established cultures in which there was little ongoing proliferation resulted in a small increase in labeling at the site of excision for all Schwann cells and a substantial increase in labeling for myelin-related cells with a peak labeling period at 4 d; (*d*) direct mechanical injury during Wallerian degeneration is mitogenic for Schwann cells; (*e*) a variety of potential mitogens failed to stimulate Schwann cell proliferation; and (*f*) replated cells have a slightly higher level of proliferation and show a small and variable response to the addition of cAMP.

KEY WORDS Schwann cell proliferation · nerve tissue culture · autoradiography · development · degeneration injury

Peripheral nerve development is an ordered process (reviewed by Webster [61]). The outgrowth of nerve fibers appears to be the initial event with naked nerve sprouts (lacking sheath cells) having been observed, for example, in the living tadpole tail fin (35, 55, 9). Subsequently, Schwann cells of neural crest origin (35, 64) migrate along the nerve fibers and begin the process of ensheathment. At first, large groups of axons are surrounded by a few Schwann cells (45, 42, 28). Subsequently, there is a burst of Schwann cell proliferation (57, 7) and invasion by the proliferating cells of the fascicles of naked axons (45, 21, among others). Eventually, a sorting of axons occurs, with the largest axons (those destined to be myelinated) segregated into a 1:1 relationship with the Schwann cells. Concomitant with the termination of Schwann cell proliferation, the myelin sheaths and connective tissue components of the peripheral nerve are formed.

Cellular interactions between neurons and supporting cells are critical during development as well as subsequently (56, 60). To elucidate the nature of these interactions, a number of experi-

mental approaches have been used. For example, in vivo studies utilizing cross anastomoses or grafting of segments of peripheral nerves have shown that the signal for myelination is neuronally mediated (54, 63, 4, 5). Also, observations on the effects of administration of nerve growth factor (NGF)[1] and its antiserum in vivo suggest that the number of axons within a nerve influence the number of Schwann cells present (6, 36).

It is evident that tissue culture techniques offer unique possibilities for the analysis of these complex cellular interactions. Recently, a tissue culture method was described for the isolation of pure populations of rat sensory ganglion neurons and Schwann cells (65). In this method, rat dorsal root ganglia (DRGs) are explanted into tissue culture dishes and briefly treated with antimitotic agents. As the ganglia mature in vitro, they recapitulate many of the developmental stages summarized above: nerve fibers grow out initially, Schwann cells migrate and proliferate along the fibers, proliferation slows considerably, and ensheathment and myelination ensue. It is possible, therefore, to analyze each of these developmental landmarks in tissue culture where cells are accessible to both direct observation and manipulation. Studies utilizing these techniques in which Schwann cells were grown in isolation or in combination with neurons have demonstrated that neurons or their attached processes (neurites) stimulate Schwann cell proliferation (66) and basal lamina production.[2]

In this paper we further characterize the mitogenic signal which the nerve fiber is known to provide to Schwann cells (66, 43). Wood and Bunge (66) presented evidence that Schwann cells grown in isolation show virtually no proliferation (<5% of the cells incorporated tritiated thymidine into DNA). When unensheathed neurites grew into the Schwann cell cultures, ~90% of the cells incorporated [^{3}H]thymidine in the region of interaction. In the present study, the pattern of Schwann cell proliferation in the region of neurite outgrowth is characterized. We have also studied the effect of axotomy on Schwann cell proliferation and demonstrated that myelin-related Schwann cells proliferate during degeneration, whereas Schwann cells related to unmyelinated nerve fibers do not. Finally, a number of known growth factors were examined for their ability to stimulate Schwann cell proliferation. In the accompanying papers of this series, we report detailed studies of the neurite mitogen; in the second paper (51), we show that a neurite membrane fraction provides a convenient assay system for the stimulation normally provided by the intact neurites and we present ultrastructural observations on interactions between Schwann cells and the neurite fraction. In the final paper (52), evidence is presented which indicates that the neurite mitogen is located on the axolemmal surface.

[1] *Abbreviations used in this paper:* CMF, Hanks' balanced salt solution without Ca^{++} or Mg^{++}; Con A, concanavalin A; DRG, dorsal root ganglion; EGF, epidermal growth factor; FGF, fibroblast growth factor; HPS, human placental serum; LI, labeling index; MEM, Eagle's minimal essential medium; NGF, nerve growth factor; PHA, leucoagglutinin; SNDRG, dorsal root ganglion composed of neurons and Schwann cells.

[2] Bunge, M. B., A. K. Williams, P. M. Wood, and J. Jeffrey. Sources of connective tissue component in peripheral nerve. I. Basal lamina and collagen fibril formation by cultured Schwann cells related to neurons. Manuscript submitted for publication.

MATERIALS AND METHODS

Source of Materials

All tissues were derived from fetal rats of the Holtzman strain. Pregnancies were accurately dated with the first appearance of sperm in a vaginal wash noted as day 0. Components for tissue culture media included Eagle's minimal essential medium (MEM), Leibovitz (L-15) medium, fetal calf serum, Hanks' balanced salt solution without Ca^{++} or Mg^{++} (CMF), and L-glutamine, all from Grand Island Biological Co. (Grand Island, N. Y.) Human placental serum (HPS) was (derived from cord blood) obtained locally. 5-Bromo-2′-deoxyuridine, cytosine-1-β-D-arabinofuranoside, and 5-fluorodeoxyuridine were obtained from Sigma Chemical Co. (St. Louis, Mo.). For Schwann cell harvesting, trypsin (three times crystallized) from Worthington Biochemical Corp. (Freehold, N. J.) and collagenase (Sigma Chemical Co., type VI) were used. Culture dishes were formed from the plastic, Aclar 33C (Allied Chemical Corp., Morristown, N. J.).

A number of reagents were tested as possible Schwann cell mitogens. These included concanavalin A (Con A), ouabain, insulin, acetylcholine chloride, L-arterenol hydrochloride, dexamethasone, dibutyryl cAMP, dibutyryl cGMP, adenosine, and 5′-AMP (all from Sigma Chemical Co.), arachidonate, $PGF_{2\alpha}$ (courtesy of Dr. P. Needleman), nerve growth factor (NGF) (courtesy of Dr. R. Bradshaw), epidermal growth factor and fibroblast growth factor (EGF and FGF) (from Collaborative Research Inc., Waltham, Mass.), tubulin (courtesy of Dr. M. Estridge), A23187 (courtesy of Dr. T. Sullivan), cholera toxin (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), proinsulin (Eli Lilly & Co., Indianapolis, Ind.) and leucoagglutinin (PHA) (from Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). To assess cell proliferation, [*methyl*-^{3}H]thymidine (40–60 Ci/mmol) from New England Nuclear (Boston, Mass.) was used at a concentration of 1 μCi/ml.

Culture Methods

CULTURE SYSTEM: To isolate pure Schwann cell populations, the general strategy was to obtain a neurite and Schwann

cell outgrowth from a sensory ganglion explant taken from an embryonic rat, and then to excise the neuronal somas (confined to the explant), allowing the neurites to degenerate, leaving an outgrowth containing only Schwann cells (65). Tissues were grown on a collagen-coated minidish (2.5 cm in diameter) of heat-molded Aclar (17). Cells in these dishes are fed with ~0.20 ml of medium. These dishes were collagen coated as described (65). Typically, Schwann cells were grown on a single layer of ammoniated collagen. However, to enhance adhesion, a second air-dried collagen layer was used for growing the initial explants of dorsal root ganglia which generated Schwann cells (SNDRGs), before transfer onto a dish with a single collagen layer.

To prepare the initial explants, DRGs were removed from 19- or 20-d embryos and treated during their initial few days in culture with a regimen of antimetabolites to eliminate any contaminating fibroblasts (65). This previous study (65) recommended two 24-h pulses of antimitotic treatment; we now include a third cycle. After this treatment, SNDRG may be transferred successively to provide several axon-Schwann cell outgrowths. To amplify this population of cells, we have sometimes replated them (see below) onto unensheathed neurites (52), on which they proliferate rapidly. All cultures containing Schwann cells were maintained on B medium consisting of 65% Eagle's MEM, 25% HPS, 10% chick embryo extract, 6 mg/ml glucose, L-glutamine (2 mM), and a concentration of NGF determined by bioassay to be 10–20 U.

CELL TRANSFER: To transfer ganglia or to initiate Wallerian degeneration by axotomy, we have used sterilized razor blade fragments to cut around the explant and into the collagen substrate. Ganglia could then be transferred with fine forceps into new dishes or be discarded.

In some experiments Schwann cells were transferred (replated) into new dishes. In this procedure, developed by Dr. P. Wood, primary Schwann cell cultures are rinsed twice with CMF. Cultures are then incubated with 0.05% collagenase (Sigma Chemical Co., type VI) in CMF (0.30 ml/culture) for 30 min at 34°C to remove the cells from their collagen substrate. During this incubation the Schwann cells aggregate into a small clump which can be rinsed with CMF. The aggregated cells are incubated for 30 min at 34°C in 0.05% trypsin (Worthington Biochemical Corp.) in CMF, the trypsin solution is withdrawn (the cells are still aggregated), B medium is added, and the aggregated cell clumps are pooled and triturated in ~5 ml of B medium. Cells are harvested by centrifugation at 100 *g* for 5 min, resuspended in B medium, and dissociated by trituration and filtering through a 15-μm pore-size nylon filter. Cell viability as assessed by trypan blue exclusion is typically 90–95%. The total number of cells is determined with a hemocytometer, and adjusted to give a final plating density of 5×10^4 cells/cm^2, approximately two-thirds of which survive. The medium is changed after 24 h and every other day thereafter.

HISTOLOGICAL TECHNIQUES: Sudan black fixation and staining procedures have been described (65). For autoradiography, cells were labeled with 1 μCi/ml of tritiated thymidine, generally for 1 d, fixed with 4% formalin, dehydrated, and the bottoms of the culture dishes containing the cells were mounted on slides. These were dipped in NTB-2 emulsion (diluted 2:1 with water). After exposure at 4°C for 5 or 6 d, the slides were developed and either viewed unstained after mounting with glycerin jelly or stained for 50 s with 0.25% toluidine blue, dehydrated through a graded series of alcohols and xylene, and mounted in DPX (Gallard-Schlessinger Chemical Mfg. Corp., Carle Place, N. Y.). To determine LI, slides were counted under phase at × 400, and cells with more than five grains/nucleus were considered labeled. A minimum of 2,000 cells for each sample were counted. The labeling index (L.I.) equals the number of labeled cells counted divided by the total number of cells counted × 100, i.e., percent labeled cells.

RESULTS

Description of the Normal Pattern of Outgrowth

Extensive descriptions utilizing both light and electron microscope analysis of developing sensory ganglia in vitro have been published (65, 16, and footnote 2). In this section, we describe the development of fibroblast-free preparations, with emphasis on the regulation of Schwann cell numbers.

After three 24-h pulses of antimitotic treatment, given on alternate days, the SNDRGs were transferred to new collagen-coated dishes. In the first few days after transfer, there is a variable but typically sparse outgrowth of nerve fibers (neurites) from the explant. These initial outgrowths are often essentially free of supporting cells. After several days the density of outgrowth increases and Schwann cells begin to migrate out from the explant, repopulating the fibers in a wave of proliferation stimulated by the neurites (66, 65). The first 2 or 3 wk are a period of intense proliferation in which numerous mitotic Schwann cells are observed throughout the entire outgrowth. Cultures labeled for 24 h with tritiated thymidine and processed for autoradiography demonstrate significant incorporation with labeling indices of ~50% throughout the outgrowth area (Fig. 1 *A*).

After this period of rapid proliferation, the rate of cell division slows considerably so that by the end of the 4th wk in culture the LI is only a few percent. The time-course for this decline in labeling is dependent on the rate and extent of neurite outgrowth and the initial number of surviving Schwann cells which repopulate the nerve fibers. Myelin first appears at about the 4th wk as thin, isolated segments, but over a period of several weeks or longer new segments appear and more mature segments thicken. By the 6th wk it is not uncommon for many of the larger nerve fibers to be myelinated along their entire length from explant to periphery. It should be noted, however, that even in mature cultures the majority of nerve fibers are ensheathed but not myelinated.

Three stages in the development of the SNDRG in vitro are shown in Fig. 1. The ganglia used in this experiment had been dissected out 2–3 mo beforehand, and had been transferred for the second time either 1–2 wk, 3–4 wk, or 5 wk before

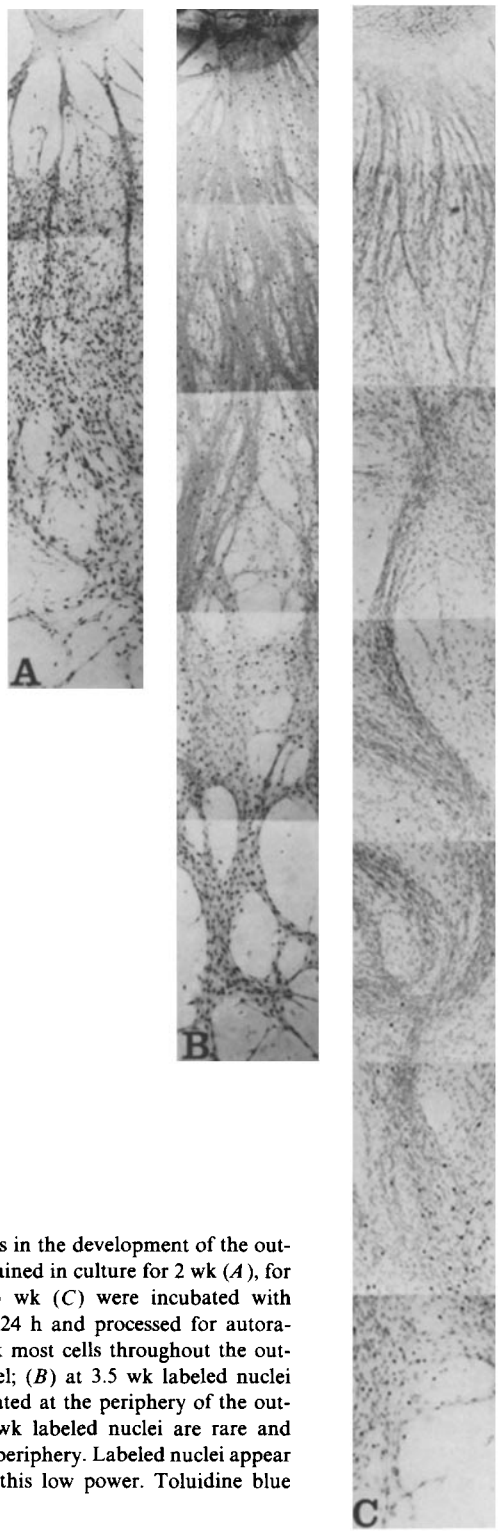

FIGURE 1 Three stages in the development of the outgrowth. Explants maintained in culture for 2 wk (*A*), for 3.5 wk (*B*), and for 5 wk (*C*) were incubated with tritiated thymidine for 24 h and processed for autoradiography. (*A*) At 2 wk most cells throughout the outgrowth incorporate label; (*B*) at 3.5 wk labeled nuclei are fewer and concentrated at the periphery of the outgrowth; (*C*) and at 5 wk labeled nuclei are rare and confined entirely to the periphery. Labeled nuclei appear as small dark spots at this low power. Toluidine blue stain. × 22.

labeling. At 1–2 wk, many cells are labeled and these appear to be randomly distributed throughout the region of outgrowth (Fig. 1*A*). At this stage, Schwann cells contact numerous fibers and appear flatter and more polygonal in shape. At 3.5 wk, labeled cells are less common and located primarily in the peripheral areas of the outgrowth (Fig. 1 *B*). By 5 wk, cells that incorporate label are rare and confined almost entirely to the tips of the neurites (Fig. 1 *C*). It is interesting to note that new neurite membrane may be inserted into this region (12, 15).

Effect of Excision of the Ganglion on Schwann Cell Proliferation

Cultures were pulsed with thymidine for 24-h periods at various times after removal of the explant containing the neuronal somas. We distinguish two situations: removal of the ganglion (*a*) from recently explanted SNDRGs or (*b*) from long-term cultures in which some myelin had formed.

Neurite degeneration was initiated by excision of the ganglion (i.e., axotomy) from young cultures (2 wk in vitro similar to the culture in Fig. 1 *a*) at either 0, 1, 2, 3, 4, etc. d before labeling with tritiated thymidine for 24 h. The results from two such experiments carried out in duplicate are shown in Fig. 2 and indicate that there is a very rapid decline in the labeling index of Schwann cells. Within 48 h after excision of the ganglia, there is virtually no incorporation of tritiated thymidine into DNA. After several weeks or more, the Schwann cells are still quiescent. In all of our studies with pure Schwann cell populations, we have used quiescent cultures in which the ganglia were excised 6 or 7 d before the experiment. In dozens of such cultures the labeling index was rarely in excess of 0.5% and usually 0.2% or less. Thus, the potent mitogenic signal provided by sensory neurites is quite labile, disappearing rapidly after axotomy.

Next, we excised ganglia from more mature cultures in which almost all proliferation had ceased (similar to Fig. 1 *C*) and myelin was present. In this experiment, autoradiographs were unstained and mounted directly with glycerin jelly, so that myelin-related cells could be visualized under phase illumination. This is a convenient system for studying Wallerian degeneration, as the same areas of the outgrowth may be followed in the living state and subsequently after fixation and

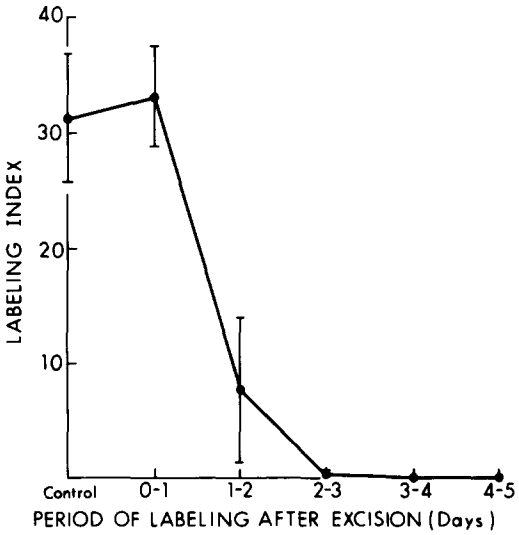

FIGURE 2 Decay in the labeling index of proliferating Schwann cells after excision of the ganglion. Schwann cells were labeled for 24 h with tritiated thymidine, either with the ganglion present (control) or at daily intervals after the ganglion was removed. This graph summarizes the results from two separate experiments carried out in duplicate with bars indicating the standard deviations.

autoradiography. Although there are some changes in cell position, the overall cellular relationships and the topography of the dish appear to be remarkably well preserved. For example, after several weeks, Schwann cells still remain aligned in the fascicles they previously formed with neurites. However, myelin shows profound changes. Within 1 d after excision, the myelin appears irregular and widening is observed at the node of Ranvier. By the 2nd d, myelin begins to fragment into ovoids, a process which continues over the next several days. By day five, a substantial amount of the myelin has been phagocytosed by the Schwann cells which elaborated it, a process termed autophagy by Asbury (8). It is interesting to note that this clearing of myelin proceeds very rapidly in our cultures, despite the absence of macrophages.

These cultures were incubated with tritiated thymidine for 4 d, starting 24 h after excision to allow the background proliferation to decay. We observed that Schwann cells that had been myelin related were stimulated to divide by axotomy. In control preparations in which the ganglion was not removed, myelin-related Schwann cells failed to incorporate thymidine. Also, Schwann cells that had not elaborated myelin remained quiescent.

These results are illustrated in Fig. 3 in which the same three myelinated fibers were photographed in the living state (Fig. 3 *A*), and after ganglion excision, labeling, and autoradiography (Fig. 3 *B*). It may be seen that those cells that were myelin related are the same cells that incorporate thymidine. There is approximately a doubling of the number of myelin-related Schwann cell nuclei (indicated with arrowheads), several of which appear as doublets, and may represent nuclei which had just divided.

The time-course for this response is shown in Fig. 4. In this experiment, Wallerian degeneration was initiated by excision and cultures were labeled for 24-h periods on successive days. Only nuclei associated with myelin or myelin debris were assessed for labeling. The response is maximal at about day four at which time ~35% of the cells were labeled. Cells almost certainly divide after this time, but the study was discontinued because much of the myelin had been resorbed and it became increasingly difficult to determine which cells had been myelin related.

In these experiments, both myelinating and nonmyelinating Schwann cells are exposed to axonal degeneration products after DRG excision, but only myelin-related cells divide. It thus seems unlikely that the stimulus for Schwann cell proliferation is solely caused by an axonal degeneration product. Also, we have observed that Schwann cells associated with isolated myelin segments may divide, but adjacent Schwann cells related to the same axon (but which have not formed myelin) remain quiescent. Therefore, some aspect of the fully differentiated Schwann cell or a breakdown product of the myelin itself may be involved in triggering division during Wallerian degeneration, rather than the breakdown of the axon per se.

Schwann Cell Response to Direct Injury

Some Schwann cell proliferation has been observed in vivo for unmyelinated peripheral nerves undergoing Wallerian degeneration but primarily in the local area of crush (49). We have also observed a slight increase in the labeling index of Schwann cells in the region immediately adjacent to the site where the DRG has been excised. This is a variable, but consistent observation. LIs vary from 1 or 2% up to 20% in the local site of excision and are essentially zero everywhere else. To test whether this may be caused by direct Schwann cell injury, we made razor blade transections in peripheral areas of an unmyelinated culture in which the ganglion was removed simultaneously. We observed a significant elevation in the LI (~25%) only in the areas of direct mechanical injury (Fig. 5). However, in experiments in which the neurites were first allowed to degenerate for 1 wk, and then razor cuts were made among Schwann cells, no stimulation was observed (data not shown). It therefore appears that degenerating neurites and direct injury must act in concert to stimulate proliferation of Schwann cells related to unmyelinated nerve fibers.

Lack of Stimulation by Known Mitogens

We have focused thus far on signals that operate during development and degeneration to influence Schwann cell numbers. We also examined a number of potential mitogens in the hope that one or more of these agents would provide a stimulus for proliferation and thereby permit propagation of Schwann cells as a continuous cell line. Some of these results were reported in preliminary form earlier (50). However, Schwann cells proved to be quite refractory to stimulation by these factors, and these results instead demonstrate the remarkable specificity of the neurite mitogen. The agents tested and the concentration used are given in Table I.

The factors tested are known mitogens for either lymphocytes or fibroblasts. The lectins Con A and PHA, which activate lymphocytes (reviewed in reference 62) and neural retinal cells (39), failed to stimulate Schwann cell proliferation. EGF and FGF lacked any stimulatory effect on Schwann cells (as was also recently reported elsewhere, 47, 34). NGF, which is normally present in our culture medium at ~50 ng/ml, failed to stimulate Schwann cells at a 10- or 100-fold higher concentration. Finally, insulin, dexamethasone, and prostaglandin $PGF_{2\alpha}$—known mitogens for fibroblasts (29, 37)—also failed to stimulate Schwann cells.

Also included in Table I are a number of factors not normally considered to be mitogens. We tested tubulin, acetylcholine, and norepinephrine because they are known to be associated with rat superior cervical ganglion neurons in vitro (26, 18). Whereas these neurons are mitogenic for Schwann cells (P. Wood, unpublished observations), these agents were not. Ouabain was demonstrated to be mitogenic for embryonic neural retina cells (40) but failed to induce Schwann cell proliferation. In several systems, an increase of

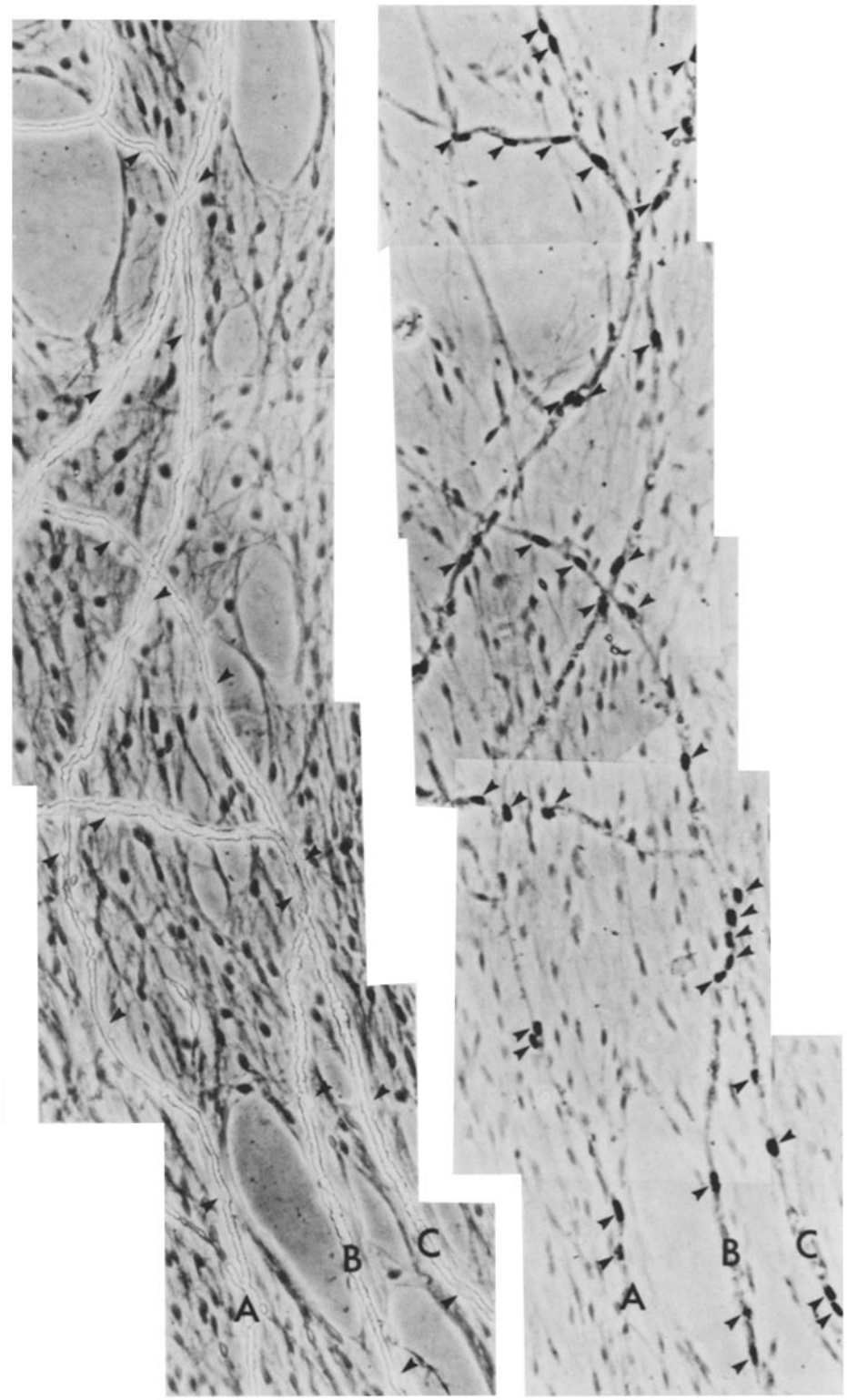

FIGURE 3 Proliferation of myelin-related Schwann cells during Wallerian degeneration in vitro. These two montages demonstrate the appearance of myelin in the living state (left panel), and an autoradiograph of the same area after excision of the ganglion, incubation with tritiated thymidine, and autoradiography (right panel). The three myelinated fibers are labeled *A*, *B*, and *C* for comparison. The original location of the nuclei and the subsequent location of the labeled nuclei are indicated by the arrowheads. Phase contrast. × 180.

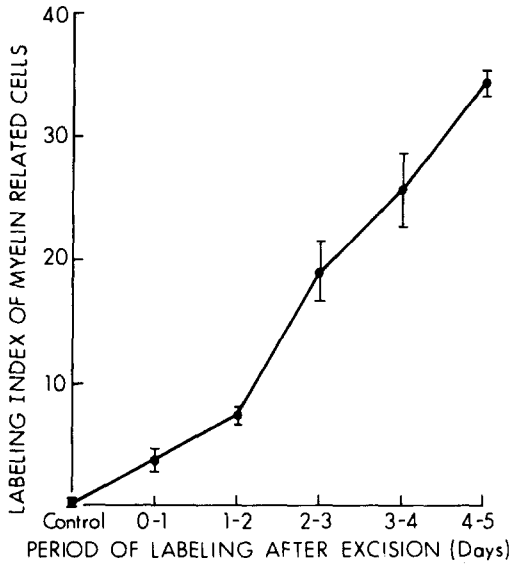

FIGURE 4 Increase in the labeling index of myelin-related cells during Wallerian degeneration in tissue culture. This graph summarizes the results from two experiments in which myelinated DRG cultures were incubated for 24 h with tritiated thymidine, either with the ganglion present (control) or at daily intervals after excision of the ganglion. After processing for autoradiography, only cells related to myelin or containing myelin debris were scored. Bars indicate range of values.

intracellular calcium has been indirectly implicated in the control of cell division (31, 25, 10, 24). Addition of A23187, a calcium ionophore, to our culture medium did not result in an increase in Schwann cell labeling (also reported by Raff et al. [47]).

Trypsin was tested as a potential mitogenic agent because of its ability to release fibroblasts from density-dependent inhibition of growth (53, 19) or serum starvation (67, 20). Pretreatment of Schwann cells with trypsin, or incubation of Schwann cells with trypsin during a 2-d labeling period failed to elevate the labeling index. In fact, as will be discussed below, cells enzymatically dissociated and replated at sparse cell densities also have a very low LI. Finally, it should be noted that these cells are grown in very high serum concentrations (25%). Shifting from low (3%) to high (25%) serum levels, or to other types of sera (fetal calf serum) does not initiate proliferation. Therefore it seems unlikely that depletion of serum factors or density-dependent inhibition of growth (contact inhibition) are the basis for the quiescent state of the Schwann cell.

Behavior of Replated Schwann Cells

A series of recent reports by Raff and his colleagues indicated that Schwann cells, prepared from sciatic nerve by methods very different from our own, display a remarkably high basal level of proliferation (13, 14, 46, 47). It seemed possible that part of this difference in proliferative behavior was caused by the use of enzymatically dissociated cells in these studies. Our initial reason for replating primary Schwann cells was to facilitate studies (described in the second paper of the series) in which neurite membranes were added to Schwann cells, by obviating mechanical shielding caused by multiple cell layers or the presence of the characteristic overlying basal lamina. The technique also allowed expansion of the number of aliquots of cells available for experimental analysis, and a direct comparison with the work cited above.

After replating, Schwann cells retain their spindle shape (although they are not so uniformly bipolar as in the primary cultures) and initially are more migratory. This may correlate with the loss of the basal lamina during enzymatic dissociation since migratory Schwann cells in vivo lack a basal lamina (9). About 48 h after dissociation and replating, cells migrate into islands or chains, often aligning dramatically end to end. The tendency of Schwann cells in vitro to form chains or ribbons of cells has been observed previously (44, 22).

Replated cells demonstrate a slight but nevertheless significant increase in labeling index compared to primary cell cultures. Schwann cells were replated into two types of culture dishes: (*a*) our conventional dish in which the average LI was ~1–2% after replating and (*b*) a micro-well chamber (described in the accompanying paper) in which for numerous reasons the labeling index was frequently lower (0.3–0.4% after replating). When these replated cells were again enzymatically harvested and replated into Aclar dishes, the LI was not increased, suggesting that repeated enzymatic harvesting is not by itself mitogenic. Therefore, replating Schwann cells does potentiate the basal level of proliferation either by removing the basal lamina or on account of subsequent maintenance of cells at low densities. Schwann cells after replating are essentially quiescent.

Is cAMP Mitogenic for Schwann Cells?

In addition to the high basal level of proliferation (15–20%), Schwann cells prepared from sciatic nerve by the method of Brockes et al. (14) are also

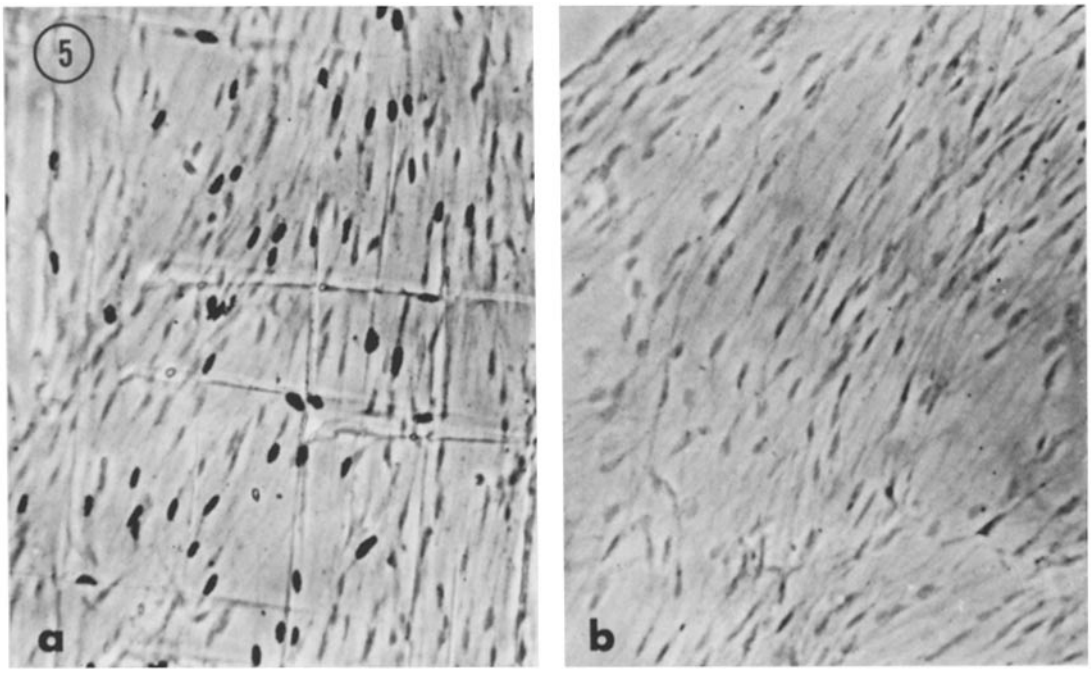

FIGURE 5 Effect of injury on Schwann cell proliferation. This figure shows two areas from the same Schwann cell culture (in vitro for 8 wk). On day 0, the ganglion was excised and a series of razor blade cuts were made in a cross-hatched pattern. Cells were incubated from days 1–4 with 0.5 μCi/ml of tritiated thymidine and processed for autoradiography. Fig. 5 *a* shows one area of cross-hatched transections demonstrating numerous labeled nuclei. Fig. 5 *b*, shown for comparison, is a contiguous area of cells which were undisturbed and unlabeled. × 225.

quite responsive to dibutyryl cAMP. Considerable stimulation of the growth rate of these cells could be observed upon addition of dibutyryl cAMP at 5×10^{-5} M or of cholera toxin (46, 47). In our initial observations with primary cultures of Schwann cells, we failed to observe any stimulation of thymidine incorporation by dibutyryl cAMP (50), or dibutyryl cGMP at concentrations ranging from 10^{-2} to 10^{-7} M. At very high concentrations of dibutyryl cAMP (10^{-3} M), this compound caused a 50% inhibition of the mitogenic effect of neurites on Schwann cells and totally abolished thymidine incorporation at a concentration of 10^{-2} M. This inhibition may be caused by a toxic effect of butyrate (32).

When dibutyryl cAMP was added to replated Schwann cells, it was weakly mitogenic in confirmation of the observations of Raff et al. (46). The maximum effect was observed at a concentration of 5×10^{-5} M. The data were highly variable, but dibutyryl cAMP consistently potentiated the background rate of thymidine incorporation. When the background was low, there was very little stimulation by dibutyryl cAMP (Table II). Neither adenosine, butyrate, or 5′-AMP can substitute for dibutyryl cAMP in this system.

In partial confirmation of the observations of Brockes et al. (14), we have also observed that cholera toxin produces a variable mitogenic stimulation for Schwann cells with the maximal effect at 5 ng/ml. This has ranged from no effect in several instances to a maximum 6–7% of primary cells labeled in a 24-h incubation with thymidine, and 18% of replated Schwann cells labeled with thymidine in 24 h. In these experiments, cells were exposed to cholera toxin for 2 d before labeling with [^{3}H]thymidine for 24 h in the presence of cholera toxin. The cause of this variability is not known. The possible differences between Schwann cells prepared from sciatic nerve by the method of Brockes et al. (14) and those prepared by the method of Wood (65) will be discussed below.

When cholera toxin was added to older SNDRG cultures, the LI increased in these cultures as well. Nevertheless, Schwann cells continued to relate to nerve fibers and myelin accrued

TABLE I

Factors That Fail to Stimulate Schwann Cell Proliferation

Factors	Concentrations tested
Concanavalin A*	0.5, 5, 50 μg/ml
Phytohemagglutinin*	0.5, 5, 50 μg/ml
NGF	0.5, 5 μg/ml
EGF	1 pg/ml to 10 μg/ml
FGF	1 pg/ml to 10 μg/ml
Acetylcholine	5×10^{-9} to 5×10^{-6} M
Norepinephrine	5×10^{-9} to 5×10^{-6} M
Insulin*	0.5–500 μg/ml
Proinsulin	0.1–10 μg/ml
Dexamethasone	5×10^{-8} to 2.5×10^{-5} M
$PGF_{2\alpha}$*	0.1–10 μg/ml
$PGF_{2\alpha}$ + insulin*	500 ng and 100 ng/ml
A23187	0.25–10 μg/ml
Arachidonic acid	0.2–25 μg/ml
Ouabain	1×10^{-5} to 5×10^{-4} M
Tubulin	1–500 μg/ml
Trypsin*	0.25–50 μg/ml

Cultures were incubated in B medium for 48 h with the additions indicated. During the last 24 h, the medium also contained 1 μCi/ml of [^{3}H]thymidine; subsequently, cells were processed for autoradiography as described in Materials and Methods.

* Incubated with Schwann cells in low serum medium, 0 or 3% for 48 h, then B medium containing [^{3}H]-thymidine was added for 24 h.

TABLE II

Effect of Dibutyryl cAMP on Schwann Cell Proliferation

	Percent labeled cells			
Dibutyryl cAMP	Exp 1	Exp 2	Exp 3	Exp 4
M				
0	5.01	1.85	0.29	0.05
10^{-5}	18.33	—	0.77	0.27
5×10^{-5}	20.0	6.97	1.43	0.95
10^{-4}	4.34	—	2.82	3.53

Replated Schwann cells were incubated with dibutyryl cAMP for 2 d at the concentrations indicated. On the 3rd d, fresh medium with dibutyryl cAMP and tritiated thymidine (1 μCi/ml) was added. After 24 h, cells were fixed and processed for autoradiography.

normally despite an overproduction of Schwann cells.

DISCUSSION

In this discussion the division of Schwann cells in tissue culture during development and degeneration is considered in the context of the literature on proliferation in vivo. The failure of response to a variety of mitogens and the stimulation by cAMP will also be discussed.

Dependency on the Neurite Mitogen

Our results on the pattern of proliferation and its rapid decay after excision of the ganglion are consistent with the previous demonstration in tissue culture that neurons are mitogenic for peripheral non-neuronal cells (66, 43). For example, evidence was presented by Wood and Bunge (66) that quiescent Schwann cells (LI <5%) were dramatically stimulated to proliferate (LI ~90%) when sensory ganglion neurites were allowed to grow into and interact with these cells. A review of the literature reveals a number of examples that suggest that neurons and axons are also mitogenic for Schwann cells in vivo during development. For example, studies of developing rat sciatic nerve indicate that axon differentiation precedes Schwann cell proliferation (45, 42). Satellite cell proliferation commences only after neuronal cell division is essentially completed (41). Schwann cell mitosis occurs in relation to the axons in a manner which maximizes surface contact between the two cells (42). For example, Diner reported that mitotic Schwann cells had axons embedded in their cytoplasm and that the plasma membranes of the Schwann cells and axons were uniformly opposed at a distance of 150–200 Å (23).

Two experimental situations also demonstrate the importance of the axon in regulating Schwann cell numbers in vivo. The administration of NGF to neonatal rats induces sympathetic nerve sprouting and hypertrophy (36). Labeling of Schwann cells with tritiated thymidine was markedly enhanced as well, a finding Hendry suggests was secondary to the neuronal hypertrophy. Conversely, anti-NGF antiserum given at birth significantly reduced the number of axons (24% of controls) and the number of Schwann cells (34% of controls) which survived (6). Also Speidel (55) found that, after sectioning the lateral line nerve (a sensory nerve) in the tadpole, there was a significant reduction in the number of sheath (Schwann) cells surviving ~3 mo after transection. Thus, when axons are hypertrophied, Schwann cells are secondarily increased, and loss of axons correspondingly diminishes the number of Schwann cells. The nature of the neuronal mitogen for Schwann cells is a subject of considerable interest. In the accompanying papers we describe

and discuss a method for analyzing this mitogen and present evidence for its surface localization.

Proliferation of Cells during Degeneration

There are two situations in which Schwann cell proliferation has been observed in vivo. During development, as we have discussed, there is a rapid burst of proliferation (7). Also there is an extensive literature describing Schwann cell proliferation during Wallerian degeneration (1, 2, 38, 11, among others). In earlier studies, it was observed that during crush injury of peripheral nerves, there was a substantial increase (up to eightfold) in the number of tubal nuclei (Schwann cell nuclei) distal to the site of injury after 25 d, which then declined slightly (11). This increase in cell number was found to be greatest in myelinated nerves and least in thinly myelinated or in unmyelinated nerves (58, 38). More recent autoradiographic studies have shown that the increase in labeling that occurs during Wallerian degeneration is greater in sciatic nerve (11, 27) than in the unmyelinated cervical sympathetic trunk (49). Finally, in one study (59), the peroneal nerve was crushed nine separate times with a significant increase in total cells with each crush.

The stimulus during injury has been proposed to be either degenerating axonal debris (2) or the vacant space left behind by the degenerating nerve fibers (38) since the final cell density achieved in Wallerian degeneration is remarkably similar in a variety of peripheral nerves. This proliferation may play a role in peripheral nerve regeneration by providing cells for the phagocytosis of degenerating myelin (33), by providing a suitable terrain for regenerating nerve fibers (48), or be required by the Schwann cell for it to regain its multipotentiality and myelination capability (4).

Our results which demonstrate Schwann cell proliferation in tissue culture during Wallerian degeneration support and extend these earlier in vivo observations. One contribution of this study is the finding that it is the myelin-related cells themselves which undergo proliferation (Fig. 3). Remarkably, a fully differentiated cell has the capability to simultaneously phagocytose its myelin and proliferate. We also observed that Schwann cells associated with isolated degenerating myelin segments (which develop naturally in a myelinating outgrowth) divide, whereas contiguous cells do not. The time-course for the peak labeling in culture is similar to that reported for the mouse sciatic nerve (11), but earlier than that reported for the rat (27). Finally, we have not observed any proximal-distal gradient for the wave of proliferation observed during degeneration. Shorter labeling periods during the earlier stages of degeneration may be useful in this regard.

These results suggest that the mitogenic stimuli during development and degeneration are distinct. During development all neurites in culture are mitogenic; after axotomy all fibers degenerate but only selected Schwann cells divide. It appears unlikely that creation of vacant space or release from some type of spatial restraint is the mitogenic signal for proliferation since there is sufficient space for all Schwann cells to divide after axotomy (especially in peripheral areas of the outgrowth). Finally, our results suggest that the stimulus is not an axonal breakdown product, per se. Schwann cells do not proliferate in response to the presence of a degenerating neurite alone, not even in situations in which the ratio of degenerating axons to Schwann cells is quite high. For example, after excision of "young" explants in which the neurites are not yet fully ensheathed, Schwann cell proliferation rapidly terminates rather than increasing (Fig. 2). Breakdown of a myelin-competent axon also does not appear to be mitogenic, Schwann cells on either side of isolated segments or between two segments did not incorporate label.

Our results are consistent with a model in which breakdown of myelin within the Schwann cell or turnover of Schwann cell membranes is the stimulus operative during degeneration. Breakdown of myelin itself as a mitogen would explain the recent observation of persistently elevated Schwann cell proliferation in the trembler mouse, a genetic mutant whose defect is confined to myelinated nerves where a continuous cycle of demyelination-remyelination was observed (3). Also in experiments to be reported elsewhere (J. Salzer, R. P. Bunge, and G. DeVries), a myelin membrane fraction was found to be somewhat mitogenic. The possibility that turnover of Schwann cell membranes during Wallerian degeneration is the stimulus must also be considered. Nonmyelinating cells can simply retract their processes during degeneration, whereas a myelin-related cell must undergo a major reabsorption and renewal of its membranes. Consistent with this model is the demonstration of the mitogenic effect of direct injury on Schwann cells in an area undergoing Wallerian degeneration both in culture and apparently in vivo (49

and footnote 3). In this sense, proliferation of myelin-related Schwann cells in Wallerian degeneration may represent a response to what is essentially a direct injury for the cell.

Lack of Response of Schwann Cells to Soluble Mitogens

In our experiments, a variety of described mitogens failed to stimulate division in Schwann cells (Table I). These results underscore the specificity of the proliferation observed during development and degeneration. In this section we discuss the response of Schwann cells to cAMP.

In experiments with cyclic AMP, we failed to observe any stimulation of primary cells. cAMP does appear to be mitogenic for replated cells although substantially less so than was reported for cells isolated by very different methods (14). cAMP has most often been described as an inhibitor of cell proliferation, although recent reports indicate it may be mitogenic for thymocytes, melanoma cells, and epidermal cells (reviewed in reference 30).

Recently a number of papers have appeared on Schwann cells isolated from rat sciatic nerve by very different methods (47, 14). These cells appear to be Schwann cells on the basis of their morphology and antigenic properties. Although they proliferate more slowly than fibroblasts, they have a remarkably high basal level of proliferation (LI of 15–20%). We believe some of this difference in basal proliferation is attributable to the manner of selection. In essence, the method of Wood (65) selects for cells which divide in association with neurites, while the method of Brockes et al. (14) selects for cells which divide independently of the nerve fiber. Both methods employ a brief exposure to antimitotic agents. In the selection method used in this paper (65) cells which proliferate before the first fibers grow out (the first day of explantation) are exposed to antimitotic agents; those which migrate away from the neuronal somata and axons are left behind after the transfer of the ganglion. In contrast, in the method of Brockes et al. (14) the cells are treated with one continuous dose of cytosine arabinoside to kill proliferating cells. This method does not remove all fibroblasts, as treatment with antitheta antibody is required to eliminate the remaining fibroblasts. Also the Schwann cells prepared by Brockes et al. (14) are derived from postnatal rat sciatic nerve; many of these cells may have been myelin related because at this stage in development the sciatic nerve is in the process of myelinating. Removal and dissociation of the sciatic nerve may inadvertently damage cells or induce changes similar to those occurring in Wallerian degeneration, to which the cells respond by dividing. As yet there has been no functional characterization of these cells. There may be functional differences, as well as differences in proliferative behavior. Finally, we have switched Schwann cells prepared by the method of Wood (65) to fetal calf serum without inducing proliferation, and therefore the difference in basal proliferation does not appear to be attributable to the use of different media (as suggested in reference 47), although conceivably different serum lots may vary in this regard.

Dibutyryl cAMP appears to amplify the level of labeling severalfold in both systems. As our background labeling is low, this is only a minor effect; however, the high background labeling in the study of Brockes et al. may account for the substantial stimulation induced by dibutyryl cAMP. These data could be interpreted most readily by assuming that dibutyryl cAMP increases the rate with which the cells already growing traverse the cell cycle, but cAMP does not recruit quiescent cells into the cell cycle. Finally, results to be reported in an accompanying paper (52), suggest that the stimulation of Schwann cells by neurites is independent of cAMP. It will be of interest to examine whether the proliferation of Schwann cells during Wallerian degeneration might have a cyclic AMP component.

It is a sincere pleasure to acknowledge the numerous suggestions and insights of Dr. Luis Glaser during the course of this work and in the preparation of this manuscript. We also gratefully acknowledge the support and expert advice of Dr. Patrick Wood in this project.

This work was supported by grants from the National Institutes of Health NS-09923 and GM 18405, and the National Science Foundation BM-77-15972. J. Salzer was supported by the Medical Scientist Training Grant TO-5-GM 02016.

Received for publication 11 July 1979, and in revised form 26 October 1979.

[3] We cannot rule out the possibility at present that direct injury to Schwann cells is lethal for those cells and these dying cells stimulate division of adjacent, uninjured cells.

REFERENCES

1. ABERCROMBIE, M., and M. L. JOHNSON. 1946. Quantitative histology of Wallerian degeneration. I. Nuclear population in rabbit sciatic nerve. *J. Anat.* **80**:37–50.
2. ABERCROMBIE, M., and J. SANTLER. 1957. An analysis of growth in

nuclear population during Wallerian degeneration. *J. Cell. Comp. Physiol.* **50**:429–450.

3. AGUAYO, A. J., G. M. BRAY, and S. C. PERKINS. 1979. Axon-Schwann cell relationships in neuropathies of mutant mice. *Ann. N. Y. Acad. Sci.* **317**:512–531.
4. AGUAYO, A. J., L. CHARRON, and G. M. BRAY. 1976. Potential of Schwann cells from unmyelinated nerves to produce myelin: A quantitative ultrastructural and radiographic study. *J. Neurocytol.* **5**:565–573.
5. AGUAYO, A., J. EPPS, L. CHARRON, and G. M. BRAY. 1976. Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: Quantitative microscopy and radioautography. *Brain Res.* **104**:1–20.
6. AGUAYO, A. J., J. B. MARTION, and G. M. BRAY. 1972. Effects of nerve growth factor antiserum on peripheral unmyelinated nerve fibers. *Acta Neuropathol.* **20**:288–298.
7. ASBURY, A. K. 1967. Schwann cell proliferation in developing mouse sciatic nerve. *J. Cell Biol.* **34**:735–743.
8. ASBURY, A. K. 1975. The biology of Schwann cells. *In* Peripheral Neuropathy. Vol. I. P. Dyck, P. K. Thomas, and E. H. Lambert, editors. W. B. Saunders Co., Philadelphia. 201–212.
9. BILLINGS-GAGLIARDI, S., H. DEF. WEBSTER, and M. O'CONNELL. 1974. *In vivo* and electron microscopic observations on Schwann cells in developing tadpole nerve fibers. *Am. J. Anat.* **141**:375–392.
10. BOYNTON, A. L., and J. F. WHITFIELD. 1976. The different actions of normal and supranormal Ca^{++} concentrations on the proliferation of BALB/c3T3 mouse cells. *In Vitro (Rockville).* **7**:479–484.
11. BRADLEY, W. G., and A. ASBURY. 1970. Duration of synthesis phase in neurolemma cells in mouse sciatic nerve during degeneration. *Exp. Neurol.* **26**:275–282.
12. BRAY, D. 1970. Surface movements during the growth of single explanted neurons. *Proc. Natl. Acad. Sci. U. S. A.* **65**:905–910.
13. BROCKES, J. P., K. L. FIELDS, and M. C. RAFF. 1977. A surface antigenic marker for rat Schwann cells. *Nature (Lond.).* **266**:364–366.
14. BROCKES, J. P., K. L. FIELDS, and M. C. RAFF. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165**:105–118.
15. BUNGE, M. 1973. Fine structure of nerve fibers and growth cones of isolated sympathetic neurons in culture. *J. Cell Biol.* **56**:713–735.
16. BUNGE, M. B., R. P. BUNGE, E. R. PETERSON, and M. R. MURRAY. 1967. A light and electron microscopy study of long-term organized cultures of rat dorsal root ganglia. *J. Cell Biol.* **32**:439–466.
17. BUNGE, R. P., and P. WOOD. 1973. Studies on the transplantation of spinal cord tissue in the rat. I. The development of a culture system for hemisections of embryonic spinal cord. *Brain Res.* **57**:261–276.
18. BUNGE, R., M. JOHNSON, and D. ROSS. 1978. Nature and nurture in the development of the autonomic neuron. *Science (Wash. D. C.).* **199**:1409–1416.
19. BURGER, M. 1970. Proteolytic enzymes initiating cell division and escape from contact inhibition of growth. *Nature (Lond.).* **227**:170–171.
20. CARNEY, D., and D. CUNNINGHAM. 1977. Initiation of chick cell division by trypsin action at the cell surface. *Nature (Lond.).* **268**:602–606.
21. CRAVIOTO, H. 1965. The role of Schwann cells in the development of human peripheral nerves. *J. Ultrastruct. Res.* **12**:634–651.
22. CRAVIOTO, H., and R. LOCKWOOD. 1968. The behavior of normal peripheral nerve in tissue culture. *Z. Zellforsch. Mikrosk. Anat.* **90**:186–201.
23. DINER, O. 1965. Les cellules de Schwann en mitose et leurs rapports avec les axones av cours du developpement du herf sciatique chez le rat. *C. R. Acad. Sci. (Paris).* **261**:1731–1734.
24. DUFFUS, J. H., and L. J. PATTERSON. 1974. Control of cell division in yeast using the ionophore, A23187 with calcium and magnesium. *Nature (Lond.).* **251**:626–627.
25. DULBECCO, R., and J. ELKINGTON. 1975. Induction of growth in resting fibroblastic cell cultures by Ca^{++}. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1584–1588.
26. ESTRIDGE, M., and R. BUNGE. 1978. Compositional analysis of growing axons from rat sympathetic neurons. *J. Cell Biol.* **79**:138–155.
27. FRIEDE, R. L., and M. JOHNSTONE. 1967. Response of thymidine labeling of nuclei in gray matter and nerve following sciatic transection. *Acta Neuropathol.* **7**:218–231.
28. GAMBLE, H. J., and A. S. BREATHNACH. 1965. An electron microscope study of human foetal peripheral nerves. *J. Anat.* **99**:573–584.
29. GOSPODAROWICZ, D., and J. MORAN. 1974. Stimulation of division of sparse and confluent 3T3 cell populations by a fibroblast growth factor, dexamethasone and insulin. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4584–4588.
30. GREEN, H. 1978. Cyclic AMP in relation to proliferation of the epidermal cell: A new view. *Cell.* **15**:801–811.
31. GREENE, W., C. M. PARKER, and C. W. PARKER. 1976. Calcium and lymphocyte activation. *Cell. Immunol.* **25**:74–89.
32. HAGOPIAN, H. K., M. G. RIGGS, L. A. SWARTZ, and V. M. INGRAM. 1977. Effect of n-butyrate on DNA synthesis in chick fibroblasts and HeLa cells. *Cell.* **12**:855–860.
33. HALL, S. M., and N. A. GREGSON. 1977. The effects of mitomycin C on the process of regeneration in the mammalian peripheral nervous system. *Neuropathol. Appl. Neurobiol.* **3**:65–78.
34. HANSON, G., and L. PARTLOW. 1978. Stimulation of non-neuronal cell proliferation *in vitro* by mitogenic factors present in highly purified sympathetic neurons. *Brain Res.* **159**:195–210.
35. HARRISON, R. G. 1924. Neuroblast versus sheath cell in the development of peripheral nerves. *J. Comp. Neurol.* **37**:123–205.
36. HENDRY, I. A. 1977. Cell division in the developing sympathetic nervous system. *J. Neurocytol.* **6**:299–309.
37. JIMENEZ DE ASUA, A., D. CLINIGAN, and P. RUDLAND. 1975. Initiation of cell proliferation in cultured mouse fibroblasts by PG F_{2a}. *Proc. Natl. Acad. Sci. U. S. A.* **72**:2724–2728.
38. JOSEPH, J. 1950. Further studies in changes of nuclear population in degenerating non-myelinating and finely myelinated nerves. *Acta Anat.* **9**:279–288.
39. KAPLOWITZ, P., and A. A. MOSCONA. 1976. Lectin mediated stimulation of DNA synthesis in cultures of embryonic neural retina cells. *Exp. Cell Res.* **100**:177–189.
40. KAPLOWITZ, P., and A. A. MOSCONA. 1976. Stimulation of DNA synthesis by ouabain and Con A in cultures of embryonic neural retina cells. *Cell Differ.* **5**:109–119.
41. LAWSON, S., K. CADDY, and T. BISCOE. 1974. Development of rat dorsal root ganglion neurones: Studies of cell birthdays and changes in mean cell diameter. *Cell Tissue Res.* **153**:399–413.
42. MARTIN, J. R., and H. DEF. WEBSTER. 1973. Mitotic Schwann cells in developing nerve: Their changes in shape, fine structure, and axon relationships. *Dev. Biol.* **32**:417.
43. MCCARTHY, K. D., and L. M. PARTLOW. 1976. Neuronal stimulation of ^{3}H-thymidine incorporation by primary cultures of highly purified non-neuronal cells. *Brain Res.* **114**:415–426.
44. MURRAY, M. R., and A. P. STOUT. 1942. Characteristics of human Schwann cells *in vitro*. *Anat. Rec.* **84**:275–293.
45. PETERS, A., and A. R. MUIR. 1959. The relationship between axons and Schwann cells during development of peripheral nerves in the rat. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **44**:117–130.
46. RAFF, M. C., A. HORNBY-SMITH, and J. BROCKES. 1978. Cyclic AMP as a mitogenic signal for cultured rat Schwann cells. *Nature (Lond.).* **273**:672–673.
47. RAFF, M., E. ABNEY, J. BROCKES, and A. HORNBY-SMITH. 1978. Schwann cell growth factors. *Cell.* **15**:813–822.
48. RAMON, Y CAJAL, S. 1928. Degeneration and Regeneration of the Nervous System. R. M. May, trans. Hafner Publishing Co., New York, 1959.
49. ROMINE, J. S., G. M. BRAY, and A. J. AGUAYO. 1976. Schwann cell multiplication after crush injury of unmyelinated fibers. *Arch. Neurol.* **33**:49–54.
50. SALZER, J. L., L. GLASER, and R. P. BUNGE. 1977. Stimulation of Schwann cell proliferation by a neurite membrane fraction. *J. Cell Biol.* **75**(2, Pt. 2): 118 *a* (Abstr.).
51. SALZER, J. L., A. K. WILLIAMS, L. GLASER, and R. P. BUNGE. 1980. Studies of Schwann cell proliferation. II. Characterization of the stimulation and specificity of the response to a neurite membrane fraction. *J. Cell Biol.* **84**:753–766.
52. SALZER, J. L., R. P. BUNGE, and L. GLASER. 1980. Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. *J. Cell. Biol.* **84**:767–778.
53. SEFTON, B. M., and H. RUBIN. 1970. Release from density dependent growth inhibition by proteolytic enzymes. *Nature (Lond.).* **227**:843–844.
54. SIMPSON, S. A., and J. Z. YOUNG. 1945. Regeneration of fiber diameter after cross-unions of visceral and somatic nerves. *J. Anat.* **79**:48–65.
55. SPEIDEL, C. C. 1964. *In vivo* studies of myelinated nerve fibers. *Int. Rev. Cytol.* **16**:173–231.
56. SPENCER, P., and H. WEINBERG. 1978. Axonal specification of Schwann cell expression and myelination. *In* Physiology and Pathobiology of Axons. S. G. Waxman, editor. Raven Press, New York. 389–305.
57. TERRY, L., G. BRAY, and A. AGUAYO. 1974. Schwann cell multiplication in developing rat unmyelinated nerves—a radioautographic study. *Brain Res.* **69**:144–148.
58. THOMAS, G. A. 1948. Quantitative histology of Wallerian degeneration. II. Nuclear population in two nerves of different fiber spectrum. *J. Anat.* **82**:135–145.
59. THOMAS, P. K. 1970. The cellular response to nerve injury. III. The effect of repeated crushes. *J. Anat.* **106**:463–470.
60. VARON, S. S., and R. P. BUNGE. 1978. Trophic mechanisms in the peripheral nervous system. *Annu. Rev. Neurosci.* **1**:327–361.
61. WEBSTER, H. DEF. 1975. Development of peripheral myelinated and unmyelinated nerve fibers. *In* Peripheral Neuropathol. P. J. Dyck, P. K. Thomas, and E. H. Lambert, editors. W. B. Saunders Co., Philadelphia. **1**:37–61.

62. WEDNER, H. J., and C. W. PARKER. 1976. Lymphocyte activation. *Prog. Allergy.* **20**:195–300.
63. WEINBERG, H. J., and P. S. SPENCER. 1976. Studies on the control of myelinogenesis. II. Evidence for neuronal regulation of myelin production. *Brain Res.* **113**:363–378.
64. WESTON, J. 1970. The migration and differentiation of neural crest cells. *Adv. Morphog.* **8**:41–114.
65. WOOD, P. 1976. Separation of functional Schwann cells and neurons from peripheral nerve tissue. *Brain Res.* **115**:361–375.
66. WOOD, P. M., and R. P. BUNGE. 1975. Evidence that sensory axons are mitogenic for Schwann cells. *Nature (Lond.).* **256**:661–664.
67. ZETTER, B. R., L. B. CHEN, and J. M. BUCHANAN. 1976. Effects of protease treatment on growth, morphology, adhesion, and cell surface proteins of secondary chick embryo fibroblasts. *Cell.* **7**:407–412.