

STUDIES OF SCHWANN CELL PROLIFERATION

III. Evidence for the Surface Localization of the Neurite Mitogen

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ABSTRACT

In the preceding paper (Salzer et al., 1980, *J. Cell Biol.* **84**:753-766), evidence was presented that a neurite membrane fraction could be used to stimulate Schwann cell proliferation in culture. In this study, we present evidence that the mitogenic signal by which intact neurites or neurite membranes stimulate Schwann cell proliferation is located at the neurite surface. This conclusion is based on the following observations: (a) stimulation of Schwann cell proliferation by neurons requires direct contact between neurites and Schwann cells, separation of the two cells by a permeable collagen diaphragm 6 μm thick prevents Schwann cell proliferation; (b) treatment of intact neurites with trypsin before preparation of neurite membranes abolishes the ability of these membranes to be mitogenic for Schwann cells; and (c) the mitogenic activity of neurite homogenates is exclusively localized in the particulate rather than the soluble fraction of the homogenate. The mitogenic component on the neurite surface is heat labile, and is inactivated by aldehyde fixation. Preliminary data suggest that the mitogenic effect of neurite on Schwann cells is not mediated by 3',5'-cyclic AMP.

KEY WORDS Schwann cells · dorsal root ganglia · mitogenic response · cell interactions · membrane localization

The development of pure populations of neurons or non-neuronal cells has recently provided unique insights into the normal cellular interactions of the peripheral nervous system (3). One such system described by Wood (38) allows the preparation of neurons and Schwann cells in isolation and led to the discovery that sensory neurites provide a mitogenic stimulus for Schwann cell division (39). A similar conclusion regarding growth control of non-neuronal cells from the chick sympathetic nervous system was reached by McCarthy and Partlow (20).

In the preceding papers of this series, this stimulation was characterized further and shown to be highly specific (26, 27). It was also found that a neurite membrane fraction could be isolated and used to stimulate Schwann cell proliferation in a manner that resembles the stimulation induced by intact neurites. The ability of neurite membranes to stimulate Schwann cell proliferation suggested that the stimulation by intact neurites might be mediated by a physical interaction between neurite surface and the surface of the Schwann cells. This concept was also consistent with the observation that neurons did not stimulate Schwann cell proliferation (39) or non-neuronal cell division (20) unless these cells were allowed to directly interact; shared media was insufficient by itself to initiate

division. In the present study, we extend these observations and provide evidence for the surface localization of the neurite mitogen.

MATERIALS AND METHODS

Enzyme and Protein Determinations

We have routinely used alkaline phosphodiesterase (PDE) as a plasma membrane marker (33, 40) and lactate dehydrogenase (LDH) as a cytoplasmic marker. LDH was measured spectrophotometrically by following the oxidation of NADH at 340 nm, during the conversion of pyruvate to lactate (2). (1 U oxidizes 1 μ mol of NADH per min at room temperature). Because of limitations in the quantity of neurite membranes, we modified the spectrophotometric assay for PDE for use in a fluorimeter as follows: The reaction was carried out at 37°C in 0.2 ml of 50 mM Tris-Cl buffer, pH 9.0, containing 0.1 mg of thymidine 5'-monophosphate- β -naphthyl ester (Sigma Chemical Co., St. Louis, Mo.). After a 15-min incubation, the reaction was slowed by dilution with 0.8 ml of 0.2 M glycine buffer, pH 10.6, and the fluorescence was determined immediately in a Farrand fluorimeter (Farrand Optical Co., Inc., Valhalla, N. Y.) using α -naphthol as a standard. 1 U of PDE activity hydrolyzes 10 nmol of the substrate per hour at 37°C. We found excellent agreement between this assay and the conventional colorimetric assay (data not shown). Parallel recoveries of PDE and Na⁺-K⁺ ATPase activities and localization of PDE to neurite membrane fractions argue for its surface membrane localization. Protein determinations were based on a spectrophotometric assay (17).

Cell Labeling and Scintillation Counting

For analysis of cell proliferation, we incubated cells with [³H]thymidine and processed cells for autoradiography as previously described (26). In some studies, we labeled dorsal root ganglia which only contained neurons (38) (NDRG) with L-[³H]fucose (10–15 Ci/mmol) obtained from New England Nuclear (Boston, Mass.). Generally, cultures were labeled for 48 h with 6 μ Ci/ml in fluorodeoxyuridine (FUDR) containing medium (described in reference 27), rinsed with minimal essential medium (MEM), and incubated overnight in a supplemented Eagle's MEM (MEM with glucose, L-glutamine, and nerve growth factor). For assay of radioactivity, cells or cell fractions were homogenized in MEM (without phenol red), an equal volume of 10% SDS was added, and an aliquot of the solution was used for scintillation counting.

Tissue culture methods

Schwann cells were replated as described in an accompanying paper (26). Typically, cells were transferred into Silastic micro-wells. We have also transferred Schwann cells onto unensheathed neurites of dorsal root ganglia free of supporting cells (NDRG) or onto collagen diaphragms (see below). For experiments in which Schwann cells were transferred onto unensheathed neurites, we initially replated the cells onto Falcon 35-mm dishes to allow recovery from enzymatic dissociation. Replated cells are much more easily dislodged from their substrate, either collagen or plastic, than primary cells presumably because they lack an extracellular matrix. Replated cells after 24 h could be gently rinsed off the Falcon dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.), triturated in medium B (27) at a concentration of 5×10^5 cells/ml, and added to the NDRG cultures. We added 0.1 ml of the cell suspension to the inside of

a glass ring (inside diameter of 0.7 cm) which had been gently placed over areas of neurite outgrowth. The medium on the outside of the ring was drawn off. This simple technique was quite effective in confining virtually all the Schwann cells to discrete areas of the dish.

In some experiments we added Schwann cells or neurons to a thin sheet of collagen. The neurons were derived from NDRG cultures whose ganglia were excised and dissociated by procedures analogous to those for Schwann cells. The collagen diaphragm was manufactured by dipping a Silastic ring into a collagen solution (38) to produce a thin film of collagen across the ring. Alternatively, a small hole could be cut into an Aclar minidish and when collagen was spread over the dish a thin film was produced across the hole. Exposure to ammonium vapors and extensive drying produced a thin, stable layer (<6 μ m) of collagen onto which neurons or Schwann cells could be introduced on either side.

Trypsinization and Fixation of Neurites

Cultures of NDRG treated with trypsin (controls were treated with bovine serum albumin) were typically rinsed with MEM three times and incubated with 0.30 ml of 0.05% trypsin (Worthington Biochemical Corp., Freehold, N. J., 180–220 U/mg three times crystallized) in MEM for 30 or 60 min at 34°C in 5% CO₂. The trypsinase was discarded, and 0.30 ml of 0.05% soybean trypsin inhibitor (Sigma Chemical Co.) in MEM was added. These neurites were used for the preparation of membranes or they were tested directly for their mitogenic activity for Schwann cells. Cultures to which Schwann cells were to be added were then rinsed twice with B medium (27) before addition of the cells.

Neurites to be treated with aldehydes were rinsed twice with BSS and twice with phosphate-buffered saline (PBS, pH 7.2, with 4% sucrose). The cultures were then incubated for 10 min at room temperature with either 1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) or 4% formaldehyde (Fischer Scientific Co., Pittsburgh, Pa.) in PBS. These cultures were rinsed three times with PBS (10 min between rinses), incubated with PBS for 30 min, incubated with BSS twice for 30 min each, and then successively with Leibovitz (L-15) medium at 34°C for 30 min, and overnight with B medium at 34°C. All operations were done under sterile conditions. Schwann cells were replated as described.

Isolation of Cytosol and Membrane-Enriched Fractions

To examine whether the neurite mitogenic activity could be localized to either cytoplasm or membranes, we modified our methods for isolating membranes (27). In these experiments, our source of neurites were NDRG preparations whose ganglia were excised as usual. Collagen and neurites were scraped together and disrupted in a Dounce homogenizer (Kontes Co., Vineland, N. J.) in 0.75 ml of MEM with 0.1% bovine serum albumin. These preparations were centrifuged at 300 g for 10 min, and the pellet, which consisted primarily of collagen, was discarded. We centrifuged the supernate at either 188,000 g for 1 h in mini-nitrocellulose tubes (total volume 0.70 mls) or in Siliclad Corex tubes (Corning Glass Works, Corning, N. Y.) at 35,000 g for 1 h. Separation of marker enzymes (LDH and PDE) and localization of mitogenic activity was similar in either procedure, although membrane recovery was substantially improved with use of Siliclad Corex tubes. The pellet was resuspended in 0.25 ml of

MEM with 0.1% bovine serum albumin and adjusted to a final PDE concentration of 200 units/ml in MEM; typically, an equal volume of MEM with 0.1% bovine serum albumin was added. Finally, an equal volume of concentrated medium was added to the supernatant and pellet fractions to give the final concentrations of medium components described previously (27).

RESULTS

Cell Contact is Required for Stimulation

It had previously been shown (39, 20) that Schwann cells proliferate only in areas in which nerve fibers have grown; Schwann cells a few millimeters away from the zone of interaction remained quiescent. Thus, it was not sufficient for the cells to share medium. However, in these experiments the distance of separation was considerable and the mitogenic stimulus might be diffusible but only exist in sufficiently large quantity in the immediate vicinity of the nerve fiber. We, therefore, reexamined the requirement for contact.

For this purpose, dissociated NDRGs were plated onto a thin sheet of collagen. After 24 h, Schwann cells were plated onto either the same side (*cis*) or opposite side (*trans*) of the collagen. Cultures were maintained for 3 d, the last 24 h in the presence of tritiated thymidine. These preparations were then fixed for autoradiography and mounted with the Schwann cells on the top.

An electron micrograph of a culture in which Schwann cells were grown *trans* collagen from the neurons is shown in Fig. 1. The collagen appears to be a uniform matrix $\sim 6 \mu\text{m}$ in thickness. The thickness is variable and probably ranges from 4 to 6 μm . We have not observed nerve fiber penetration into the collagen matrix. It can be seen that, after only 4 d, neurites have already formed fascicles composed of numerous individual processes. Several Schwann cells in cross section are positioned opposite the neurites.

In general, the appearance of cells on this collagen surface is comparable to that on our usual collagen substrate. The dissociated neurons extended processes within 24 h of plating, and by 4 d have established an extensive network. Ganglia extend neurites as well, although there may be more retraction on this surface. When Schwann cells are replated onto the neurons, they attach to the neurites preferentially and within a few days form an organized network (Fig. 2*a*). By contrast, when Schwann cells are replated on the surface opposite from the neurons they form chains of cells which appear to be unrelated to the orientation of the neuronal networks (Fig. 2*b*).

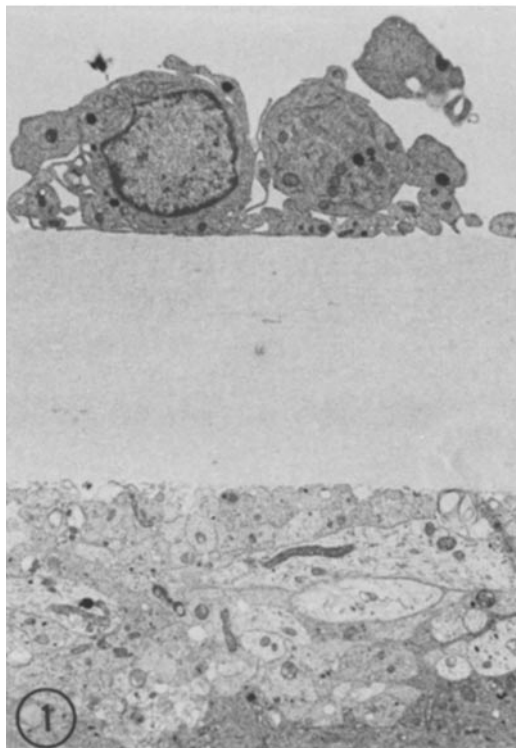


FIGURE 1 Schwann cells and neurons on opposite sides of a collagen diaphragm. This electron micrograph shows a group of Schwann cells above, the interposed collagen, and numerous neurite processes directly below the Schwann cells. $\times 5,500$.

Autoradiographs of Schwann cells *cis* and *trans* to neurons are shown in Fig. 2. As described previously (39), neurites provide a potent mitogenic signal for Schwann cells, and cells replated onto neurons show a very high labeling index ($>50\%$). By contrast, cells plated on the opposite side (*trans*) do not display an increased incorporation of tritiated thymidine. Thus stimulation appears to require cell-cell contact.

This interpretation depends on the permeability characteristics of the ammoniated collagen diaphragm. In experiments carried out to determine the permeability properties of the collagen membrane, we used the following procedure: Both sides of the collagen diaphragm were hydrated; the top solution contained a molecular weight standard in a solution containing two other markers: phenol red (mol wt 380) which freely diffused across, and yeast (3–5 μm in diameter) which were excluded if the diaphragm was intact. After the phenol red equilibrated, an aliquot was taken from the bottom

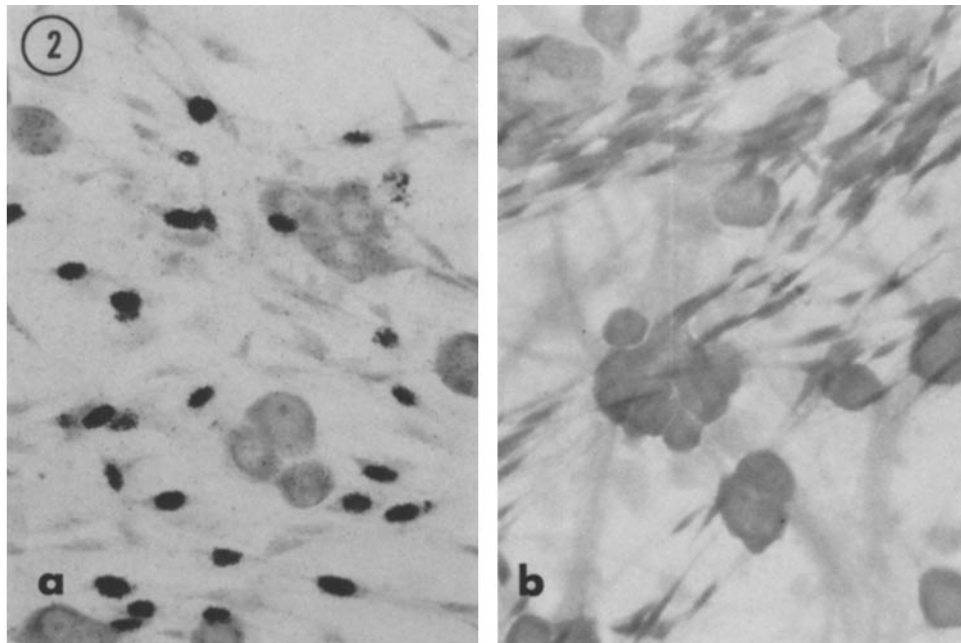


FIGURE 2 Autoradiographs of Schwann cells and neurons. (a) Dissociated neurons were added to the same surface of the collagen diaphragm as the Schwann cells. After 3 d, cells were incubated for 24 h with tritiated thymidine, and processed for autoradiography. Labeled nuclei appear black, on account of high grain density. (b) Dissociated neurons were added to the opposite side of a collagen diaphragm from the replated Schwann cells. After 3 d cells were incubated for 24 h with thymidine as before. Cells were processed for autoradiography in parallel with the cells in Fig. 2a (Schwann cells on top). Toluidine blue stain. $\times 550$.

solution for analysis. We found that extremely large molecules such as blue dextran (mol wt 2×10^6) diffused across the collagen very slowly, but that proteins such as cytochrome *c* (mol wt 12,000) or lactate dehydrogenase (mol wt 137,000) readily diffused across the diaphragm. Thus, if the mitogen were soluble (unless it were of extremely large molecular weight and highly asymmetrical) it should have gained access to the Schwann cells. These results strongly suggest that contact is required between neurites and Schwann cells for the mitogenic signal.

Mitogenic Activity of Neurites is in the Membrane Fraction and not in the Cytoplasm

Analysis of the neurite membrane fraction revealed that there was significant contamination with cytoplasm and cytoplasmic organelles. Electron micrographs of neurite preparation demonstrate that membrane vesicles often enclose some protein and occasional organelles such as lysosomes (27). Consistent with these electron micro-

scope observations is the finding of significant levels of acid phosphatase activity, a lysosomal marker (7), and detectable succinate dehydrogenase activity, a mitochondrial marker (8), in our neurite membrane preparations.

To quantitate the cytoplasmic contamination of these membranes, we have measured lactic dehydrogenase activity. We generally recover 65–75% of the PDE activity of the homogenized neurites in the 35,000 g pellet, but also ~12–17% of the LDH activity. While this fraction is enriched approximately fivefold for plasma membrane markers, there is clearly substantial cytoplasmic contamination in this fraction. This LDH activity was not removed by repeated homogenization and centrifugation of the membranes, and the activity was virtually totally latent, i.e., it was only demonstrable in the presence of a detergent, suggesting that it is trapped inside the membrane vesicles. We have routinely used 0.05% Triton X-100 in our LDH measurements, although virtually all the activity was released at 0.018% Triton X-100.

A variety of procedures did not deplete our

membrane preparations of LDH activity. Use of sonication, freeze-thaw cycles, or osmotic shock of the membranes did not substantially reduce the contaminating LDH activity (data not shown). We have therefore carried out parallel assays in which we determined the mitogenic activity of the soluble and particulate fractions obtained by homogenization of the neurites to ascertain whether the mitogenic activity of the neurite membrane fraction could be accounted for by cytoplasmic contamination. The ratio of LDH/PDE is 50-fold higher in the supernatant fraction than in the membrane fraction. In four separate experiments, we have failed to observe any significant mitogenic stimulation by the cytoplasmic fraction (Fig. 3). The mitogenic activity of the pellet therefore appears to reflect its content of neurite membranes rather than the contaminating cytoplasm.¹

Trypsin Sensitivity of the Neurite Mitogen

Having demonstrated that the mitogenic activity can be isolated in a membrane fraction, we investigated whether this activity might be localized to the surface of the neurite. We examined this question by the use of trypsin, which in relatively short incubations should be active primarily on proteins located on the exterior surface of the plasma membrane.

As described in Materials and Methods, we incubated intact NDRG for either 30 or 60 min at 34°C with 0.05% trypsin. The medium was discarded and soybean trypsin inhibitor (0.05%) was added to inhibit any residual trypsin. Neurites were harvested as usual. Parallel cultures treated simultaneously with 0.05% trypsin and 0.05% soybean inhibitor served as controls for the trypsin activity. Membranes were standardized to 1.2 PDE units and added to primary cultures of Schwann cells as usual. Because PDE is somewhat sensitive to the action of trypsin (see below), trypsin-treated neurites were actually added in slight excess com-

¹ It is important to note that the actual LDH activity (cytoplasm) in the Schwann cells' microenvironment is very difficult to estimate. Most of the cytoplasm appears to be located inside membrane vesicles, which would decrease the cytoplasm available to the Schwann cells. However, with time these vesicles settle onto the cells and, as described in the preceding paper (27), are eventually phagocytosed; this would tend to increase the local concentration of cytoplasm near (or in) the Schwann cells, although it would seem highly unlikely that cytoplasmic material localized in lysosomal vesicles is mitogenic.

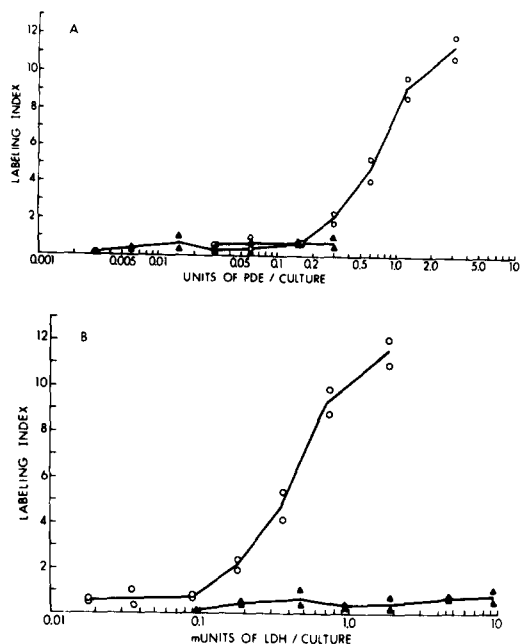


FIGURE 3 The mitogenic activity of neurite homogenates is localized to the membrane fraction. In this experiment, we assayed both the supernatant fraction (\blacktriangle) and the membrane fraction (\circ) obtained by homogenization of neurites. The data are presented in two forms. In *A*, the mitogenic response is presented as function of the PDE content of the fractions. In *B*, it is presented as a function of the LDH content of the fractions. Although the mitogenic stimulation correlates with PDE activity, it does not correlate with LDH activity and is therefore presumed to be located on the neurite membrane rather than in the cytoplasm.

pared to controls. The results of four such experiments are summarized in Fig. 4.

Control beds (no addition) had an average labeling index of $<0.2\%$. Addition of neurites incubated simultaneously with trypsin and soybean inhibitor increased the labeling index to 12.5% in these four experiments. Neurite membranes isolated from NDRG treated with trypsin for 30 min increased the labeling index only to 1.80% ; neurite membranes from NDRG treated for 60 min increased the labeling index only to 0.98% .

Similar observations were made with replated Schwann cells. In this experiment control neurite membranes at 1.2 PDE units stimulated Schwann cells to a labeling index of $17.65\% (\pm 1.1\%)$, whereas trypsin-treated neurites (0.05% for 30 min) resulted in a labeling index of $5.32\% (\pm 0.9\%)$. Control beds in this experiment had a labeling index of 1.5% .

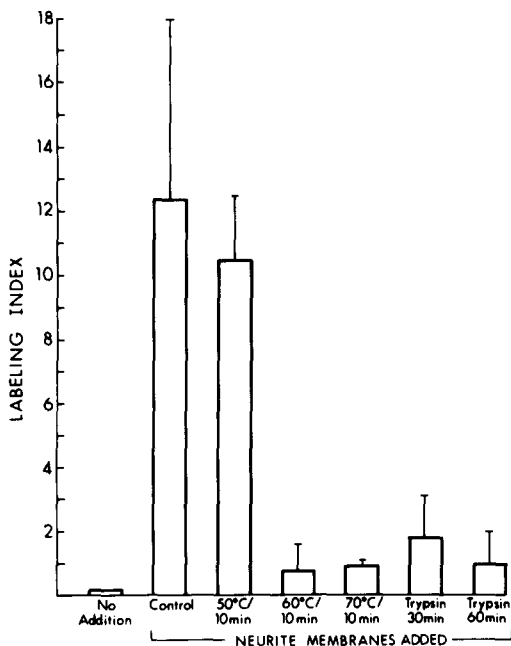


FIGURE 4 Trypsin and heat inactivation of the neurite mitogen. In four separate experiments, addition of neurite membranes to primary Schwann cell cultures increased the labeling index from <0.2 to 12.5% after a 2-d incubation with membranes. If, however, the ganglia and neurites were first incubated with trypsin (0.05% at 34°C) for 30 or 60 min before isolating the membranes, most of the mitogenic activity was abolished. Heating neurite membranes at 60°C for 10 min also inactivates their mitogenic effect.

The effect of trypsin suggests that the mitogenic activity on the neurite surface is a protein; in agreement with this assumption, we find (Fig. 4) that the mitogenic activity is sensitive to heat denaturation and is abolished by heating the membranes at 60°C for 10 min.

Because neurites extend up to 1 cm in length and as neurites are known to actively pinocytose a variety of materials in culture (4), we considered it possible that trypsin might be acting intracellularly. We therefore characterized the effect of trypsin on [3 H]fucose-labeled proteins (which are exteriorly located) and on LDH, an intracellular marker.

We used [3 H]fucose to label proteins as most surface proteins are glycosylated; and in previous experiments with superior cervical ganglion neurites, nine particulate-associated proteins but only one soluble protein were found to be labeled with fucose and very little glycolipid labeling was de-

tected with this precursor (9). In initial experiments, we examined the time-course for release of [3 H]fucose into the medium during incubation of NDRG with 0.05% trypsin at 34°C. After 60 min, ~35–40% of the total counts was released into the medium; slightly more counts were released after incubation with 0.2% trypsin at 34°C for 60 min. Both neurites and ganglia contributed equally to the counts appearing in the trypsin. In a control incubation with 0.05% bovine serum albumin, only 3% of the total counts were released. Finally, the appearance of counts in the trypsin was exponential, with approximately half the counts released after 10 min.

Approximately 60% of the [3 H]fucose counts were trypsin insensitive. Of these counts, ~20% represent TCA-soluble material, presumably [3 H]fucose or phosphorylated derivatives. The remainder represents material inaccessible to trypsin which may or may not be on the cell surface.

By contrast to [3 H]fucose-labeled proteins, LDH appears to be relatively insensitive to external trypsin. In two experiments in which intact NDRG were treated with 0.05% trypsin (controls were incubated with 0.05% bovine serum albumin) for 1 h, we found no significant decrease in neurite LDH activity ($98.0 \pm 6\%$ compared to control). These findings, i.e., the decrease of neurite counts and the insensitivity of neurite LDH activity, with time of trypsin treatment, are graphically illustrated in Fig. 5. In addition, PDE activity also decreases ~25% after a 60-min incubation with 0.05% trypsin (data not shown). If, however, neurites were disrupted first, either by homogenizing in 0.05% trypsin or by incubating a supernatant fraction (cytoplasm-enriched fraction) in 0.05% trypsin, LDH values were reduced to 20% of control values (incubated with 0.05% bovine serum albumin). Thus, if the trypsin had been taken up in any significant quantity, we would have observed a decrease in LDH activity.

In summary, the trypsin appears to be acting on externally located markers ([3 H]fucose and PDE) under conditions in which the neurite mitogen is almost completely inactivated. We conclude that at least one component involved in the mitogenic effect of neurite on Schwann cells is a trypsin-sensitive protein located on the neurite surface, in a manner that makes it readily accessible to external trypsin.

Finally, we determined whether any mitogenic activity might be in the trypsin. In this experiment, after a 30-min incubation with 0.05% tryp-

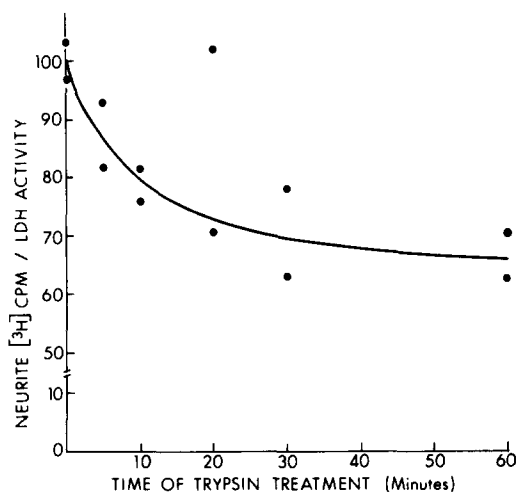


FIGURE 5 Release of neurite fucopeptides during trypsin treatment. NDRGs labeled with [^3H]fucose as described in Materials and Methods were treated with 0.05% trypsin at 34°C for the times indicated. Neurites were collected as described in Materials and Methods, and their content of [^3H]fucose and LDH was determined. LDH recovery was 98% after 60-min incubation with trypsin. This graph demonstrates that there is a progressive decline in the radioactive content of [^3H]fucose-labeled neurites during incubation with trypsin, but that the LDH activity is not affected, i.e., it is trypsin insensitive. Results from two experiments are shown.

sin, 0.05% soybean inhibitor and concentrated medium were added to the trypsin and reconstitute medium. This material was added to Schwann cells. After 24 h the procedure was repeated in the presence of tritiated thymidine, and the cells were processed for autoradiography. We observed no increase in the labeling index over background in this experiment. This is not a result of a toxic effect of trypsin on Schwann cells, as this level of trypsin in medium does not inhibit proliferation, even in the absence of soybean trypsin inhibitor. Apparently, trypsin inactivates the mitogenic signal rather than simply releasing it in active form from the neurite surface.

Recovery of Neurite Mitogen from Trypsin Treatment

Experiments carried out to determine the recovery time of the neurite mitogen also demonstrate its trypsin sensitivity. In this experiment, Schwann cells were plated onto Falcon dishes for 24 h and then rinsed off and replated onto unensheathed neurites which were treated with 0.1% trypsin or

0.1% trypsin with 0.1% soybean inhibitor for 1 h. Results are shown in Table I. It can be seen that stimulation of Schwann cells by the trypsin-treated neurites lags behind the control neurites treated simultaneously with trypsin and soybean inhibitor by 24–30 h. Control cells had a labeling index of 0.05% in this experiment. Essentially similar results were obtained with a 0.05% trypsin treatment, i.e., stimulation lagged behind controls by ~30 h. The lag period in control cultures represents both (a) recovery time from replating for Schwann cells and (b) the lag period after interaction with neurites for the cells to progress into S phase. In the case of trypsin-treated neurites, Schwann cells have presumably recovered from replating long before the mitogen has been renewed, and the additional lag period observed with these neurites must therefore represent the time required by the neurites to regenerate the mitogenic signal after trypsin treatment; this process appears to require 1–2 d.

Schwann cells replated onto trypsin-treated neurites initially lack the longitudinal orientation of cells plated onto control neurites. Cells settle onto trypsin-treated neurites but assume a random orientation with respect to the neurites. After 10–15 h, Schwann cells begin to orient longitudinally, but there is a long lag before division ensues. Therefore, whereas binding and stimulation may be related they appear to be separable events at least in their time-course for recovery. We also investigated whether the reexpression of the mitogen demonstrated any particular spatial distri-

TABLE I
Schwann Cell Interaction with Trypsin-Treated Neurites

Time of labeling h	Trypsin-treated neurites	
	Control neurites	labeling index
18–36	4.20	0.19
36–48	22.23	0.62
48–60	40.75	4.56
60–72	ND	39.63

Schwann cells were replated onto unensheathed neurites which had been pretreated with 0.1% trypsin for 1 h, or with 0.1% trypsin-0.1% trypsin inhibitor for 1 h in MEM. After removal of the medium, Schwann cells were added as described in Materials and Methods. At the times indicated, the cultures were incubated with [^3H]thymidine, fixed for autoradiography, and the percent of labeled nuclei was determined. ND, not determined.

bution. Thus, the appearance of labeled cells on the neurite could be used as a biological assay to determine where the mitogen was reinserted into the plasma membrane: near the cell soma, all along the length of the nerve fiber, or at the periphery. We did not observe any spatial distribution, i.e., labeled cells appeared randomly along the length of the nerve fiber. This suggests that new mitogen is expressed simultaneously (within our temporal resolving ability) along the whole length of the neurite or that if it is preferentially inserted in one location it rapidly diffuses along the fiber (12).

Additional experiments suggest that the mitogen is a protein. Schwann cells replated onto aldehyde-fixed neurites (1% glutaraldehyde or 4% formaldehyde for 10 min) fail to bind or divide. Results from an experiment in which cells were seeded onto control or glutaraldehyde-fixed neurites are shown in Fig. 6 *a* and *b*. It may be seen that the cells were unlabeled with [³H]thymidine and ran-

domly oriented to the aldehyde-fixed neurites; in this regard, they resemble trypsin-treated neurites. Cells seeded onto aldehyde-fixed neurites could still proliferate after the addition of cholera toxin, a known mitogen (22), which indicates that the lack of cell division in the presence of fixed neurites was not simply a toxic phenomenon. Because a variety of treatments that denature or destroy proteins (external trypsin, heat, and aldehyde fixation) inactivate the mitogenic signal, we conclude that one or more proteins on the neurite surface are necessary for the mitogenic response.

Is the Stimulation by Neurites Mediated by Cyclic AMP?

Cyclic AMP is not mitogenic for primary Schwann cells and is weakly mitogenic for replated Schwann cells (26), although it is a more potent mitogen for the Schwann cells prepared by Raff et al. (22). We have examined whether cAMP may

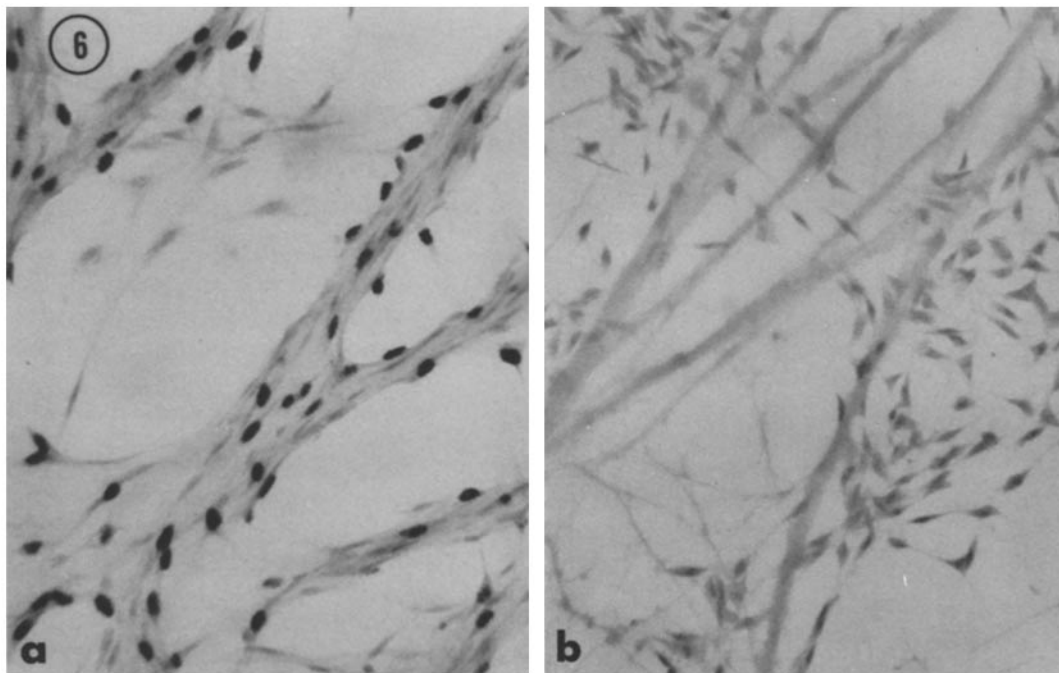


FIGURE 6 Schwann cell interaction with control or glutaraldehyde-fixed neurites. (a) Schwann cells were replated onto living neurites and, after 44 h, incubated with tritiated thymidine for 12 h. It may be seen that cells dramatically align and form fascicles with the neurites. There is a substantial increase in the number of labeled nuclei. Schwann cells which do not appear to have established contact with neurites remained unlabeled. (b) Schwann cells were replated onto glutaraldehyde-fixed neurites and, after 60 h, were incubated for an additional 24 h with tritiated thymidine. Schwann cells do not incorporate label under these conditions and do not bind or interact with the fixed neurites. In most areas, cells appear to maintain a completely random orientation with respect to the fixed neurites. Toluidine blue stain. $\times 270$.

be involved in the mitogenic response to neurite membranes, possibly as a "second messenger," by examining whether inhibition of cAMP phosphodiesterase can potentiate the response to membranes. Inhibition of cAMP phosphodiesterase (the enzyme that degrades cAMP) by theophylline or isobutylmethyl xanthine (IBMX) should potentiate effects that are mediated by cAMP (32). The results in Table II show that these inhibitors had no effect on the mitogenic response. These results suggest that the mitogenic response is cAMP independent. These results are consistent with the lack of response by these cells to dibutyl cAMP (26) and studies in which stimulation of chick non-neuronal cell proliferation by sympathetic neurons (34) or of rat Schwann cells by a pituitary growth factor (22) are independent of changes in intracellular levels of cAMP.

DISCUSSION

There is a growing body of evidence that many cell-cell interactions may represent contact-mediated phenomena (for review, see reference 11). The results presented in this paper are consistent with this hypothesis: the neuronal stimulation of Schwann cell proliferation appears to require contact and be mediated by a protein(s) on the surface of the neurite. This is indicated by the lack of stimulation across a thin diaphragm of collagen, recovery of mitogenic activity from neurite ho-

TABLE II
Effect of Inhibitors of cAMP PDE on Mitogenic Response

Additions	% Labeled nuclei		
	Exp 1	Exp 2	Exp 3
None	1.56-1.45	0.26	0.12-0.20
IBMX	2.26-1.71	ND	0.16-0.07
Theophylline	ND	0.25-0	0.05-0.01
Neurites	8.59-11.47	2.24-1.67	3.34-3.48
Neurites + IBMX	8.43-7.21	2.82-3.88	1.29-0.94
Neurites + theophylline	ND	1.88-1.49	1.21-1.51

Schwann cells were replated into Silastic microwells. 3 d after plating, neurite membranes (0.30 PDE units) were added with or without the phosphodiesterase inhibitors IBMX (4×10^{-5} M) or theophylline (10^{-3} M). After 24 h the procedure was repeated in the presence of 1 μ Ci/ml of tritiated thymidine, and cultures were subsequently processed for autoradiography. All experiments were carried out in duplicate. Individual determinations are shown.

ND, not determined.

mogenates in the pellet (membrane) fraction but not supernatant fraction, and inactivation of the mitogen by external trypsin. These experimental approaches are similar to ones used in other interacting systems and will be considered in that context.

A requirement for cell contact has often been demonstrated in transfilter experiments. For example, successful transfilter induction by spinal cord of mouse kidney tubules was demonstrated across thin Millipore filters (Millipore Corp., Bedford, Mass.) with pore sizes down to 0.1 micron (13, 14). These results were taken as evidence for cell induction via diffusible factors. However, recently, results from this type of experiment have been questioned, as refinement in fixation techniques has shown that cellular processes do penetrate into Millipore filters (35, 36).

In the case of sensory neurite stimulation of Schwann cell proliferation (39) or sympathetic neuronal stimulation of non-neuronal cells (20), co-cultivation of cells in the same dish was insufficient to induce division, suggesting that contact might be important for stimulation. We reexamined this issue by culturing cells on a thin collagen sheet that allowed diffusion of relatively large molecules but prevented penetration of neurites and therefore cell-cell contact. Use of this collagen "filter" has several advantages. Because collagen is essentially transparent under these conditions, cells are readily visualized. Collagen can be prepared as a very thin sheet (6 μ m or less) and provides an important and physiological substrate for these cells (5). The lack of stimulation of Schwann cells when neurons are grown transfilter supports the concept that contact is necessary for stimulation. Also, in experiments in which Schwann cells are replated onto bare neurite ganglia, it is our impression that only cells that establish contact with the neurites subsequently incorporate tritiated thymidine; cells a short distance away from the neurites remain unlabeled (see for example, Fig. 6a).

A more recent approach to the study of cell interactions has been the use of isolated membranes as a model for these interactions. For example, cell membranes prepared for chick neural retina competitively blocked, in a stage and site specific manner, homotypic cell aggregation (21). In another study the normal developmental pattern of the slime mold, *Dictyostelium discoideum*, was altered by the addition of plasma membranes (30). Finally, in a series of studies which comple-

ment our own it was found that plasma membranes from 3T3 cells inhibit fibroblast proliferation; membranes isolated from transformed cells (SV40 transformed 3T3 cells, SV3T3) which do not exhibit contact inhibition of growth showed lower inhibitory activity when added to 3T3 cells, and SV3T3 cells did not respond to the addition of membranes (40). Recent work indicates that the membranes can bind to the 3T3 fibroblasts in a saturable manner (M. A. Lieberman and T. Woolsey, unpublished observations, summarized in reference 6). An extract from solubilized membranes has been prepared and shown to inhibit growth in a manner that resembles the activity of membranes (41).

We have found that stimulation of Schwann cell proliferation by neurite membranes is highly specific and provides a convenient model for the interaction of Schwann cells with neurites (27). We have also failed to observe any mitogenic activity associated with cytosol-enriched fractions. Furthermore, we have presented suggestive evidence that stimulation of Schwann cell proliferation by neurite membranes is independent of cAMP.

Our results differ somewhat from those recently described for a chick sympathetic non-neuronal cell population. Hanson and Partlow (16) reported that sonicates or homogenates of neurons stimulated non-neuronal cell division. However, their non-neuronal cells show a higher basal level of proliferation and undoubtedly represent a mixed cell population. Also, it is unclear whether the stimulation in these experiments resides in membrane fragments or in cytoplasm. Finally, this stimulatory activity has significant heat stability and is not completely specific since similar preparations from non-neuronal cells are stimulatory (15).

There are a number of parallels between our findings and studies on pancreatic epithelial-mesenchymal interaction (reviewed in reference 28), especially studies on mesenchymal factor. Particulate fractions from mesodermal tissues but not other tissues stimulate the differentiation and division of pancreatic epithelium (25). Also, this activity is heat labile and trypsin sensitive, and could not be replaced by a variety of soluble mitogens (23). Experiments in which mesenchymal factor was covalently linked to Sepharose beads strongly suggested that it stimulated proliferation by an interaction with the epithelial cell membrane (19). It may be, therefore, that cell-cell

interactions controlling cell proliferation during development are generally mediated by surface proteins.

Part of the evidence for the surface localization of the neurite mitogen is based on experiments with external trypsin. We have used a concentration of 0.05% trypsin, which is a relatively large quantity. It is important to note, however, that cells are grown on a collagen substrate, and neurites are tightly packed together into fascicles so that there is a large amount of protein in each dish and the membranes may not be completely accessible to the medium. We have not systematically studied the effect of different concentrations of trypsin on the inactivation of the mitogen, but there is a more complete inactivation of the mitogen when NDRGs are incubated with trypsin for 60 min (rather than 30 min), and there is good agreement with the kinetics of [³H]fucose release. These results suggest that, for this particular tissue culture system, we are just below saturating levels of trypsin. Also, under the conditions of treatment, neurites remain attached to the collagen and show normal viability, again suggesting that the level of trypsin used is not toxic.

The action of trypsin is likely to be confined to the cell surface. These results do not distinguish whether the mitogenic effect is caused by a single protein on the neurite surface, or whether several proteins are required for this effect (42). The stimulation of Schwann cells by neurites might require several surface proteins acting together, and these would all be inactivated by trypsin. Alternatively, it is possible that the mitogen is not a protein and that the action of trypsin is to disrupt the general architecture of the neurite membrane, and in this way another component such as a glycolipid is indirectly affected. Heat treatments and aldehyde fixation could also exert their effects in this manner. Trypsin might eliminate a component necessary for the normal cellular recognition of neurites by Schwann cells, and stimulation might be affected secondarily. For example, Schwann cells do not appear to interact with trypsin-treated neurites or bind treated membranes. It is likely that binding of the neurite membranes is necessary for the mitogenic effect of the membranes and that the effect of trypsin may be primarily to block binding (and possible internalization) and in this way abolish the mitogenic activity of these preparations. Resolution of these alternative interpretations will require solubilization and ultimately purification of the mitogenic activity; nevertheless, they each

postulate a critical role for the neurite cell surface in the mitogenic stimulation of Schwann cells.

Most cell surface proteins appear to be glycosylated (18), and glycoproteins have been postulated to be important in mediating cell-cell interactions (24). For these reasons, we are presently examining whether the mitogen is a glycoprotein. In preliminary studies, in which neurite membranes were treated with periodate, under conditions in which carbohydrates are known to be oxidized (10, 31), the mitogenic effect of the neurite membranes was substantially reduced. Our studies also suggest that this treatment inactivated wheat germ agglutinin (WGA) binding activity of the membranes, whereas addition of glycerol together with periodate protected both the mitogenic and WGA-binding activity of the neurite membranes. These data suggest that the mitogen may indeed be a glycoprotein. Further work will be necessary to establish this point.

The neurite also controls the production of the basal lamina² and myelin (1, 29, 37). It will be of interest to determine whether the factor that regulates myelination is distinct from the mitogenic signal. The ability of sympathetic nerve fibers to stimulate division and their inability to induce myelination is one of several situations which suggest that these signals are dissociable. We are hopeful that approaches similar to those described in this paper will help elucidate whether the control of myelin formation is also mediated by contact between the axon and the Schwann cell.

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² Bunge, M. B., A. K. Williams, P. M. Wood, J. Uitto, and J. J. Jeffrey. 1980. Comparison of nerve cell and nerve cell plus Schwann cell cultures, with particular emphasis on basal lamina and collagen formation. *J. Cell Biol.* **84**:184-202.

REFERENCES

1. AGUAYO, A. J., 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.
2. BERGMAYER, H. U. 1974. *Methods of Enzymatic Analyses*. Vol. 1. Academic Press, Inc., New York. 481-482. 2nd edition.
3. BUNGE, R. P. 1975. Changing uses of nerve tissue culture 1950-1975. In *The Nervous System*. D. B. Tower, editor. Raven Press, New York. 31-41.
4. BUNGE, M. B. 1977. Initial endocytosis of peroxidase or ferritin by growth cones of cultured nerve cells. *J. Neurocytol.* **6**:407-439.
5. BUNGE, R. P., and M. B. BUNGE. 1978. Evidence that contact with connective tissue matrix is required for normal interaction between Schwann cells and nerve fibers. *J. Cell Biol.* **78**:943-950.
6. BUNGE, R. P., L. GLASER, M. A. LIEBERMAN, D. RABEN, J. SALZER, B. WHITTENBERGER, and T. WOOLSEY. 1979. Growth control by cell to cell contact. *J. Supramol. Struct.* In press.
7. DeDUVE, C. 1965. The separation and characterization of subcellular particles. *Harvey Lect.* **59**:49-87.
8. DEPIERRE, J. W., and M. L. KARNOVSKY. 1973. Plasma membranes of mammalian cells: A review of methods for their characterization and isolation. *J. Cell Biol.* **56**:275-303.
9. ESTRIDGE, M., and R. BUNGE. 1978. Compositional analysis of growing axons from rat sympathetic axons. *J. Cell Biol.* **79**:138-155.
10. FILOSA, S., R. PICTET, and W. J. RUTTER. 1975. Positive control of cyclic AMP on mesenchymal factor controlled DNA synthesis in embryonic pancreas. *Nature (Lond.)*, **257**:702-705.
11. FRAZIER, W., and L. GLASER. 1979. Cell surface components involved in cell recognition. *Annu. Rev. Biochem.* **48**:491-523.
12. FRYE, C. D., and M. EDIDIN. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* **7**: 319.
13. GROBSTEIN, C. 1956. Trans-filter induction of tubules in mouse metanephrogenic mesenchyme. *Exp. Cell Res.* **10**:424-440.
14. GROBSTEIN, C., and A. J. DALTON. 1957. Kidney tubule induction in mouse metanephrogenic mesenchyme without cytoplasmic contact. *J. Exp. Zool.* **135**:57-73.
15. HANSON, G. R., and L. M. PARTLOW. 1977. Stimulation of thymidine incorporation by cell sonicates. Transactions of the American Society of Neurochemistry. **8**:142 (Abstr.).
16. 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.
17. KALB, V. F., and R. W. BERNLOHR. 1977. A new spectrophotometric assay for protein in cell extracts. *Anal. Biochem.* **82**:362-371.
18. KORNFIELD, R., and S. KORNFIELD. 1976. Comparative aspects of glycoprotein structure. *Annu. Rev. Biochem.* **45**:217-237.
19. LEVINE, S., R. PICTET, and W. J. RUTTER. 1973. Control of cell proliferation and cytodifferentiation by a factor reacting with the cell surface. *Nat. New Biol.* **246**:49-51.
20. MCCARTHY, K. D., and L. M. PARTLOW. 1976. Neuronal stimulation of ³H-thymidine incorporation by primary cultures of highly purified nonneuronal cells. *Brain Res.* **114**:415-426.
21. MERRELL, R., D. GOTTLIEB, and L. GLASER. 1976. Membranes as a tool for the study of cell surface recognition. In *Neuronal Recognition*. S. Barondes, editor. Plenum Press, New York. 249-273.
22. RAFF, M. C., E. ABNEY, J. BROCKES, and A. HORNBY-SMITH. 1978. Schwann cell growth factors. *Cell.* **15**:813-822.
23. RONZIO, R. A., and W. J. RUTTER. 1973. Effects of a partially purified factor from chick embryos on macromolecular synthesis of embryonic pancreatic epithelium. *Dev. Biol.* **30**:307-320.
24. ROSEMAN, S. 1975. Sugars of the cell membrane. In *Cell Membranes Biochemistry, Cell Biology and Pathology*. G. Weissmann and R. Claiborne, editors. Hospital Practice Publishing Co., Inc., New York. 55-64.
25. RUTTER, W. J., N. K. WESSELLS, and C. GROBSTEIN. 1964. Control of specific synthesis in the developing pancreas. *Nat. Cancer Inst. Monogr.* **13**:51-65.
26. SALZER, J. L., and R. P. BUNGE. 1980. Studies of Schwann cell proliferation. I. An analysis of tissue culture of proliferation during development, Wallerian degeneration, and direct injury. *J. Cell Biol.* **84**:739-752.
27. SALZER, J., A. WILLIAMS, L. GLASER, and R. 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.
28. SAXEN, L., M. KARKINEN-JAAKELAINEN, E. LEHTONEN, S. NORDLING, and J. WARTIOVAARA. 1976. Inductive tissue interactions. In *The Cell Surface in Animal Embryogenesis and Development*, G. Poste and G.

- L. Nicolson, editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 331-407.
29. 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.
 30. SMART, J., and J. TUCHMAN. 1976. Inhibition of the development of *D. discoideum* by isolated plasma membranes. *Dev. Biol.* **51**:63-76.
 31. STAHL, P., H. SIX, J. RODMAN, P. SCHLESINGER, D. TULSIANI, and O. TOUSTER. 1976. Evidence for specific recognition sites mediating clearance of lysosomal enzymes *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **73**:4045-4049.
 32. SUTHERLAND, E. W. 1972. Studies on the mechanism of hormone action. *Science (Wash. D. C.)* **177**:401-408.
 33. TOUSTER, O., N. N. ARONSON, JR., J. DULANEY, and H. HENDRICKSON. 1970. Isolation of rat liver plasma membranes: Use of nucleotide pyrophosphatase and phosphodiesterase I as marker enzymes. *J. Cell Biol.* **47**:604-618.
 34. WALLACE, L., L. PARTLOW, and J. FERRENDELLI. 1978. Comparison of levels of adenosine 3',5'-monophosphate in highly purified and mixed primary cultures of neurons and non-neuronal cells from embryonic chick sympathetic ganglia. *J. Neurochem.* **31**:801-807.
 35. WARTIOVAARA, J., E. LEHTONEN, S. NORDLING, and L. SAXEN. 1972. Do membrane filters prevent cell contacts? *Nature (Lond.)* **238**:407-408.
 36. WARTIOVAARA, J., S. NORDLING, E. LEHTONEN, and L. SAXEN. 1974. Transfilter induction of kidney tubules: Correlation with cytoplasmic penetration into nucleopore filters. *J. Embryol. Exp. Morphol.* **31**:667-682.
 37. 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.
 38. WOOD, P. 1976. Separation of functional Schwann cells and neurons from peripheral nerve tissue. *Brain Res.* **115**:361-375.
 39. WOOD, P. M., and R. P. BUNGE. 1975. Evidence that sensory axons are mitogenic for Schwann cells. *Nature (Lond.)* **256**:662-664.
 40. WHITTENBERGER, B., and L. GLASER. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface membranes. *Proc. Natl. Acad. Sci. U. S. A.* **74**:2251-2255.
 41. WHITTENBERGER, B., D. RABEN, M. D. LIEBERMAN, and L. GLASER. 1978. Inhibition of growth of 3T3 cells by extract of surface membranes. *Proc. Natl. Acad. Sci. U. S. A.* **75**:5457-5461.
 42. 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.