Differential Proliferative Responses of Cultured Schwann Cells to Axolemma- and Myelin-enriched Fractions. I. Biochemical Studies

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ABSTRACT Cultured rat Schwann cells were treated for 72 h with axolemma- and myelin-enriched fractions prepared from rat brainstem. [^{3}H]Thymidine was added to the cultures 48 h before the termination of the experiment. Although, both fractions produced a dose-dependent uptake of label into Schwann cells, the shape of the dose response curves and rates at which [^{3}H]thymidine was incorporated were different. The axolemma-enriched fraction produced a sigmoid dose response curve with a Hill coefficient of 2.05. The dose response curve for myelin rose sharply and saturated at a level that was ~50% of the maximal response observed with axolemma. Schwann cells that had been treated with axolemma exhibited little change in the rate of [^{3}H]thymidine incorporation from 36–72 h after the addition of the membranes. In contrast, Schwann cells accumulated label three times faster during the 48–72-h period following the addition of myelin to the cultures when compared with the rate during the preceding 12-h interval. Furthermore, the mitogenic activity of the myelin-enriched fraction was decreased by the addition of ammonium chloride, a lysosomal inhibitor, whereas the activity of the axolemmal fraction was not impaired.

Schwann cells proliferate during development (2, 20) and Wallerian degeneration (4, 10). Using cultured neurites and Schwann cells from rat dorsal root ganglia, Salzer and Bunge (15) demonstrated that the developmental signal was provided by the growing axon. Morphological studies (1, 12, 21) suggested that the increase in the number of cells during Wallerian degeneration was proportional to the size of the myelinated fiber. Indirect evidence for myelin as a mitogenic signal was provided by Salzer and Bunge (15) who found that only Schwann cells that had produced myelin proliferated during Wallerian degeneration.

The nature of the axonal signal has been investigated using membrane fractions derived from cultured neurites (6, 16, 17) or PC 12 cells (14) and axolemma-enriched fractions prepared from central and peripheral nervous tissue (6, 8, 9, 19). Two studies (6, 9) have reported that myelin-enriched fractions were slightly mitogenic for cultured Schwann cells; however, the authors were unable to determine whether the activity was intrinsic to myelin membranes or due to contaminating axolemmal membranes.

In the present study, we demonstrate the presence of two distinct mitogenic activities in fractionated nervous tissue. Axolemma- and myelin-enriched fractions prepared from rat brainstem were found to stimulate the incorporation of [^{3}H]-thymidine by cultured Schwann cells. The two mitogenic activities could be differentiated by their dose response curves, the rate at which they stimulate uptake of label into the Schwann cells, and their sensitivity to the lysosomal inhibitor, ammonium chloride.

MATERIALS AND METHODS

HF media[1] is Dulbecco's modified Eagle's Medium (Gibco Laboratories, Grand Island, NY) with sodium bicarbonate and 10% fetal calf serum (Sterile Systems, Logan, UT). Saline I is a balanced salt solution containing 138 mM NaCl, 5.4 mM KCl, 1.1 mM Na_2PO_4, 1.1 mM KH_2PO_4, and 22 mM dextrose, pH 7.0.

Preparation of Schwann Cells: Schwann cells were prepared according to the methods of Brockes et al. (5). Briefly, sciatic nerves from 2-d-old rat pups were excised and treated with trypsin and collagenase. The mixture was triturated with a Pasteur pipette and filtered through 209-μm Nitex (Tetko Inc., Elmsford, NY). Cells were collected by centrifugation (900 *g*, 5 min) and suspended in 10 ml of HF and plated at a density of 3–4.5 × 10^6 cells per 10-cm dish. 24 h later the antimitotic drug, cytosine arabinoside, was added at a final concentration of 10^{-5} M. 3 d later the media was removed and replaced

[1] *Abbreviations used in this paper:* HF (media), Dulbecco's modified Eagle's medium with sodium bicarbonate and 10% fetal calf serum.

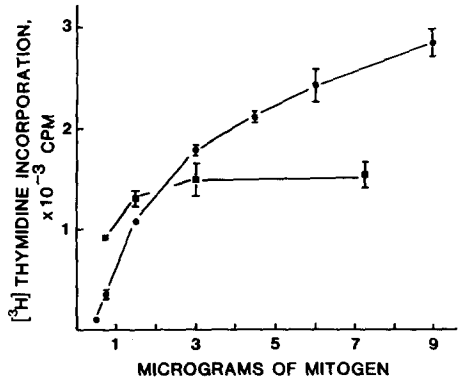

FIGURE 1 [^{3}H]Thymidine incorporation by Schwann cells stimulated with membrane fractions. Values have been corrected for controls (50–100 cpm) and are means ± SEM for n = three or four determinations. Axolemma- (●) or myelin- (■) enriched fractions were added to the Linbro well to give a final volume of 100 μl. 24 h later, 0.3 μCi of [^{3}H]thymidine in 25 μl of HF was added. 72 h after the addition of the mitogen, the cells were harvested and counted.

with HF. To eliminate any fibroblasts that survived the incubation with cytosine arabinoside, we treated the cultures with anti-Thy 1.1 (New England Nuclear, Boston, MA) and rabbit complement (Cappel Laboratories, Inc., Cochranville, PA). This procedure produced cultures that contained 99.5% Schwann cells.

[^{3}H]Thymidine Incorporation Assay: After the cultures were treated with anti-Thy 1.1, the cells were plated in 96-well dishes (Linbro Chemical Co., Hamden, CT) at a density of 10,000 cells per well. 24 h later the mitogen or HF was added to bring the total volume in each well to 100 μl. 1 d later 0.3 μCi of [^{3}H]thymidine (New England Nuclear, 15 Ci/mmol) in 25 μl of HF was added to each well. 48 h after the addition of the label, the cells were treated with 0.005% trypsin and 0.02% EDTA and were collected on filter paper using a Titertek (Flow Labs, Inc., McLean, VA) cell harvester. The radioactivity on the filters was determined in an Intertechnique SL 3-scintillation counter (in/US Service Corp., Fairfield, NJ).

Autoradiography of Mitogen-treated Schwann Cells: Schwann cells were plated onto glass coverslips in 24-well plastic culture dishes at a density of 40,000 to 50,000 cells per well after treatment with anti-Thy 1.1. Mitogens or HF were added to give a final volume of 500 μl per coverslip. 24 h after mitogen addition, 1.5 μCi of [^{3}H]thymidine in 125 μl of HF was added to each coverslip. 2 d later the cells were washed and processed for autoradiography as previously described (8). A minimum of eight randomly selected fields with at least a total of 1,000 cells were counted for each coverslip.

Preparation of Axolemma- and Myelin-enriched Fractions: Axolemma- and myelin-enriched fractions were prepared from 1 gram of rat brain stem as previously described (7) with minor modifications. The shocked myelinated axons were separated on a 34-ml linear sucrose gradient (10–40% sucrose [wt/wt], in 10 mM *N*-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid with 0.2% sodium azide) by centrifugation at 85,000 *g* for 15–18 h. Those fractions that were between 28 and 32% sucrose (wt/wt) and those between 17 and 19% sucrose (wt/wt) were pooled for the axolemma- and myelin-enriched fractions, respectively. The samples were collected by centrifugation and resuspended in sterile Saline I to give a concentration of membrane protein between 1 and 3 mg/ml. Protein was determined using the microassay of Bradford (3) in the presence of 0.1 N NaOH with a gammaglobulin standard.

RESULTS

Stimulation of [^{3}H]Thymidine Incorporation by Cultured Schwann Cells with Axolemma- and Myelin-enriched Fractions

Axolemma-enriched fractions stimulated a dose-dependent incorporation of [^{3}H]thymidine into Schwann cells (Fig. 1). The myelin-enriched fraction also increased the levels of [^{3}H]thymidine uptake into Schwann cells in a dose-dependent manner; however, the shape of the curve was different from the results obtained when axolemma was used as the mitogen. At lower concentrations, myelin stimulated a larger accumulation of [^{3}H]thymidine into Schwann cells than the corresponding dose of axolemma. As the levels of the mitogens were increased, the axolemma-enriched fraction became more potent than myelin in promoting the accumulation of label within the cells.

Radioautography of Schwann Cells Stimulated with Axolemma- and Myelin-enriched Fractions

Schwann cells stimulated with the two mitogens were examined by radioautography to confirm the results obtained with the cell harvester. The axolemma-enriched fraction stimulated a dose-dependent increase in the number of labeled Schwann cells (Fig. 2). The myelin-enriched fraction was more mitogenic for Schwann cells than the axolemma at lower doses, which agreed with the results observed with the cell harvester. Furthermore, higher concentrations of myelin were less mitogenic than a comparable level of axolemma.

Is the Mitogenic Activity of the Myelin Fraction Due to Contaminating Axolemmal Membranes?

The axolemma- and myelin-enriched fractions appear to represent separate mitogenic signals based on their different dose response curves. The dose-dependent incorporation of label produced by the myelin, however, may be due to contaminating axolemmal membranes and to an intrinsic inhibitory factor in myelin that, at high concentrations, would prevent the stimulatory effect of the axolemma.

Schwann cells were incubated with different concentrations of the axolemma-enriched fraction and harvested 12, 24, and 48 h after the addition of the [^{3}H]thymidine (Fig. 3). Myelin was also added to cultures in parallel and harvested at the same intervals. 12 h after the addition of the label, Schwann cells that had been treated with 8 μg of the myelin-enriched fraction (arrowhead, Fig. 3) accumulated the same amount of

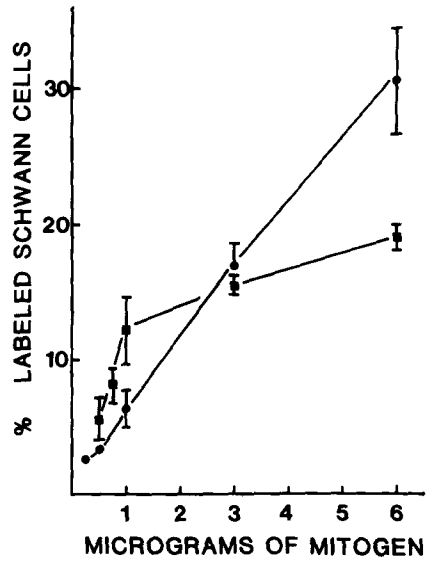

FIGURE 2 Autoradiography of Schwann cells stimulated with axolemma- or myelin-enriched fractions. Values are means ± SD for n = 3. Between 0.2 and 0.4% of the untreated Schwann cell nuclei were labeled with [^{3}H]thymidine. To facilitate comparison with Fig. 1, the abscissa was chosen to express the amount of mitogen, axolemma- (●) or myelin- (■) enriched fractions, present per 100 μl of media.

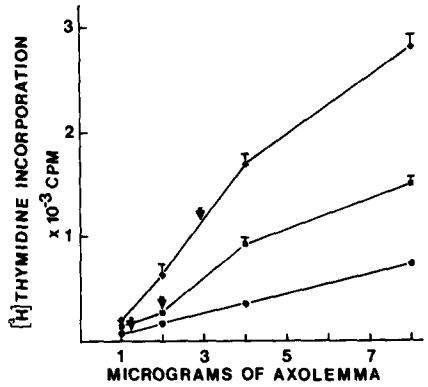

FIGURE 3 Dose-dependent incorporation of [³H]thymidine by Schwann cells stimulated with an axolemma-enriched fraction. Values have been corrected for controls (50–100 cpm) and are the means ± SEM for n = three or four determinations. Schwann cells were incubated 24 h with the axolemma-enriched fraction before the addition of [³H]thymidine. The cells were harvested 12 h (●), 24 h (■), and 48 h (◆) after the addition of the label. 8 μg of a myelin-enriched fraction (arrowheads) was added to sister cultures and harvested at the same intervals.

[³H]thymidine as Schwann cells that had been incubated with ~1 μg of the axolemma. 24 and 48 h after the addition of the label, the identical concentration of myelin (arrowheads, Fig. 3) produced mitogenic responses equivalent to ~2 and 3 μg, respectively, of the axolemma-enriched fraction.

The mitogenic activity of the myelin fraction may be due to axolemmal contamination and myelin membranes may act to delay or inhibit the ability of the axolemma to express its stimulatory effect. Schwann cells were treated with the myelin-enriched fraction to allow the myelin to interact with the cells. After 24 h, [³H]thymidine and the axolemmal fraction were then added to these cultures. The experiment was terminated 24 h after the addition of the label, because the myelin membranes, if inhibitory, appeared to exert the greatest influence up to 24 h after the addition of the [³H]thymidine (Fig. 3). The combination of the myelin and axolemma stimulated the incorporation of [³H]thymidine into 10.2% of the Schwann cells, whereas myelin alone labeled 6.9% of the cells (Fig. 4). The axolemma-enriched fraction, when added simultaneously with the label, stimulated the incorporation of [³H]thymidine into 4.7% of the Schwann cells.

Rate of [³H]Thymidine Incorporation by Schwann Cells

[³H]Thymidine was added to Schwann cells that had been treated with either axolemma- or myelin-enriched fractions for 24 h. The cells were harvested at 12-, 24-, or 48-h intervals following the addition of the label (Fig. 5). The rates of thymidine incorporation appeared linear with time for the axolemma-treated Schwann cells. In contrast, the rate of label accumulated by the Schwann cells stimulated with myelin appeared to increase during the last 24 h. The rates of [³H]-thymidine incorporation were calculated for four levels of mitogen (Table I). The myelin-treated Schwann cells on the average incorporated [³H]thymidine three times faster during the final 24-h labeling period when compared with the rate during the prior 12-h interval. When a similar comparison was made for the axolemma-treated Schwann cells, the average rate for the two periods was the same.

Effect of Ammonium Chloride on the Mitogenic Activity of Axolemma- and Myelin-enriched Fractions

During Wallerian degeneration the appearance of myelin in lysosomes of Schwann cells (18) and increased lysosomal activity (11) accompanied a period of rapid proliferation of the Schwann cells. If the mitogenic activity of the myelin-enriched fraction for cultured Schwann cells were dependent upon a similar pathway, the addition of the lysosomal inhibitor ammonium chloride (13) should decrease the incorporation of [³H]thymidine. To examine this we added axolemma- or myelin-enriched fractions to Schwann cells in the presence of 6 or 9 mM ammonium chloride (Fig. 6). Whereas the mitogenic activity of the myelin fraction was significantly decreased by the addition of ammonium chloride, the ability of the axolemma-enriched fraction to stimulate incorporation of label was not compromised. We also added myelin- and

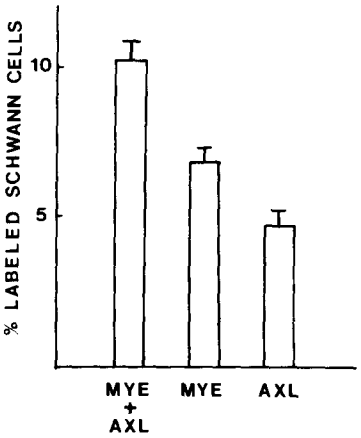

FIGURE 4 Effect of myelin membranes on the mitogenic activity of axolemma. Values are the means ± SD for n = 3. The myelin-enriched fraction was added to the Schwann cells to achieve a final concentration of 5 μg/0.1 ml. After 24 h, [³H]thymidine and the axolemma-enriched fraction (final concentration 4 μg/0.1 ml) were added to the cultures. The cells were washed and processed for radioautography 24 h later. Sister cultures were incubated with only the myelin- or the axolemma-enriched fraction at the concentrations described above. The axolemmal fraction and the label were added to the sister culture simultaneously.

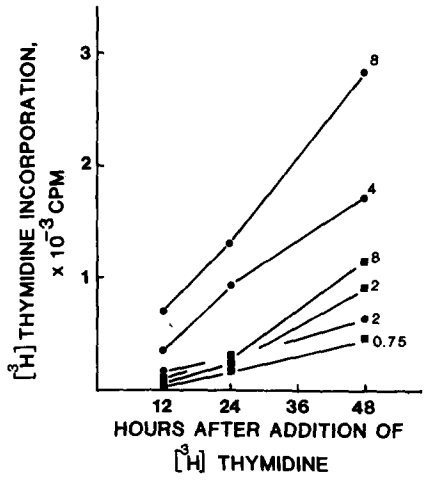

FIGURE 5 Rates of [³H]thymidine incorporation by Schwann cells stimulated with axolemma- or myelin-enriched fractions. Values are the means of three or four determinations. Axolemma- (●) or myelin- (■) enriched fractions were added at the level (micrograms) indicated on the figure. 24 h later, [³H]thymidine was added to the wells as described previously. The cells were harvested and counted at the intervals indicated.

axolemma-enriched fractions simultaneously to Schwann cells in the presence or absence of 6 mM ammonium chloride. In the presence of the inhibitor, the combined fractions produced labeling of the Schwann cells equivalent to that observed with the axolemma-enriched fraction alone.

Cooperative Nature of [^{3}H]Thymidine Incorporation by Cultured Schwann Cells Stimulated with the Axolemma-enriched Fraction

The dose-dependent incorporation of label into Schwann cells stimulated with axolemma was examined more carefully (Fig. 7) because the data in Fig. 1 did not generate a simple curve that would pass through the origin. The results from Fig. 7*A* were replotted in the appropriate Hill coordinates which produced a straight line with a slope of 2.05. Three other dose response curves were analyzed in a similar fashion and produced slopes of 1.65, 2.1, and 2.4. A Hill coefficient (the value of the slope) greater than unity describes a reaction that exhibits positive cooperativity.

TABLE I

Rates of [^{3}H]Thymidine Accumulation by Schwann Cells Stimulated with Axolemma- or Myelin-enriched Fractions

Mitogen concentration	Axolemma			Myelin		
	Rate I*	Rate II‡	II/I	Rate I*	Rate II‡	II/I
μg/well	cpm/h			cpm/h		
1.0	4.0	3.1	0.7	5.0	19.2	3.8
2.0	7.8	15.0	1.9	14.6	28.1	1.9
4.0	48.0	33.0	0.7	10.5	39.8	3.8
8.0	48.0	63.0	1.3	15.4	35.5	2.3
	$\bar{x} = 1.2 \pm 0.6$§			$\bar{x} = 3.0 \pm 1.0$§‖		

* Rate of [^{3}H]thymidine incorporation from 12–24 h following addition of label (36–48 h after addition of mitogen).
‡ Rate of [^{3}H]thymidine incorporation from 24–48 h following addition of label (48–72 h after addition of mitogen).
§ These values represent the means ± SD of rate II/rate I.
‖ The means are statistically different by Student's *t* test ($p < 0.01$).

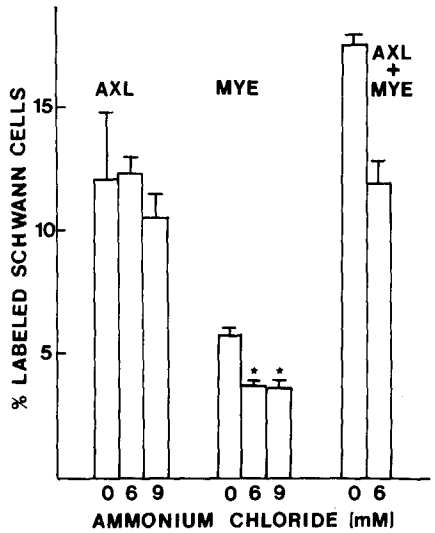

FIGURE 6 Effect of ammonium chloride on the mitogenic activities of the axolemma- or myelin-enriched fractions. The values are the means ± SD for $n = 3$. Schwann cells were treated with axolemma (5 μg/0.1 ml) or with myelin (2 μg/0.1 ml) in the presence or absence of ammonium chloride. After 24 h [^{3}H]thymidine was added to the cultures, and 48 h later the cells were washed and processed for radioautography. The means (asterisk) are statistically different by Student's *t* test ($P < 0.01$) when compared with cells that were not treated with ammonium chloride. A set of Schwann cells was treated with both axolemma and myelin (same doses as above) in the presence or absence of 6 mM ammonium chloride. Control cultures typically had one or two cells labeled per 1,000. Labeling of Schwann cells that were treated with only ammonium chloride was approximately five to seven cells per 1,000.

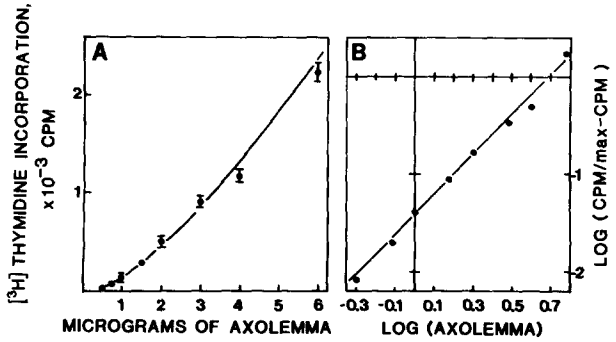

FIGURE 7 Dose response curve and Hill plot for Schwann cells stimulated with axolemma-enriched fractions. (*A*) Values are means ± SEM for $n = 4$. Axolemma was added to give a final volume of 100 μl in each Linbro well. (*B*) Results from *A* were replotted with the appropriate Hill coordinates. The slope of the curve was 2.05, and 3,500 cpm was chosen as the value for the maximum.

DISCUSSION

Both axolemma- and myelin-enriched fractions prepared from rat brain stem stimulated the incorporation of [^{3}H]-thymidine into cultured Schwann cells. Increasing concentrations of axolemma produced a sigmoid curve that possessed a Hill coefficient of 2 when the results were replotted in the appropriate Hill coordinates. The simplest interpretation of this observation is that the binding of two molecules in the axolemmal fraction to the Schwann cell is required to stimulate uptake of label into Schwann cells. Other studies (6, 19) have described a dose-dependent relationship between the concentration of the axolemma-enriched fraction and the incorporation of labeled material into Schwann cells, but too few points were examined in the linear region of the curve to permit a detailed analysis of the response. Also, Schwann cells in our study were incubated 24 h longer with both the axolemma-enriched fraction and [^{3}H]thymidine.

The relationship between increasing concentrations of myelin and the accumulation of [^{3}H]thymidine within Schwann cells was not identical to the response elicited by the axolemma-enriched fraction. Although myelin was a more potent stimulus at lower concentrations than the axolemma, the actual mass added to the cultures may be equivalent because of the imbalance in the lipid-to-protein ratios in these fractions. Nonetheless, the maximum incorporation of label produced by the myelin membranes was approximately one half that observed with the axolemma. Similar curves were obtained when Schwann cells stimulated with the mitogens were examined by radioautography.

However, the possibility exists that the stimulatory effect of the myelin-enriched fraction may result from contaminating axolemmal membranes. The mitogenic activity of a fixed concentration of the myelin fraction was determined at different intervals following the addition of the [^{3}H]thymidine and compared with the corresponding dose response curve generated by the axolemma-enriched fraction. Prolonged ex-

posure of a myelin fraction to cultured Schwann cells cannot lead to a threefold increase of axolemmal contamination; more likely, the observed mitogenicity is intrinsic to the myelin membranes themselves.

Alternatively, myelin membranes may be acting to delay or inhibit the expression of mitogenic activity of any contaminating axolemmal membranes in the myelin-enriched fraction. Schwann cells were incubated with the myelin-enriched fraction before addition of the axolemma-enriched fraction to allow the myelin membranes to interact first with the cells. When these cultures were compared with others treated with either myelin or axolemmal fractions alone, the myelin membranes did not substantially diminish the ability of the axolemma to stimulate the incorporation of [^{3}H]thymidine.

The rates of [^{3}H]thymidine incorporation by Schwann cells were not equivalent when myelin-treated cells were compared with those incubated with axolemma. The rate of [^{3}H]thymidine accumulation by Schwann cells stimulated with axolemma during the last 24 h (48–72 h after addition of mitogen) was essentially unchanged compared with the preceding 12-h interval (36–48 h following addition of mitogen). In contrast, the myelin-treated Schwann cells incorporated label three times faster during the final 24-h period than during the preceding 12 h. This sudden increase coincides temporally with an increase in Schwann cell proliferation during Wallerian degeneration in vitro (4) and in vivo (15).

The accumulation of myelin debris within Schwann cell lysosomes (18) and an increase in lysosomal activity (11) accompanies the period of intense Schwann cell proliferation during Wallerian degeneration. If myelin acts as a mitogenic signal for Schwann cells, the membranes may require processing through the lysosomal compartment of the cell prior to activating cell division. Cultured Schwann cells were treated with axolemma- or myelin-enriched fractions in the presence of the lysosomal inhibitor ammonium chloride. The mitogenic activity of the myelin fraction was significantly decreased by the inhibitor, whereas the activity of the axolemma was not. When axolemma- and myelin-enriched fractions were added to the same cultures with ammonium chloride, the labeling of the Schwann cells was equivalent to that observed when only axolemma was present. The presence of endocytosed myelin in Schwann cells, which may not be degraded as a consequence of impaired lysosomal function, does not appear to inhibit the mitogenicity of the axolemma-enriched fraction.

Two distinct mitogenic signals for cultured Schwann cells can be distinguished in fractionated nervous tissue. Our results provide direct evidence for the participation of myelin membranes in Schwann cell proliferation in vitro which may correspond to the mitogenic events occurring during Wallerian degeneration. Whether the mitogenic activity of the axolemma-enriched fraction is related to the axonal stimulus for Schwann cells during development remains to be resolved. Purification of the mitogen from both fractions will demonstrate whether the differences are due to the molecular structure of the mitogen or to the environment in which the mitogen resides. Future studies will extend our present observations and examine the mechanisms by which the two membrane fractions exert their respective mitogenic influence.

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