

# Factors Influencing the Release of Proteins by Cultured Schwann Cells

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**ABSTRACT** Cultured rat Schwann cells grown in association with sensory neurons when labeled with [<sup>3</sup>H]leucine, [<sup>3</sup>H]glucosamine, or [<sup>35</sup>S]methionine release labeled polypeptides into the culture medium. Analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the culture medium reveals a reproducible pattern of >20 polypeptides with molecular weights ranging from 15,000 to >250,000. Five major polypeptides (apparent molecular weights 225,000, 210,000, 90,000, 66,000, 50,000, and 40,000) account for ~40% of the leucine or methionine radioactivity in medium polypeptides. Schwann cells grown in a serum-free defined medium, in which Schwann cells do not relate normally to axons, release approximately four times less labeled medium polypeptides than cultures grown in medium supplemented with serum and chick embryo extract. In addition, there is a qualitative difference in the pattern of medium polypeptides resolved by SDS-PAGE, so that a single polypeptide (mol wt 40,000) accounts for nearly all of the label in medium polypeptides. Switching of cultures grown in defined medium to supplemented medium for 2 d results in a fourfold increase in the amount of labeled polypeptides appearing in the culture medium, and a return to the normal pattern of medium polypeptides as resolved by SDS-PAGE. This change in the pattern of polypeptides released by Schwann cells is accompanied by changes in the association between Schwann cells and axons. An early step in the establishment of normal axon-Schwann cell relations appears to be an inward migration of Schwann cells into axonal bundles and spreading of Schwann cells along neurites. These changes are evident within 48 h after medium shift. Our results thus suggest that the release of proteins by Schwann cells may be important for the development of normal axonal ensheathment.

The development of peripheral nerves requires the cooperative activity of three different cell types: peripheral neurons, Schwann cells, and fibroblasts. The study of peripheral nerve development has been aided greatly by the recent development of tissue culture techniques that allow one to obtain and culture separately or in combination each of these three cell types (21). With this tissue culture system it has been possible to show, for example, that sensory axons are mitogenic for Schwann cells (16, 22) and that this mitogen is probably a neurite membrane protein (17, 18). It has also been demonstrated that Schwann cells synthesize and secrete several collagen types and that the basal lamina that covers the axon-Schwann cell unit is synthesized by Schwann cells in the absence of fibroblasts (5), but only in the presence of neurons (20).

Several lines of evidence suggest that secretory activity of Schwann cells is required for their normal functioning. Culturing of Schwann cells and neurons in medium that contains the

proline analogue *cis*-hydroxyproline (which inhibits collagen hydroxylation and secretion) causes abnormal Schwann cell behavior: the basal lamina coverage becomes patchy and incomplete, and in addition, abnormal and incomplete ensheathment of the axons by Schwann cells occurs (8). Culturing of Schwann cells and neurons in a serum-free defined medium developed by Bottenstein and Sato for growth of neuroblastoma cells (2) results in a complete failure of the Schwann cells to ensheath the neurites (13). That these Schwann cells are failing to secrete is suggested by the observations that no basal lamina is formed and the granular endoplasmic reticulum of the Schwann cells is swollen and filled with flocculent material (13); this condition is reversed by the addition of embryo extract and serum to the culture medium and myelination is subsequently observed.

Observations made on the peripheral nerves of the mutant mouse dystrophic also suggest an apparent correlation between

Schwann cell secretion and normal ensheathment. In this mouse, portions of the roots of the spinal and cranial nerves exhibit an almost complete failure of Schwann-cell ensheathment with a corresponding failure of development of the extracellular matrix materials normally found in the endoneurium (3, 4, 19). In more distal regions of these nerves the lesion is not as severe, consisting of areas of incomplete ensheathment of axons by Schwann cells, as well as abnormal nodes of Ranvier (9). A defect in Schwann cell secretory activity is suggested by the finding of abnormalities in the Schwann cell basal lamina throughout the peripheral nervous system (9, 12).

These observations suggesting a link between Schwann cell secretion and normal Schwann cell function have led us to examine more directly the secretory activity of Schwann cells. This paper reports observations on the amounts and types of polypeptides secreted by cultured Schwann cells and changes in Schwann cell activity associated with alterations in this secretory activity.

## MATERIALS AND METHODS

### Culture Media

Three different culture media were used in these experiments. Supplemented medium is 65% Eagle's minimum essential medium (EMEM), 25% human placental serum (HPS) (obtained from a local hospital), 10% 9 d chick embryo extract, 2 mM glutamine, 6 mg/ml glucose and nerve growth factor (NGF, 10 U/ml) (1). Antimitotic medium is 90% EMEM, 10% HPS, 2 mM glutamine,  $2 \times 10^{-6}$  fluorodeoxyuridine,  $2 \times 10^{-5}$  uridine, and NGF (10 U/ml). The defined medium used was a modification of that used by Bottenstein and Sato (2), and consisted of 50% Dulbecco's modified EMEM, 50% Ham's F-12, insulin (5  $\mu$ g/ml), transferrin (100  $\mu$ g/ml), progesterone (20 nM), putrescine (100 nM), selenium (50 nM), and NGF (10 U/ml). Culture media components were purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.; Sigma Chemical Co., St. Louis, Mo.; or Collaborative Research Inc., Waltham, Mass. Collagen substrata were prepared from reconstituted rat tail collagen.

### Preparation of Cultures

Explant cultures of dorsal root ganglia from embryonic Holtzman rats, from which fibroblasts had been eliminated and which contained only sensory neurons and Schwann cells were prepared essentially as described previously (5, 16, 21).

Cultures containing only Schwann cells were prepared from Schwann cell-neuron explant cultures by excising the ganglia (containing the neuronal somas) with sterile razor blade fragments as described earlier (21). For some experiments these Schwann cells were replated after collagenase and trypsin treatment as described previously (16).

### Radiolabeling of Cultures

Cultures were labeled with 4, 5- $^3$ H]leucine (60 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), 6- $^3$ H]glucosamine (30 Ci/mmol; Amersham Corp.), or  $^{35}$ S]methionine (1000 Ci/mmol; New England Nuclear, Boston, Mass.) either overnight in supplemented medium or for 3 h in EMEM containing NGF. The two labeling procedures gave similar results. Labeling with  $^3$ H]leucine and  $^{35}$ S]methionine was done in medium free of leucine and methionine, respectively. Generally labeling was performed with four dorsal root ganglion explants per "mini-dish" with a feed volume of 0.2 ml. Radiolabels were used at concentrations of 200 to 250  $\mu$ Ci/ml. We estimate that in these cultures each ganglion is surrounded by an outgrowth containing  $10^5$  Schwann cells. Under the labeling conditions used the rate of accumulation of  $^{35}$ S]methionine labeled medium polypeptides is 10,000 cpm/explant/3 h, but this amount varies depending upon the size of the explants and length of time in culture.

### Harvesting of Medium and Cell Layer Fractions

Labeled medium was removed from the culture dishes with a Pasteur pipet, and the cultures were rinsed twice with 0.25 ml of phosphate buffered saline (PBS) (0.1 M K-phosphate 0.15 M NaCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; purchased from Sigma Chemical Co.). The media and washes were pooled and the labeled proteins collected by 1 of 2 methods. By the first method an equal volume of 20% TCA was added to the pooled media and washes, and the proteins were collected by centrifugation. The protein pellets

were washed three times with 5% TCA, once with 50% methanol, air dried, and then dissolved in a small volume of 2% SDS, 5% 2-mercaptoethanol, 0.0625 M Tris-Cl, pH 6.8 by brief heating. Alternatively, the pooled media and washes were dialyzed against the same buffer (three changes of 500 vol) and then concentrated by dialysis with Aquacide-2 (Calbiochem-Behring Corp., San Diego, Calif.). In either case appropriate aliquots were counted in 10 ml of Scintiverse (Fisher Scientific Co., Pittsburgh, Pa.) in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.) to determine the total incorporation of radiolabel into medium polypeptides.

To analyze incorporation into the cell layer of the cultures, the washed cells were scraped by hand into 0.5 ml of PBS (with 1 mM PMSF) and treated with TCA as above.

### SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 7.5 to 15% gradient gels using the buffer system of Laemmli (10). After running, the gels were stained for 1 h in 0.25% Coomassie Blue, 50% methanol, 7% acetic acid, destained overnight in 35% methanol, 7% acetic acid, and prepared for fluorography by impregnation with Enhance (New England Nuclear, Boston, Mass.) according to the manufacturer's instructions. The dried gels were exposed for 4 d to 2 wk at  $-70^\circ\text{C}$  to Kodak XR film that had been preflashed to increase sensitivity (11). Microdensitometry of developed x-ray film was performed with a Joyce-Loebl microdensitometer. For some experiments the dried gels were cut into 1-mm slices, dissolved in 0.5 ml of 30% hydrogen peroxide, and counted by liquid scintillation spectrometry.

### Microscopic Analysis

Living cultures were photographed using phase optics with a Zeiss inverted microscope. Cultures to be examined by light and electron microscopy after embedding and sectioning were fixed and prepared by methods previously described (5).

## RESULTS

When cultures containing Schwann cells and neurons that have been grown in medium supplemented with serum and embryo extract are incubated as described above with either  $^3$ H]leucine,  $^3$ H]glucosamine, or  $^{35}$ S]methionine, they are found to release acid insoluble radioactivity into the culture medium. Analysis by SDS-PAGE of this culture medium reveals a reproducible pattern of labeled polypeptides, which is distinct from the pattern of bands observed in the cell layer of these cultures.

Fig. 1 *a* shows a fluorogram of a gel of the medium (lane 1) and cell layer (lane 2) polypeptides of a Schwann cell-neuron culture labeled for 18 h with  $^{35}$ S]methionine. The medium polypeptides labeled with  $^3$ H]leucine and  $^3$ H]glucosamine are shown in Fig. 1 *b*. As can be seen, the medium polypeptides are resolved into >20 distinct bands, with molecular weights ranging from 15,000 to >250,000. More than half of the leucine- and methionine-labeled bands are also labeled by glucosamine, the most heavily labeled band being a polypeptide with an apparent molecular weight of 66,000. Six major bands (mol wt 225,000, 210,000, 90,000, 67,000, 50,000, and 40,000) account for ~40% of the radioactivity in leucine- and methionine-labeled medium polypeptides.

We have found that although the presence and positions of the labeled bands are reproducible among different culture preparations, the intensity of labeling of some of the bands varies (e.g. compare Fig. 1 with Fig. 4). The appearance of the medium polypeptides is not dependent on the conditions of labeling because cultures labeled for 18 h in supplemented medium and cultures grown in supplemented medium but labeled for 3 h in serum-free medium have given similar results. Because the mechanism by which the medium proteins are produced is unknown, they will be referred to in this paper as "cell-released proteins" rather than secreted proteins.

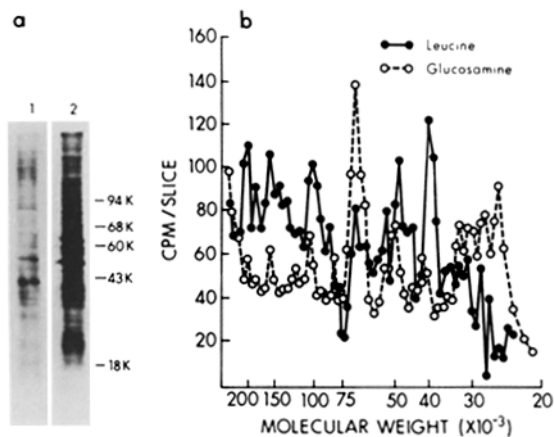


FIGURE 1 SDS-PAGE of labeled Schwann cell-neuron cultures. Schwann cell-neuron cultures grown for 3 weeks in supplemented medium were labeled for 18 h with (a) [ $^{35}\text{S}$ ]methionine or (b) [ $^3\text{H}$ ]leucine or [ $^3\text{H}$ ]glucosamine. The medium and cell layer proteins were processed for SDS-PAGE as described in Materials and Methods. *a*, lane 1:  $^{35}\text{S}$ -methionine-labeled medium polypeptides; lane 2: [ $^{35}\text{S}$ ]methionine-labeled cell layer polypeptides; shown is a fluorogram of the dried gel. *b*: leucine and glucosamine-labeled medium proteins were subjected to SDS-gel electrophoresis in adjacent lanes of a polyacrylamide slab gel. The dried gel was cut into 1 mm slices, dissolved in hydrogen peroxide and counted. (●) [ $^3\text{H}$ ]leucine-labeled medium polypeptides; (○) [ $^3\text{H}$ ]glucosamine-labeled medium polypeptides.

### Schwann Cells are the Major Source of the Cell Released Proteins

To test whether the cell-released proteins were coming from Schwann cells, neurons, or both, the following experiments were performed. Sister explant cultures of neurons and Schwann cells were grown and, immediately before labeling, the ganglia, which contain all of the neuronal somas, were removed from one-half of the cultures. These cultures with ganglia removed (which contained Schwann cells still in contact with axons) plus their intact sister cultures were then tested for their abilities to release labeled polypeptides into the culture medium. Results of experiments with leucine and glucosamine labeling are shown in Table I. The results demonstrate that removal of the neuronal somas does not cause a substantial decrease in the amounts of labeled polypeptides appearing in the culture medium.

When the pattern of medium polypeptides was analyzed by SDS-PAGE (Fig. 2), the pattern of leucine-labeled polypeptides appearing in the culture medium was found not to be substantially altered by removal of the neuronal somas. Quantitation of the areas under densitometric traces of the fluorograms revealed no significant differences between the two sets of labeled polypeptides. Similar results have been obtained with [ $^{35}\text{S}$ ]methionine-labeled cultures (data not shown). Because the cell body is the site of neuronal protein synthesis, these results suggest that the Schwann cells are the major source of the labeled medium polypeptides. This conclusion is further strengthened by the observation that labeling of cultures containing only neurons results in the release of only small amounts (<10% of that released by Schwann cells) of medium polypeptides. Further analysis of the putative neuron-released polypeptides is in progress.

TABLE I  
Secretion of Polypeptides by Schwann Cell-neuron Cultures:  
Effect of Removal of Neuronal Somas\*

Radiolabel	Labeling time h	Medium polypeptides	
		Intact cultures $\ddagger$	Ganglia removed $\S$
CPM/Explant			
Leucine	2	1,606	1,690
Leucine	18	19,190	21,380
Glucosamine	18	15,187	14,700

\* After labeling the culture media were harvested and the polypeptides precipitated with TCA. One culture dish containing four explants was used for each analysis. Shown is a representative example of three separate experiments.

$\ddagger$  Schwann cell-neuron cultures grown for 2 wk in supplemented medium.

$\S$  The ganglia, containing the neuronal somas, were excised from the cultures just before start of labeling.

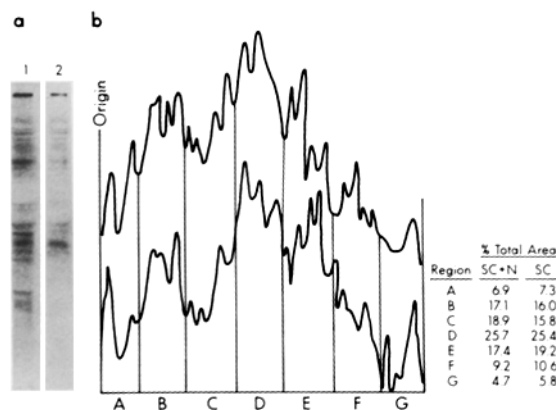


FIGURE 2 Effect of ganglion excision on the pattern of labeled medium polypeptides. Intact Schwann cell-neuron cultures and sister cultures from which the ganglia had been removed were labeled for 18 h with [ $^3\text{H}$ ]leucine and the medium polypeptides were subjected to SDS-gel electrophoresis. (a) Fluorogram of leucine-labeled medium polypeptides released by intact cultures (lane 1) and cultures from which the ganglia had been excised (lane 2). (b) Densitometric tracings of the fluorograms shown in (a); top: intact Schwann cell-neuron cultures; bottom: cultures from which the ganglia had been removed. In the table the areas under the densitometer tracings in (b) were quantitated in the regions shown.

### Effect of Neurons on the Appearance of Schwann Cell-released Proteins

Contact with both axons and a proper extracellular matrix is required for expression of some Schwann cell functions (5, 6, 20). For this reason we analyzed the appearance of Schwann cell-released proteins in cultures of Schwann cells that had been isolated from neurons and replated onto a substratum of reconstituted rat tail collagen or tissue culture plastic. The results of these experiments are shown in Table II. Replated Schwann cells that had been isolated from neurons for 9 d release methionine-labeled polypeptides into the culture medium in amounts comparable to control Schwann cell-neuron cultures. Schwann cells replated onto tissue culture plastic gave results similar to cells replated onto collagen. The pattern of labeled medium proteins released by these three kinds of cultures (Schwann cell-neuron cultures, and isolated Schwann cells replated on collagen or plastic) were very similar on SDS-

TABLE II  
Effect of Replating on Schwann Cell-released Proteins

Culture type	Substratum	Medium polypeptides* CPM/Explant
Schwann cells plus neurons	collagen	8,150
Replated Schwann cells	collagen	8,478
Replated Schwann cells	plastic	7,100

\* Results are the average of two dishes each containing 4 ganglia for each type of preparation.

PAGE analysis (data not shown). Thus the absence of neurons and lack of a collagen substratum do not appear to affect the appearance of Schwann cell-released proteins.

### The Pattern of Schwann Cell-released Proteins is Altered in Cultures Grown in Defined Medium

Schwann cells cultured in defined medium fail to ensheath or myelinate axons. Morphological observations of these Schwann cells suggest that their inability to relate normally to axons results, at least in part, from a failure to secrete (13).

To test this hypothesis, we have compared the proteins released by Schwann cells grown in defined medium with proteins released by Schwann cells shifted to supplemented medium. Sister Schwann cell-neuron cultures were grown for 2 to 2.5 wk in defined medium, after which time half the cultures were switched to supplemented medium. 2 d later both sets of cultures were washed free of culture medium and labeled with [<sup>35</sup>S]methionine for 3 h in methionine-free EMEM. The results of these experiments are shown in Fig. 3. Cultures that had been fed supplemented medium for 2 d released ~2.5–4 times more labeled polypeptides into the medium than cultures fed only defined medium. This difference is not due to a decreased amount of incorporation of [<sup>35</sup>S]methionine into proteins in general, because the amounts of radioactivity in the cell layers were similar in the two types of cultures (Fig. 3).

SDS-PAGE analysis of the labeled medium polypeptides appearing in these cultures is shown in Fig. 4. Schwann cells grown in defined medium release predominantly a single major polypeptide (mol wt 40,000) that is also released by Schwann cells fed supplemented medium. All the other normally appearing polypeptides, however, are either reduced in amount or undetectable. In contrast, the pattern of labeled polypeptides released by the cultures fed supplemented medium for 2 d after 2 wk in defined medium was indistinguishable from cultures fed supplemented medium continuously (Fig. 4 c). This striking difference was specific to the medium polypeptides and was not seen when the labeled proteins in the cell layers were examined (Fig. 4 b). These results suggest that cultures grown in defined medium are deficient in their ability to release polypeptides into the culture medium; this defect can be corrected, however, by switching these cultures to serum and embryo extract supplemented medium. We also tested whether the supplemented medium-induced change in the pattern of medium polypeptides released by Schwann cells required the presence of neurons. For these experiments defined-medium-grown cultures were prepared by excising the ganglia from Schwann cell neuron cultures. 2 wk after removing the ganglia, one-half of the cultures were switched to supplemented medium, and after 2 d the cultures were labeled with [<sup>35</sup>S]methionine. As shown in Table III, the Schwann cells that were

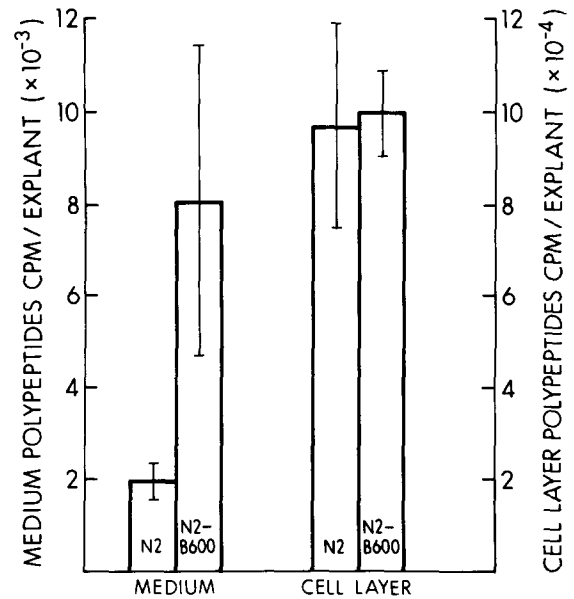


FIGURE 3 Effect of culture conditions on Schwann cell released proteins. Schwann cell-neuron cultures were grown for 2 to 2.5 wk in defined medium. After this time half the cultures were switched to supplemented medium. Two days later the two sets of cultures were labeled with [<sup>35</sup>S]methionine in methionine-free EMEM. The medium and cell layer polypeptides were processed as described in Materials and Methods. The results are averaged from four separate experiments, each with one or two cultures of four explants each per culture type. The bars indicate standard deviation. (N2) defined medium. (B600) supplemented medium.

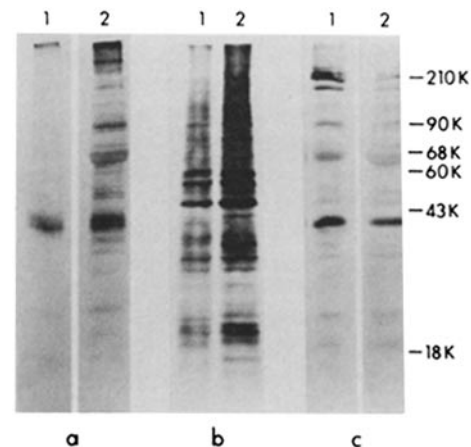


FIGURE 4 SDS-gel electrophoretic analysis of the effect of supplemented medium on Schwann cell-neuron cultures. Schwann cell-neuron cultures were labeled with [<sup>35</sup>S]methionine as described in the legend to figure 3. (a) Medium polypeptides of cultures grown in defined medium (lane 1) and cultures grown in defined medium switched to supplemented medium 2 d before labeling (lane 2); (b) cell layer polypeptides of defined medium (lane 1) and supplemented medium switched (lane 2) cultures; (c) medium polypeptides of cultures grown in defined medium switched to supplemented medium 2 d before labeling (lane 1) and cultures grown for 3 wk in supplemented medium only (lane 2). Shown are fluorographs of the dried gels.

switched to supplemented medium in the absence of neurons responded nearly identically to Schwann cells switched in the presence of neurons. Analysis by SDS-PAGE of the medium of these cultures produced results similar to those presented above for Schwann cell-neuron cultures (data not shown). Thus

TABLE III  
Effect of Supplemented Medium on Proteins Released by Isolated Schwann Cells

Culture Type	Feeding regimen	Medium polypeptides* CPM/Explant
Schwann cells	defined medium (3 wk)	903
Schwann cells	defined medium (3 wk); supplemented medium (2d)	4,456
Schwann cells plus neurons	defined medium (3 wk); supplemented medium (2d)	3,660

\* Results are from duplicate cultures each containing four explants. The experiment was performed twice with identical results.

the stimulation by supplemented medium of the appearance of Schwann cell-released proteins does not require the presence of neurons.

### Switching of Schwann Cell-Neuron Cultures Grown in Defined Medium to Supplemented Medium Causes Changes in the Relation of Schwann Cells to Axons

Our results on the change in the pattern of Schwann cell-released proteins upon feeding defined medium cultures supplemented medium led us to investigate the sequence of events that lead to the establishment of a normal axon-Schwann cell relationship.

A series of photographs of a living culture demonstrating these changes is shown in Fig. 5. In cultures grown in defined medium the Schwann cells appear as small, rounded cells, intensely birefringent in phase contrast (Fig. 5 *a*). The Schwann cells are associated with neurite bundles but are perched on the outside of these bundles rather than being spread longitudinally along the neurites. By 16 h after switching to supplemented medium the Schwann cells lose their highly rounded shape and appear to be spreading out along the neurites (Fig. 5 *b*). In addition, the Schwann cells associated with single neurites or small neurite bundles disappear and presumably become associated with larger bundles. This change is concomitant with an increase in size of many neurite bundles. Over the next few days the changes visible in living cultures are more subtle, as the Schwann cells increasingly spread on the neurites and penetrate into the neurite bundles (Fig. 5 *c*).

This latter change is strikingly apparent in transverse sections of fixed and stained cultures viewed by light (Fig. 6) or electron microscopy (not shown). In defined medium, Schwann cells appear as either flat cells lying on the collagen substratum underneath the neurites or round cells sitting on top of neurite bundles or on the collagen substratum. Only rarely are Schwann cells found within a neurite bundle. In contrast, in cultures fixed 2-d after switching from defined to supplemented medium, there is a striking increase in the number of Schwann cell nuclei within bundles of neurites, with a concomitant decrease in the number of flat cells and round cells. Thus, an early step in the establishment of normal Schwann cell-axon relationships appears to be an inward migration of Schwann cells into the neurite bundles. This Schwann cell migration is apparent at a time when the ensheathment of axons is only beginning and is accompanied by the appearance of small patches of basal lamina at the Schwann cell surface (not shown). Earlier work has established that subsequent to this

early change upon medium shift, many Schwann cells will progress to myelinate the larger axons (13).

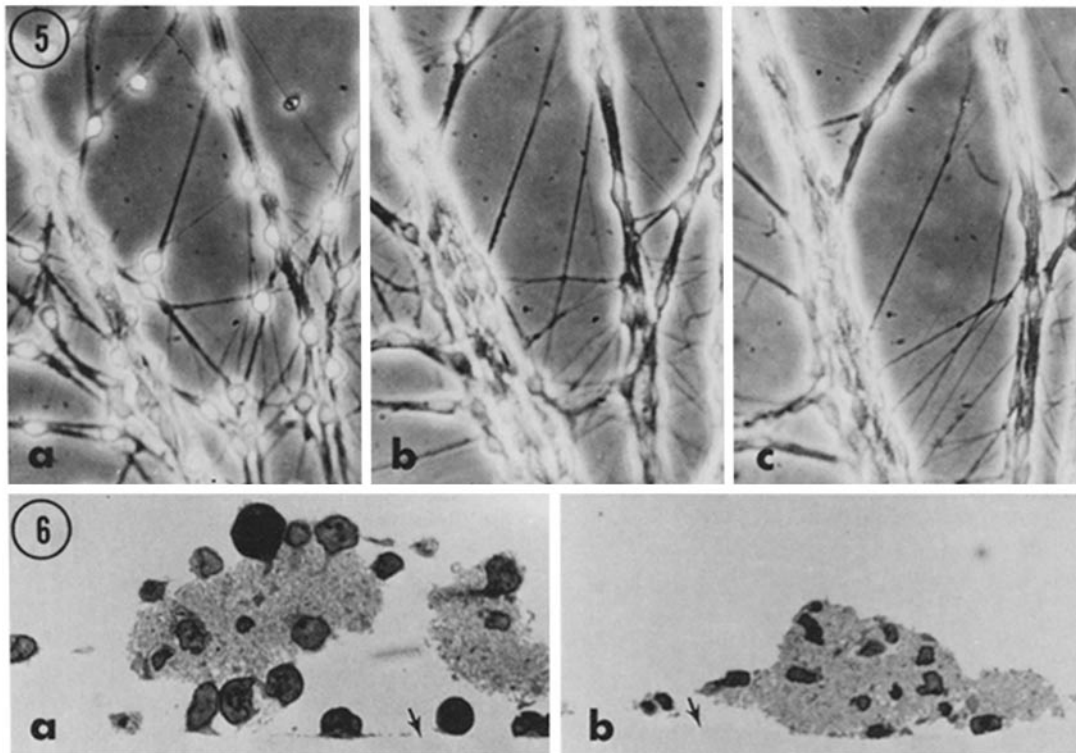
## DISCUSSION

The results presented here demonstrate that rat Schwann cells grown in culture release a reproducible pattern of polypeptides into the culture medium. Although the mechanism by which Schwann cells release these polypeptides is not known, several lines of evidence suggest that they are the result of a physiological process and are not released, for example, by dead or dying cells: (a) medium polypeptides are reproducible both in amount and in the patterns observed after SDS-PAGE; (b) the medium polypeptides are distinct from the labeled polypeptides seen in the cell layers of the cultures; (c) the appearance of the medium polypeptides is independent of the labeling conditions (e.g. short term labeling in serum-free medium vs. long term labeling in supplemented medium); (d) the appearance of the medium polypeptides can be reversibly altered by modifying the culture conditions in ways that affect Schwann cell differentiation; and (e) the medium polypeptides are produced in cultures in which there is no visible sign of Schwann cell death.

In a previous paper evidence was presented which demonstrated that Schwann cells in association with axons synthesize and secrete several types of collagen polypeptides (5). The number and size distribution of polypeptides as analyzed by SDS-PAGE in the present study suggest that Schwann cells release other components in addition to collagens. These may include basal lamina components (other than collagens), ligands for Schwann cell-axon recognition and adhesion, enzymes (e.g. collagen-modifying enzymes) and trophic factors. Identification of some of the Schwann-cell-released proteins by immunological and other methods is now in progress. Our observation that changes in the relation of Schwann cells to axons accompanies the alteration in the pattern of polypeptides released by Schwann cells suggests that one or a set of these polypeptides may be involved in Schwann cell-axon interactions. Direct support of this hypothesis must await further experimentation, however, because the complex media components that must be added to promote Schwann cell secretion may themselves foster the observed Schwann cell response.

The mechanism responsible for the differences in medium polypeptides between Schwann cells grown in defined and supplemented media is not known. Two observations suggest that the decrease seen in cultures grown in defined medium is not due to an increased rate of degradation of released polypeptides caused by the absence of protease inhibitors present in serum. The first is that the pattern and amount of medium polypeptides observed in cultures grown in supplemented medium is not changed when radiolabeling is performed in serum-free medium or serum-containing medium. Secondly, ultrastructural observations have shown that Schwann cells grown in defined medium contain elements of rough endoplasmic reticulum that are swollen and filled with flocculent material (13), a feature consistent with a decreased rate of secretion of polypeptides.

One unexpected result to come from these studies is that the release of proteins by Schwann cells does not appear to require the presence of neurons. Previously it had been shown by electron microscopy analysis that Schwann cells form a basal lamina only when cultured in the presence of neurons, suggesting that a neuronal signal was required to turn on Schwann cell secretion (5, 20). It now appears, however, that the effect of neurons on basal lamina formation is more subtle. A possible



FIGURES 5 and 6 Fig. 5 shows light micrographs of living cultures of Schwann cells and neurons demonstrating changes in appearance after switching from defined medium to supplemented medium. (a) culture after 3 wk in defined medium. (b) same area of this culture 16 h after switching to supplemented medium; (c) same area 67 h after switching to supplemented medium. Phase contrast optics.  $\times 325$ . Fig. 6 shows light micrographs of transversely sectioned Schwann cell-neuron cultures demonstrating changes in appearance after switching from defined medium to supplemented medium. Cultures were grown for 3 wk in defined medium, after which time one half were switched to supplemented medium. Two days later the cultures were fixed in glutaraldehyde and processed as for electron microscopy. Semi-thin sections were stained with toluidine blue and methylene blue and examined by bright field oil immersion optics. (a) defined medium culture; (b) defined medium culture 2 d after switching to supplemented medium. Preliminary counts of the distribution of Schwann cell nuclear profiles in these cultures show that  $<4\%$  (3 out of 94 counted) of defined medium-grown Schwann cell nuclei are within neurite bundles, whereas 2 d after switching to supplemented medium  $>33\%$  (29 out of 80 counted) of Schwann cell nuclei have migrated to positions within bundles. Arrows indicate collagen substratum.  $\times 640$ .

explanation is that neurons modulate the secretion of a minor component that is required for basal lamina formation, or that the neurons themselves secrete a required component. It is also possible that the effect of neurons is on the Schwann cell membrane, either through the synthesis of new membrane components that act as basal lamina "receptors", or through a reorganization of existing membrane components into a surface onto which a basal lamina can form. Further work will be required to clarify this problem.

Our observations that an early step in the establishment of normal axon-Schwann cell relationships in these cultures is the inward migration of Schwann cells from positions peripheral to the axonal bundles is reminiscent of the inward migration of Schwann cells that occurs during the formation of peripheral nerves in embryonic development (15). Cultured Schwann cells grown in defined medium appear to be blocked in their development at this migratory step or an obligatory prior step. There is a striking similarity in this regard between Schwann cells grown in defined medium and the Schwann cells found in the roots of the spinal nerves of the dystrophic mouse. In these nerves the Schwann cells, deficient in basal lamina coverage, appear to be unable to carry out this migratory step and remain in the peripheral portion of the axon bundles, resulting in a nearly complete lack of ensheathment of axons located in the

central portion of the bundles. This similarity, and the finding that Schwann cells grown in defined medium are deficient in their ability to release polypeptides into the culture medium, lend support to the hypothesis that the dystrophic mouse lesion results from a secretory abnormality of the Schwann cells. Additional evidence for this hypothesis has been published previously (7, 14). The availability of tissue culture preparations of these cells should allow this hypothesis to be tested directly.

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