

## Identification of proteins secreted from axons of embryonic dorsal-root-ganglia neurons

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Secretion of proteins from the growth cone has been implicated in axon growth and synapse formation and might be involved in the transmission of a variety of axon-derived regulatory signals during neurogenesis. In order to identify axonally secreted proteins, dorsal-root-ganglia neurons from chicken embryos were cultured in a compartmentalized cell culture system that allows separate access to neuronal cell somas and axons. The proteins synthesized by the neurons were metabolically labeled by addition of [<sup>35</sup>S]methionine to the compartment containing the cell somas; the proteins released from the axons were harvested from the culture medium of the axonal compartment. Two-dimensional gel electrophoresis revealed two axonally secreted proteins with apparent molecular mass of 132–140 kDa and 54–60 kDa; they were termed axonin-1 and axonin-2, respectively. Both axonins were found to be secreted from a variety of neuronal cell cultures, but not from any of the nonneuronal cultures investigated, and hence might be neuron-specific. Virtual absence of these proteins from the axonal protein pattern suggests constitutive secretion. The information acquired on coordinates and spot morphology of these proteins in two-dimensional gel electrophoresis provides a useful assay for their purification.

The elaboration of the information-processing network that characterizes the nervous system includes the extension of axons to span the distance to target cells along a prespecified path, the establishment of specific synapses and the differentiation of glial cells interposed between axons or ensheathing them. These processes involve many well-established interactions between axons and their environment. Axons not only receive and respond to signals of their environment in order to tune their developmental activities, but also actively shape their environment. The external activities of axons comprise two modes of operation. By emission of signals, axons regulate developmental processes of adjacent cells and, by deposition of new molecules or post-translational modification of pre-existing molecules, they directly accomplish modifications in the extracellular space.

Virtually all types of cells in contact with the shaft of axons or encountered by the moving growth cones have been reported as targets of axonal regulatory signals. Axons exert a mitogenic effect on apposed Schwann cells [1] and oligodendrocytes [2], promote survival of the common progenitor of oligodendrocytes and type-2 astrocytes [3–5], affect astroglial differentiation [6] and contribute to the development of postsynaptic specialization during synaptogenesis, as indicated by the aggregation of receptors in the membrane of target cells [7]. The possibility of a direct impact of axons on the extracellular matrix was first revealed by studies on the neuromuscular junction. After nerve and muscle degeneration, the synaptic extracellular matrix left behind at the original synaptic site was found to be sufficient to induce the reestablishment of the presynaptic terminal [8] as well as the

postsynaptic specialization of the neuromuscular end plate [9]. A local structural specialization of the extracellular matrix with synapse-organizing activity was implicated. The detection of antigenic determinants specific for the synaptic basal lamina further supported this hypothesis [10]. The suggestion that the synaptogenic features could be conferred on an originally nonspecific extracellular matrix by axons came from the virtually concurrent observation that axons of motor neurons growing over cultured myotubes induce dense aggregates of acetylcholine receptors in the myotube membrane on their path of growth [7, 11]. A possible mode of generation of the axon-dependent local specialization of the synaptic extracellular matrix is suggested by the recent finding that axons growing over cultured myotubes remove components from the extracellular matrix of their growth path and deposit new ones [12].

The involvement of axonally secreted molecules in extroverted axonal activities has recently obtained support from a number of experimental data. (a) Krystosek and Seeds [13, 14] have shown that plasminogen activator is produced in neurons and released from growth cones. Using substrate gels as a means of detection, the molecular characteristics of the neuron-released plasminogen activator have been determined and an additional protease, a calcium-dependent metalloprotease, which is also released, has been identified [15]. A large proportion of both proteases were found to be released by the distal part of the neurites and involvement in growth cone functions was suggested. These data, in conjunction with the work of Monard and collaborators [16–18], suggest an interplay of axon-derived proteases and glia-derived protease inhibitors in the regulation of neurite extension. (b) Deposition in the synaptic extracellular matrix of a nerve-terminal-derived proteoglycan, initially detected in synaptic vesicles of the electric organ of marine rays, has recently been reported

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[19]. It has been suggested to serve as an anchor for the nerve terminal. (c) Soluble factors from a variety of neural sources induce the aggregation of acetylcholine receptors in the membrane of noninnervated myotubes *in vitro*. A role in the early events of synapse formation is inferred (for a recent review cf. [20]).

In this work presented here, we identified proteins secreted from dorsal-root-ganglia axons of chicken embryos by their coordinates in two-dimensional SDS/PAGE using selective metabolic labeling in a compartmentalized cell culture system (for a schematic cf. Fig. 1). In this system, originally devised by Campenot [21, 22], the surface of the cell culture dish is subdivided into three compartments by a Teflon piece, which is joined onto the dish by silicon grease. Adjacent compartments are connected by a thin film of medium. If dissociated neurons are plated into the central compartment, their somas remain confined to this area, whereas the outgrowing axons extend into the adjacent compartments. Since axons are not equipped for protein synthesis but obtain their proteins by axonal transport from the cell soma, the separate access to neuronal somas and processes can be exploited for selective metabolic labeling of axonal and axonally secreted proteins. In order to label selectively the proteins secreted by axons into the side compartment, [<sup>35</sup>S]methionine was added to the medium of the center compartment containing the cell somas. After an incubation period allowing protein synthesis and axonal transport, the proteins that had been released from the axons and accumulated in the medium of the axonal compartment were harvested and subjected to two-dimensional SDS/PAGE, followed by fluorography. Two proteins, of apparent molecular mass 54–60 kDa and 132–140 kDa, were found to be secreted from axons of embryonic chicken dorsal-root-ganglia neurons.

## MATERIALS AND METHODS

### *Compartmentalized cell cultures*

A version of the compartmentalized cell culture system, originally devised by Campenot [21, 22], was used, as modified for selective metabolic labeling of axonal proteins by Sonderegger et al. [23–26].

### *Cell culture*

White Leghorn chick embryos, staged according to Hamburger and Hamilton [27], were used throughout the study. Dorsal-root-ganglia neurons from 10-day-old embryos were grown in the compartmentalized cell culture system as previously described [23–25]. Ventral and dorsal halves of spinal cords were dissected from 6-day-old embryos as described by Masuko et al. [28]. Culture conditions were as given by Sonderegger et al. [24]. Retinas were obtained from 7-day-old embryos and cultured according to Brackenbury et al. [29]. Ciliary ganglia were dissected from 7-day-old embryos according to Tuttle et al. [30] and cultured on a layer of lysed fibroblasts according to Nishi and Berg [31]. Superior cervical ganglia from 10-day-old chicken embryos were cultured as reported by Zurn and Mudry [32]. Embryonic fibroblasts from 10-day-old chicken embryos were grown as described by Jaussi et al. [33]. The cultivation and characterization of nonneuronal cells of the peripheral and the central nervous system have been described by Sonderegger et al. [25]. Skeletal muscle was dissected on embryonic day 10 and myotubes were grown according to Fischbach [34] and cardiac muscle cells

according to Varon et al. [35]. For cultivation of liver cells of 8-day-old embryos, the technique of Braun et al. [36] was adopted.

### *Selective metabolic labeling of axonal proteins and two-dimensional gel electrophoresis*

Selective metabolic labeling of the newly synthesized axonal proteins was carried out as previously described [23–25]. However, horse serum was omitted during the labeling period in both the center and the side compartments, in order to reduce the protein content to quantities compatible with subsequent two-dimensional SDS/PAGE. The labeling medium added to the center compartment was composed of methionine-free growth medium substituted with 1 mCi/ml [<sup>35</sup>S]methionine (approx. 1000 Ci/mmol, New England Nuclear, Boston, MA) and 15 μM unlabeled methionine. The medium in the side compartments was identical, except that the radioactive methionine was replaced by 8 mM unlabeled methionine. To make the hydrostatic situation simple and reproducible, the volume of medium and the liquid surface level of the center compartment were matched to that of the side compartments. This was obtained by fitting a grease plug between the outer rim of the teflon inset and the wall of the culture dish on each side of the opening slit of the center compartment (Fig. 1, stippled area). All incubations were for 40 h.

Diffusion of radioactive methionine was assessed in all labeling experiments by direct liquid scintillation counting, since the contribution of soluble proteins to the total radioactivity in the medium was less than 1%. At the end of the labeling period, 5 μl medium from both the center and side compartments was removed and the radioactivity determined by liquid scintillation counting. Cultures in which the side compartment accumulated more than 10% of the radioactivity of the center compartment, were considered 'leaky' and discarded. Usually nine out of ten plates were 'tight'. These were processed further by collecting the medium of both the center and side compartments. Proteins were precipitated [37], and precipitates were air-dried and resuspended in sample buffer for two-dimensional SDS/PAGE.

To harvest the cell-bound proteins, the cellular material was washed by rinsing the plates with minimal essential medium (MEM, Gibco). The cellular material was dissolved in 2% SDS and 5% 2-mercaptoethanol, at a temperature of 95°C, precipitated [37] and resuspended in sample buffer for two-dimensional SDS/PAGE.

To control for local incorporation of diffused radioactive amino acid by cells of the side compartments, labeling was carried out with reversed media. The labeling medium, containing the radioactive amino acid, was added to the side compartment, whereas the medium containing 8 mM unlabeled methionine (which was added to the side compartment under standard labeling conditions) was added to the center compartment. Side-compartment concentrations of [<sup>35</sup>S]-methionine employed were 0.1 mCi/ml and 1 mCi/ml, respectively. All other experimental parameters were exactly the same as those used under standard labeling conditions.

For labeling under conditions of blocked axonal transport, either colchicine or vinblastine was added selectively to the medium of the side compartments 1 h prior to the addition of radioactive methionine to the center compartment. Colchicine was used at 5 μM and vinblastine at 10 μM, concentrations reported to be effective for blockade of axonal trans-

port in cultured neurons [38]. All other parameters were identical to those used under the standard labeling conditions.

#### *Two-dimensional SDS/PAGE and fluorography*

Two-dimensional SDS/PAGE was carried out according to O'Farrell [39]. In each experiment, the entire protein content of either the center compartment or the pooled protein content of both side compartments was subjected to two-dimensional SDS/PAGE. The ampholine solution of the isoelectric focusing step was composed of 1.6% pharmalyte 5/8, 0.4% pharmalyte 3/10 and 0.8% pharmalyte 4/6.5 (all from Pharmacia). The second dimension was run in 10% acrylamide with 0.02% linearly polymerized polyacrylamide (BDH). [ $^{14}\text{C}$ ]Methylated molecular mass markers carbonic anhydrase (30 kDa), albumin (69 kDa), phosphorylase (97 kDa), and myosin (200 kDa) were purchased from NEN. [ $^{14}\text{C}$ ]Aspartate aminotransferase (45 kDa) was generated by modification of mitochondrial aspartate aminotransferase with [ $^{14}\text{C}$ ]N-ethylmaleimide. Fluorography was carried out according to the principles of Bonner and Laskey [40] and Laskey and Mills [41]; however, the commercially available acetic-acid-based enhancer (En3Hance, NEN) was used. The dried gels were exposed to Fuji medical X-ray film (Fuji Photo Film Co.). Exposures were for four weeks at  $-70^{\circ}\text{C}$ , followed immediately by a second exposure for 2 weeks.

#### *Computerized analysis of two-dimensional electropherograms*

To compare the amount of individual secreted proteins in the center compartment and the side compartments, the GELLAB system for computerized analysis of two-dimensional SDS/PAGE was used [25, 26, 42–46]. This system uses data acquired by scanning fluorographic replicas of the two-dimensional electropherograms, along with a neutral density calibration wedge. A high resolution black and white Television camera was used to scan the autoradiographs into  $512 \times 512$  picture element images with 256 gray values (white to black). The images were then calibrated in terms of absorbance units and thereafter all measurements were given in terms of integrated absorbance. GELLAB then segments individual spots in each gel image. There were four experiments and two different exposures were made of the two different experimental conditions yielding 16 fluorograms. A reference gel (denoted the Rgel) was selected from the set of eight ( $n$ ) gels. A small number of landmark spots were defined on the Rgel. Corresponding landmark spots were then manually identified on both replicas of each of the remaining  $n-1$  gels and entered into a landmark file. Because a number of spots consisted of putative horizontal post-translational-modification trains of proteins, GELLAB was enhanced to allow the specification of composite groups of spots at the time the landmarking is performed. Pairing of the remaining spots between the different gels was then performed automatically. Corresponding spots from different gels were automatically merged into a composite gel data base. Such corresponding spots for all gels were called Rspot sets. They consisted of those spots in the gels which corresponded to the same spot in the Rgel. Those regular Rspots which were determined to be in composite-spot lists were then added together for each composite spot (for each gel) to form additional synthetic Rspot set called composite groups. For statistical purposes, a composite Rspot was treated as if it was a simple Rspot. Its integrated density and area equalled the sum of the Rspots it included and its position (pI, molecular mass) reflected the

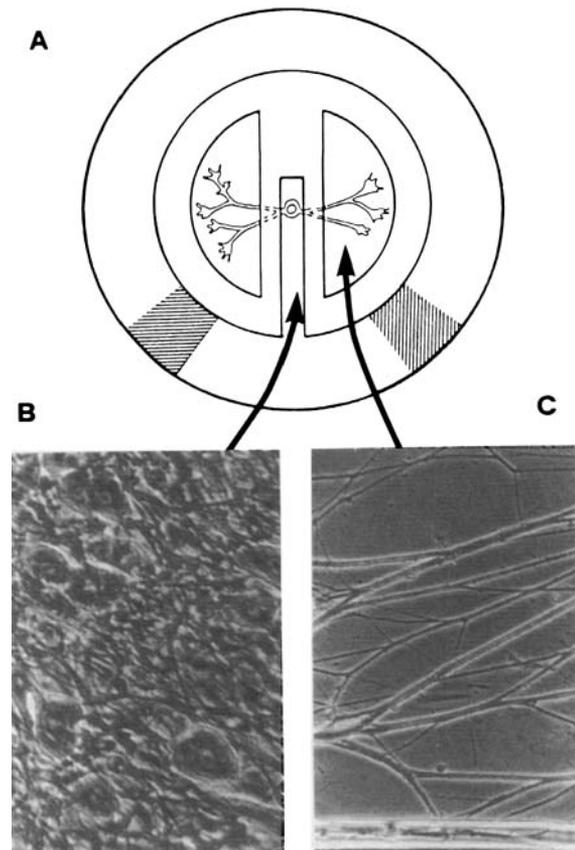


Fig. 1. Morphological aspects of chicken embryonic dorsal-root ganglia cultured in the compartmentalized cell culture system. (A) Schematic top view of the compartmental cell culture system devised by Campenot [21, 22]. To match volume and surface level of the center compartment with the side compartment during the metabolic labeling period, the volume of medium accessible from the center compartment was controlled by two grease plugs placed between the outer rim of the teflon inset and the wall of the cell culture dish (stippled areas). (B) Micrographs of the neuronal somas and surrounding nonneuronal cells of the center compartment. Phase contrast optics,  $\times 324$ . (C) Axon fascicles of the side compartment after 1 week in culture; note the virtual absence of nonneuronal cells. Phase contrast optics,  $\times 324$ .

integrated density-weighted mean of its member spots. The minimum and maximum absorbance were taken over all of the Rspots included; data on the spots used to construct the composite-group spots were not considered in isolation. The ratios between the two experimental classes were computed for 19 spots which were present in all gels. These 19 spots included 14 single spots and five composite spots. The ratio histogram function was used to compute the density ratios of a set of spots for the two experimental classes and to summarize the ratios in a histogram table.

The CGELP program in GELLAB has facilities for interactively searching the composite-spot data base as well as saving the results of searches and visualizing them with Rmaps, i.e. a copy of any of the original gel images with selected Rspots labeled. GELLAB also provides the facility to generate so-called mosaic images. A mosaic image is an image composed of panels of subregions of the gel images surrounding a particular spot ordered by increasing normalized density. The assumed coordinates of the center of the spot to be investigated were used to position the center of

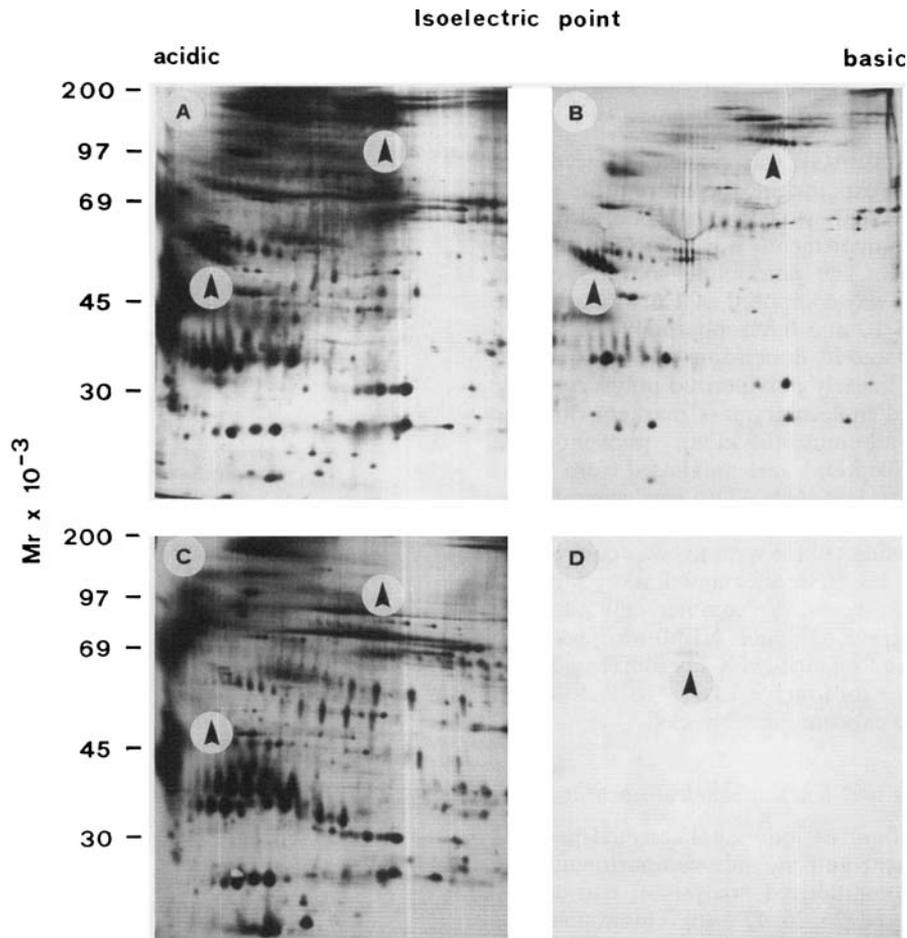


Fig. 2. Metabolically labeled proteins of the medium conditioned by dorsal-root-ganglia neurons in the compartmentalized cell culture system. Fluorographs were made by exposure of two-dimensional electropherograms of conditioned medium proteins after a 40-h incubation period with radiolabeled methionine (all panels reduced to 35%). (A), (B) Radiolabeled proteins of the center compartment and the side compartment, respectively, of a standard labeling experiment with 1 mCi/ml [ $^{35}\text{S}$ ]methionine in the center compartment. Side compartment proteins (B) are a subset of the center-compartment proteins (A). Note the equal distribution between center and side compartments of two proteins of 54–60-kDa and 132–140-kDa (arrows). The other side-compartment proteins have markedly more abundant counterparts in the center compartment. (C) Proteins of nonneuronal cells of dorsal-root ganglia cultured in the center-compartment medium. Note the absence of the 54–60-kDa and the 132–140-kDa proteins from this gel (expected coordinates are indicated by arrows). (D) Control for local protein synthesis and secretion in the side compartment by interchange of the media of the center compartment and the side compartment. Metabolic labeling was performed with 0.1 mCi/ml [ $^{35}\text{S}$ ]methionine in the side compartment. Covalent labeling of albumin (arrow) is a known artefact at high concentrations of [ $^{35}\text{S}$ ]methionine

each panel relative to that spot and were labeled with a white dot. Each panel represents a different gel and 16 gels can be displayed in one mosaic image. The spots of the surrounding region give a morphological context which allowed verification that the spot indicated was in fact the one under investigation. Hence, as mispaired spots were easily detected by visual inspection of these mosaic images, this facility provided an excellent tool for the control of accurate spot pairing. We made extensive use of the mosaic image facility in checking for correct pairing of every spot that was included in the pairwise comparisons between experimental classes (gels of the side compartment versus gels of the center compartment).

#### Manual excision of radioactive spots from gels and scintillation counting

Selected radioactive protein spots were cut from gels and, for liquid scintillation counting, dissolved in Lumasolve/Lipoluma/Water (Lumac Application Laboratory) at a ratio

of 10:1:0.2 and incubated at 20°C for four days. Measurement of [ $^{35}\text{S}$ ]methionine was performed at a counting efficiency of 70%. For background subtraction, two or three gel areas, free of detectable proteins, were excised 3–5 mm from the protein spot under investigation and their radioactivity determined. Background radioactivity was normalized with respect to unit area and subtracted from the measurements of the protein spots according to the area of the corresponding gel piece.

#### Labeling of mitochondrial aspartate aminotransferase with [ $^{14}\text{C}$ ]N-ethylmaleimide

Mitochondrial aspartate aminotransferase was a generous gift of Drs H. Gehring and P. Christen. For covalent radioactive labeling, 1 mg aspartate aminotransferase in 50 mM sodium phosphate, pH 7.5, was incubated with 7.5  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]N-ethylmaleimide (NEN) for 30 min at 20°C in the presence of 20 mM  $\alpha$ -methylaspartate. Small molecular mass components

were removed by two washes with phosphate-buffered saline in a Centricon microconcentrator (Amicon), followed by gel filtration on a Superose-12 column (Pharmacia).

## RESULTS

### *The radioactively labeled proteins in the medium of the side (axonal) compartment are a subset of those of the central (cell soma) compartment*

In the compartmentalized cell culture system employed, a proportion of the axons of neurons added to the center compartment grow out into the adjacent side compartments (cf. Fig. 1A). In the central, plating compartment the neuronal somas enlarge to a diameter of up to 30  $\mu\text{m}$  within a week after plating and are surrounded by numerous nonneuronal cells (Fig. 1B). In the side compartment, the predominant elements are axon fascicles extending up to 8 mm, often reaching the outer rim of the teflon inset (Fig. 1C). After exposure to fluorodeoxyuridine from day two to day five after plating, only isolated nonneuronal cells survive in the side compartment (for details cf. [24]).

After incubation with [ $^{35}\text{S}$ ]methionine in the center compartment, the radioactively labeled proteins of the medium in the central and side compartments were analyzed by two-dimensional SDS/PAGE followed by fluorography. As can be seen in Fig. 2, many labeled proteins are found in the medium of the center compartment (Fig. 2A), whereas relatively few labeled proteins are present in the side compartment (Fig. 2B). This is not surprising because the center compartment is occupied by a dense layer of neuronal and nonneuronal cells, which have access to radioactive methionine, whereas the axons, reaching out from neuronal cell somas of the center compartment, are the only cellular elements of the side compartment that are expected to secrete labeled proteins. The proteins of the medium in the side compartments are a subset of the proteins of the medium in the center compartment. This is in agreement with the assumption that quite a number of the axons do not cross into the adjacent compartments, but remain in the center compartment, and fulfils the expectation that axonally secreted proteins would occur in both the somal and the axonal compartments.

### *Local incorporation of diffused radioactive amino acid does not affect the pattern of side-compartment proteins*

Diffusion of soluble medium components between adjacent compartments along the connecting film of medium is an inherent feature of the compartmentalized cell culture system used. Indeed, by the end of a standard labeling experiment, the concentration of radioactive methionine in the side compartment had reached 1.4–5.3% of the concentration in the center compartment (Table 1). Hence, it was important to know whether the radioactive methionine accumulated in the side compartment could contribute to the pattern of side compartment proteins by incorporation into proteins of resident cells.

For controls, the media in the center and side compartments were reversed. The axons and cells of the side compartment were exposed to 0.1 mCi/ml [ $^{35}\text{S}$ ]methionine. Hence, the concentration of labeled methionine in the side compartment medium was approximately twice the concentration measured at the end of the incubation period in the most 'leaky' standard labeling experiments (Table 1). All other experimental parameters were identical. A representative fluorograph is

Table 1. Diffusion of soluble medium components in the compartmentalized cell culture system

[ $^{35}\text{S}$ ]Methionine and [ $^{14}\text{C}$ ]aspartate aminotransferase (AspAT) were added to the center compartment and their concentrations in the side compartment and center compartment determined, after incubation for 40 h, by liquid scintillation counting. Data on the quantification of the endogenous labeled proteins (except 132–140-kDa and 54–60-kDa proteins) are taken from the metabolic labeling experiments (Fig. 3, spots 3–19). The average radioactivity, in the side compartment is expressed as a percentage of that in the center compartment  $\pm$  standard error (SE); the range also is given

Labeled compound	n	Side/center compartment	
		mean $\pm$ SE	range
		%	
[ $^{35}\text{S}$ ]Methionine	7	3.2 $\pm$ 0.5	1.4–5.3
[ $^{14}\text{C}$ ]AspAT	6	1.7 $\pm$ 0.7	0.1–4.0
Endogenous labeled proteins	4	12.8 $\pm$ 1.7	5.0–36.2

shown in Fig. 2D. The faintly labeled protein in the middle of this gel corresponds to serum albumin and represents a known artefact due to covalent modification of residual serum proteins at high concentrations of [ $^{35}\text{S}$ ]methionine [47]. No other labeled proteins were detected, indicating that local incorporation of diffused radioactive amino acid into cells resident in the side compartment was below the detection limit. In a second series of controls, the concentration of [ $^{35}\text{S}$ ]methionine used was 1 mCi/ml, corresponding to the concentration in the center compartment of standard labeling experiments. Under these conditions, labeling of serum albumin was markedly enhanced and additional radioactive spots were seen at the locations of other major serum proteins, such as transferrin and apolipoprotein A-I, as identified by coordinates and spot morphology according to Anderson and Anderson [48] (data not shown). Again, no radioactive spots were detectable at locations corresponding to the coordinates of the side-compartment proteins occurring under standard labeling conditions. The absence of any detectable local contribution in the side compartment leads to the conclusion that all labeled proteins of the side compartment are synthesized in the center compartment. Absence of labeling at 1 mCi/ml [ $^{35}\text{S}$ ]methionine indicates that, under the standard conditions (given by cell number and amount of diffused [ $^{35}\text{S}$ ]methionine in the side compartment) protection against contaminating proteins of local origin is granted by a safety factor of at least 10-fold.

### *The two major proteins, one of 54–60 kDa and one of 132–140 kDa, are actively accumulated in the side compartment*

Visual estimation of the density ratio (side to center compartment) of individual proteins found in the side compartment suggested that they subdivide into at least two groups (Fig. 2A, B). Two proteins, one of 54–60 kDa and one of 132–140 kDa are present in both compartments in about equal amounts (arrows in Fig. 2A, B). Both are major proteins in the side-compartment medium and are not detectable among the proteins released by nonneuronal cells of dorsal-root ganglia (Fig. 2C). All other proteins of the side compartment have markedly more pronounced corresponding spots in the center compartment, hence displaying a steep gradient,

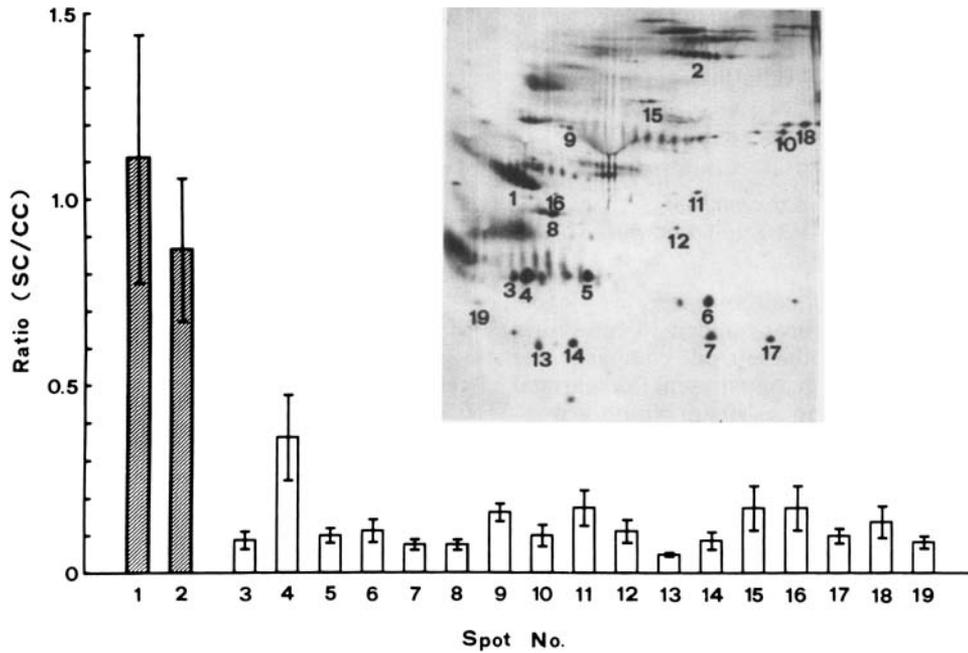


Fig. 3. Ratios of protein quantities in the side and center compartments, revealing two populations of side-compartment proteins. The newly synthesized, radiolabeled proteins were quantified using GELLAB, a computerized system for the quantitative analysis of two-dimensional SDS/PAGE and the ratio of the quantities in the side compartment (SC) and the center compartment (CC) was determined. For each protein included, mean and standard error of the ratio are given ( $n = 4$ ). In the inset, each protein included is labeled by an arbitrary number. The ratios of the 54–60-kDa (1) and the 132–140-kDa (2) proteins are represented by stippled bars

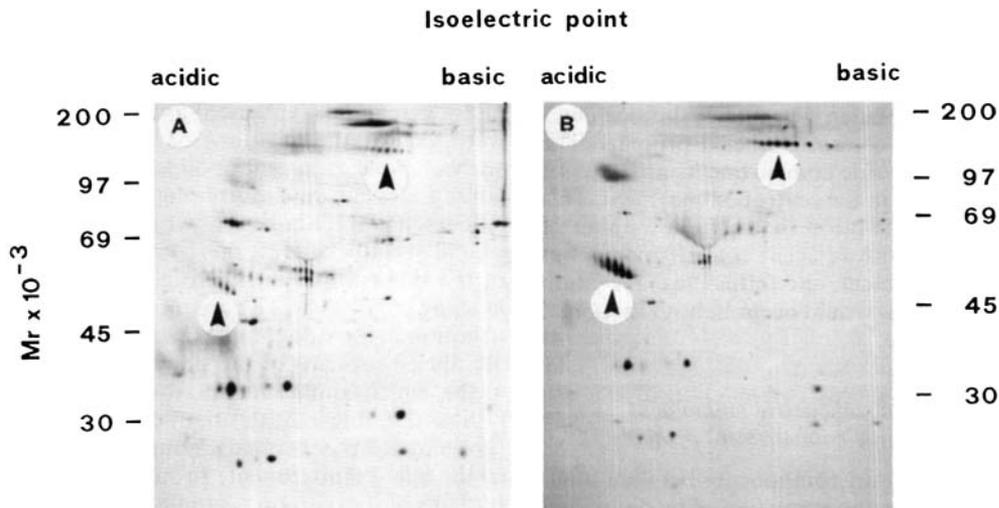


Fig. 4. Influence of blockade of axonal transport on the pattern of side-compartment proteins, suggesting that only the 54–60-kDa and the 132–140-kDa proteins depend on axonal transport. Fluorography of two-dimensional electropherograms (both panels reduced to 35%). Conditions for metabolic labeling, SDS/PAGE and fluorographic exposure were identical to the standard labeling experiment. (A) Side-compartment-medium proteins generated in the presence of 5  $\mu$ M colchicine; (B) side-compartment-medium proteins of control experiment with intact axonal transport. A pronounced reduction of the 54–60-kDa and the 132–140-kDa proteins (arrows) during blockade of the axonal transport is obvious from comparison with the remaining spots of the same gel (A) as well as from comparison with 54–60-kDa and the 132–140-kDa proteins of the control (B)

and all of them are found among the proteins released from nonneuronal cells of dorsal-root ganglia (Fig. 2C). The incomplete separation between the compartments, with diffusion along the connecting medium film, could sufficiently explain this occurrence. Diffusion of radioactively labeled proteins from the medium of the center compartment along the connecting medium film was assessed by means of [ $^{14}$ C]aspartate aminotransferase. When [ $^{14}$ C]aspartate aminotrans-

ferase was added to the center compartment, 0.1–4.0% crossed the barrier to the side compartment by the end of a standard incubation period (Table 1). Hence, diffusion of [ $^{14}$ C]aspartate aminotransferase in the control experiments, in terms of the concentration ratio between the compartments, was fairly close to the values obtained with [ $^{35}$ S]methionine, which served as an internal standard for diffusion throughout all experiments.

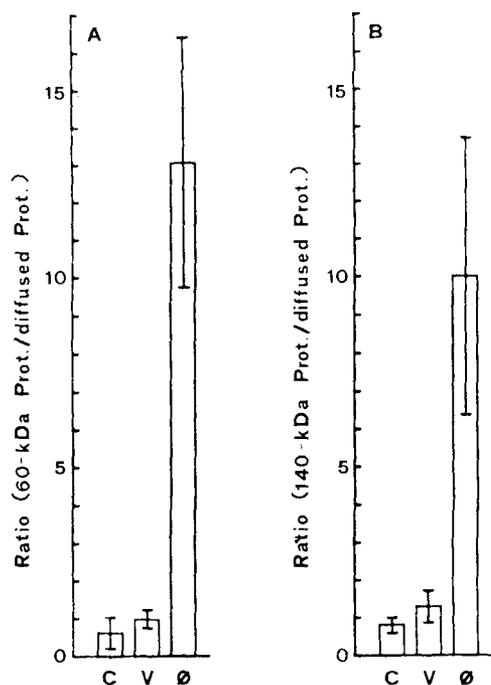


Fig. 5. Comparison of the side-compartment/center-compartment ratios, suggesting that the residual quantities of the 54–60-kDa and the 132–140-kDa proteins during blockade of the axonal transport result from diffusion. Metabolic labeling experiments were carried out under standard conditions. Blockade of axonal transport was achieved by local addition to the side-compartment medium of 5  $\mu$ M colchicine (C) or 10  $\mu$ M vinblastine (V). Controls were without any added drug ( $\emptyset$ ). Each experimental condition was carried out in duplicate. Quantification was by excision of radioactive spots from gels and liquid scintillation counting. The four proteins taken as representative of the group of diffused proteins were spots 4–7 in Fig. 3. They were selected because of their isolated location on the gels, which allowed them to be cut out selectively. The side/center-compartment ratios were determined and the average ratio of the four diffused proteins was calculated. As an index for active accumulation in the side compartment, the ratios of the 54–60-kDa (A) and the 132–140-kDa (B) proteins were divided by the average ratio of the diffused proteins. Hence, active accumulation in the side compartment is indicated by a ratio (side-compartment/center-compartment ratios) greater than one. A ratio of one indicates the distribution between the side and center compartments to be identical to the distribution of the diffused proteins, hence absence of active accumulation.

The endogenously synthesized proteins were quantified on the basis of their integrated absorbance on the fluorograms, using GELLAB, a computerized image-processing system for gel analysis; the densitometric data were confirmed by manual excision of selected spots and liquid scintillation counting. The two proteins mentioned above, of apparent molecular mass 54–60 kDa and 132–140 kDa and which are the most prominent proteins in the side-compartment medium (Fig. 3, spots 1 and 2), exhibit side/center-compartment ratios of 1.11 and 0.87, respectively. Obviously, such distributions cannot be explained by diffusion. In the absence of any detectable local protein synthesis or secretion by cells resident in the side compartment, active accumulation by the axons, which are the only cellular elements spanning from the center to the side compartments, is inferred.

No evidence for active accumulation can be derived from the distribution of other proteins. The 17 proteins of this

Table 2. Screening for the 54–60-kDa and the 132–140-kDa protein in the conditioned medium of neuronal and nonneuronal cell cultures. For metabolic labeling, 0.1 mCi/ml [ $^{35}$ S]methionine was added to the medium of dissociated cultures. Incubation was for 40 h. The conditioned medium was harvested, the proteins precipitated and subjected to two-dimensional SDS/PAGE. Cell densities were matched among the neuronal cultures and among the nonneuronal cultures. Nonneuronal cultures were at least five times more dense than neuronal cultures. The time of dissection for tissue cultivation is given in embryonic days; CNS, central nervous system; PNS, peripheral nervous system

Cell type	Source	Embryonic age	54–60-kDa protein	132–140-kDa protein
		days		
Neuronal	dorsal root ganglion	10	+	+
	ventral spinal cord	6	+	+
	dorsal spinal cord	6	+	+
	retina	10	+	+
	ciliary ganglion	7	+	+
	superior cervical ganglion	9	+	+
Non-neuronal	glia (CNS)	10	–	–
	glia (PNS)	10	–	–
	fibroblasts	10	–	–
	skeletal muscle	10	–	–
	cardiac muscle	8	–	–
	liver	8	–	–

group have ratios in the range 0.05–0.36 (Fig. 3, spots 3–19 and Table 1). All proteins of this group can be found among the proteins released from nonneuronal cells of dorsal-root ganglia (Fig. 2C), which densely populate the center compartment and the area underneath the barrier which separates the compartments (for an illustration cf. [24], Fig. 2).

#### *Inhibitors of axonal transport abolish active accumulation of the 54–60-kDa and the 132–140-kDa proteins in the compartment*

Axonally secreted proteins are synthesized in the neuronal cell soma and transported to their site of secretion by axonal transport [49]. Hence, experimental manipulations affecting axonal transport should result in changes in axonal protein secretion. In order to test this assumption, metabolic labeling experiments were carried out under conditions in which axonal transport was blocked by colchicine or vinblastine (for a review cf. [50]), which were selectively added to the side compartments. As illustrated in Fig. 4, markedly reduced amounts of both the 54–60-kDa and the 132–140-kDa proteins appeared in the side compartments during blockade of axonal transport, whereas the amounts of the diffused spots remained unaffected.

The residual amounts of the 54–60-kDa and the 132–140-kDa proteins in the side compartment medium during local blockade of axonal transport were accompanied by a persistently high quantity in the center compartment, the relatively steep gradient implying diffusion. In order to test this, the side/center-compartment ratios of the 54–60-kDa and the 132–140-kDa proteins were compared with the average of the ratios of four representative diffused proteins (Fig. 3, spot 4–7). The 54–60-kDa and the 132–140-kDa proteins and the four proteins of the diffused group were quantified

by excising the appropriate radioactive spots from the gel and determining their ratio between the side compartment and center compartment by liquid scintillation counting. The results are shown in Fig. 5 for both the 54–60-kDa protein (A) and the 132–140-kDa protein (B). Under standard conditions, the 54–60-kDa and the 132–140-kDa proteins had side/center-compartment ratios which were 13 times and 10 times, respectively, higher than the ratios of the diffused proteins. This difference disappeared, when the axonal transport was inhibited by colchicine or vinblastine, as indicated by ratios (side/center-compartment) of close to one. The distribution of the 54–60-kDa and the 132–140-kDa proteins between side and center compartments then matched that of the diffused proteins. This clearly documents the dependence of the accumulation of the 54–60-kDa and the 132–140-kDa protein on axonal transport.

*Both the 45–60-kDa and the 132–140-kDa proteins are not detectable among the proteins of the axons*

Although no evidence for death of axons during the entire length of the experiment was obtained by visual inspection, we tested whether the occurrence of the 54–60-kDa and the 132–140-kDa proteins in the side compartment could be explained by release from axons that had died and disintegrated after incorporating radioactive methionine. To control for this possibility, we compared the proteins of the medium bathing the axons with the axonal proteins. As shown in Fig. 6, neither the 54–60-kDa protein nor the 132–140-kDa protein is present in the cellular material of the axonal compartment.

*Both the 54–60-kDa and the 132–140-kDa proteins occur in the supernatant of all neuronal cell cultures tested, but are absent from all nonneuronal cultures*

We were interested to know whether the axonally secreted proteins identified in dorsal-root-ganglia cultures would be secreted from other neuronal cell types, and whether or not they were neuron-specific (Table 2). Six types of dissociated neuronal and six nonneuronal cultures were investigated. All nonneuronal cultures contained at least five times as many cells as the neuronal cultures. Among the neuronal cultures, neurons from both the peripheral and central nervous system were included. Among the nonneuronal cultures were glia of both the central and the peripheral nervous system, as previously characterized [25]. The data are summarized in Table 2. Among six neuronal cell cultures, all contained both the 54–60-kDa and the 132–140-kDa protein. On the other hand, none of six nonneuronal cell cultures secreted these proteins.

## DISCUSSION

The unequivocal assignment of secretory proteins to a particular cell type and, further, the assessment of the secretion from a particular area of the cell surface presents problems that, in most experimental systems, are hard to overcome. The axons, by their characteristic morphology as long, tube-like processes and by their logistic dependence on the cell soma for their supply of proteins, offer a means for identifying the proteins they secrete. Axonally secreted proteins are labeled along with all neuronal proteins when a radioactive amino acid is added to the cell somas. Because of

their post-translational translocation, they can be separately harvested at the remote sites of their secretion. The *in vitro* approach presented, utilizing a compartmentalized cell culture system, permits separate access to neuronal somas and axons for selective metabolic labeling of axonally secreted proteins. A thin film of medium connecting adjacent compartments serves as a path for the extension of axons into adjacent compartments. If a radioactive amino acid is added exclusively and remains confined to the center compartment, the axonally transported and secreted proteins are, in the ideal case, the only proteins in the side-compartment medium that appear radioactively labeled. However, the incomplete separation between the compartments, which is inherent to this cell culture system, carries with it the possibility of exchange between compartments, such as diffusion of medium components.

The contribution of local protein synthesis in the side compartment (from incorporation of diffused radioactive amino acid by nonneuronal cells resident in the side compartments) was minimized by preventive measures: (a) the number of nonneuronal cells in the side compartment was reduced by addition of an antimetabolic agent and (b) the diffused radioactive amino acid was diluted by addition of an excess of its unlabeled counterpart to the side compartment. Under the conditions employed here, which have been elaborated previously for the selective metabolic labeling of cell-bound proteins of axons [24], local incorporation of radioactive amino acid into secretory proteins synthesized by cells of the side compartment did not occur.

The large number of nonneuronal cells exposed to the radioactive amino acid generate relatively high concentrations of secretory proteins in the medium of the center compartment. Thus, we had to consider whether proteins could diffuse to the side compartment and add to the proteins secreted from axons. Indeed, when the distribution ratio between side compartment and center compartment was determined for the proteins present in the side compartment, two significantly distinct groups of proteins emerged. Two proteins, one of 54–60-kDa and the other of 132–140-kDa, were present in both the center and side compartments at about equal concentrations. This distribution clearly indicates an active accumulation mechanism. The remaining side-compartment proteins were present in much lower amounts and had side/center-compartment distribution ratios in the range 0.05–0.36. A diffusional origin of this group of proteins is suggested by several pieces of evidence: (a) no detectable local synthesis or secretion of metabolically labeled proteins by cells resident in the side compartment occurs, (b) all of these proteins can be identified in the supernatant medium on nonneuronal cells of dorsal-root ganglia, which densely populate the center compartment, and (c) pharmacological blockade of axonal transport does not affect the accumulation of these proteins in the medium of the side compartments.

Initially, it might appear curious that this group of proteins displays a distribution ratio between the compartments which, on average, is about seven times higher than the values obtained with an extrinsic protein ( $[^{14}\text{C}]$ aspartate aminotransferase). However, the parameters contributing to diffusion of externally added and endogenously synthesized proteins are too different to allow comparison. In the case of the proteins synthesized during the experiment, a hardly predictable, possibly major, contribution to protein diffusion into the side compartment may come from local synthesis and secretion by cells of the confluent monolayer beneath the separating barrier. Especially in the proximal half of the barrier, where

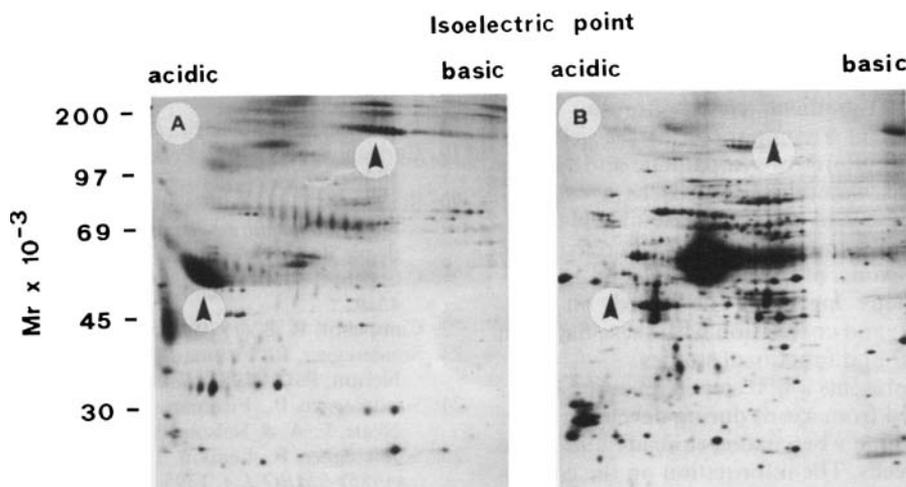


Fig. 6. Comparisons of cell-bound and released proteins of dorsal-root-ganglia axons, revealing absence of the 54–60-kDa and the 132–140-kDa proteins from the cellular material. Fluorographs of two-dimensional SDS/PAGE of a standard labeling experiment. The proteins of the conditioned medium and the axons of the two side compartments of one culture system were loaded onto the gels. (A) Radiolabeled proteins of the conditioned medium of the side compartment; (B) radiolabeled proteins of the cellular material (mainly axons) of the side compartment. Arrows in A point to the 54–60-kDa and the 132–140-kDa proteins; arrows in (B) indicate the coordinates of the expected location of these proteins

the supply of radioactive amino acid may be virtually unlimited (for an illustration cf. [24], Fig. 2), the extremely narrow extracellular space available for the secreted proteins might lead to a local protein concentration which is considerably higher than that of the medium of the center compartment.

The divergence of the distribution ratio of these proteins from those of externally added diffusion probes could be explained by their secretion not only from nonneuronal cells, but from both nonneuronal cells and axons. The distribution ratios of such proteins would be expected to be between those of exclusively nonneuronal and exclusively neuronal proteins. One protein (spot no. 4 in Fig. 3), whose distribution ratio differs significantly from both the 54–60-kDa/132–140-kDa group and the remaining side-compartment proteins, might fall into this class. Whereas the occurrence of the 54–60-kDa and the 132–140-kDa proteins has not been observed in any of the purely nonneuronal cultures, the evidence for an exclusively nonneuronal origin of the other proteins can hardly be established, since a complete separation of neuronal and nonneuronal cells in culture has proven extremely difficult. The compactness of this group of proteins with respect to their distribution ratios suggests a common origin. Hence, the assumption of secretion by both nonneuronal cells and axons would include all proteins of this group. At any rate, the issue is not essential for the conclusion drawn on the 54–60-kDa and the 132–140-kDa proteins, for which the data unambiguously indicate their active accumulation in the side compartment.

The implication of an active process in the accumulation of the 54–60-kDa and the 132–140-kDa proteins was convincingly confirmed by the unequivocal and complete response to blockade of axonal transport. Under local blockade of axonal transport, the accumulation of these proteins in the side compartment decreased to the level of the diffused proteins. Although axonally transported, both the 54–60-kDa and the 132–140-kDa proteins were not found in the axonal material in substantial amounts. If secreted by the regulated pathway, characterized by an intracellular half-life of about 10 h [51], about half the amount of a protein secreted

during 40 h should be in the intracellular stores by the end of the radioactive pulse. Hence, processing by the constitutive type of secretion, characterized by small intracellular stores and short intracellular half-life [51], is more likely. The absence of large amounts in the axons precludes the interpretation that these proteins are released from axons which died during the labeling period. Even death of all axons before the end of the experiment with quantitative release of these two proteins could not have yielded the quantities found in the culture medium. Hence, we consider it established that the presence of these proteins in the medium resulted from axonal secretion.

Since the most prominent and intriguing feature of these proteins is their axonal secretion, we propose to refer to the 132–140-kDa protein as axonin-1 and to the 54–60-kDa protein as axonin-2. Axonin-1 and axonin-2 are apparently the major secretory proteins of the axons of cultured chicken embryonic dorsal-root-ganglia neurons. However, in heavily overloaded gels, at least one additional candidate for an axonally secreted protein with apparent molecular mass of about 70 kDa is present (data not shown). The presence of both proteins in all neuronal cultures tested and their absence from all nonneuronal cultures suggests them to be neuron-specific; their occurrence in all neuronal cultures argues for expression by several, and possibly all, neuronal subpopulations. These data, however, have been obtained *in vitro* and in a study limited to six cultures each of neuronal and nonneuronal cells; the conclusions suggested will have to be verified in more extensive studies.

To the best of our knowledge, this is the first study on the identification of axonally secreted proteins by metabolic labeling and two-dimensional SDS/PAGE. Hence, data of similar studies are not available for comparison. A number of proteins released by dissociated cultures of sympathetic and sensory neurons have been identified on two-dimensional SDS/PAGE [52, 53] and nonequilibrium pH-gradient electrophoresis [15]. They are expected to include proteins secreted by axons; and both axonin-1 and axonin-2 could have their counterparts among the reported proteins. Differences in a variety of experimental parameters, including the animal

species used (rat versus chicken), the cell culture system (dissociated cultures versus the compartmentalized cell culture system), and the electrophoretic technique of the first dimension (nonequilibrium pH-gradient electrophoresis versus isoelectric focusing), prevent a comparison of the gel data. Two proteins released by cultures of sympathetic and various types of sensory neurons were characterized as a calcium-dependent metalloprotease and an urokinase-like plasminogen activator; both were demonstrated to be secreted from the distal part of the axons of sympathetic neurons [15]. Neither of these proteins has been identified on two-dimensional SDS/PAGE, and correlation with these data will require further molecular and functional studies.

The present study represents a first step in an approach to studying proteins secreted from axons during development of the nervous system, the time when axons elongate and make connections with target cells. The information on the coordinates and spot morphology of these proteins in two-dimensional SDS/PAGE provides an unequivocal assay for their purification. In an accompanying paper [54], we report on the purification of one of the axonally secreted proteins identified here.

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