Characterization of a Factor that Promotes Neurite Outgrowth: Evidence Linking Activity to a Heparan Sulfate Proteoglycan

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ABSTRACT Rat sympathetic neurons, plated onto extracellular matrix produced by cultured bovine corneal endothelial cells, rapidly extended neurites in the absence of nerve growth factor (NGF). The response was unaffected by antiserum to NGF. Rapid outgrowth also occurred when sympathetic neurons were plated onto polylysine-coated surfaces that had been exposed to serum-free medium conditioned by corneal endothelial cells (CM_{SF}). A response was seen even when the neurons were cultured without serum. When plated onto a polylysine-coated dish treated with CM_{SF} over half its surface, only the neurons on the treated half extended neurites.

The active factor in CM_{SF} was destroyed by trypsin, acid (pH 1.6), base (pH 12.7), or heating to 80°C; it was stable to heating to 60°C, collagenase, deoxyribonuclease, and neuraminidase. The factor elutes just after the void volume of a Sepharose 6B column. In associative cesium chloride gradients, it sediments as a peak centered at a density of 1.36–1.37, corresponding to a peak of material that can be biosynthetically labeled with [^{35}S]sulfate or [^{3}H]leucine. Material from this fraction was inactivated by heparinase, but not chondroitinase ABC, implying that a heparan sulfate proteoglycan is essential for the factor's activity. Inactivation by contaminants in the heparinase preparation was ruled out. Further purification indicated that the active factor may exist as an aggregate containing a heparan sulfate proteoglycan and other molecules.

CM_{SF} also promoted neurite outgrowth by other types of neurons. Furthermore, a variety of cell types were shown to produce factors similar to that in CM_{SF}.

An important determinant in the development of multicellular organisms is the extracellular matrix (ECM) upon which cells attach, migrate, and differentiate. It seems likely that a class of substances that affect neuronal development will be found to be associated with this extracellular matrix. The importance of the interaction between neuron and substratum has already been demonstrated in vitro (1–3). In vivo, axons appear to follow routes determined by the substratum (4). In some cases, this reflects the association of axons with already oriented cells, e.g., radial glia in the cerebellum (5) and the pioneering optic nerve fibers of *Daphnia* (6).

The existence of factors necessary for the normal development of certain neurons is established. Sympathetic and sensory neurons require nerve growth factor (NGF) for survival and development in vivo and in vitro (7). Certain other factors appear to be necessary for the survival and development of cultured parasympathetic neurons (8, 9). Other factors, few of which have been significantly purified, have recently been reported to promote the development of sympathetic, parasympathetic, sensory, and spinal cord neurons in culture (10–24). If such factors are supplied by the cells that innervate or are innervated by certain types of neurons, it may explain why appropriate intercellular contacts must be made in order for many types of neurons to survive in vivo (25, 26).

Among these factors, a few are likely candidates for regulatory substances associated with the ECM in vivo (16, 17, 21). The factors in this group all promote the outgrowth of neurites from certain types of neurons in vitro. Some have been shown to be effective only when attached to the culture substratum (17, 21). The response they induce is rapid, and those factors that have been tested on sympathetic neurons promote neurite outgrowth from these cells, even when they are cultured in the

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 SEPTEMBER 1982 574-585

absence of NGF.

In this report, a neurite outgrowth-promoting factor found in medium conditioned by bovine corneal endothelial cells is described. The factor acts on cultured sympathetic, sensory and spinal cord neurons. Its activity requires attachment to the culture surface. The factor has been characterized and partially purified. It appears to be a very large molecule or a molecular aggregate. Evidence is presented that a heparan sulfate proteoglycan is an essential component of this factor.

MATERIALS AND METHODS

Materials

Hyaluronic acid (grade IV), heparin (grade I) and chondroitin sulfate (mixed isomers, grade III) were obtained from Sigma Chemical Co., St. Louis, MO. Dermatan sulfate, keratan sulfate, and heparan sulfate were reference standards that were generously donated by Dr. Martin B. Mathews and Dr. J. A. Cifonelli (Dept. of Pediatrics, University of Chicago, Chicago, Ill.). Heparan sulfate was also supplied by Upjohn Laboratories (Kalamazoo, MI), and it was this heparan sulfate that was coupled to Sepharose. Antiserum against NGF was the generous gift of Dr. Eric M. Shooter (Stanford Medical School, Stanford, CA). Crude heparinase was kindly donated by Dr. Alfred Linker (Veterans' Administration Hospital, Salt Lake City, UT). Other reagents, where not specified, were obtained from commercial sources.

Dissociated Neurons

Superior cervical ganglia from 1–2-d-old Sprague-Dawley rats were dissociated by a modification of an enzymatic procedure (27). Ganglia were cleaned, stripped, and incubated for 30 min at 37°C in 0.25% trypsin (Sigma Chemical Co.) in a holding solution of basal L-15 Medium with added glucose (6 g/L), penicillin (100 U/ml), streptomycin (100 μg/ml) and glutamine (2 mM). Ganglia were washed three times with holding solution, resuspended in medium containing serum, and triturated using a 9-in. pasteur pipette that had been flame-polished to about half its original tip diameter. Fragments were allowed to settle, and the supernatant liquid, containing dissociated cells, was removed. For serum-free cultures, ganglia that had been incubated with trypsin were washed twice with holding solution containing soybean trypsin inhibitor (2.5 mg/ml, Sigma Chemical Co.) and twice with holding solution alone. The ganglia were then triturated in serum-free medium. For cultures of cells dissociated without trypsin, ganglia were dispersed mechanically (28).

Enzymatically dissociated dorsal root ganglion cells were prepared from newborn Sprague-Dawley rats (29) and 8-d chick embryos (30). Lumbar sympathetic chains from 11-d chick embryos were dissociated enzymatically (31). Cerebella and olfactory bulbs were removed from newborn Sprague-Dawley rats and dissociated mechanically (32).

Rat sympathetic and sensory neurons were cultured in the "complete L-15-CO_2" medium of Hawrot and Patterson (28), containing 5% adult Sprague-Dawley rat serum. Methocel (methylcellulose) was omitted and NGF was added only in those experiments where noted. In these cases, the 7S form of NGF, prepared from male mouse salivary glands (33), was used at a concentration determined to promote optimal neurite outgrowth in cultures of rat sympathetic neurons. For cultures without serum, transferrin (100 μg/ml), progesterone (20 nM), putrescine (100 μM), selenium (30 nM), and insulin (5 μg/ml) were added (34).

Chick sympathetic and sensory neurons were cultured in Dulbecco's Modified Eagle's Medium (DME) containing 0.5% glucose, supplemented with 5% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). Rat cerebellar and olfactory bulb cells were cultured as described (32).

Routinely, dissociated cells were plated at a density of 1–2 × 10^2 cells/mm^2, as determined by hemacytometer counts. Cultures were incubated at 37°C in a humidified atmosphere containing either 5% CO_2 (for cultures in L-15-CO_2-based medium) or 10% CO_2 (for other cultures).

Preparation of Culture Substrata

Dissociated neurons were cultured in polystyrene dishes or multiwell plates (Falcon) that had been treated for tissue cultures. Culture surfaces were exposed to poly-D-lysine (Sigma Chemical Co.; type I-B, 1 mg/ml in 0.1 M sodium borate, pH 8.4) overnight, washed with twice-distilled water, and sterilized under ultraviolet light. Cells were either plated directly onto these surfaces, or onto polylysine-coated surfaces that had been further treated with solutions to be tested for neurite outgrowth-promoting activity. These solutions were applied for 8–16 h at 4°C, after which the culture surfaces were washed at least three times in basal L-15, in which they could be stored. Complete medium was added shortly before cells were plated. Polylysine-coated dishes that had been further treated in this way on only half of their surfaces were prepared as described (17).

Evaluating Neurite Outgrowth

Since the neuronal cultures studied also contained non-neuronal cells, only relatively large, round, phase-bright cells were counted as neurons whenever neurite outgrowth was assessed by phase-contrast microscopy. Of these, only cells bearing processes greater than two cell diameters in length and possessing either a well-visualized growth cone or at least one bifurcation were scored as neurite-bearing neurons. Since the aim of these assays was to quantify cell-substratum interactions, neurons whose cell bodies or processes were confined predominantly to the surface of non-neuronal cells (e.g., fibroblasts) were eliminated from counting. Using these criteria, reliable and reproducible results were obtained. For photography, cultures were rinsed gently in Dulbecco's phosphate buffered saline (PBS), and fixed in PBS containing 2% glutaraldehyde and 5% sucrose.

Non-neuronal Cell Culture

Cultures of bovine corneal endothelial cells were established from steer eyes (35, 36). Stocks were maintained in DME containing 0.1% glucose, and supplemented with 10% calf serum and 5% FCS (Irvine Serum Company, Irvine, CA), glutamine (2 mM), Fungizone (Squibb & Sons, Inc., Princeton, NJ, 2.5 μg/ml), and gentamycin (Garamycin, Schering Corp., Kenilworth, NJ, 50 μg/ml). Fibroblast growth factor (FGF), prepared as described (37), was added at 100 ng/ml every other day until the cells reached confluence.

Bovine vascular endothelial cell and vascular smooth muscle cell cultures were established from bovine aortic arch (38, 39). Cell stocks were maintained in DME (0.1% glucose) with 10% calf serum for endothelial cells, or 5% bovine serum for smooth muscle cells. Glutamine, antibiotics, and FGF were added as above. Human foreskin fibroblasts obtained from the UCSF culture facility were grown in DME (0.5% glucose) with 10% FCS, and glutamine and antibiotics as above. Bovine adrenal cortical cells (40) were grown in F12 medium, with 5% heat-inactivated horse serum (Colorado Serum Co., Denver, CO), 5% FCS, and glutamine and antibiotics as above. PTK-1 cells (American Type Culture Collection, Rockville, MD) were grown in DME (0.1% glucose) with 10% newborn calf serum, penicillin (100 U/ml) and glutamine and streptomycin as above. C_2 cells (41) were grown in DME (0.1% glucose) with 5% heat-inactivated horse serum, glutamine, penicillin, and streptomycin as above. A-431 cells (42) and N-18 cells (43) were grown in DME (0.1% glucose), with 10% FCS, and glutamine, penicillin, gentamycin, and streptomycin as above.

Plastic dishes coated with ECM produced by corneal endothelial cultures were prepared as described (44). Briefly, confluent cultures were first washed with distilled water and then exposed to 0.02 M NH_4OH in distilled water for 5 min, followed by washing and storage in PBS. Nuclei and cytoskeletal elements have not been detected on these plates (45). Only trace amounts of the FGF used to feed cells were detectable in the ECM (46).

Preparation of Conditioned Media

Conditioned medium from corneal endothelial cells (CM) refers to complete culture medium in which confluent monolayers of cells were grown for 5–7 d. Serum-free conditioned medium (CM_{SF}) was prepared by incubating confluent monolayers for 5 d in DME, without serum, supplemented with glutamine (2 mM), gentamycin (50 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and Fungizone (2.5 μg/ml). CM and CM_{SF} were filtered through a 0.45 um filter before storage at 4°C. Conditioned media from confluent cultures of other cell types were similarly prepared. The abbreviations CM and CM_{SF}, however, are reserved for material derived from corneal endothelial cell cultures.

Physical, Chemical, and Enzymatic Treatments

Heat: CM_{SF} was heated in a water bath for 30 min to either 60°C or 80°C, and then cooled on ice.

Acid and Base: CM_{SF} was brought to pH 1.6 with 1 N HCl or pH 12.7 with 1 N NaOH. After 1 h at room temperature, samples were neutralized with 1 N NaOH and 1 N HCl, respectively. As a control for the slight increase in salt concentration and volume in both, a third sample, to which acid and base were premixed and then added, was used.

Enzymes: Trypsin (Sigma Chemical Co., type III) was used at 100 μg/ml for 2 h at 37°C, after which soybean trypsin inhibitor was added to a final concentration of 500 μg/ml. As a control, trypsin inhibitor was added before the trypsin. Deoxyribonuclease I (Sigma Chemical Co., type III) was used at 100 μg/ml for 2 h at 37°C. Collagenase (Worthington Biochemical Corp., Freehold, NJ, 200

U/mg) was used at 20 U/ml for 2 h at 37°C. Neuraminidase (Sigma Chemical Co., type IX) was used at 2.5 U/ml for 5 h at 37°C. Chondroitinase ABC (Sigma Chemical Co.) was used at 2 U/ml for 6 h at 37°C. Crude heparinase was used at indicated concentrations and times, at 30°C.

Gel Filtration

A column (31 × 0.67 cm) of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated at 4°C in 50 mM Tris-HCl, pH 7.4. The void volume was measured as the volume from which blue dextran 2000 (Pharmacia Fine Chemicals) was completely excluded. 0.5-ml samples were applied and fractions were eluted at 2.5 ml/h.

Metabolic Labeling

For labeling with [^{3}H]leucine, confluent monolayers of corneal endothelial cells were washed into the serum-free medium prepared with leucine-free DME. After 2 h of incubation, this medium was removed and more of the same medium, now containing L-[4,5-^{3}H]leucine (Amersham, 50.6 Ci/mmol) at 25 μCi/ml was added. After 2 d of incubation, cold leucine was added to a final concentration of 10 μg/ml. The dishes were incubated two more days and the medium was collected and filtered through a 0.22 μm filter.

For labeling with [^{35}S]sulfate, cultures were washed into serum-free medium as described above, using sulfate-free rather than leucine-free DME. After 2 h of incubation, this medium was removed and more of the same medium, now containing $Na_2[^{35}S]O_4$ (Amersham Corp., Arlington Heights, IL, carrier-free) at 40 μCi/ml, was added. After 4 d of incubation, the medium was collected and filtered through a 0.22 μm filter.

During the entire course of these incubations, cells appeared healthy, and did not detach from the dish. After 4 d, most of the radioactivity had still not been incorporated into macromolecules. Batches of labeled CM_{SF} were always tested and shown to possess neurite outgrowth-promoting activity. All radioactive samples were counted in Aquasol (New England Nuclear, Boston, MA). When ^{3}H and ^{35}S were counted in the same samples, appropriate corrections were made.

Isopycnic Centrifugation

Samples were concentrated 5- to 10-fold by dialysis against polyethylene glycol ("20-M", Union Carbide Corp., New York, NY) or sucrose. Two types of gradients were prepared, as defined by Sajdera and Hascall (47): dissociative gradients, for which samples were dialyzed exhaustively against 4 M guanidinium chloride (GuHCl) in 0.05 M Tris-HCl, pH 7.4, and associative gradients for which samples were dialyzed exhaustively against 0.4 M GuHCl in 0.05 M Tris-HCl, pH 7.4. Solid CsCl was then added to a final concentration of 31% (wt/wt) for dissociative gradients, or 37% (wt/wt) for associative gradients. These samples were centrifuged at 5°C to equilibrium (~48 h) at 40,000 rpm using a SW 50.1 rotor (DuPont Instruments, Newtown, CT) (48). Gradients were eluted and the density of fractions was determined by weighing volumes in preweighed 10 μl micropipettes; the radioactivity of these samples was then counted. Each fraction was dialyzed against two changes of 0.05 M Tris-HCl before further testing.

Immobilization of Glycosaminoglycans on Sepharose

Oxirane-activated Sepharose 6B was prepared as described (49). Glycosaminoglycans were resuspended at 10 mg/ml in sodium carbonate buffer (0.1 M, pH 9.5). Activated Sepharose was rinsed in this buffer, and for each gram of Sepharose (suction-dried weight) 1 ml of glycosaminoglycan solution was added. After 24 h at 37°C, the liquid was eluted, and the Sepharose washed and blocked with ethanolamine (49). The Sepharose was then packed into columns and equilibrated in 0.1 M sodium acetate, pH 7.0. Coupling was quantified by measuring the disappearance of free glycosaminoglycans from the reaction mixture using the carbazole method for the determination of uronic acids (50). In this manner, heparan sulfate-Sepharose and chondroitin sulfate-Sepharose were prepared containing 1 mg/ml and 0.7 mg/ml glycosaminoglycan of packed Sepharose, respectively.

RESULTS

Effect of Corneal Endothelial Extracellular Matrix on Sympathetic Neurons

Neonatal rat sympathetic neurons require NGF for survival and development in vivo (7). Similarly, in vitro, only when NGF is present do these cells survive and extend neurites when cultured on traditional substrata, such as tissue culture plastic, and collagen- or polylysine-coated surfaces. In the absence of NGF, they fail to put out processes, and quickly die (e.g., 33). In the present study, rat sympathetic neurons were cultured on ECM produced by cultured corneal endothelial cells. Under these conditions, neurite outgrowth was seen whether or not NGF was provided. Fig. 1 *a* shows cells plated on ECM and cultured without NGF. For comparison, cells from an NGF-supplemented culture grown on polylysine are shown in Fig. 1 *b*. In the latter type of culture, neurite formation could be completely blocked by antiserum to NGF (Fig. 1 *d*). Outgrowth on ECM, however, was unaffected when anti-NGF was preincubated with the ECM and, in addition, added to the culture medium at a concentration sufficient to bind 0.5 μg/ml of β-NGF, the active subunit of NGF (Fig. 1 *c*). This NGF concentration is 50 times that required for an optimal biological response.

The behavior of neurons on ECM was unusual in other ways (Table I and Fig. 2). Neurites appeared earlier and grew more rapidly on ECM than on polylysine-coated plastic. Within 6 h after plating onto ECM, >80% of the neurons had neurities, many of them several cell diameters in length. In contrast, neurons plated onto polylysine and cultured with NGF had very few processes by 6 or even 12 h, although most cells extended neurites by 24 h.

Although neurites appeared early and grew rapidly on ECM in the absence of NGF, their rate of growth slowed dramatically after 24 h and cell viability (estimated by morphological criteria) fell steadily thereafter. By 72 h, <20% of the neurons appeared alive; none survived over 5 d. Massive cell death could be avoided only if NGF was present in the culture medium. Then, good viability was maintained for over a week, the longest time assayed. It appears, therefore, that despite the outgrowth-promoting effects of ECM, rat sympathetic neurons still require NGF for survival.

An additional characteristic of cultures grown on ECM was the morphology of the neurons. When compared to controls (cells grown on polylysine with NGF), the average number of neurites projecting from each cell body was greater, many processes were more highly branched, and a significant number of processes appeared unusually thin.

Sympathetic neurons prepared by mechanical rather than enzymatic dissociation responded equally well to ECM, and had a similar viability, indicating that the effects observed did not depend on some change in cell function caused by exposure to trypsin (not shown).

Surfaces Treated with Conditioned Medium Can Substitute for ECM

To see whether the neurite outgrowth-promoting component(s) of ECM could be recovered in soluble form, medium conditioned by corneal endothelial cells was prepared. Polylysine-coated tissue culture dishes were exposed to this conditioned medium (CM) and washed thoroughly. Sympathetic neurons plated onto this substratum responded as they did on ECM, by rapidly extending neurites. Serum-free conditioned medium (CM_{SF}) applied in this way also produced an active substratum (Table II). Therefore the active substance(s) is synthesized and secreted by corneal endothelial cells, and is not a modified or concentrated component of serum.

Table II also shows the results obtained when neurons plated on CM_{SF}-treated polylysine surfaces were cultured without serum. Since rapid neurite outgrowth was also observed under

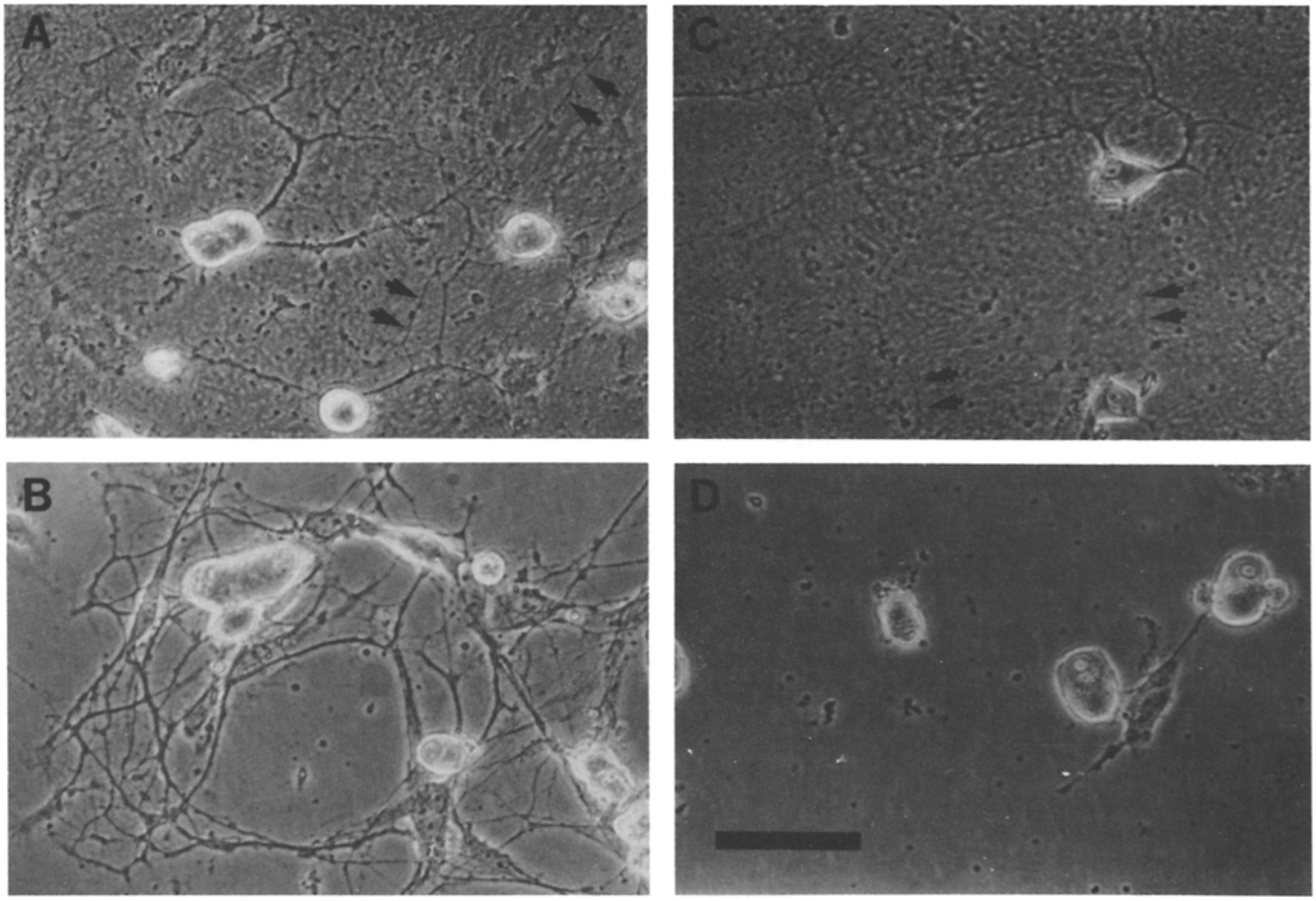

FIGURE 1 Effect of anti-NGF on the response of neurons to ECM and NGF. Rat sympathetic neurons were grown on ECM without NGF (*a*) and on a polylysine substratum with NGF (*b*). In *c*, cells were grown as in *a*, except ECM was preincubated for 2 h at 37°C with anti-NGF and anti-NGF was present in the culture medium. In *d*, cells were grown as in *b*, except anti-NGF was present in the culture medium. Representative groups of cells were photographed after 24 h in culture. The substratum-attached material constituting ECM appears, by phase microscopy, as a mottled background. Thick neurites are easily seen above this background. Fine neurites, some of which are marked with arrows, are more difficult to discern. Bar, 50 μm. × 380.

TABLE I

Comparison of the Response of Neurons to NGF and ECM

		Hours after plating				
Substratum	Medium	6	12	24	48	72
		% Neurite outgrowth				
Polylysine	+ NGF	2	6	75	86	84
ECM	− NGF	86	78	73	40	2
		% Neuronal survival				
Polylysine	+ NGF	94	94	86	87	85
ECM	− NGF	99	93	78	49	17

Rat sympathetic neurons cultured with NGF on polylysine-coated tissue culture plastic and without NGF on ECM were fixed at various times after plating. Neurite outgrowth was measured by counting random fields and determining the percentage of presumptive neurons with neurites. Survival was estimated crudely as the percentage of presumptive neurons lacking morphological signs of cell death or injury, including cell swelling, loss of adhesiveness, retraction of neurites, and accumulation of intracytoplasmic "granules." Over 100 neurons were counted for each point shown above.

these conditions, the CM_{SF}-coated surface was not acting merely by adsorbing and concentrating some serum component onto the substratum. Instead it appears to act directly on sympathetic neurons. Fig. 3 shows the appearance of cells on CM_{SF}-treated polylysine-coated surfaces when grown in the presence and absence of serum.

When dishes of tissue-culture plastic or petri plastic that had not been coated with polylysine were exposed to CM_{SF}, they did not support rapid neurite outgrowth. When only half of a polylysine-coated dish was treated with CM_{SF}, only the neurons plated on that half responded with rapid outgrowth (not shown). This indicates that the active component(s) affects neurons as a substratum-bound factor, not by diffusing into the medium. Indeed, binding of the component(s) to the substratum must be tight, since several days of intermittent washing and storage did not diminish the activity of treated dishes.

Preliminary Characterization of the Active Factor(s)

A standard assay for neurite outgrowth-promoting activity was developed. Polylysine-coated microwells were treated with samples to be assayed, sympathetic neurons were plated, and the percentage of neurons with neurites was scored at 12–18 h. Fig. 4 shows a dose-response curve for dilutions of CM_{SF}. Titers calculated from these curves were similar for different batches of CM or CM_{SF}. Even when the neurons were supplied with NGF, the dose-response was unchanged, provided outgrowth was assayed at ~12 h (not shown).

To characterize its active factor(s), CM_{SF} was subjected to various treatments and assayed. As shown in Table III, it was

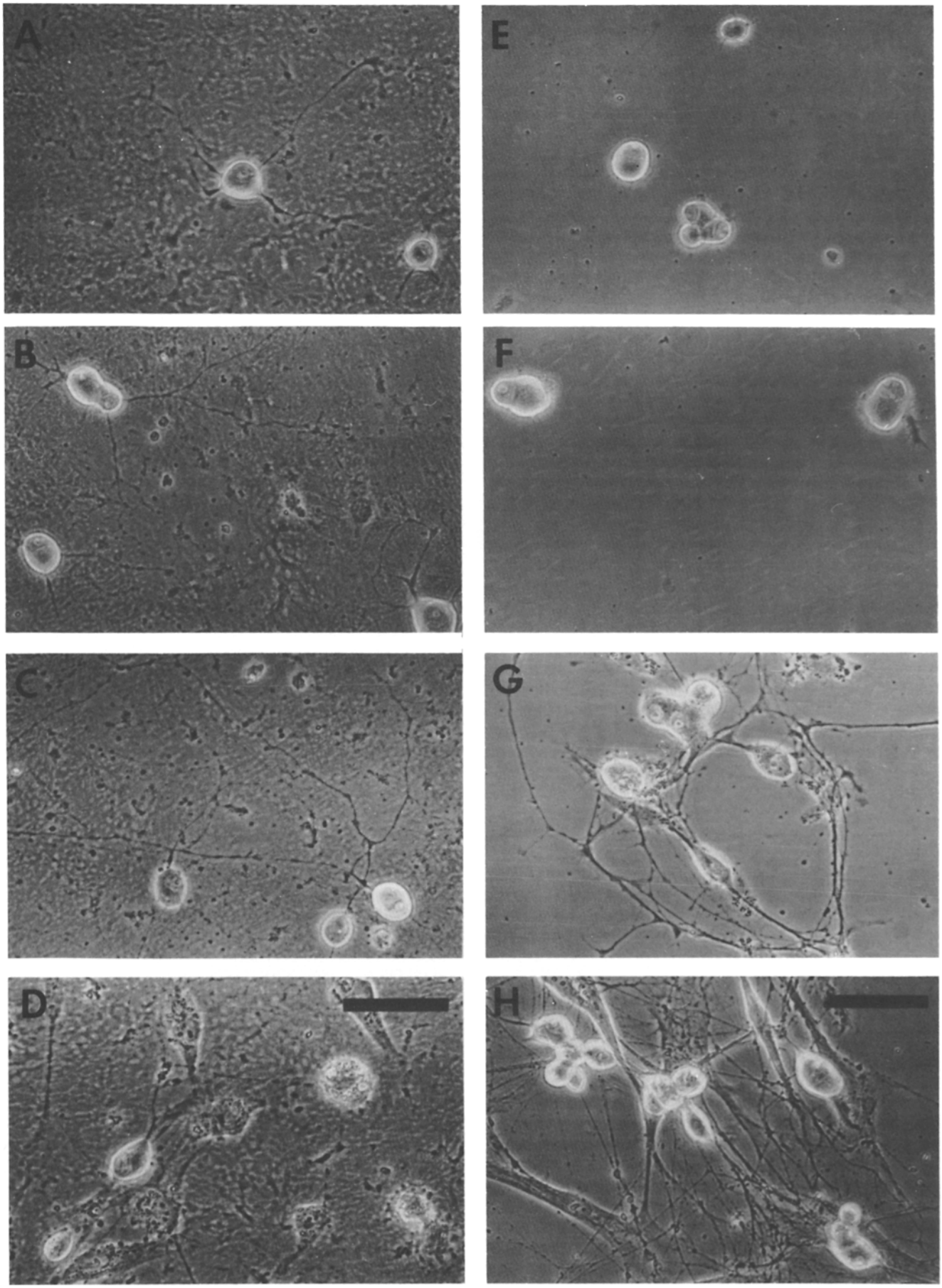

FIGURE 2 Comparison of responses to NGF and ECM. Rat sympathetic neurons grown on ECM without NGF (*a–d*) and on a polylysine substratum with NGF (*e–h*) were fixed at various times after plating, and photographed (*a*, *e*–6 h; *b*, *f*–12 h; *c*, *g*–24 h; *d*, *h*–48 h). Bars, 50 μm. × 380.

TABLE II

Neuronal Response to CM_{SF} in the Presence and Absence of Serum

Substratum	Medium	Hours after plating 5	13	24	48
		% Neurite outgrowth			
CM_{SF}-treated polylysine	+ serum	18	56	63	35
	− serum	57	58	48	21
		% Neuronal survival			
CM_{SF}-treated polylysine	+ serum	95	96	91	48
	− serum	89	89	81	25

Rat sympathetic neurons were plated on polylysine-coated tissue culture plastic that had been treated with CM_{SF}. Neurons were cultured without NGF, and with or without serum. Neurite outgrowth and survival were determined as in Table I.

inactivated by incubation at low or high pH, or by heating to 80°C for 30 min. Heating to 60°C, however, did not reduce activity. Trypsin inactivated CM_{SF}, and this was the result of specific proteolysis, since inactivation could be blocked by trypsin inhibitor. No decrease in the activity of CM_{SF} was seen after exposure to deoxyribonuclease, collagenase, or neuraminidase.

Partial Purification of the Active Factor

CM_{SF}, concentrated fivefold by dialysis against polyethylene glycol, was fractionated on a column of Sepharose 6B (exclusion limit 4×10^6 for globular proteins; 1×10^6 for polysaccharides). Fractions were collected and assayed. As shown in Fig. 5, neurite outgrowth-promoting activity eluted just after the void volume in a broad peak.

CM_{SF} was also fractionated by isopycnic sedimentation in CsCl density gradients, under nondissociating conditions. In preliminary experiments, activity was found at densities between 1.3 and 1.4. Since this is between the densities of pure proteins and polysaccharides, it appeared that the active factor might contain carbohydrate as well as protein. To obtain more detailed information, CM_{SF}, metabolically labeled with [^{3}H]-leucine and [^{35}S]sulfate, was prepared. With some qualifications (51), the sulfate label is specific for glycosaminoglycans and, therefore, marks the position of proteoglycans in these gradients. The leucine label marks proteins and, therefore, also glycoproteins and proteoglycans. Concentrated CM_{SF}, to which aliquots of leucine-labeled and sulfate-labeled CM_{SF} were added, was then centrifuged in CsCl as before. Fractions were collected and assayed for density, radioactivity, and neurite outgrowth-promoting activity. As illustrated in Fig. 6, this activity was confined to a single peak that matched a major peak of sulfate- and leucine-labeled material. Centered at a density of 1.36–1.37, this peak was clearly distinct from the broad peak of lower density representing the bulk of proteins and glycoproteins present in CM_{SF}. These experiments were repeated several times using different batches of CM_{SF} and labeled CM_{SF}, and this collection of corresponding peaks of sulfate, leucine, and activity was always seen centered at about the same density (1.35–1.39). These results indicate that a sulfated proteoglycan is present in the active fractions and may represent the active factor.

Characterization of the Partially Purified Factor

Further evidence implicating a proteolgycan as the active factor was obtained when active fractions from the CsCl gradients described above were pooled and incubated with the enzymes heparinase or chondroitinase ABC, and then assayed for activity. The results in Table IV *A* demonstrate that neurite outgrowth-promoting activity is lost after treatment with heparinase, which digests heparan sulfate and heparin, but not after treatment with chondroitinase ABC, which digests hyaluronic acid, chondroitin 4- and 6-sulfate, and dermatan sulfate (52).

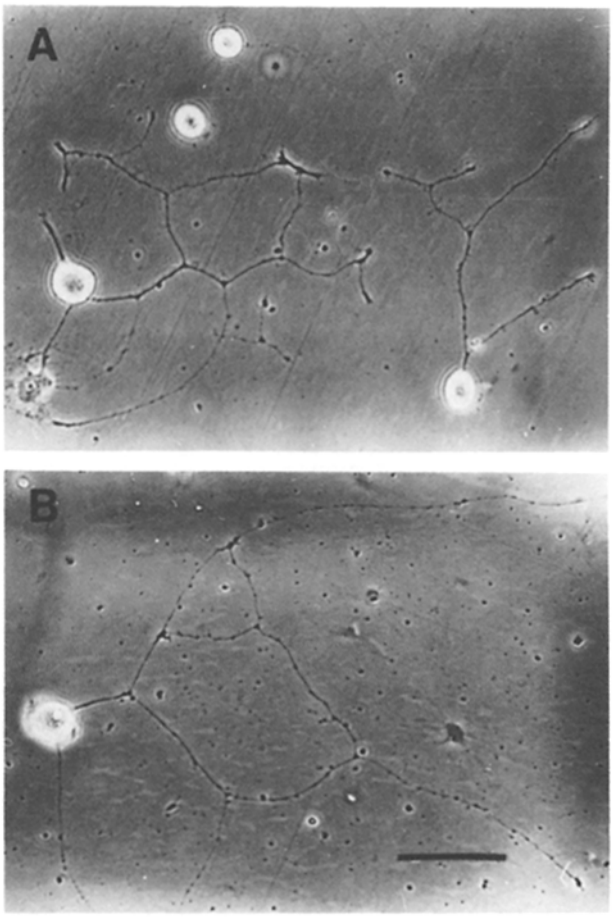

FIGURE 3 Response of neurons to CM_{SF}-treated substrata. Rat sympathetic neurons plated on CM_{SF}-treated polylysine substrata, cultured without NGF for 13 h in serum-containing (*a*) and serum-free medium (*b*). Bar, 50 μm. × 280.

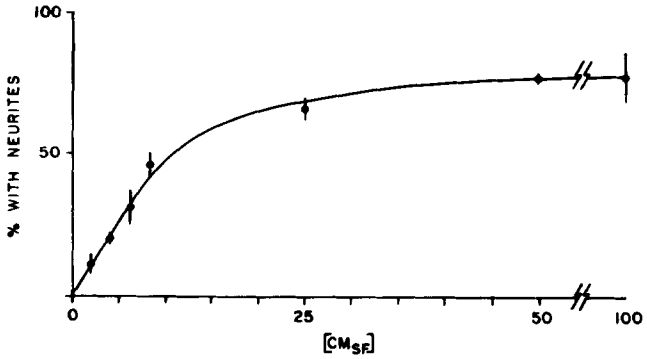

FIGURE 4 Dose-response curve for CM_{SF}-promoted neurite outgrowth. Dilutions of CM_{SF} were assayed for neurite outgrowth-promoting activity as described. Concentration is expressed as a percentage of undiluted CM_{SF}. Neurite outgrowth is expressed as percentage of total neurons with neurites. Assays were performed in triplicate; the data are graphed as averages ± 1 SD

TABLE III

Partial Characterization of CM_{SF}

Treatment	% Neurite Outgrowth
None	38 ± 2
Acid (pH 1.6)	3 ± 3
Base (pH 12.7)	12 ± 3
Acid-Base control (pH 7.4)	46 ± 6
60°C	35 ± 9
80°C	4 ± 1
Trypsin	0 ± 1
Trypsin + trypsin inhibitor	41 ± 6
Deoxyribonuclease	40 ± 8
Collagenase	39 ± 1
Neuraminidase	43 ± 6

CM_{SF} was exposed to various treatments and then assayed, as described, for neurite outgrowth-promoting activity. Data, averages of duplicate assays each representing counts of 50–100 neurons, are presented ± the deviation of each assay from the mean.

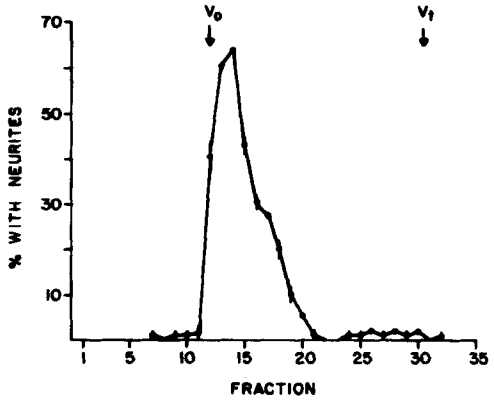

FIGURE 5 Sepharose 6B chromatography of CM_{SF}. CM_{SF} was fractionated by gel filtration, as described. Fractions were assayed for neurite outgrowth-promoting activity.

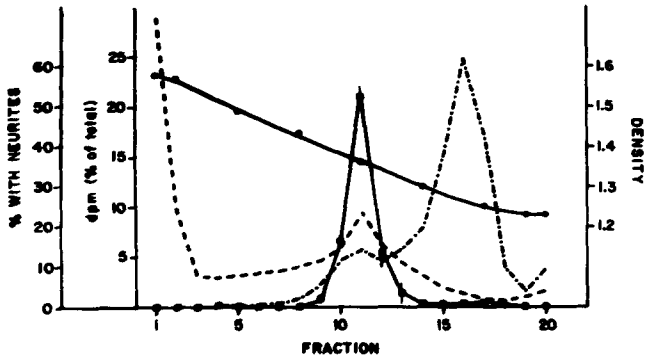

FIGURE 6 Isopycnic sedimentation in associative cesium chloride gradients. CM_{SF}, labeled with [^{3}H]leucine and [^{35}S]sulfate, was centrifuged in CsCl containing 0.4 M GuHCl, as described in Materials and Methods. The density (—●—) and radioactivity of fractions were measured. ^{3}H (-·-·-) and ^{35}S (-----) were expressed as a percentage of total dpm. After being dialyzed, fractions were assayed for neurite outgrowth-promoting activity (—■—).

This result suggests that the active factor is a heparan sulfate-containing proteoglycan. However, since the heparinase used was a crude preparation it may have been contaminated with other activities (e.g. proteolytic enzymes and enzymes that degrade other glycosaminoglycans). Therefore, two experiments were performed to confirm that specific digestion of heparan sulfate was responsible for inactivation of the factor.

First, serial dilutions of heparinase were tested for their ability to inactivate the factor. Appropriate dilutions were then retested in the presence and absence of bovine serum albumin (0.2 mg/ml). The data are presented graphically in Fig. 7. Since the ability of heparinase to inactivate the factor was not diminished by this concentration of albumin, it is unlikely that nonspecific proteolysis, if present, could account for the observed effects of heparinase.

Second, aliquots of heparinase were passed, at 4°C, over columns of either heparan sulfate-Sepharose or chondroitin sulfate-Sepharose (Table IV *B*). The eluent from the heparan sulfate column no longer possessed the ability to inactivate the factor; the eluent from the chondroitin sulfate column retained this ability, as did an equivalent aliquot of heparinase which was not run on either column but simply diluted to the same volume as the eluted samples. Therefore it is only that component of heparinase which binds heparan sulfate, and does not bind chondroitin sulfate, that is responsible for inactivating the factor.

The observation that activity was somewhat increased after treatment with the fraction of heparinase that did not bind heparan sulfate requires explanation. Perhaps this fraction contains molecules which modify the active factor, altering the way in which it presents itself to neurons; alternatively, this fraction may contain enzymes which digest molecules that are present in the partially purified factor and inhibit its active component.

Although enzymatic digestion of heparan sulfate apparently inactivated the factor isolated from CM_{SF}, neither heparan sulfate, nor any other purified glycosaminoglycan, showed any

TABLE IV

Effect of Glycosaminoglycan-degrading Enzymes on the Neurite Outgrowth-promoting Factor

A	Crude Heparinase*	Chondroitinase ABC‡	Control§
	% Neurite Outgrowth		
Partially purified factor	1 ± 1	44 ± 10	53 ± 7
CM_{SF}	3 ± 3	46 ± 5	47 ± 2

B	
Treatment of Crude Heparinase	*% Neurite Outgrowth*
Control‖	38 ± 0.2
Eluted from heparan sulfate-Sepharose	48 ± 0.6
Eluted from chondroitin sulfate-Sepharose	13 ± 2.4
Crude heparinase¶	2 ± 0.4
Crude heparinase**	28 ± 0.3

In *A*, Pooled active fractions from an associative gradient and CM_{SF} were treated with heparinase and chondroitinase ABC for 6 h and assayed. In *B*, 50-μl aliquots of heparinase (6.0 mg/ml in 0.1 M sodium acetate, pH 7.0) were applied to columns containing 100 μl of glycosaminoglycan-conjugated Sepharose and eluted, at 4°C, in 200 μl of buffer. Controls were not chromatographed, but were simply diluted with buffer to 1.5 and 0.15 mg/ml. Samples were added to 4 vol of partially purified factor and the mixture was incubated for 4 h at 30°C and assayed for neurite outgrowth-promoting activity. As in Fig. 7, a dilution of the factor affording maximal sensitivity was used. Data are averages of duplicate assays ± deviation from the mean.

* 1 mg/ml.
‡ 2 U/ml.
§ No enzyme.
‖ No heparinase.
¶ Final dilution, 300 μg/ml.
** Final dilution, 30 μg/ml.

neurite outgrowth-promoting activity when bound to polylysine-coated plastic and assayed (Table V).

In the light of these results, it seems likely that a heparan sulfate proteoglycan present in the partially purified material is responsible for neurite outgrowth-promoting activity. Whether it alone is responsible for this activity, however, was investigated subsequently.

Further Purification of the Active Factor

Proteoglycans which form complexes with hyaluronic acid have been described (47) and glycosaminoglycans are known to bind to collagens and fibronectin (53, 54). It seemed possible that the neurite outgrowth-promoting factor might have been isolated as part of a complex with such molecules, since aggregates would probably not dissociate during purification by gel filtration or density sedimentation in CsCl gradients containing 0.4 M GuHCl (associative gradients). To investigate this possibility, labeled, partially purified factor was recentrifuged in CsCl containing 4 M GuHCl (a dissociative gradient). Under these conditions, previously characterized proteoglycan complexes have dissociated (e.g. reference 47).

When fractions from such a gradient were collected (Fig. 8), radioactivity was no longer found only in the density range 1.34–1.39, i.e., the range from which the applied material had originally been pooled. Some of the ^{35}S appeared in a peak, also containing ^{3}H, that sedimented at a higher density (1.40–1.45). This behavior would be expected of a proteoglycan that had formerly been associated with less dense molecules, such as proteins. In agreement with this, a substantial peak of ^{3}H was found at a lower density appropriate for a protein or glycoprotein. Some of the ^{35}S and ^{3}H found between these two peaks was in a position consistent with proteoglycans that had remained at their original apparent density—these may or may not have been associated with other molecules. Some of the ^{35}S sedimented as a peak with a density so low (1.29–1.31) that it might represent sulfate-labeled material other than proteoglycans (cf. reference 51).

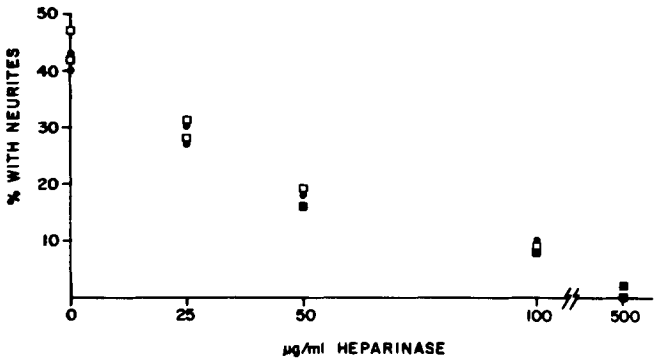

FIGURE 7 Effect of heparinase concentration on inactivation of the partially purified factor. Partially purified factor (pooled active fractions from an associative gradient) was incubated with various concentrations of heparinase in the presence (●) and absence (□) of bovine serum albumin (0.2 mg/ml), and tested for neurite outgrowth-promoting activity. To ensure maximal sensitivity, partially purified factor was used at a dilution, the activity of which fell on the steeply rising portion of the dose-response curve (cf. Fig. 4).

TABLE V

Activity of Purified Glycosaminoglycans

Substance applied	Amount bound	Neurite outgrowth
	$\mu g/cm^2$	%
Hyaluronic acid	0.31	<1
Dermatan sulfate	0.54	<1
Keratan sulfate	—	<1
Chondroitin sulfate (mixed isomers)	0.63	<1
Heparan sulfate	0.45	<1
Heparin	0.73	<1
CM_{SF}	—	74 ± 3

Glycosaminoglycans, at 1 mg/ml in PBS, were assayed for neurite outgrowth-promoting activity. Some of the glycosaminoglycan-treated polylysine-coated surfaces were not used for culturing neurons, but were treated with concentrated sulfuric acid to remove bound material. These samples were then assayed for total uronic acid by the carbazole method (50). By correlating the results obtained with standard curves prepared for each glycosaminoglycan, it was possible to calculate the data shown above. Since keratan sulfate does not contain uronic acid, its binding was not assayed. Data are averages of duplicate assays ± deviation from the mean.

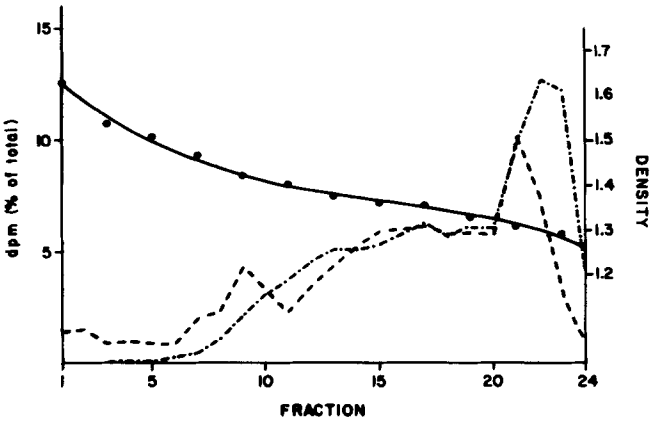

FIGURE 8 Fractionation of the partially purified factor by isopycnic sedimentation under dissociating conditions. Active fractions from associative gradient centrifugation (fractions 10–12 in Fig. 6) were pooled and recentrifuged in CsCl containing 4 M GuHCl. Density (—●—) and radioactivity were measured. ^{3}H (-·-·-) and ^{35}S (- - - - -) were expressed as a percentage of total dpm.

Attempts to discover which of these components was responsible for neurite outgrowth-promoting activity were unsuccessful. None of the gradient fractions showed biological activity, nor did any combination of pooled fractions that was tested. Indeed, simply exposing either CM_{SF} or the partially purified factor to 4 M GuHCl, followed by dialysis, eliminated activity. Similarly, when ECM was extracted with 4 M GuHCl, activity disappeared from the ECM and could not be recovered from the extract after dialysis (not shown). Thus, it appears that active factor is inactivated by GuHCl at this high concentration. This was not unexpected, considering the strongly chaotropic properties of this agent.

Preliminary characterization of material isolated from dissociative gradients has indicated that nitrous acid-degradable polysaccharides are present in both of the peaks of ^{35}S believed to represent proteolgycans (not shown). This implies that heparan sulfate proteoglycans are present in these peaks, since heparin and heparan sulfate are the only glycosaminoglycans degraded by nitrous acid (55). Failure to recover biological activity after dissociation, however, has made it impossible to identify which of the proteoglycans present in the partially purified factor is (are) necessary for activity. Neither has it been possible to determine whether protein associated with the partially purified factor is related to the factor's activity.

Effect of CM_{SF}-treated Substrata on Other Types of Neurons

In addition to rat sympathetic neurons, other neuronal cultures were plated onto CM_{SF}-treated, polylysine-coated sub-

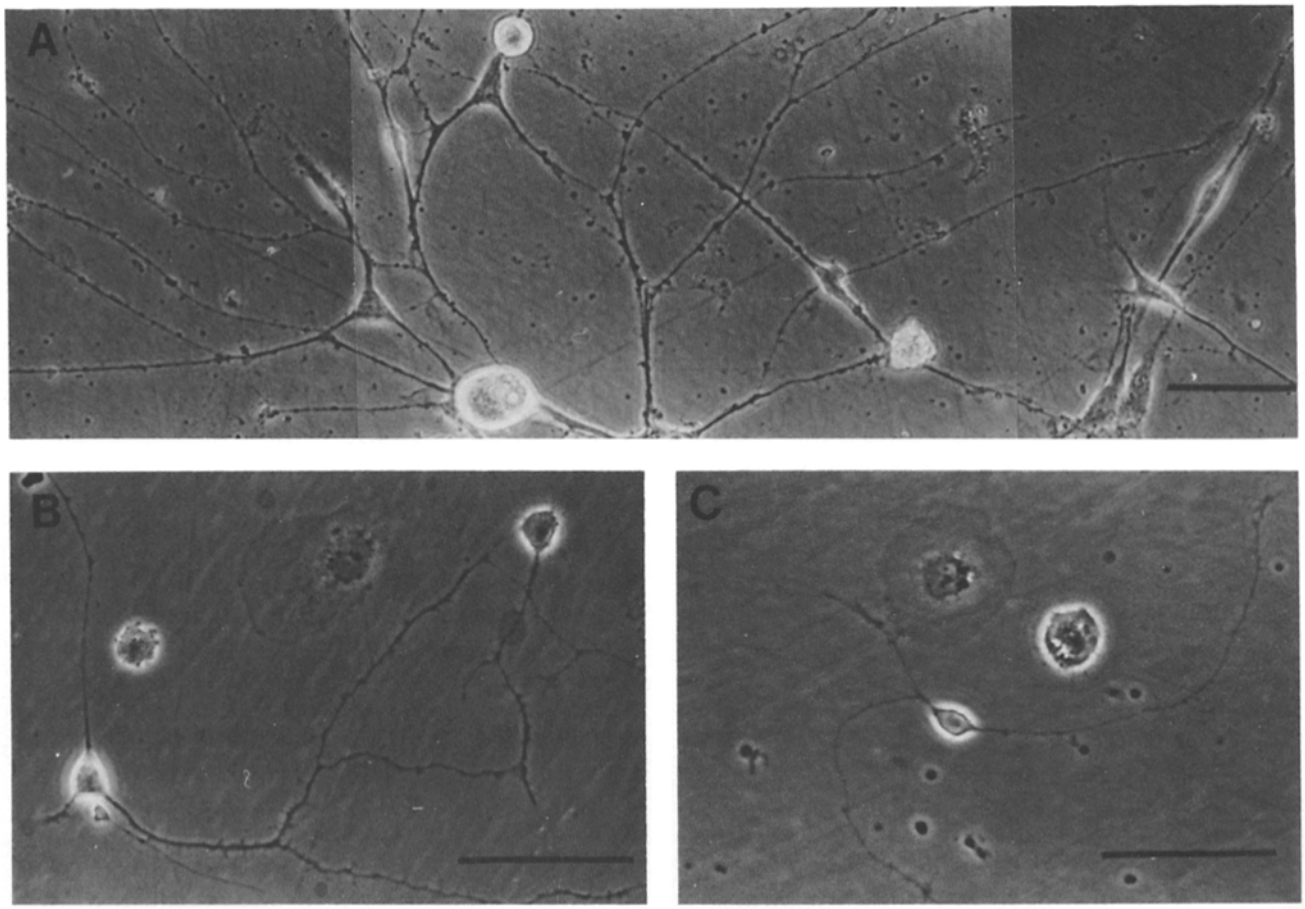

FIGURE 9 Response of other neurons to CM_{SF}. Other types of neurons were plated onto CM_{SF}-treated polylysine-coated substrata and cultured without NGF. Rat sensory neurons (*a*) were photographed after 18 h, chick sensory neurons (*b*) after 13 h, and chick sympathetic neurons (*c*) after 11 h. Bars, 50 μm. *a*, × 340; *b* and *c*, × 460.

strata. Rat and chick sensory neurons and sympathetic neurons all responded in the characteristic manner already described, i.e., with early, rapid, extensive neurite outgrowth (Fig. 9). NGF, which these cells normally require for outgrowth and survival, was not required for the response. Thus, two classes of peripheral NGF-dependent neurons from two different species respond to the factor contained in CM_{SF}.

When a motoneuron-enriched population of cells from embryonic chick spinal cord (56) was plated on CM_{SF}-treated substrata, neurite outgrowth in the first 12 h was considerably more extensive than on control substrata, such as polylysine and collagen (Calof, Lander, Fujii, Gospodarowicz, and Reichardt. Unpublished observations.) In contrast, when dissociated cells from cerebellum and olfactory bulb of neonatal rats were plated on CM_{SF}-treated substrata, no positive or negative effects on growth were observed (not shown). When examining cultures not requiring NGF, such as these, negative results may be equivocal, since neurite outgrowth may normally be quite rapid and growth-promoting factors may effect only a minimal increase. With this in mind, however, it is interesting to note that of the cell types tested, all of those with peripheral axons in vivo responsed to CM_{SF}; those with processes only in the central nervous system were not noticeably affected.

Production of Similar Factors by Other Cell Types

Serum-free media conditioned by confluent cultures of various cell types were prepared as described and assayed for neurite outgrowth-promoting activity in the standard manner, using rat sympathetic neurons. Media conditioned by bovine vascular endothelial cells, bovine vascular smooth muscle cells, bovine adrenal cortical cells, human skin fibroblasts, and the cell lines C_2 (mouse skeletal muscle), PTK-1 (kangaroo rat epithelium), A-431 (human vulva carcinoma), and N-18 (mouse neuroblastoma) all possessed activity indistinguishable from that of CM_{SF}. The titers of the activity of vascular endothelial and smooth muscle conditioned media were measured and were comparable to that of CM_{SF} (not shown). When serum-free, conditioned medium produced by vascular smooth muscle cells was fractionated by associative CsCl density sedimentation, neurite outgrowth-promoting activity was found in a peak located at about the same density at which the active factor in CM_{SF} sedimented.

The ability to produce factors that promote neurite outgrowth seems, therefore, to be shared by a large class of cells, of which the bovine corneal endothelial cell is a member. Further work will be necessary to determine whether all cells that grow attached to surfaces can produce such factors, and what characteristics these factors share.

DISCUSSION

Evidence has been presented that an extracellular matrix produced by bovine corneal endothelial cells can induce rapid neurite outgrowth by rat sympathetic neurons in vitro. In medium conditioned by these endothelial cells, a soluble factor has been detected which, when bound to an appropriate sub-

stratum, has a similar outgrowth-promoting effect on several types of neurons. Media conditioned by several cell types from diverse sources also possess this activity.

The active factor in bovine corneal endothelial cell-conditioned medium has been characterized and partially purified. Several lines of evidence establish the link between neurite outgrowth-promoting activity and a heparan sulfate proteoglycan: the factor binds to a polycationic surface, as do glycosaminoglycans. In associative CsCl gradients, it cosediments with a major peak of [^{35}S]sulfate- and [^{3}H]leucine-labeled material at a density appropriate for a proteoglycan. Most of the classes of proteoglycans, namely hyaluronic acid, dermatan sulfate, and chondroitin 4- and 6-sulfate, are digested by the enzyme chondroitinase ABC. The fact that the neurite outgrowth-promoting factor is resistent to this enzyme, suggests that one of the remaining two proteoglycan classes, heparan sulfate or keratan sulfate, is involved; inactivation of the factor by heparinase implies it is heparan sulfate. This is in agreement with the observation (57) that ECM produced by bovine corneal endothelial cells contains heparan sulfate, but not keratan sulfate.

Although the crude heparinase used has been reported not to contain proteolytic activity (58), controls were done which verified that inactivation of the factor by heparinase was not caused by contaminating proteases. Other controls, using Sepharose-conjugated glycosaminoglycans, demonstrated that the material in the heparinase preparation which inactivates the factor is bound by heparan sulfate, and not chondroitin sulfate.

Proposed hypotheses regarding the molecular nature of the factor are summarized in Fig. 10.

A heparan sulfate proteoglycan alone may possess neurite outgrowth-promoting activity. In conditioned medium, this molecule may exist free in solution (Fig. 10*a*)—in which case it has a large size and density (ca. 1.36)—or it may be a smaller factor that happens to associate with other products of the corneal endothelial cell (Fig. 10*b*).

Alternatively, the factor could consist of a complex formed by a heparan sulfate proteoglycan and other molecules, with the integrity of the complex being required for activity (Fig. 10*c*). Such other molecules probably would not include hyaluronic acid or other proteoglycans, since the factor is resistant to chondroitinase ABC. A protein(s) or glycoprotein(s) would be more likely. To explain why only an intact complex would be active, it is not necessary to assume that neurons must recognize more than one element of the complex. One possibility is that neurons recognize only the protein or glycoprotein component, but that the presence of a proteoglycan is necessary to anchor the complex to an appropriate substratum (e.g. polylysine, extracellular matrix). Certain molecules known to be secreted by corneal endothelial cells, such as fibronectin (59), are plausible candidates for the nonproteoglycan portion of such a complex. Fibronectin alone, however, has no neurite outgrowth-promoting activity in this system, and CM_{SF} is not significantly depleted of activity when preincubated with gelatin-Sepharose, which binds fibronectin (unpublished observations). One worker studying a factor similar to the one described here has found antibodies against fibronectin to have no effect on the factor's activity (60).

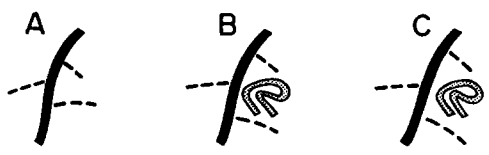

FIGURE 10 Hypotheses regarding the molecular nature of the factor. In each drawing, a heparan sulfate proteoglycan is represented by a protein core (▬) to which heparan sulfate side chains (- - - - -) are attached. Neurite outgrowth-promoting activity may be associated with a free proteoglycan (*a*), or with one that is bound to a protein or glycoprotein (▭) that is not essential for activity (*b*). The third drawing (*c*) depicts a similar complex, in which both the proteoglycan and the glycoprotein are essential for activity.

If, as in Fig. 10*a* and *b*, a proteoglycan is the only molecule essential for promoting neurite outgrowth, the protein core of the proteoglycan must be necessary for the molecule's function, since activity is eliminated by trypsin. Compatible with this is the observation that heparan sulfate alone is not active. The observation that 4 M GuHCl also destroys activity suggests that the proteoglycan may be subject to denaturation that is not readily reversible; this would almost certainly involve the core protein.

If, as in Fig. 10*c*, the factor is a complex active only in its intact form, then inactivation by trypsin may reflect the loss of any protein-containing component. Purified heparan sulfate would be expected to lack activity, since it lacks components of the complex. Exposure to 4 M GuHCl could destroy activity either because a component of the complex is denatured by these conditions, or because the complex is unable to reassemble efficiently after being dissociated.

The properties of the neurite outgrowth-promoting factor characterized in this report suggest that it is one of a group of factors that have recently been described (16, 17, 19, 21). These factors have been reported to affect a variety of neuronal types, promoting neurite outgrowth without affecting other parameters, such as survival. Some of the factors have been shown to require attachment to a polycationic surface, to elicit a neuronal response (17, 21). For example, one factor prepared from chick heart cell conditioned medium induces neurite outgrowth, in some situations, within 20 minutes (17, 61). The factor promotes growth of only those neurites in contact with the polycationic substratum to which it is has been bound (62); similar activity is found in the substratum-attached material left behind by cultured heart cells (60). Activity of this factor is not blocked by antiserum to nerve growth factor, nor by antiserum to fibronectin, a glycoprotein important in cellular adhesion (63). The factor is inactivated by trypsin.

A similar factor has been discovered in mouse heart cell conditioned medium (16); it purifies as a large molecule, and is inactivated by trypsin and by incubation at 100°C, but not by hyaluronidase, collagenase, neuraminidase, or incubation at 60°C. Adler et al. (21) have noted the production of comparable factors by a very wide variety of mammalian tissues—heart, kidney, lung, Schwann cells, astroglia—and several avian tissues. These factors promote neurite outgrowth from sympathetic, sensory, and some spinal cord neurons. Of those tested, none are inactivated by antiserum to NGF or fibronectin.

So far, none of the previously described neurite outgrowth-promoting factors has been significantly purified. No suggestion has been made that proteoglycans play a role in the activity of these factors. Because they share many of the properties of the factor described in this report, however, it seems likely they, too, will be found to involve heparan sulfate proteoglycans.

Heparan sulfate proteoglycans are ubiquitous molecules which may have a great variety of biological functions. They are widely distributed in tissues including the brain (64), are found on the surfaces of many cell types (65), and are present

in basement membranes (cf. 48, 66). The polysaccharide portions of heparan sulfate proteoglycans are diverse in length, degree of sulfation, and uronic acid composition (67–69). Some appear to be integrally associated with cell membranes; others may bind cell surface receptors (70, 71). Heparan sulfate may be a negative regulator of cell proliferation (72–74). It may play a role in neuronal development since levels are much higher in developing animals than in adults (75).

Heparan sulfate proteoglycans may also function in the control of cell motility and adhesion, since heparan sulfate is present in the newly formed adhesion sites of fibroblasts, glioma cells, and neuroblastoma cells (e.g. 76, 77). The factor described in this report may, in fact, function by increasing adhesion between the neuronal plasmalemma and the substratum.

It is reported here that a variety of cell types produce a conditioned medium capable of promoting neurite outgrowth from rat sympathetic neurons. Among these types are neuroblastoma cells, tumor cells with neuronal properties. If neurons, too, can produce neurite outgrowth-promoting factors in culture, the initiation and rate of synthesis of such factors may limit the speed with which neurons extend neurites in vitro. Supplying neurons with a suitable factor from an exogenous source—as was done in this study—would then be expected to promote earlier, more extensive production of neurites. It would be interesting to discover whether NGF and other trophic factors induce the synthesis of neurite outgrowth factors by the neurons they affect. If synthesis of neurite outgrowth factors occurs in vivo, and is similarly regulated, these factors may be important in determining the pattern and density of innervation of the tissues neurons contact.

ADDENDUM

Experiments have been done with CM_{SF} prepared from bovine corneal endothelial cells grown in the presence of 2.5 mM *p*-nitrophenyl β-D-xyloside. Because β-D-xylosides compete with xylosylated core proteins as templates for glycosaminoglycan polymerization, cells exposed to these drugs secrete proteoglycans containing fewer and shorter glycosaminoglycan chains, in addition to secreting free glycosaminoglycans synthesized onto the drug (78, 79). CM_{SF} produced under these conditions retained neurite outgrowth-promoting activity. However, when fractionated on associative CsCl gradients in preliminary experiments, the biological activity banded at a decreased density (~1.30 instead of 1.36–1.37). Since β-D-xylosides promote the synthesis of proteoglycans which contain less carbohydrate and are therefore less dense, this result corroborates the other data linking biological activity to a proteoglycan. The result, however, does not distinguish among the three models presented.

This work was supported by U. S. Public Health Service (USPHS) grant GM-07618 (A. D. Lander), USPHS grant EY-02186 (D. Gospodarowicz), and grants from the National Institutes of Health, the National Science Foundation, the Muscular Dystrophy Association, and the March of Dimes (L. F. Reichardt).

Received for publication 24 August 1981, and in revised form 10 April 1982.

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