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PURIFICATION AND CHARACTERIZATION OF "NEURITE OUTGROWTH-PROMOTING FACTORS"

by

ARTHUR D. LANDER

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

NEUROSCIENCE

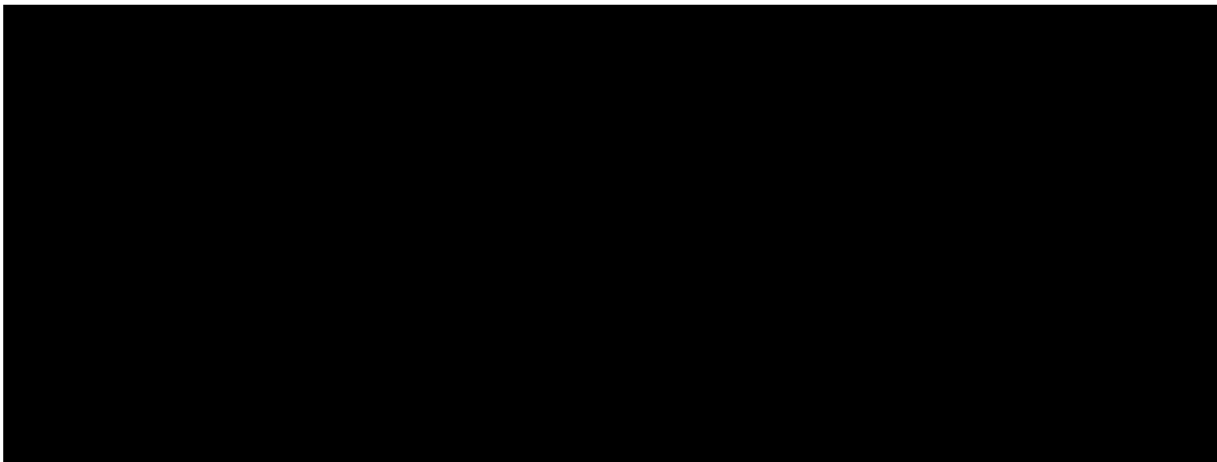
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



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To Anne

ACKNOWLEDGEMENTS

Finding the patience, motivation, and blind faith needed to face the daily challenges of being a graduate student is too much for anyone to do alone. To all those who helped me, I owe a debt of gratitude. In particular, I wish to thank:

Louis Reichardt, my advisor, for his encouragement, and for giving me freedom--perhaps more than I deserved--to follow my own hunches, and develop a sense of what is feasible,

Bill Matthew, who as a graduate student combined intelligence, compassion, level-headedness, and remarkable productivity, setting a standard for me to try to live up to,

Ralph Greenspan, for insisting that thoughtful consideration of one's work is still a respectable endeavor,

Anne Calof, for more support and assistance than I could begin to acknowledge, including merciless, but much needed criticism of every stupid idea I ever had, and every awful line I ever penned,

Kevin Tomaselli, for never passing up the chance for a good scientific argument, even if it meant playing the Devil's advocate more often than anyone should have to,

Other members of the Reichardt lab, such as Dave Shelton and Janet Winter, whose helped me with their encouragement and by their examples,

Dennis Fujii, my collaborator, for being an endless source of enthusiasm, as well as a seemingly endless source of conditioned medium,

Tim Mitchison, for letting me pick his brain many a late night,

Marc Kirschner, for demonstrating that the theory behind ultracentrifugation is fun,

Bruce Alberts, for putting so much effort into refereeing my P.N.A.S. manuscript,

The members of my dissertation committe, Reg Kelly, Zena Werb, Steve Rosen and David Bentley, for reading so much verbiage, and for their helpful comments,

Members of the Medical Scientist Training Program, including Marion Nestle, Dave Cox, Jana Toutolmin (and all of Jana's predecessors), Larry Mirels and Bob Willet, for their moral--as well as financial--support,

Mike Bishop, for helping me make up my mind about what I wanted to be,

My brother, Eric, who encourages me to aim high,

and my Mother, whose requests for copies of all my reprints, past, present and future, are appreciated.

Finally, I would like to express my gratitude to the Liquor Barn, the 22nd & Irving Produce Market, the Inner Sunset Community Food Store, Dave Shelton's blender, Charis Bennett's chile con queso, and the lime-squeezing efforts of the devoted members of the Reichardt lab whose semi-regular walks on the wild side helped me preserve my sanity.

PURIFICATION AND CHARACTERIZATION "NEURITE OUTGROWTH-PROMOTING FACTORS"

Arthur D. Lander

ABSTRACT

The outgrowth of axons and their guidance to targets constitutes a crucial phase of neuronal development. The role that molecules in the neuronal environment play in influencing axonal growth and guidance may be studied _in vitro_, by examining the effects of particular substances on neurite outgrowth. The studies reported here concern the effects of substances known as "neurite outgrowth-promoting factors", which are found in media conditioned by many cell types. "Neurite outgrowth-promoting factors" act by adsorbing to appropriate culture substrata and enhancing the initiation and extension of neurites.

The "neurite outgrowth-promoting factor" in bovine corneal endothelial conditioned medium was purified, and characterized through physical and enzymatic treatments, metabolic labeling, denaturing gel electrophoresis, and immunochemical analysis. The "neurite outgrowth-promoting factors" in other conditioned media were also studied. Purified molecules derived from the extracellular matrix were screened for activity resembling that of "neurite outgrowth-promoting factors".

The results indicate that the bovine corneal endothelial "neurite outgrowth-promoting factor" consists of three associated molecules: the basement membrane glycoprotein laminin, a proteoglycan of the heparan sulfate class, and entactin, another extracellular matrix glycoprotein. Of these molecules, laminin appears primarily responsible for

mediating neurite outgrowth-promoting activity, although the proteoglycan plays an important role in attaching the "neurite outgrowth-promoting factor" to the culture substratum. Studies of the "neurite outgrowth-promoting factors" in media conditioned by other cells indicated they were similar in composition. Interestingly, immunochemical differences between the neurite outgrowth-promoting activity of a purified preparation of laminin and the neurite outgrowth-promoting activity in conditioned media suggested that different forms of laminin exist, or that molecules bound to laminin alter its immunoreactivity.

These findings suggest that laminin may play a role in axonal growth and guidance _in vivo_.

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I N T R O D U C T I O N

A great variety of morphological events contribute to the embryonic development of neural structures (Jacobson, 1978). Among these events, the establishment of neuronal connections through the growth and appropriate guidance of axons is particularly suited to experimental study. It is not only possible to characterize the complex and stereotyped patterns of axonal connections that are found in vertebrate and invertebrate nervous systems, it is also possible in many cases to disturb growing axons experimentally, and observe how these patterns change. Studies of amphibian retinotectal projections (e.g. Fraser and Hunt, 1980), avian peripheral nerve development and muscle innervation (Morris, 1978; Lance-Jones and Landmesser, 1980; Swanson and Lewis, 1982), regeneration of mammalian motor axons (Nitkin _et al._, 1983), and establishment of insect nerve connectives (Bentley and Caudy, 1983; Raper _et al._, 1983) have been among the many studies that suggest the kinds of interactions that influence axonal growth and guidance.

Through such studies, evidence has been found that the timing and pattern of axon growth from a neuron depend both on information specified by the neuron and cues supplied by the axonal environment (reviewed by Johnston and Wessells, 1980). Among the types of environmental cues that might be expected to influence axon growth and guidance, _in vivo_ studies of vertebrates and invertebrates support major roles for the interaction of growing nerve fibers with other nerve fibers (e.g. Raper _et al._, 1983; Thanos _et al._, 1984), nerve cell bodies (Bentley and Caudy, 1983), target tissues (LanceJones and

Landmesser, 1980; Thanos *et al.*, 1984), tissues through which axons grow (Katz and Lasek, 1979, 1981; Morris, 1978; Swanson and Lewis, 1982; Bentley and Caudy, 1983), and the extracellular matrix (e.g. Nitkin *et al.*, 1983).

While these interactions appear to influence the establishment of appropriate axonal connections, the mechanisms by which this influence is exerted are still largely unclear. To understand these mechanisms, it would seem particularly important to identify molecules that are present on axons or in their environments and participate in these interactions. A few techniques exist that can lead directly from the perturbation of axonal behavior *in vivo* to the identification of relevant molecules. For example, it has been possible to isolate mutants of *Drosophila* that display abnormal neural connections as a result of the alteration of a single gene (Wyman and Thomas, 1983). It may also be worthwhile to search for monoclonal antibodies that identify environmental structures used as landmarks by navigating axons (cf. Raper *et al.*, 1983). For the most part, however, the circumstances in which these techniques can be used are limited. More commonly, problems of access to cell populations, and limitations on experimental manipulations that can be performed make it difficult to begin a search for relevant molecules with *in vivo* experiments only.

In vitro techniques overcome several of these problems. Indeed, the technique of tissue culture appears to have been invented to settle an issue about axonal growth, namely, whether axons are extensions of single nerve cells, or form by the fusion of processes from many cells (Harrison, 1907). Over the years, much information has been gathered about axonal growth and guidance. It has become clear, for

instance, that the growth cone, the organelle found at the tip of every growing axon *in vivo* and neurite *in vitro*[1], plays a crucial role in sampling the cellular environment and navigating through it. Morphologically, a growth cone is a broad cytoplasmic flattening from which emanate needle-like filopodia (microspikes) and broad "veils" or lamellipodia. Microtubules extend into the base of the growth cone but not into the filopodia or lamellipodia, where unusual arrays of actin filaments are found (Yamada *et al.*, 1971; Johnston and Wessels, 1980). *In vitro* studies have been particularly helpful in showing that growth cones are a site of new membrane insertion and that growth cones are also actively motile organelles, capable of altering their rate and direction of growth in response to the composition and topography of their substratum, to contact with other cells and other growth cones, to diffusible molecules such as nerve growth factor, to electric fields, and to local differences in mechanical tension (e.g. Weiss, 1955; Letourneau, 1975a, 1975b; Bray, 1982; reviewed by Johnston and Wessels, 1980).

While these findings illustrate the utility of the *in vitro* technique, the drawbacks are equally apparent. In particular, it is clear that a substance identified as affecting neurite growth *in vitro* is only a candidate for a substance of biological relevance. *In vivo*, the substance may not be appropriately localized, or it may not elicit

---

[1] The term "axon" entails certain functional properties and morphological specializations that distinguish it from a dendrite. *In vitro*, the distinction between axon and dendrite is usually too difficult to make, and the neutral term "neurite" is universally applied.

a neuronal response, or it may even elicit a neuronal response that is entirely different from the one it brings about in vitro. However, it must nevertheless be conceded that the study of neurite outgrowth in vitro is still an excellent way to screen for molecules that affect axonal growth an guidance.

Among the screening that has been done along these lines, several studies have focused on effects mediated by the extracellular matrix (ECM). These studies have been motivated, in part, by observations of neuromuscular regeneration in vivo, which indicate that cues found in ECM are sufficient to guide axons to particular synaptic sites (reviewed by Nitkin et al., 1983). Indeed, there is evidence that particular molecules "label" these sites (e.g. Sanes and Chiu, 1983). Other important studies further confirm that ECM plays a major role in the morphogenesis of neuronal (e.g. Toole, 1976; Thiery et al., 1982; Boucaut et al., 1984) and non-neuronal tissues (e.g. Hay, 1981a, 1981b; Toole, 1981, Hall et al., 1982; Reddi, 1984).

Another motivation for studying the effects of ECM on neurite outgrowth is the simplicity of the approach. The ECM is a non-living structure consisting of collagens, other glycoproteins, glycosaminoglycans, and proteoglycans (Hay, 1981a). It may be analyzed, modified, or fractionated by any of a large number of biochemical techniques. Since ECM in vivo forms a substratum for cell attachment and growth, the effects of ECM in vitro may be looked at by applying it to a culture substratum. More specifically, culture substrata may be treated with whole ECM, molecules purified from ECM, or substances believed to be derived from or related to components of ECM, and the effects these substances have on the formation or growth of neurites may be observed.

Some studies of the effects of ECM components on neurite outgrowth began as deliberate inquiries into the functions of particular ECM molecules, such as glycosaminoglycans, fibronectin, collagens and laminin (e.g. Akers et al., 1981; Baron-Van Evercooren et al., 1982; Carbonetto et al., 1983). Others started out as practical attempts to define the growth conditions of certain classes of neurons, such as ciliary ganglion neurons (Helfand et al., 1978), sympathetic neurons (Edgar and Thoenen, 1982) and spinal motoneurons (Calof and Reichardt, 1984). The results presented in the following chapters comprise one such study. The experiments that are described concern themselves with the composition and function of substances known as "neurite outgrowth-promoting factors". Most of these data have been previously published or are in press (Lander et al., 1982, 1983, 1985a, 1985b).

It is important to point out that "neurite outgrowth-promoting factors" have been under study by several other groups, originally in the laboratory of Wessels (Helfand et al., 1978) and more recently by groups led by Collins (Collins, 1978a, 1978b, 1980; Collins and Garret, 1980; Collins and Lee, 1984), Varon (Adler et al., 1981; Adler and Varon, 1980, Manthorpe et al., 1981, 1983; Davis et al., 1984a, 1984b, 1985) Coughlin (Coughlin et al., 1981, 1982), and Thoenen (Edgar and Thoenen, 1982, Edgar et al., 1984). The observations reported by these laboratories contributed significantly to the conceptual development of the study presented here. Many of these observations are discussed in the following chapters.

M E T H O D S

Materials

Hyaluronic acid (grade IV, from bovine vitreous humor), heparin (grade I, from porcine intestinal mucosa) and chondroitin sulfate (mixed isomers, grade III, from whale and shark cartilage) were obtained from Sigma. Dermatan sufate, 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). Heparan sulfate (from bovine lung) was also supplied by Upjohn Laboratories (Kalamazoo, MI). 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). Dr. Linker also generously provided a stock of Flavobacterium heparinum, and heparitinase was purified from heparin-induced cultures of this organism by hydroxylapatite chromatography, as described by Linker and Hovingh (1972). This procedure freed the heparitinase from all detectable chondroitinase activity, in addition to cleanly separating it from heparinase activity, so that further purification of the enzyme by cellulose phosphate chromatography was not required (Linker and Hovingh, 1972).

Gelatin-Sepharose was prepared by mixing 100 ml of packed CNBr-activated Sepharose 4B (Pharmacia) (March et al., 1974) with 25 ml of a warmed 50 mg/ml solution of swine skin gelatin (Sigma) in 0.25 M NaCl, 0.05 M $NaHCO_3$, and shaking the slurry overnight. The resin was then washed extensively with buffers of pH 4 and pH 9.5, then stored at 4°C in PBS (recipe below) containing 0.02% NaN_3. The amount of gelatin coupled was estimated as 1 mg/ml resin by comparing the

OD_{280} of the unbound solution (including all washes) with the OD_{280} of the initial coupling solution. Before each use, the gelatin-Sepharose was washed extensively with PBS.

Laminin was purified from the Engelbreth-Holm-Swarm (EHS) sarcoma, maintained in the thigh muscles of C57/Bl-6 mice, using a modification of the procedures of Timpl et al. (1979), and Kleinman et al. (1982). All steps were performed at 4°C. Briefly, tumors were homogenized in 3.4 M NaCl, 0.05 M Tris, pH 7.4, 4mM EDTA, 1 mM phenylmethylsulfonyl-fluoride (PMSF) and 2 mM N-ethylmaleimide (NEM). Insoluble material was collected by centrifugation 23,500 g for 30 minutes. Homogenization in the same buffer and centrifugation was repeated twice. The insoluble residue was extracted overnight in 0.5 M NaCl, 0.05 M Tris, pH 7.4, 4 mM EDTA, 1 mM PMSF, 2 mM NEM, and centrifuged at 23,500 g for 30 minutes. The supernatant liquid was saved. The pellet was resuspended in more of the same buffer, centrifuged again, and this supernatant pooled with the first. To the pooled supernatants was added saturated ammonium sulfate, dropwise with stirring, to 30% of saturation. After 1 hour, the precipitate was collected by centrifugation at 23,500 g for 30 minutes. The pellet was dissolved in 0.15 M NaCl, 0.05 M Tris, pH 7.4. It required several hours of stirring to dissolve. It was then dialyzed exhaustively against more of the same buffer. Solid NaCl was then added to a final concentration of 1.7 M. After stirring 1 hour, the small precipitate, consisting primarily of contaminating type IV collagen, was removed by centrifugation at 23,500 g for 30 minutes. To the supernatant, solid NaCl was added to a final concentration of 3.4 M. After 1 hour of stirring, the precipitate was collected, and the supernatant discarded. The pellet was

redissolved in 0.15 M NaCl, 0.05 M Tris, pH 7.4, and dialyzed exhaustively against freshly prepared 2M urea, 0.5 M NaCl, 0.05 M Tris, pH 8.6. It was centrifuged at 23,500 g for 30 minutes, and the supernatant was diluted with an equal volume of 2M urea, 0.05 M Tris, pH 8.6. The solution was applied to a column of DEAE-cellulose, equilibrated with 2M urea, 0.25 M NaCl, 0.05 M Tris, pH 8.6. Unbound protein was saved, dialyzed exhaustively against 2M urea, 0.05 M Tris, pH 8.6, and applied to a column of DEAE-cellulose equilibrated with 2M urea, 0.05 M Tris, pH 8.6, and again, unbound protein was saved. This material was dialyzed into 0.5 M NaCl, 0.05 M Tris, pH 7.4, made 20% in glycerol, quick frozen in 1 ml aliquots in a dry ice-ethanol bath, and stored at -70°C. Except for the presence of a very small amount of low molecular weight contaminants, this laminin appeared homogeneous by SDS-gel electrophoresis.

A rabbit antiserum directed against laminin was prepared (Timpl et al., 1979; Hudson and Hay, 1980). Anti-laminin antibodies were affinity-purified from this antiserum by chromatography on a column of laminin (coupled to Affigel 10 (Bio-Rad) at 0.8 mg/ml of gel). The coupled laminin had been previously chromatographed on Biogel A 1.5M (Bio-Rad) to free it of low molecular weight contaminants (Rohde et al., 1980).

Fibronectin, purified from human plasma, was generously donated by Dr. Janet Winter. Rat tail collagen was prepared as described by Hawrot (1980). Platelet factor 4 was prepared from "fresh" human platelet concentrate (Peninsula Memorial Blood Bank, Burlingame, CA.) by chromatography on heparin-epsilon-aminocaproic acid-Sepharose, as described by Levine and Wohl (1976). Cell culture media were prepared

by the UCSF cell culture facility. Radiochemicals were obtained from Amersham. Non-immune rabbit immunoglobulin (Ig) was prepared by batch absorption and elution from DEAE-cellulose (Hudson and Hay, 1980). Goat anti-rabbit Ig was from Cappel. Phosphate buffered saline (PBS) consisted of 137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 adjusted to pH 7.4. Tris buffered saline (TBS) consisted of 100 mM NaCl, 50 mM Tris base, adjusted with concentrated HCl to pH 7.4 at 4°C. A rabbit antiserum against entactin, which did not cross react with laminin was supplied by Dr. Hynda Kleinman (National Institutes of Health, Bethesda, MD). Other reagents, where not specified, were obtained from commercial sources.

Dissociation and Culture of Primary Neurons

Superior cervical ganglia from 1-2 day old Sprague-Dawley rats were dissociated by a modification of an enzymatic procedure (Wakshull *et al.*, 1979). Ganglia were cleaned, stripped, and incubated for 30 minutes at 37° C in 0.25% trypsin (Sigma) in a holding solution of Leibovitz L-15 medium with added glucose (6 g/L), penicillin (100 U/ml), streptomycin (100 ug/ml) and glutamine (2 mM)[2]. Ganglia were washed three times with holding solution, resuspended in medium containing serum, and triturated using a nine inch pasteur pipette that had been flame-polished to about half of its original tip diameter. Fragments were allowed to settle, and the supernatant liquid, con-

---

[2] In the experiments in chapters 3-5, a holding solution of Dulbecco's Modified Eagle Medium containing 0.4% glucose (DME-0.4), no bicarbonate, 44 mM HEPES, penicillin, streptomycin and glutamine, was used.

taining 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) 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 (Hawrot and Patterson, 1979).

Enzymatically dissociated dorsal root ganglion cells were prepared from newborn Sprague-Dawley rats (Fields, et al., 1978) and 8-day chick embryos (Varon and Raiborn, 1971). Lumbar sympathetic chains from 11-day chick embryos were dissociated enzymatically (Varon and Raiborn, 1972). Cerebella and olfactory bulbs were removed from newborn Sprague-Dawley rats and dissociated mechanically (Sotelo, et al., 1980).

Rat sympathetic and sensory neurons were either cultured in the "complete L-15-CO_2" medium of Hawrot and Patterson (1979), containing 5% adult Sprague-Dawley rat serum (for experiments in chapters 1 and 2) or in Dulbecco's Modified Eagle's Medium with 0.4% glucose (DME-0.4) containing 5% fetal calf serum, glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml) (for experiments in chapters 3-5). Methocel (methylcellulose) was not used, and NGF was added only in those experiments where noted. In these cases, the 7S form of NGF, prepared from male mouse salivary glands (Chun and Patterson, 1977), was used at a concentration determined to promote optimal neurite outgrowth in cultures of rat sympathetic neurons. For cultures without serum, transferrin (100 ug/ml), progesterone (20 nM), putrescine (100 uM), selenium (30 nM) and insulin (5 ug/ml) were added

(Bottenstein and Sato 1979).

Chick sympathetic and sensory neurons were cultured in DME-0.4 supplemented with 5% fetal calf serum (FCS), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 ug/ml). Rat cerebellar and olfactory bulb cells were cultured as described (Sotelo, et al., 1980).

Routinely, dissociated cells were plated at a density of 1-2 x 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 8% CO_2 (for other cultures).

Preparation of Culture Substrata

Dissociated neurons were cultured in polystyrene dishes or multiwell plates (Costar) that had been treated for tissue culture. Culture surfaces were exposed to poly-D-lysine (Sigma type I-B, 1 mg/ml in 0.1M sodium borate, pH 8.4) overnight, washed with twice-distilled water, and sterilized for 30-45 minutes under ultraviolet light. Cells were either plated directly onto these surfaces, or after these surfaces had been further treated with solutions to be tested for neurite outgrowth-promoting activity. Such solutions were applied for 8-16 hours at 4°C, after which the culture surfaces were washed at least three times in PBS, basal L-15, or basal DME, in which they could be stored. Antisera, if applied, were diluted in complete culture medium and added for 5 hr at room temperature. Antisera were either washed off extensively, or were left in the wells during the culture period (as indicated in the text). Complete culture 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 (Collins, 1978b).

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 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 fixed in PBS containing 2% glutaraldehyde and 5% sucrose. Where not otherwise indicated, cultures were fixed after 11-13 hours of growth, and the data presented represent averages of duplicate wells (± the deviation) from a typical assay. In certain experiments, the quantity of biological activity in each sample was expressed in "neurite outgrowth-promoting units". This represents the titer of the sample--i.e. the inverse of the dilution that gave a half maximal response--multiplied by the volume, in ml., of the sample.

Non-neuronal Cell Culture

Cultures of bovine corneal endothelial cells were established from steer eyes (Gospodarowicz et al., 1977b; Gospodarowicz and Greenberg,

1978). Stocks were maintained in DME containing 0.1% glucose (DME-0.1) and supplemented with 10% calf serum and 5% FCS (Irvine Serum Company, Irvine, CA), glutamine (2 mM), Fungizone (Squibb, 2.5 ug/ml), and gentamycin (Garamycin, Schering, 50 ug/ml). Fibroblast growth factor (FGF), prepared as described (Gospodarowicz, et al., 1978), 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 (Gospodarowicz, et al., 1976; Gospodarowicz et al., 1977c). Cell stocks were maintained in DME-0.1 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-0.4 with 10% FCS, and glutamine and antibiotics as above.

Bovine adrenal cortical cells (Gospodarowicz et al., 1977a) were grown in F12 medium, with 5% heat-inactivated horse serum (HS) (Colorado Serum Co.), 5% FCS, and glutamine and antibiotics as above.

PTK-1 cells (American Type Culture Catalog, Rockville, MD) were grown in DME-0.1 with 10% newborn calf serum, penicillin (100 U/ml) and glutamine and streptomycin as above.

A-431 cells (Giard, et al., 1973) and N-18 cells (Amano et al. 1972) were grown in DME-0.1, with 10% FCS, and glutamine, penicillin, gentamycin, and streptomycin as above.

RN22 cells and PC12 cells were grown in DME-0.4 with 10% horse serum (HS) 5% fetal calf serum (FCS), glutamine, penicillin and streptomycin as above.

PFHR9 cells were grown in DME-0.1 with 10% FCS, glutamine, penicillin and streptomycin as above.

C_2 cells (Yaffe and Saxel, 1977) were maintained in DME-0.4 with 20% FCS, glutamine, penicillin and streptomycin as above, grown to near-confluence, switched to the same medium with 10% HS instead of the FCS, and allowed 2-3 d to fuse into myotubes.

Primary embryonic chick muscle cultures were prepared, fused, and maintained in DME-0.4 supplemented with 10% HS, 25 ug/ml ovotransferrin (Calof and Reichardt, 1984), glutamine, penicillin and streptomycin as above.

Preparation of ECM-coated dishes

Plastic dishes coated with ECM produced by corneal endothelial cultures were prepared as described (Gospodarowicz et al., 1980). Briefly, confluent cultures were first washed with distilled water and then exposed to 0.02 M NH_4OH in distilled water for 5 minutes, followed by washing and storage in PBS. Nuclei and cytoskeletal elements have not been detected on these plates. (Gospodarowicz, et al., 1981b). Only trace amounts of the FGF used to feed cells were detectable in the ECM (Gospodarowicz and Ill, 1980).

Preparation of Conditioned Media (CM's)

Conditioned media were prepared from confluent or near confluent non-neuronal cultures by washing the cells into fresh culture medium, incubating for a fixed period of time, removing the medium, filtering it through a 0.2u filter, and storing it at 4°C.

For BCE cells, the fresh medium used for conditioning contained

either 10% calf serum and 5% FCS (for BCE-CM used in chapters 1 and 2), 0.5-1.0% FCS (chapters 3-5), or no serum at all, in which case it is referred to as serum-free conditioned medium (CM_{SF}). CM and CM_{SF} were harvested after 5 days of conditioning.

For primary chick muscle cultures, CM was prepared from fused myotubes using DME-0.4 supplemented with ovotransferrin (25 ug/ml), insulin (10 ug/ml) and polyvinylpyrrolidone-360 (Sigma, 0.5 mg/ml), and harvested after 3-5 days.

For all other cell types, the same medium used to maintain the cells was used for conditioning, which was carried out for 5 days for bovine vascular endothelial cells, human foreskin fibroblasts, bovine adrenal cortical cells, PTK-1 cells, and A-431 cells; for 2-3 days for RN22, PC12 and PFHR9 cells; and for 2 days for cultures of already fused C_2 myotubes.

Physical, Chemical, and Enzymatic Treatments of BCE-CM_{SF}

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

Acid and Base: BCE-CM_{SF} was brought to pH 1.6 with 1N HCl or pH 12.7 with 1N NaOH. After one hour at room temperature, samples were neutralized with 1N NaOH and 1N 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 pre-mixed and then added, was used.

Enzymes: Trypsin (Sigma, type III) was used at 100 ug/ml for 2 hours at 37°C, after which soybean trypsin inhibitor was added to a final concentration of 500 ug/ml. As a control, trypsin inhibitor was

added before the trypsin. Deoxyribonuclease I (Sigma, type III) was used at 100 ug/ml for 2 hours at 37°C. Collagenase (Worthington, 200 U/mg) was used at 20 U/ml 2 hours at 37°C. Neuraminidase (Sigma, type IX) was used at 2.5 U/ml for 5 hours at 37°C. Chondroitinase ABC (Sigma) was used at 2 U/ml for 6 hours at 37°C. Crude heparinase was used at indicated concentrations and times, at 30°C. Samples to be digested with purified heparitinase were made 2.5 mM in $CaCl_2$, and 35 ug/ml of purified heparitinase in TBS was added. Controls received TBS in place of enzyme. After incubation at 43°C for 2 hours (Linker and Hovingh, 1972), the reaction was stopped by cooling to 4°C or if appropriate, by the addition of SDS-containing electrophoresis buffer.

Metabolic Labeling

For labeling of BCE-CM with [^{3}H]-leucine, cells were washed into the serum-free medium prepared with leucine-free DME-0.1. After two hours 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 uCi/ml was added. After two days of incubation, cold leucine was added to a final concentration of 10 ug/ml. The dishes were incubated two more days and the medium was collected and filtered through a 0.22um filter.

For labeling of BCE-CM with [^{35}S]-sulfate in the experiments described in chapter 2, cultures were washed into serum-free medium as described above, using sulfate-free rather than leucine-free DME. After two hours of incubation, this medium was removed and more of the same medium, now containing $Na_2[^{35}S]O_4$ (Amersham, carrier-free) at 40 uCi/ml was added. After four days of incubation, the medium was

collected and filtered through a 0.22um filter. For labeling of BCE-CM with [^{35}S]-sulfate in the experiments described in chapter 4, cultures were rinsed twice in sulfate free DME-0.1, then adding sulfate-free DME-0.1 containing carrier-free [^{35}S]-sulfate (40 uCi/ml), unlabeled sulfate ($MgSO_4$, 4 uM), a reduced amount of FCS (0.5%) and glutamine and penicillin. After 4 days of culture, the CM was harvested, treated, and stored as described above.

CM's metabolically labeled with [^{35}S]methionine were prepared by rinsing cultures with methionine-free DME-0.1, and labeling in DME-0.1 containing glutamine and antibiotics as above, [^{35}S]methionine (40 uCi/ml, 1110 Ci/mmol, Amersham), unlabeled methionine (final methionine 21 uM), and reduced levels of serum (for BCE, 0.5% FCS; for PC12 and RN22, 0.5% FCS and 1% HS; for PFHR9, 1% FCS; for C_2 and chick muscle cells, 1% HS). Cultures were maintained 3 days (BCE) or 2 days (all others), then fed with 1 ml DME-0.1 per 10 ml culture fluid, supplying an additional 20 uM methionine. After 24 hr, the labeled CM's were harvested, filtered (0.2 u), and treated with the protease inhibitors phenylmethylsulfonyl fluoride (1mM), EDTA (3 mM), pepstatin (10^{-7} M) and N-ethyl maleimide (2 mM). Labeled CM's were used at once or stored at -20°C.

During the entire course of these incubations cells appeared healthy, and did not detach from the dish. Batches of labeled CM's were always tested and shown to possess neurite outgrowth-promoting activity. All radioactive samples were counted in Aquasol or Aquasol-II (New England Nuclear). When ^{3}H and ^{35}S were counted in the same samples, appropriate corrections were made.

Isopycnic Centrifugation

Samples were concentrated 5-10 fold by dialysis against polethylene glycol ("20-M", Union Carbide) or sucrose. Two types of gradients were prepared, as defined by Sajdera and Hascall (1969): 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% (w/w) for dissociative gradients, or 37% (w/w) for associative gradients. These samples were centrifuged at 5°C to equilibrium (about 48 hours) at 40,000 rpm using a SW 50.1 rotor (Hassel, et al., 1980). Gradients were eluted and the density of fractions was determined by weighing volumes in preweighed 10 ul micropipettes; the radioactivity of these samples was then counted. Each fraction was dialyzed against two changes of 0.05 M Tris-HCl, pH 7.4 before further testing.

Immobilization of Glycosaminoglycans on Sepharose

Oxirane-activated Sepharose 6B was prepared according to Sundberg and Porath (1974). Briefly, 10 g suction-dried Sepharose was washed with water, then mixed with 10 ml 1,4-butanediol bis(diglycidyl ether) and 10 ml of a solution of 0.6M NaOH containing 2 mg sodium borohydride per milliliter. The suspension was mixed by rotation for 8 hours at room temperature. The reaction was stopped by washing the gel with large volumes of water. Coupling was done as follows: 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 hours at 37°C, the liquid was eluted, and the Sepharose washed and blocked with ethanolamine (Sundberg and Porath, 1974). 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 (Bitter and Muir, 1962). In this manner, heparan sulfate-Sepharose and chondroitin sulfate-Sepharose containing, respectively, 1 mg and 0.7 mg glycosaminoglycan per ml of packed Sepharose, were prepared. The heparan sulfate used in this procedure was that supplied by Upjohn laboratories.

Immunochemical techniques

Enzyme-linked immunoassays (EIAs) were performed on samples by adsorbing them to polylysine coated tissue culture plastic wells (as described above) overnight, washing and blocking the wells with EIA buffer (5% Newborn calf serum in PBS) and applying antibodies (affinity-purified anti-laminin at 1 ug/ml in EIA buffer) overnight. After washing three times in EIA buffer, peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG, Cappel) was added at 1:2500 in EIA buffer for 2 hours. After washing four times with EIA buffer and twice with PBS, each well received 0.1 ml of peroxidase substrate, which consisted of a freshly mixed solution of 20 ml of 0.1 M Sodium Acetate, 0.05 M NaH_2PO_4, 1 ml of a 2.19% aqueous solution of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid)(stored frozen and freshly thawed), and 0.25 ml of 0.83% H_2O_2 (aqueous)(prepared fresh from 30% H_2O_2 stored

at 4°C). After two hours at room temperature, results were quantified spectrophotometrically at 415 nM (Reading, 1982).

For immunoprecipitations, samples of CM's (0.3 ml), mixed with affinity-purified anti-laminin (amounts as indicated) and carrier non-immune rabbit Ig (5-25 ug), were incubated overnight at 4°C. Precipitating antiserum (goat anti-rabbit Ig) was added to the equivalence point (determined to be 2.5 ul/ug carrier Ig). After 12-24 hr at 4°C, the precipitate was removed by centrifugation, the supernatant decanted, and the pellet washed once with TBS. For electrophoretic analysis, the pellet was resuspended in an equal volume of 9 M urea, and allowed to remain at 4°C overnight, with occasional agitation, until completely dissolved. It was then mixed with an equal volume of double-strength electrophoresis sample buffer without glycerol.

Immunoblotting was carried out on a sample of BCE-CM that was depleted of fibronectin by gelatin-Sepharose absorption (described below) and concentrated by two rounds of ultracentrifugation (described below). The sample was subjected to non-reducing SDS-PAGE, electrophoretically transferred to nitrocellulose (Batteiger et al., 1982), and probed with affinity-purified anti-laminin (1 ug/ml) or rabbit anti-entactin serum at 1:100 diluted into 2% normal goat serum in PBS. Bound antibody was detected by incubation of the nitrocellulose in a solution of ^{125}I-goat anti-rabbit IgG, extensive washing, and autoradiographic exposure of Kodak XAR-2 film at -70°C.

Purification of the "Neurite Outgrowth-Promoting Factor" in BCE-CM

Freshly prepared labeled CM was mixed with gelatin-Sepharose in PBS (6 ml resin/100 CM), stirred slowly at room temperature for 2

hours, then filtered through a Bio-Rad "Econocolumn" containing a porous polypropylene filter. The resin was washed with 1 bed volume of PBS and this was pooled with the filtrate. CM absorbed in this way was cooled to 4°C and stirred, as saturated $(NH_4)_2SO_4$ was added, dropwise, to 48% of saturation. This, and all subsequent steps were carried out at 4°C. The solution was stirred overnight, then centrifuged at 20,000g for 1 hr. The pellet was resuspended in Tris-buffered saline (TBS) containing 0.1% Triton X-100, and protease inhibitors (1 mM PMSF, 10^{-7} M pepstatin, 2mM NEM, and 2mM EDTA). This material was dialyzed overnight against 400 volumes of TBS containing protease inhibitors (EDTA was reduced in this instance to 0.2mM). The retained material was centrifuged at 25,000g for 1 hour to remove the slight precipitate that remained. The supernatant was then mixed with DEAE cellulose (DE52, Whatman) that had been equilibrated in TBS containing 0.1% Triton X-100. 1 ml of resin was added for each 100 ml of labeled CM used at then start of purification. The suspension, sealed within a disposable polypropylene column (Bio-Rad), was mechanically rocked for 2 hours. The unbound material was eluted from the column bottom, and the ion-exchange matrix washed with 2 bed volumes of TBS containing 0.1% Triton X-100. Bound material was eluted with 1M NaCl, 0.05M Tris-HCl, 0.1% Triton X-100, pH 7.4. Fractions were collected manually, and the appearance of eluted radioactivity was monitored by liquid scintillation counting (Aquasol-II, New England Nuclear, was used as a fluor). Greater than 90% of the bound radioactivity eluted within the first 1.3 ml, and this material was pooled. To this sample was added solid sucrose (ultra-pure, Schwarz-Mann) to 5% (w/v), in order to increase its density, and sufficient phenol red (concentrated aqueous solution)

to color it lightly. The sample was then applied to a column (1.5 cm x 28.5 cm) of Sepharose CL4B (Pharmacia) equilibrated in 0.5M NaCl,0.05 M Tris-HCl pH 7.4, 0.1% Triton, 0.2 mg/ml hemoglobin (human, 2X re-crystalized, Sigma) containing the aforementioned four protease inhibitors (EDTA at 2 mM). The increased density, and light coloring of the sample facilitated appplication to the column by underlaying. The column was run at 3.5 ml/hr (i.e. 2 ml/cm^2-hr) and 1 ml fractions were collected. Calibration of the column was performed separtely using three markers: β -galactosidase was detected enzymatically (Craven et al., 1965), and high molecular weight aggregates of the enzyme served as a convenient void volume (V_o) marker (Clarke, 1975). Laminin was iodinated using chloramine-T (Hudson and Hay, 1980) and detected by gamma counting. [^{35}S]-sulfate was used to determine the total volume (V_t) and was detected by liquid scintillation counting.

Fractions from gel filtration with neurite outgrowth-promoting activity (see text) were pooled, placed into dialysis tubing, and embedded in dry Sephadex (Pharmacia). Once sufficiently concentrated the sample was dialyzed against TBS containing protease inhibitors (EDTA was 2 mM). To 0.4 ml of recovered material was added sedimentation velocity standards, β -galactosidase 2.5 units (Worthington) and intestinal alkaline phosphatase, 3.4 units (Sigma). The solution was layered onto a preformed density gradient (10.7 ml of a 5-20% sucrose gradient in TBS containing 0.1% Triton X-100, resting atop 0.4 ml of 60% sucrose in the same buffer) in a polyallomar ultracentrifuge tube (Beckman) and centrifuged in an SW41 Ti rotor at 40,000 rpm for 11.7 hours (at 4°C). Fractions were eluted by puncture of the tube bottom. Sedimentation standards were located by enzymatic assay

(Craven *et al.*, 1965, Schlesinger & Barret, 1965).

An Alternative Purification

An alternative method for a rapid, but partial, purification of the "neurite outgrowth-promoting factor" in BCE-CM was also devised: BCE-CM labeled with ^{35}S-methionine (54 ml) was absorbed with gelatin-Sepharose (3 ml), as described above. After removal of the resin by filtration, the CM was centrifuged in a 45 Ti rotor at 42,000 rpm (140,000 g) for 11 hr. The pellet was resuspended in TBS containing 0.1% Triton X-100 and 2 mM EDTA, and re-centrifuged at 44,000 rpm (153,500 g) for 10 hr. The pellet was resuspended in 0.53 ml of the same buffer, and insoluble material removed by brief low speed centrifugation. The supernatant material was mixed with sedimentation standards (alkaline phosphatase, 3.4 U, and β-galactosidase, 2.5 U) and layered onto a gradient of sucrose (5-20%) in TBS containing 0.1% Triton X-100 and centrifuged in an SW 41 Ti rotor at 38,000 rpm for 12 hr. The gradient was eluted by puncture of the tube bottom.

Electrophoresis

The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) system of Laemmli (1970) was used. Samples were prepared in buffer without 2-mercaptoethanol, except where indicated. Gels were cast on GelBond PAG supports (Marine Colloids) and were 2.8% acrylamide in the stacking portion and 2.9-15% exponential gradients of acrylamide in the separating portion. Gradient gels were poured using a two-chambered gradient maker in which the lower chamber--the one out of which the formed gradient flowed--was held at a constant volume. The concen-

tration of acrylamide at any point in such a gradient was calculated according to the formulae:

$C_x \quad = [(C_0 - B) \exp(-x/V)] \; + \; B$

and $C_{final} = [(C_0 - B) \exp(-W/V)] \; + \; B$

where B = acrylamide concentration in upper chamber

C_0 = acrylamide concentration in lower chamber

W = volume of upper chamber

V = volume of lower chamber

C_x = acrylamide concentration in the gradient at a position "x" ml up from the bottom.

and C_{final} = acrylamide concentration at the top of the gradient, i.e. "W" ml up from the bottom.

The gradients described in these studies were prepared with the following parameters: B = 0; C_0 = 15% acrylamide, 0.4% Bis; W = 17.25 ml; V = 10.5 ml; C_{final} = 2.9% acrylamide. Gels were cast with a width of 13 cm., a stacking region 2.5 cm. in length, and a separating region 15.5 cm. in length.

After electrophoresis, gels were fixed in 10% acetic acid, 7% ethanol in water. Non-radioactive marker proteins, when used, were visualized by staining with Coomassie brilliant blue. Staining solutions were adjusted to contain no more than 30% methanol, in order to prevent excessive shrinkage which could tear gels from their Gel-Bond supports. Stained gels were destained in 10% acetic acid, 7% ethanol in water, and the position of stained standards recorded by fine scratches in the back of the GelBond support. Gels were then processed for fluorography by three 3-hour soaks in dimethylsulfoxide (DMSO), one 3-hour soak in 16% (w/w) 2,5-diphenyloxazole in DMSO, and

soaks in multiple changes of distilled water lasting from 3 hours to overnight (Bonner and Laskey 1974). Gels were then equilibrated in 5% glycerol in water, and dried under vacuum, with heating only after the first hour of drying was completed. Gels were then exposed to preflashed Kodak XAR-2 film at -70°C (Laskey and Mills, 1975). Fluorographic exposure over labeled bands was measured by the method of Suissa (1983), in which exposed silver grains were eluted from the autoradiograph with 1M NaOH, and quantified turbidimetrically at 500 nm.

C H A P T E R 1

DETECTION OF A "NEURITE OUTGROWTH-PROMOTING FACTOR" IN EXTRACELLULAR MATRIX AND CONDITIONED MEDIA

SUMMARY

In this chapter, evidence is presented that bovine corneal endothelial ECM exerts a dramatic stimulatory effect on the outgrowth of neurites by rat sympathetic neurons. This effect occurs in the absence of nerve growth factor and is insensitive to anti-nerve growth factor antibodies. When polylysine-coated substrata are exposed to medium conditioned by bovine corneal endothelial (BCE) cells, they acquire a similar neurite outgrowth-promoting activity. Substrata treated in this manner promote neurite outgrowth not only by rat sympathetic neurons, but by other classes of rat and chicken neurons. Polylysine-coated substrata can acquire neurite outgrowth-promoting activity not only by exposure to BCE-conditioned medium, but also by exposure to any of several conditioned media produced by a variety of cultured cells.

RESULTS

Effect of Corneal Endothelial Extracellular Matrix on Sympathetic Neurons

Neonatal rat sympathetic neurons require NGF for survival and development *in vivo* (Levi-Montalcini and Booker, 1960). 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 (Levi-Montalcini and Angeletti, 1963; Chun and Patterson, 1977). 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. Figure 1.1a shows cells plated on ECM and cultured without NGF. For comparison, cells from an NGF-supplemented culture grown on polylysine are shown in Figure 1.1b. In NGF-supplemented cultures, neurite formation could be completely blocked by antiserum to NGF (Figure 1.1d). Outgrowth on ECM, however, was unaffected by anti-NGF treatment, even when anti-NGF was preincubated with the ECM and then added to the culture medium at a concentration sufficient to bind 0.5ug/ml of *beta*-NGF, the active subunit of NGF (Figure 1.1c). 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 1.1 and Figure 1.2). Neurites appeared earlier and grew more rapidly on ECM than on polylysine-coated plastic. Within 6 hours after plating onto ECM, more than 80% of the neurons had neurites, many of them several cell diameters in length. In contrast, neurons plated onto polylysine and

Figure 1.1. Effect of anti-NGF on the response of neurons to ECM and NGF. Rat sympathetic neurons were grown for 24 hours 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 hours 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 hours 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 equals 50 um.

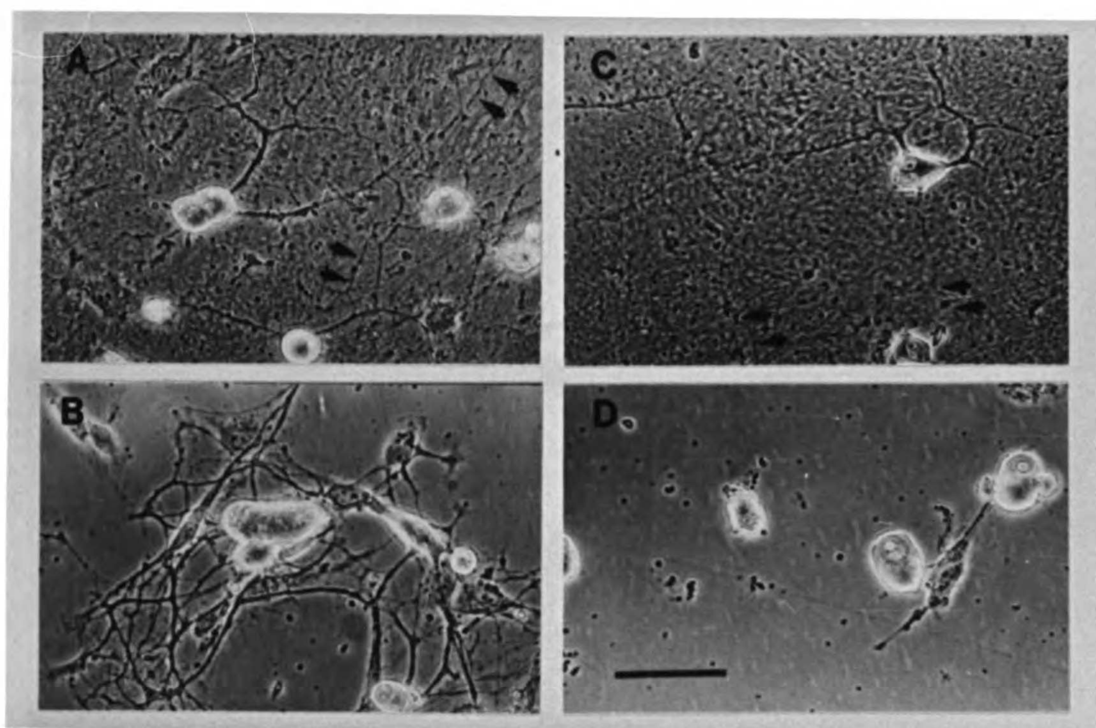

A
B
C
D

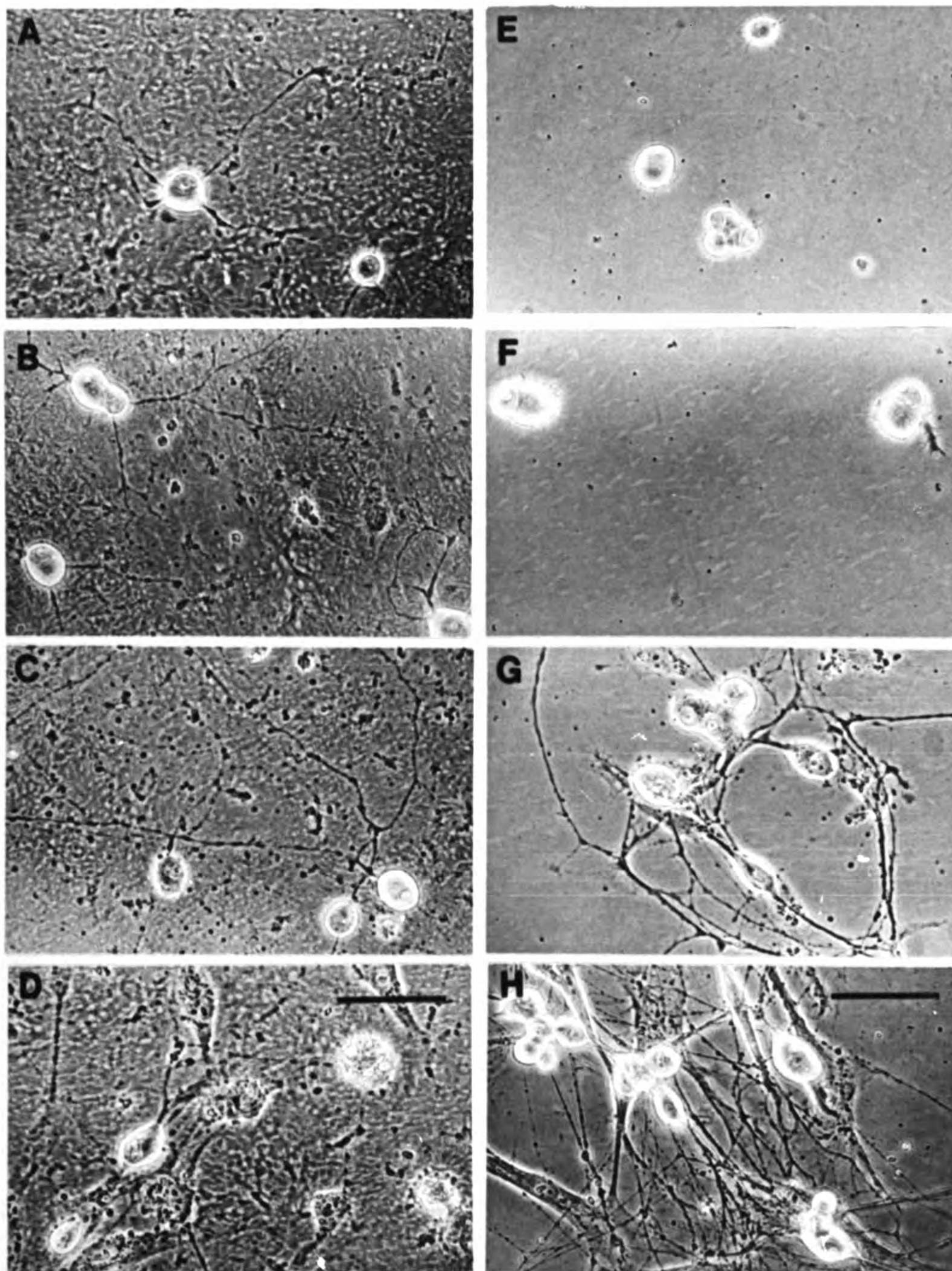
TABLE 1.1

Comparison of the response of neurons to NGF and ECM

Neurite Outgrowth		Hours after plating				
Substratum	Medium	6	12	24	48	72
Polylysine	+ NGF	2	6	75	86	84
ECM	- NGF	86	78	73	40	2
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.

Figure 1.2. 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 hours; b,f -12 hours; c,g -24 hours; d,h -48 hours). Bars equal 50 um.


A
B
C
D
E
F
G
H

cultured with NGF had very few processes by 6 or even 12 hours, although most cells extended neurites by 24 hours.

Although neurites appeared early and grew rapidly on ECM in the absence of NGF, their rate of growth slowed dramatically after 24 hours and cell viability (estimated by morphological criteria) fell steadily thereafter. By 72 hours, less than 20% of the neurons appeared alive; none survived over five days. 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 neurite outgrowth-promoting effects of ECM, rat sympathetic neurons still require NGF for survival.

Another characteristic of cultures grown on ECM was the particular morphology of the neurons (Figures 1.1 and 1.2). When compared to control cultures (cells grown on polylysine with NGF), the average number of neurites projecting from each cell body was greater in cultures grown on ECM. In addition, many neuritic processes were more highly branched, and a significant number of processes appeared unusually thin in cultures grown on ECM.

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).

<u>Surfaces Treated with Conditioned Medium Can Substitute for ECM</u>

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 (BCE-CM) and washed thoroughly. Sympathetic neurons plated onto this substratum responded as they did on ECM, by rapidly extending neurites (Figure 1.3). Serum-free conditioned medium (BCE-CM_{SF}) applied in this way also produced an active substratum (Figure 1.4, Table 1.2). Therefore the active substance(s) is synthesized and secreted by corneal endothelial cells, and is not a modified or concentrated component of serum.

Table 1.2 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 these conditions, the BCE-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. Figure 1.4 shows the appearance of cells on BCE-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 BCE-CM_{SF}, they did not support rapid neurite outgrowth. When only half of a polylysine-coated dish was treated with BCE-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.

When polylysine-coated dishes were exposed to serum (e.g. 10% fetal calf serum) for several hours in order to "block" absorbent sites on the substratum, subsequent application of BCE-CM or -CM_{SF} did

Figure 1.3. Response of neurons to BCE-CM-treated substrata. Rat sympathetic neurons were prepared and cultured without NGF for 11 hours on a polylysine substratum (A) treated with BCE-CM or (B) untreated. Bar = 50 um.

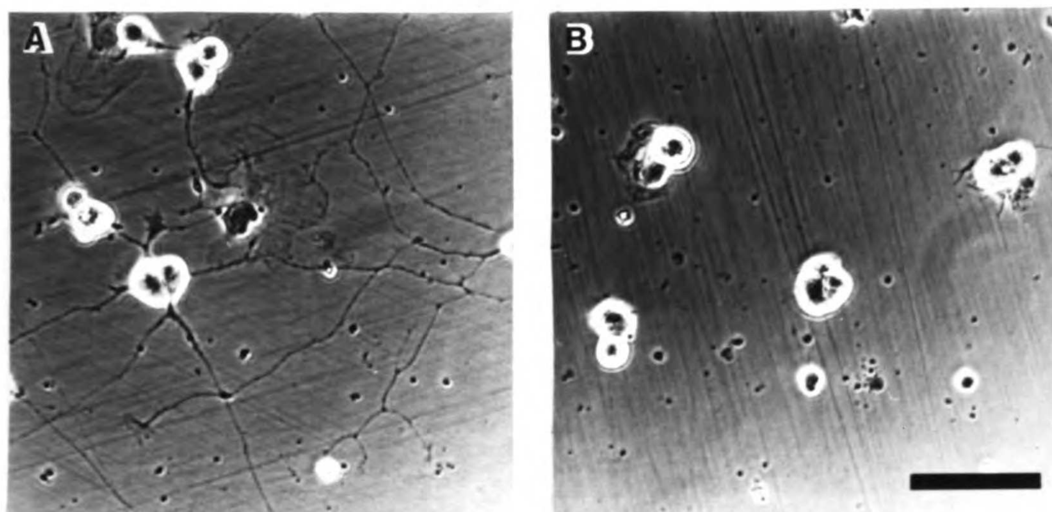

A
B

Figure 1.4. Response of neurons to BCE-CM_{SF}-treated substrata. Rat sympathetic neurons plated on BCE-CM_{SF}-treated polylysine substrata, cultured without NGF for 13 hours in serum-containing (a) and serum-free medium (b). Bar equals 50 um.

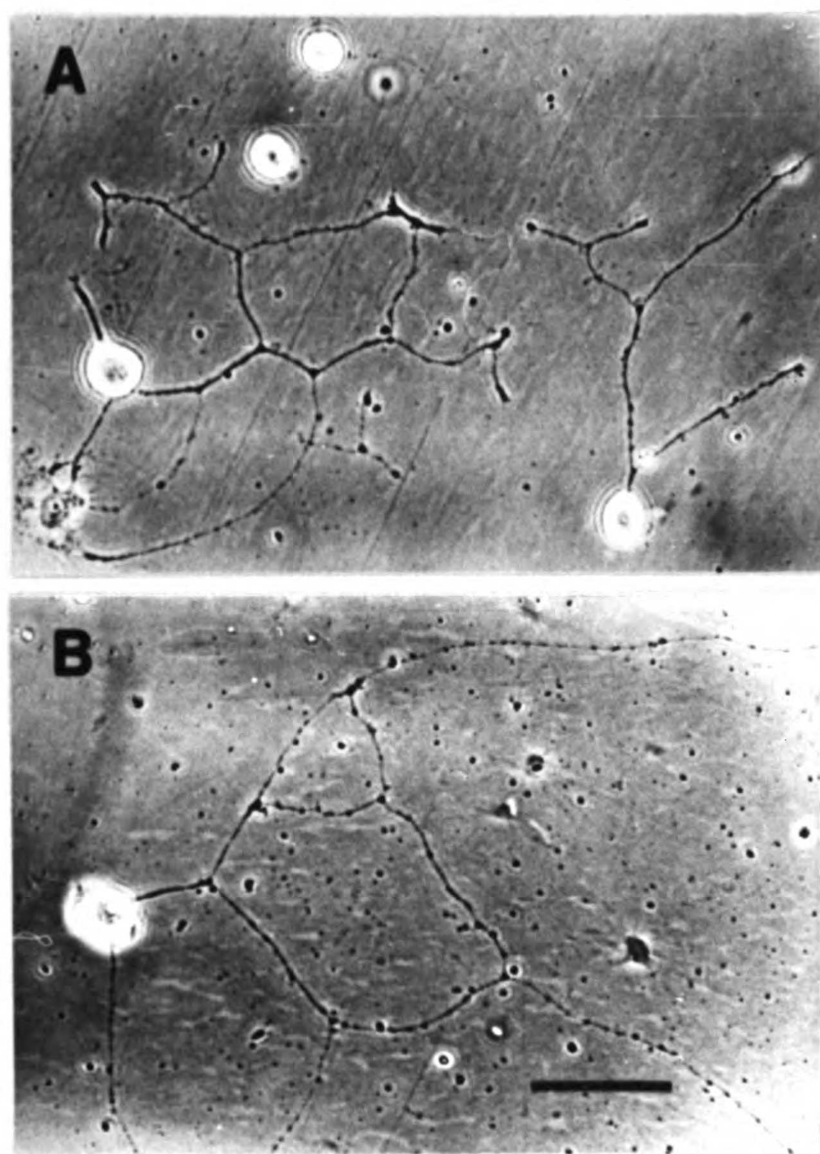

A
B

TABLE 1.2

Neuronal response to BCE-CM_{SF} in the presence and absence of serum

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

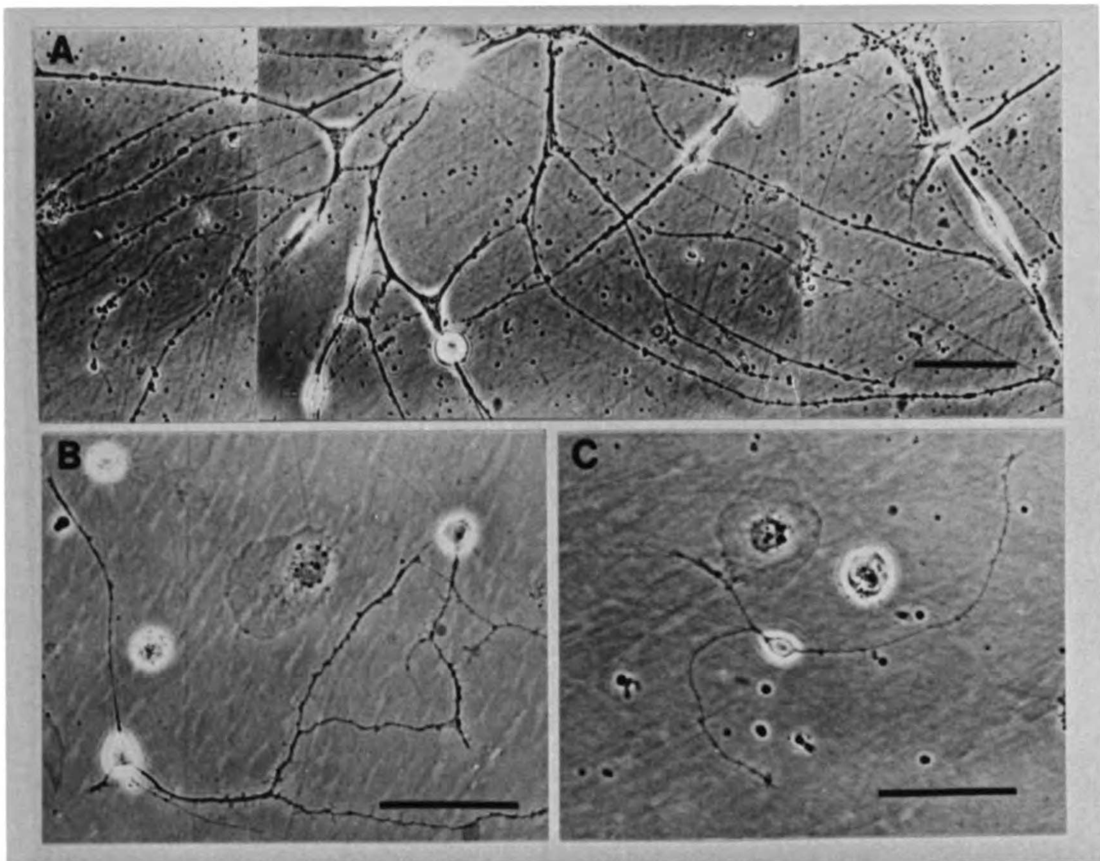
Rat sympathetic neurons were plated on polylysine-coated tissue culture plastic that had been treated with CM_{SF}. Neurons were cultured without NGF, and with or without serum. Neurite outgrowth and survival were determined as in Table 1.

not result in a substratum that promoted rapid neurite outgrowth. In fact, on such a "blocked" substratum, BCE-CM or $-CM_{SF}$ that was added into the neuronal culture medium was unable to promote neurite outgrowth (unpublished observations). These results imply that in order to elicit an appropriate neuronal response, factor(s) in BCE-CM and BCE-CM_{SF} must adsorb to the substratum, being ineffective when present in solution only.

<u>Effect of CM_{SF}-Treated Substrata on Other Types of Neurons</u>

In addition to rat sympathetic neurons, other neuronal cultures were plated onto BCE-CM_{SF}-treated, polylysine-coated substrata. Rat and chick sensory neurons and sympathetic neurons all responded in the characteristic manner already described, i.e., with early, rapid, extensive neurite outgrowth (Figure 1.5). NGF, which all of 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 BCE-CM_{SF}. Other cell types that respond to BCE-CM_{SF}-treated substrata with rapid neurite outgrowth include motoneurons purified from embryonic chick spinal cords (Calof and Reichardt, 1984). In contrast, dissociated neurons from neonatal rat cerebellum and neonatal rat olfactory bulb (prepared by the method of Sotelo *et al.*, 1980) did not respond to BCE-CM_{SF}-treated substrata. However, these results are difficult to interpret, since neurite outgrowth by these two cell types is rapid even on untreated substrata.

Figure 1.5. Several types of neurons respond to BCE-CM_{SF}-treated substrata. Different types of neurons were plated onto BCE-CM_{SF}-treated polylysine-coated substrata and cultured without NGF. Rat sensory neurons (a) were photographed after 18 hours, chick sensory neurons (b) after 13 hours, and chick sympathetic neurons (c) after 11 hours. Bars equal 50 um.


A
B
C

Production of Similar Factors by Other Cell Types

Serum-free and serum-containing 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, primary embryonic chick myotubes, and the cell lines C_2 (mouse skeletal muscle), PTK-2 (kangaroo rat epithelium), A-431 (human vulva carcinoma), N-18 (mouse neuroblastoma), RN22 (rat Schwannoma), PC12 (rat pheochromocytoma) and PFHR9 (endodermally differentiated mouse teratocarcinoma cells) all possessed activity indistinguishable from that in BCE-CM and $BCE\text{-}CM_{SF}$. Some of these results are shown in Table 1.3.

TABLE 1.3

Substrata treated with other CM's also promote neurite outgrowth.

Substratum	% with Neurites
BCE-CM	65 ± 3.8
PC12-CM	53 ± 0.4
RN22-CM	49 ± 1.0
C_2-CM	31 ± 2.9
Chick Muscle CM	73 ± 3.4
PFHR9-CM	59 ± 0.6
Control	<2

Rat sympathetic neurons were cultured for 11 hours on polylysine-coated substrata treated with several different CM's, and neurite outgrowth was scored as described.

DISCUSSION

Neurite Outgrowth-Promoting Effects of ECM and CM

The data presented above demonstrate that some component or components of an ECM produced *in vitro* exerts a dramatic neurite outgrowth-promoting effect on rat sympathetic neurons. ECM elicited early initiation of rapid extensive neurite outgrowth, yet it did not support neuronal survival. ECM thus appears to act differently from NGF, and it was not surprising that anti-NGF did not affect the neurite outgrowth-promoting activity of ECM.

These observations suggested that ECM produced *in vitro* may contain a factor that, if present in ECM *in vivo*, might be of importance in regulating the growth or guidance of axons during development. It was clear that this hypothesis could be tested only if the molecule or molecules in ECM responsible for promoting neurite outgrowth could be identified. It was therefore extremely fortunate that the neurite outgrowth-promoting activity of ECM could be reproduced reasonably well by substrata that had been exposed to BCE-CM, since CM is a considerably better starting point for purification and biochemical characterization than ECM. This is true largely because the molecules in CM are already in solution, while those in ECM may require harsh, potentially denaturing, conditions for extraction. The principal hazard of studying the neurite outgrowth-promoting activity of CM is that a polylysine substratum treated with CM is only a crude approximation of an *in vitro* ECM (which is itself only an approximation of ECM *in vivo*). There was always the chance that purification of a substratum-binding, neurite outgrowth-promoting molecule from BCE-CM

would yield a molecule not actually found in ECM's. Fortunately, certain of the observations described above were reassuring: First, the neurite outgrowth-promoting activity of BCE-CM is also found in BCE-CM_{SF}, and it is completely functional when assayed in the absence of serum. Consequently, the active factor(s) cannot be a serum component that is concentrated or modified by BCE cells. Second, when a polylysine substratum is treated with BCE-CM or -CM_{SF}, neurite outgrowth-promoting activity becomes a property of the substratum; neurons on a "blocked" substratum that are then exposed to BCE-CM or -CM_{SF} in solution are not stimulated to extend neurites. This suggests that the active factor(s) in BCE-CM and -CM_{SF} must be immobilized on the substratum to be effective; such a property would be particularly appropriate for an ECM molecule.

Sources and Actions of "Neurite Outgrowth-Promoting Factors"

It is clear from the experiments described above that rat sympathetic neurons are not the only class of neurons that extends neurites in response to BCE-CM or -CM_{SF}. It is also clear that BCE cells are not unique in producing a CM with neurite outgrowth-promoting activity. In fact, the data shown here are only a part of a body of results assembled by several laboratories, some of it preceding the work described here; these studies indicate that a great variety of mammalian and avian cells, of numerous morphological and functional types, release into CM a substance or substances that can adsorb preferentially to polycationic substrata (i.e. polylysine and polyornithine) and promote profuse and rapid extension of neurites by many types of neurons. Those neurons which respond to CM-treated substrata

include mammalian and avian neurons of the peripheral nervous system (e.g., sympathetic and parasympathetic neurons), neurons that maintain connections in both the peripheral and central nervous systems (dorsal root ganglion neurons and spinal motoneurons), and even some classes of neurons that are confined to the central nervous system (e.g., hippocampal neurons) (Helfand *et al.*, 1978; Collins 1978a; Adler *et al.*, 1981; Coughlin *et al.*, 1981; Manthorpe *et al.*, 1983; Calof and Reichardt, 1984).

Those cells that release a "neurite outgrowth-promoting factor" into CM's also defy categorization, except in that they do not include cells such as lymphocytes and lymphoid cell lines, which do not attach very firmly to their own culture substrata (Adler *et al.*, 1981) These observations raise the possibility that the active factor in these numerous CM's is a ubiquitous molecule that plays a general role in cell-substratum interactions. This view could explain why, among the cell types that produce "neurite outgrowth-promoting factors", there are some that do not normally encounter neurons or their processes *in vivo*.

Interestingly, two of the cell types reported in this chapter to produce a CM with neurite outgrowth-promoting activity are neuronal cell lines with neuronal properties (N18 neuroblastoma and PC12 pheochromocytoma). It is not known whether primary neurons can also produce a "neurite outgrowth-promoting factor", but it may be safely concluded that, *in vitro*, most neurons do not make much of such a "factor"--if they did they ought to promote their own neurite outgrowth on untreated polylysine substrata. It remains a possibility, however, that primary neurons produce a small amount of such a "factor", or

might be able to induce the synthesis of such a "factor" under certain circumstances. This last possibility is intriguing, since the production of an endogenous "neurite outgrowth-promoting factor" could conceivably be involved in the neurite extension that occurs in response to "trophic" agents such as NGF. This point is discussed further in the Conclusions.

C H A P T E R 2

INITIAL CHARACTERIZATION OF A "NEURITE OUTGROWTH-PROMOTING FACTOR"

SUMMARY

In this chapter, preliminary characterization of the "neurite outgrowth-promoting factor" in BCE-CM_{SF} is described. The "factor" is sensitive to treatments that digest or denature proteins, such as trypsin, prolonged heating, and extremes of pH. Evidence from CsCl density centrifugation also suggests that sulfated carbohydrate material is associated with the "neurite outgrowth-promoting factor". Consistent with this, heparinase, an enzyme that degrades the sulfated glycosaminoglycan heparan sulfate, inactivates the "factor" in BCE-CM_{SF}. The issue of whether the "neurite outgrowth-promoting factor" is a heparan sulfate proteoglycan or a complex that contains a heparan sulfate proteoglycan is discussed. Consistent with the latter possibility, the "factor" has a very high apparent molecular weight ($> 10^6$ daltons), and is associated, in CsCl density gradients, with a peak of material that appears to contain protein-proteoglycan complexes.

RESULTS

Preliminary Characterization of the "Neurite Outgrowth-Promoting Factor" Produced by BCE cells

A standard assay for neurite outgrowth-promoting activity in CM's 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 hours. Figure 2.1 shows a dose-response curve, assayed after 13 hours, for dilutions of BCE-CM_{SF}. Titers calculated from these curves were similar for different batches of BCE-CM or -CM_{SF}. Even when the neurons were supplied with NGF, the dose-response was unchanged, provided outgrowth was assayed at about 12 hours (not shown).

In order to characterize its active factor(s), BCE-CM_{SF} was subjected to various treatments and assayed. As shown in Table 2.1, it was inactivated by incubation at low or high pH, or by heating to 80°C for 30 minutes. Heating to 60°C, however, did not reduce activity. Trypsin inactivated BCE-CM_{SF}, and this was the result of specific proteolysis, since inactivation could be blocked by trypsin inhibitor. No decrease in the activity of BCE-CM_{SF} was seen after exposure to deoxyribonuclease, collagenase or neuraminidase.

Fractionation of BCE-CM_{SF}

BCE-CM_{SF}, concentrated five-fold by dialysis against polyethylene glycol, was fractionated on a column of Sepharose 6B. Fractions were collected and assayed. As shown in Figure 2.2, neurite outgrowth-promoting activity eluted just after the void volume in a broad peak.

Figure 2.1. Dose-response curve for BCE-CM_{SF}-promoted neurite outgrowth. Dilutions of BCE-CM_{SF} were assayed for neurite outgrowth-promoting activity as described. Concentration is expressed as a percentage of undiluted BCE-CM_{SF}. Neurite outgrowth is expressed as percentage of total neurons with neurites after 13 hours in culture. Assays were performed in triplicate; the data are graphed as averages $\pm$ 1 standard deviation.

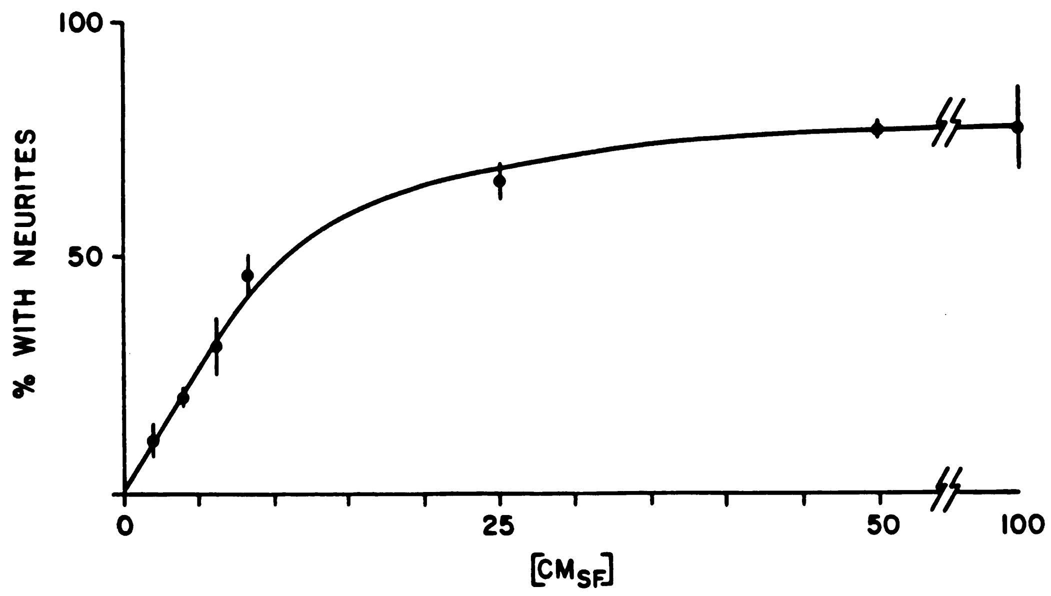

100
50
% WITH NEURITES
0
25
50
100
[CMSF]

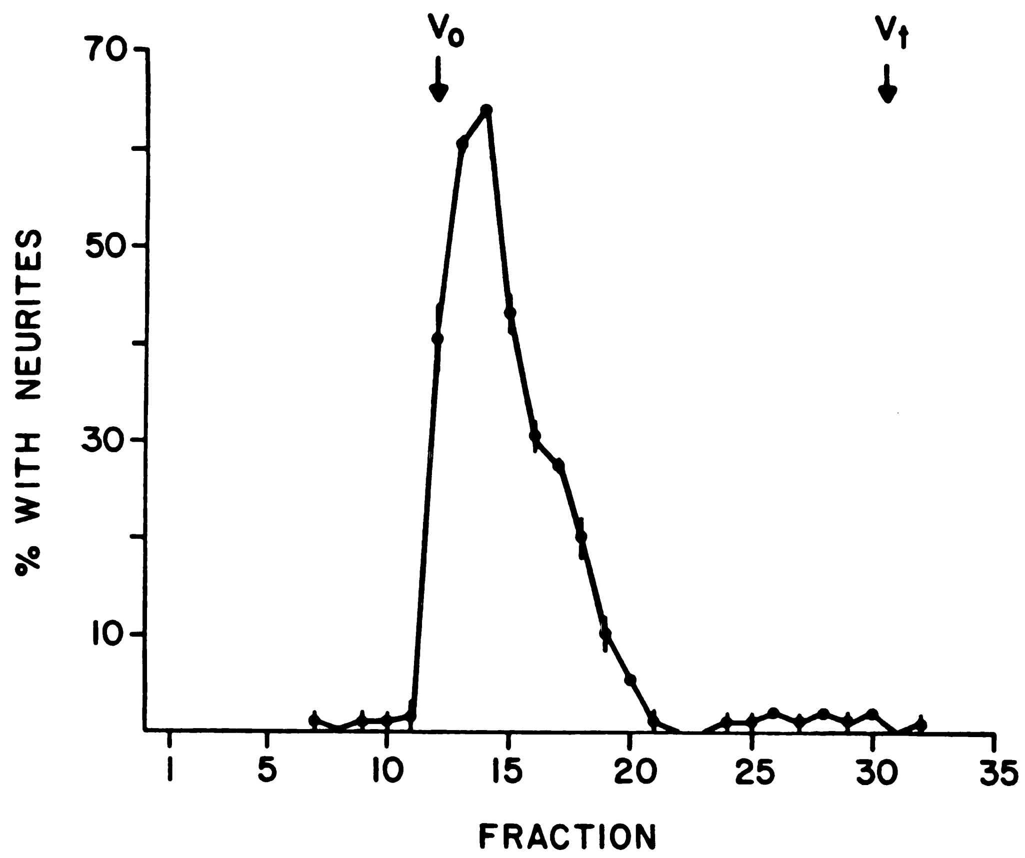
TABLE 2.1

Partial characterization of the "neurite outgrowth-promoting factor" in $BCE\text{-}CM_{SF}$

Treatment	Neurite Outgrowth	% of control
None	38 ± 2	(100)
Acid (pH 1.6)	3 ± 3	8
Base (pH 12.7)	12 ± 3	32
Acid-Base Control (pH 7.4)	46 ± 6	120
60°C	35 ± 9	92
80°C	4 ± 1	11
Trypsin	0 ± 1	0
Trypsin + Trypsin Inhibitor	41 ± 6	108
Deoxyribonuclease	40 ± 8	105
Collagenase	39 ± 1	103
Neuraminidase	43 ± 6	113

$BCE\text{-}CM_{SF}$ 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.

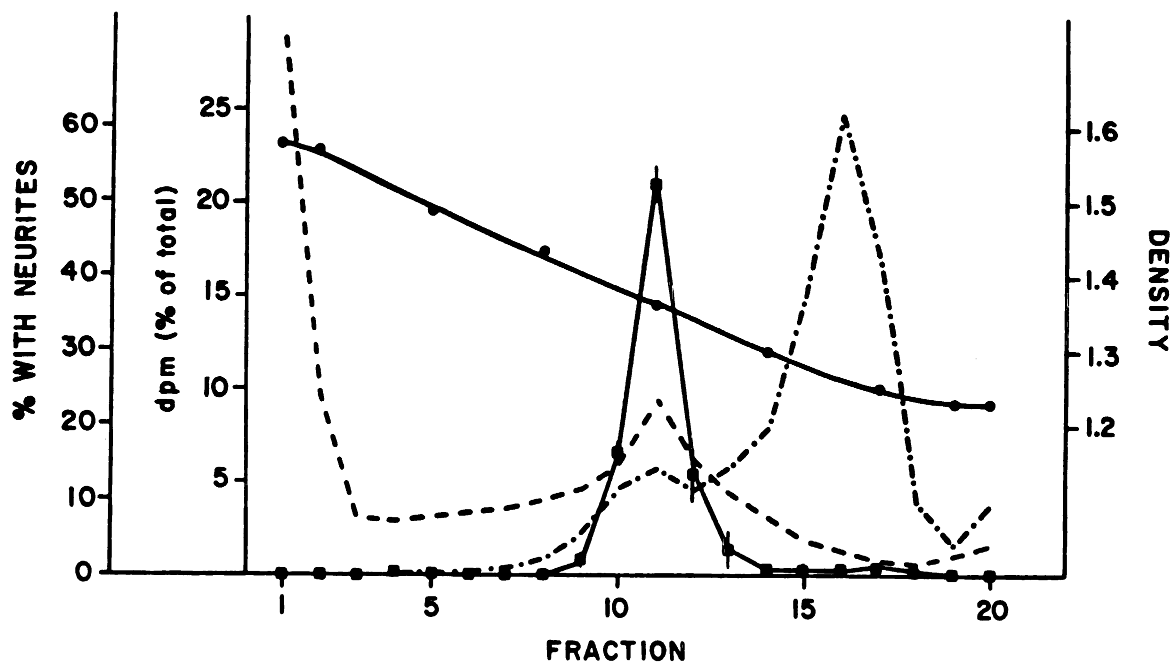
Figure 2.2. Sepharose 6B chromatography of BCE-CM_{SF}. BCE-CM_{SF} was fractionated on a column (31 x 0.67 cm) of Sepharose CL6B equilibrated in 50 mM Tris-HCl, pH 7.4. A 0.5-ml sample was applied, and fractions were eluted at 2.5 ml/hour.



V_o
V_t
70
50
30
10
% WITH NEURITES
1
5
10
15
20
25
30
35
FRACTION

$BCE\text{-}CM_{SF}$ was also fractionated by isopycnic sedimentation in CsCl density gradients. 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. In order to obtain more detailed information, $BCE\text{-}CM_{SF}$, metabolically labeled with $[^3H]$-leucine and $[^{35}S]$-sulfate, was prepared. With some qualifications (Branford-White, 1980), the sulfate label is specific for sulfated glycosaminoglycans and, therefore, marks the position of proteoglycans in these gradients. The leucine label marks proteins and, therefore, also glycoproteins and proteoglycans. Concentrated $BCE\text{-}CM_{SF}$, to which aliquots of leucine-labeled and sulfate-labeled $BCE\text{-}CM_{SF}$ 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 Figure 2.3, this activity was confined to a single peak that matched a significant 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 $BCE\text{-}CM_{SF}$. These experiments were repeated several times using different batches of $BCE\text{-}CM_{SF}$ and labeled $BCE\text{-}CM_{SF}$, 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 suggest that a sulfated proteoglycan is present in the active fractions.

Figure 2.3. Isopycnic sedimentation in a cesium chloride gradient. BCE-CM_{SF}, labeled with [^{3}H]leucine and [^{35}S]sulfate, was centrifuged in CsCl containing 0.4 M GuHCl, as described in 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 (—■—).



% WITH NEURITES
60
50
40
30
20
10
0
dpm (% of total)
25
20
15
10
5
DENSITY
1.6
1.5
1.4
1.3
1.2
1
5
10
15
20
FRACTION

Characterization of the Partially Purified "Neurite Outgrowth-Promoting Factor"

Further evidence of the association of a proteoglycan with 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 2.2a demonstrate that neurite outgrowth-promoting activity is lost after treatment with crude 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 (Linker and Hovingh, 1977).

This result suggests that heparan sulfate is a necessary component of the "neurite outgrowth-promoting factor". The chondroitanse control is important because crude heparinase contains some chondroitinase as an impurity. To further control for effects due to contaminating glycosaminoglycan lyases or proteases present in crude heparinase, two experiments were performed:

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 Figure 2.4. Since the ability of heparinase to inactivate the factor was not diminished by this concentration of albumin, it is unlikely that non-specific 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 2.2b). The eluant from the heparan sulfate column no longer

TABLE 2.2

Effect of glycosaminoglycan-degrading enzymes on the "neurite outgrowth-promoting factor"

A.

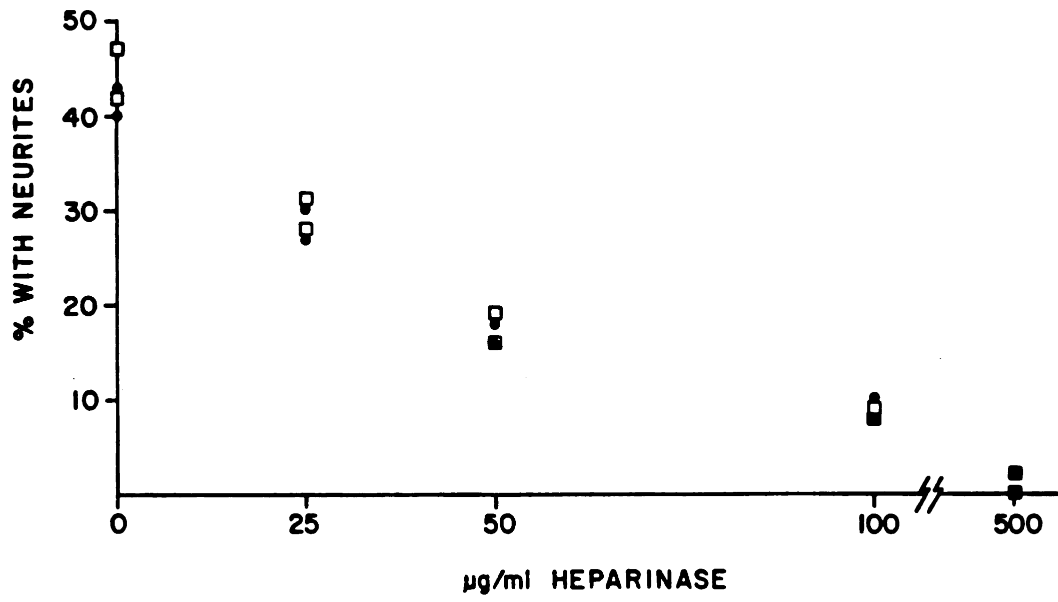
	Neurite outgrowth		
	Crude Heparinase 1 mg/ml	Chondroitinase ABC 2 U/ml	Control no enzyme
Partially purified factor	1 ± 1	44 ± 10	53 ± 7
CM_{SF}	3 ± 3	46 ± 5	47 ± 2

B.

Treatment of Crude Heparinase	Neurite outgrowth
Control (no heparinase)	38 ± 0.2
Eluted from heparan sulfate-Sepharose	48 ± 0.6
Eluted from chondroitin sulfate-Sepharose	13 ± 2.4
Crude heparinase (final dilution 300 ug/ml)	2 ± 0.4
Crude heparinase (final dilution 30 ug/ml)	28 ± 0.3

In A, Pooled active fractions from an associative gradient and CM_{SF} were treated with heparinase and chondroitinase ABC for 6 hours and assayed. In B, 50 ul aliquots of heparinase (6.0 mg/ml in 0.1 M sodium acetate, pH 7.0) were applied to columns containing 100 ul of glycosaminoglycan-conjugated Sepharose and eluted, at 4°C, in 200 ul 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 volumes of partially purified factor and the mixture was incubated for 4 hours at 30°C and assayed for neurite outgrowth-promoting activity. As in Figure 2.4, a dilution of the factor affording maximal sensitivity was used.

Figure 2.4. Effect of heparinase concentration on inactivation of the partially purified "neurite outgrowth-promoting factor". Partially purified "factor" (pooled active fractions from a CsCl 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, the partially purified "factor" was used at a dilution, the activity of which fell on the steeply rising portion of the dose-response curve (cf. Figure 2.1).


50
40
30
20
10
% WITH NEURITES
0
25
50
100
500
µg/ml HEPARINASE

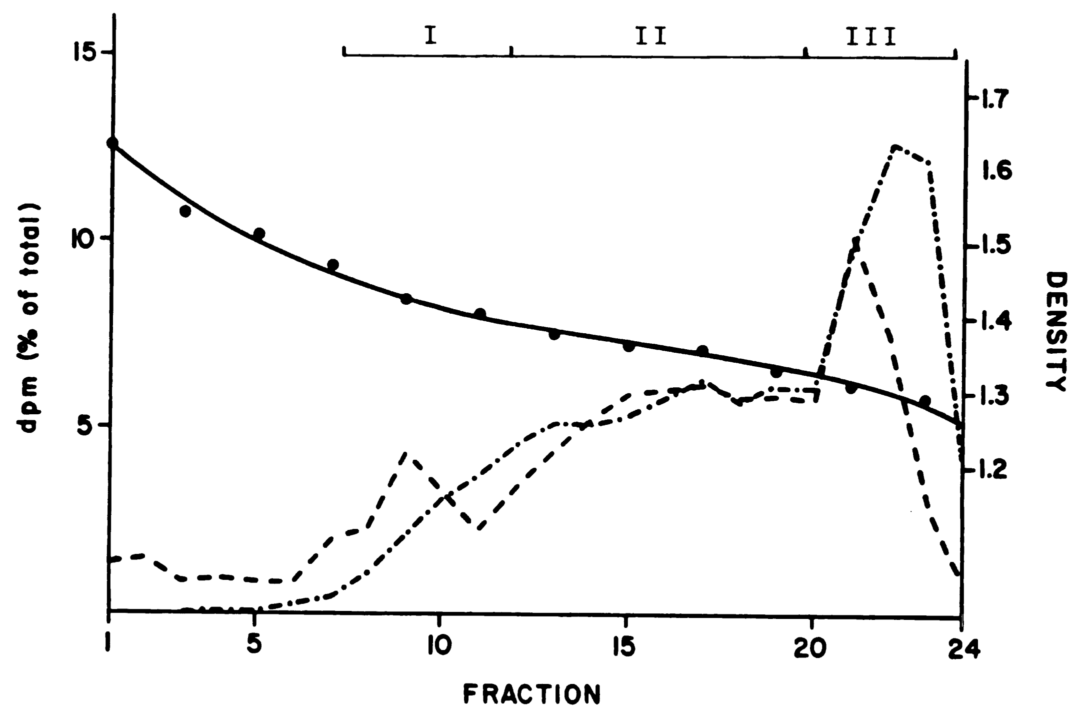
possessed the ability to inactivate the factor; the eluant from the chondroitin sulfate column retained this ability, as did an equivalent aliquot of heparinase which was not run on either column but simply diluted to the same volume as the eluted samples. Therefore, a component of heparinase that binds heparan sulfate, and does not bind chondroitin sulfate is necessary for inactivating the factor.

Further Purification of the "Neurite Outgrowth-Promoting Factor"

Although these results imply that heparan sulfate plays a role in the activity of the "neurite outgrowth-promoting factor", they do not imply that the factor consists, entirely, of a heparan sulfate proteoglycan. Proteoglycans are known to form complexes with hyaluronic acid (Sajdera and Hascall, 1969) and other ECM molecules (Lindahl and Höök, 1978; Yamada et al., 1980; Del Rosso et al. 1981; Hedman et al., 1982; Höök et al., 1984). It seemed possible that the neurite outgrowth-promoting factor might have been isolated as part of a complex with such molecules. To investigate this possibility, the labeled, partially purified "neurite outgrowth-promoting factor" was recentrifuged in CsCl containing 4 M GuHCl. Under these strongly chaotropic conditions, previously characterized proteoglycan complexes have dissociated (e.g. Sajdera and Hascall, 1969).

When fractions from such a gradient were collected (Figure 2.5), 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, marked 'I' in Figure 2.5, also containing ^{3}H, that sedimented at a higher density (1.40 - 1.45). This behavior would be expected of a proteoglycan that had

Figure 2.5. Fractionation of the partially purified "neurite outgrowth-promoting factor" by isopycnic sedimentation under dissociating conditions. Active fractions from associative gradient centrifugation (fractions 10-12 in Figure 2.3) 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. The roman numerals I, II and III demarcate regions of the gradient that are referred to in the text.


I
II
III
15
10
5
dpm (% of total)
1.7
1.6
1.5
1.4
1.3
1.2
DENSITY
1
5
10
15
20
24
FRACTION

formerly been associated with less dense molecules, such as proteins. In agreement with this, a substantial peak of ^3H was found at a lower density (region 'III' in Figure 2.5) appropriate for a protein or glycoprotein. Some of the ^{35}S and ^3H found between these two peaks was in a position consistent with proteoglycans that had remained at their original apparent density (region 'II' in Figure 2.5)--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; region 'III' in Figure 2.5) that it might represent sulfate-labeled material other than proteoglycans (cf. Branford-White, 1980), or proteoglycans associated with very low density molecules such as lipids.

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 BCE-CM_{SF} or the partially purified "neurite outgrowth-promoting 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 the active factor is inactivated by GuHCl at this high concentration.

Preliminary characterization of material isolated from CsCl gradients containing 4M GuHCl has indicated that nitrous acid-sensitive polysaccharides are present in both of the peaks of ^{35}S believed to represent proteoglycans (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

(Kosher and Searls, 1973). Because 4M GuHCl eliminated biological activity, it was not possible to determine which of the proteoglycans and proteins resolved in Figure 2.5 are involved in promoting neurite outgrowth.

DISCUSSION

Characterization of the "Neurite Outgrowth-Promoting Factor" Reveals the Presence of Protein and Heparan Sulfate

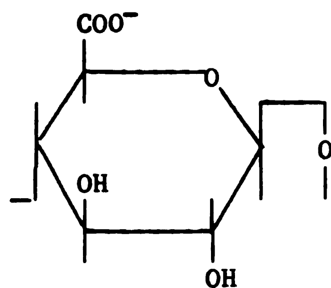
The experiments described in this chapter were carried out in order to obtain some basic information about the biochemical nature of the "neurite outgrowth-promoting factor" in BCE-CM_{SF}. The effect of trypsin (Table 2.1) establishes that a protein is involved, and the effects of prolonged heating and extremes of pH suggest that denaturation of this protein destroys its activity. The results of isopycnic centrifugation provide evidence that carbohydrate--sulfated carbohydrate in particular--is also a component of the "neurite outgrowth-promoting factor". The results of digestion with heparinase and chondroitinase indicate that this carbohydrate is the glycosaminoglycan heparan sulfate.

Like all glycosaminoglycans, heparan sulfate is a polymer of repeating disaccharide units composed of a hexose or hexuronic acid linked to a hexosamine, and which may be modified by N-acetylation, O-sulfation and/or N-sulfation. Different classes of glycosaminoglycans are distinguished by the particular sugars that comprise the disaccharide unit. Heparan sulfate and heparin form a class of glycosaminoglycan whose disaccharide unit consists of D-glucuronic acid or L-iduronic acid, linked $\beta(1 \rightarrow 4)$ to α-D-glucosamine. Positions where sulfation and acetylation may occur are shown in Figure 2.6. Up to five sulfate groups may be added to each disaccharide unit, although it is most common for heparan sulfate to contain 0.4-2 sulfates per disaccharide (Lindahl and Höök, 1978). The term "heparin" refers

Figure 2.6. Sugar composition of the heparan sulfate/heparin class of glycosaminoglycans (adapted from Höök *et al.*, 1984).

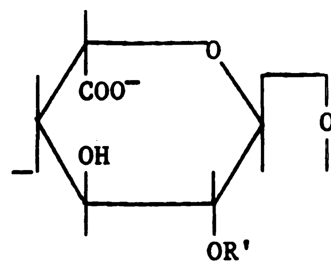
HEXURONIC ACID

HEXOSAMINE

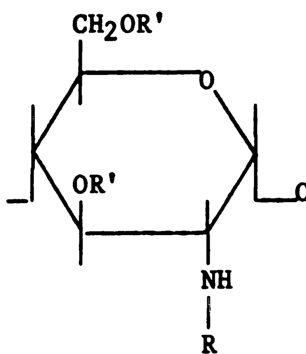


β -D-glucuronic acid

OR



α -L-iduronic acid



α -D-glucosamine

n

Where R = $-C(=O)CH_3$ or $-SO_3^-$

and R' = $-H$ or $-SO_3^-$

to low molecular weight glycosaminoglycans of this class that are derived from mast cells; they are heavily sulfated, and are not found covalently attached to proteins. With the exception of these molecules, heparan sulfate chains are always found attached to the proteins upon which they were synthesized, the entire molecule being referred to as a heparan sulfate proteoglycan (Lindahl et al., 1977).

Heparan sulfate proteoglycans are ubiquitous molecules which may have a great variety of biological functions. They are widely distributed in tissues including the brain (Toledo and Dietrich, 1977), are found on the surfaces of many cell types (Höök et al., 1984), and are present in the ECM in vivo (Hassell et al., 1980; Kanwar and Farquhar, 1979), and in vitro (e.g. Culp et al., 1978, 1980; Lark and Culp, 1983). The polysaccharide portions of heparan sulfate proteoglycans are diverse in length, degree of sulfation, and ratio of glucuronic to iduronic acid (Lindahl et al., 1977; Oldeberg et al., 1979; Radhakrishnamurthy et al., 1980). Some appear to be integrally associated with cell membranes; others may bind cell surface receptors (Kjellén et al., 1980; Hurst et al., 1981). Heparan sulfate may be a regulator of cell proliferation (Chiarugi and Vannucchi, 1976; Kraemer and Tobey, 1972; Cohn et al., 1976; Castellot et al., 1981; Fritze et al., 1985) and adhesion (e.g. Culp, 1978; Laterra et al., 1983; Cole et al., 1985). It may play a role in neuronal development since levels are much higher in the brains of developing animals than adults (Margolis et al., 1975).

The association of heparan sulfate with the neurite outgrowth-promoting activity in BCE-CM_{SF} is exciting because it is consistent with the active factor in BCE-CM_{SF} being a component of the ECM, which

could be appropriately disposed *in vivo* to interact with growing axons. Interestingly, since the publication of the results presented in this chapter, data have been obtained that also implicate a heparan sulfate proteoglycan in the neurite outgrowth-promoting activity of other CM's. For example, when several CM's were subjected to isopycnic centrifugation such as that shown in Figure 2.3, neurite outgrowth-promoting activity sedimented as a peak of about the same density as that observed for BCE-CM_{SF} (Lander *et al.*, 1983 and unpublished observations; Calof and Reichardt, 1984; Davis *et al.*, 1985). The "neurite outgrowth-promoting factor" in several CM's has also been shown to be sensitive to heparinase degradation (Lander *et al.*, 1983; Calof and Reichardt 1984; M. Gurney, personal communication). For one cell type, PC12 pheochromocytoma cells, monoclonal antibodies exist that recognize a heparan sulfate proteoglycan released into CM. This antibody has been shown to be able to remove, by immunoprecipitation, the "neurite outgrowth-promoting factor" in PC12-CM (Matthew *et al.*, 1985).

Is the "Neurite Outgrowth-Promoting Factor" a Heparan Sulfate Proteoglycan?

While the data presented in this chapter strongly suggest that a heparan sulfate proteglycan is involved in the activity of the "neurite outgrowth-promoting factor" in BCE-CM_{SF}, it would be incorrect to conclude at this point that the "neurite outgrowth-promoting factor" is a heparan sulfate proteoglycan. Instead, that possibility must be entertained along with the alternative possibility that the "factor" consists of two or more molecules associated with each other, only one

of which need be a heparan sulfate proteoglycan.

Both of these hypotheses are plausible. If the factor is a proteoglycan, it must require both its core protein and its heparan sulfate chains for activity, since both trypsin and heparinase abolish activity. Inactivation of the factor by 4M GuHCl would then most likely be interpreted as due to denaturation of the core protein.

Alternatively, if the factor is a complex consisting of more than one molecule, then the roles of different components of the complex in promoting neurite outgrowth may be difficult to ascertain. For example, the neurite outgrowth-promoting activity may depend on the presence of both a heparan sulfate proteoglycan and an associated molecule, with the integrity of the complex being required for biological activity. Such an associated molecule would most likely be a protein or glycoprotein, rather than another proteoglycan or a glycosaminoglycan such as hyaluronic acid, since the "neurite outgrowth-promoting factor" is resistant to chondroitinase ABC. If the associated molecule is a (glyco)protein, then degradation of it, rather than degradation of the proteoglycan core protein, could account for the ability of trypsin to inactivate the "neurite outgrowth-promoting factor". Likewise, inactivation of the "factor" by 4M GuHCl could reflect dissociation of a proteoglycan-containing complex, provided that the complex does not readily re-form after removal of the chaotropic salt.

It is interesting to consider how a complex of two or more molecules might be needed to mediate the promotion of neurite outgrowth. One possibility is that neurons must interact with two molecules simultaneously in order to respond. A second possibility is that the complexing of two molecules generates a single active site with which

neurons interact. A third possibility is that only one of the components of the complex is directly responsible for promoting neurite outgrowth, but it requires that another component be bound to it in order for it to express its activity. This third possibility is important to consider, because the neurite outgrowth assay described in this chapter requires that the "neurite outgrowth-promoting factor" be capable of doing two things: it must adsorb to polylysine substrata, and it must promote neurite outgrowth. It is easy to imagine that one component of the "factor" might mediate binding to polylysine, while another interacts with neurons. A proteoglycan would be ideally suited to the first task, because of its strongly negative charge. Thus, the action of the "neurite outgrowth-promoting factor" on neurons could relate more directly to a molecule associated with a heparan sulfate proteoglycan, rather than to a heparan sulfate proteoglycan itself.

These considerations make it clear that, in order to understand the action of the "neurite outgrowth-promoting factor" on neurons, it is necessary to determine whether it consists of a proteoglycan, or a proteoglycan-containing complex, and, if it is a complex, which molecules are responsible for its biological activity. Unfortunately, the data presented in this chapter do not resolve this question. Certain observations suggest that a complex may be involved--for example, the "factor" has a very large molecular size (Figure 2.2), and proteoglycan-containing complexes do appear to be present in a partially-purified neurite outgrowth-promoting fraction (see Figure 2.5, and discussion above). However, the definitive answer to this question required further purification and characterization of the "neurite outgrowth-promoting factor", the results of which are presented in Chapter 4.

C H A P T E R 3

LAMININ CAN ACT AS A "NEURITE OUTGROWTH-PROMOTING FACTOR"

SUMMARY

In this chapter, experiments are described in which various purified molecules derived from the ECM are screened for neurite outgrowth-promoting activity. One of these molecules, the glycoprotein laminin, is shown to be as effective as the "neurite outgrowth-promoting factor" in BCE-CM. However, whereas the neurite outgrowth-promoting activity of laminin-treated substrata is blocked by the application of anti-laminin antibodies, the neurite outgrowth-promoting activity of BCE-CM—treated substrata is not. In fact, of six different CM-treated substrata tested, the neurite outgrowth-promoting activity of only one of them is diminished by treatment with anti-laminin antibodies. What these results imply about the relationship between laminin and CM-derived "neurite outgrowth-promoting factors" is discussed.

RESULTS

The results in Chapters 1 and 2 indicate that the "neurite outgrowth-promoting factor present in BCE-CM and other CM's may be composed of a heparan sulfate proteoglycan and possibly other molecules found in ECM. To investigate this question, two complementary approaches were taken. First, certain ECM molecules that have been identified and are available in pure form were screened for neurite outgrowth-promoting activity when after they had been adsorbed to a polylysine-coated substratum. These results are presented below. The second approach, the results of which are presented in the following chapter, involved the use of standard fractionation methods to purify the "neurite outgrowth-promoting factor" from BCE-CM, and determine its composition directly.

Screening of molecules for neurite outgrowth-promoting activity

The molecules tested for the ability to promote neurite outgrowth are shown in Table 3.1. The list is not at all exhaustive, being greatly limited by availability. As the data in Table 3.1 indicate, the six classes of glycosaminoglycans, fibronectin, and rat tail collagen (which consists predominantly of interstitial-type collagens), all components of ECM, had no neurite outgrowth-promoting activity, at least not for neonatal rat sympathetic neurons grown in the absence of NGF. The basement membrane glycoprotein laminin, however, was very effective in promoting neurite outgrowth. In fact, the response of rat sympathetic neurons to a laminin-treated substratum was essentially equal to that seen on a BCE-CM-treated substratum. There was also no

TABLE 3.1. Neurite outgrowth-promoting activity of purified molecules.

Substance Applied	Concentration Applied	Amount Bound (ug/cm^2)	Neurite Outgrowth (%)
1. Glycosaminoglycans			
Hyaluronic Acid	1 mg/ml	0.31	< 1
Dermatan Sulfate	1 mg/ml	0.54	< 1
Keratan Sulfate	1 mg/ml	N.D.	< 1
Chondroitin Sulfate (mixed isomers)	1 mg/ml	0.63	< 1
Heparan Sulfate	1 mg/ml	0.45	< 1
Heparin	1 mg/ml	0.73	< 1
2. Fibronectin, plasma	450 ug/ml	N.D.	< 1
	28 ug/ml	N.D.	< 1
3. Collagens, rat tail	*	N.D.	< 1
4. Laminin	5 ug/ml	N.D.	77 ± 1.2
5. Platelet Factor 4	1 mg/ml	N.D.	< 1
6. BCE-CM	undiluted	N.D.	74 ± 3

Samples diluted in PBS were applied to polylysine-coated surfaces overnight, and neurite outgrowth-promoting activity assayed. Duplicates of the glycosaminoglycan-treated surfaces were treated with concentrated sulfuric acid, to remove bound material, and assayed for total uronic acid by the carbazole method (Bitter and Muir, 1962). By correlating these results with standard curves for each glycosaminoglycan, it was possible to calculate the amount bound. Since keratan sulfate does not contain uronic acid, its binding was not assayed. N.D. = not done.

* Collagen was applied as a 1:4 dilution of rat tail tendon extract (Hawrot, 1980) in distilled water, and a thin film was air dried.

qualitative difference seen, in terms of neuronal morphology or average apparent neurite length, between neurons cultured on laminin-treated substrata and on BCE-CM-treated substrata.

The remaining molecule tested that is shown in Table 3.1 is platelet factor 4. This protein is not a component of ECM, but it tightly binds heparin and heparan sulfate (Levine and Wohl, 1976), a function that it shares with several ECM molecules including fibronectin and laminin. As shown in Table 3.1, platelet factor 4 did not promote neurite outgrowth. This is interesting, because rat sympathetic neurons appear to contain a heparan sulfate proteoglycan on their surfaces (Matthew et al., 1985). This suggests that the presence on the culture substratum of a molecule that binds neuronal cell surfaces is not by itself sufficient to promote neurite outgrowth by rat sympathetic neurons in the absence of NGF. This view is borne out by the observations that neurons are not stimulated to extend neurites when they are plated onto substrata treated with cell-surface-binding antibodies, such as anti-heparan sulfate proteoglycan (data not shown) or anti-"neural cell adhesion molecule" (K. Tomaselli, personal communication).

Thus, of all the molecules tested, only laminin promoted neurite outgrowth from rat sympathetic neurons. Indeed, the effects of laminin were not limited to these neurons, but were also demonstrated for rat sensory neurons (data not shown) and chicken motoneurons (Calof and Reichardt, 1984). In order to determine the quantity of laminin required to promote outgrowth, different concentrations of laminin were applied to polylysine substrata overnight, and these substrata were tested for neurite outgrowth-promoting activity. The data form

the dose-response curve shown in Figure 3.1, and indicate that half-maximal activity is achieved at a concentration of applied laminin between 50 and 100 ng/ml. The amount of laminin actually binding to the substratum under these conditions was not determined. Interestingly, it was found that, unless freshly thawed laminin solutions were used in these assays, a greater concentration of laminin was needed to achieve half-maximal activity. For example, solutions of laminin that had remained for 1 day at 4°C showed half maximal activity anywhere between 100 ng/ml and 1 ug/ml. As such samples did not appear to be undergoing proteolysis (as judged by SDS-gel electrophoresis, data not shown), it seems likely that this effect reflected the tendency of laminin to self-aggregate (Engel et al., 1981). Perhaps self-aggregation decreases the number of laminin molecules available to promote neurite outgrowth.

Effects of anti-laminin antibodies on neurite outgrowth-promoting activity

Because substratum-bound laminin mimics the effects of the substatum-bound BCE-CM on neurons, it seemed possible that laminin is related to, or is a component of, the "neurite outgrowth-promoting factor" in BCE-CM and other CM's. As a preliminary test of this hypothesis, a rabbit antiserum was generated against purified mouse laminin. As Table 3.2 shows, the response of rat sympathetic neurons to a laminin-treated substratum is effectively blocked by this anti-laminin antiserum. This was observed not only if the antiserum was present throughout the period of neuronal culture, but also if the antiserum was just exposed to the substratum, and then washed away

Figure 3.1. Response of rat sympathetic neurons to polylysine-coated substrata treated with different concentrations of laminin. Laminin solutions, stored at -70°C, were thawed and diluted to the indicated concentrations in TBS. 50 ul samples were applied to polylysine-coated wells of a 96-well microtiter plate (0.32 cm^2/well) and incubated overnight. Wells were washed and assayed for neurite outgrowth-promoting activity as described (see Methods).

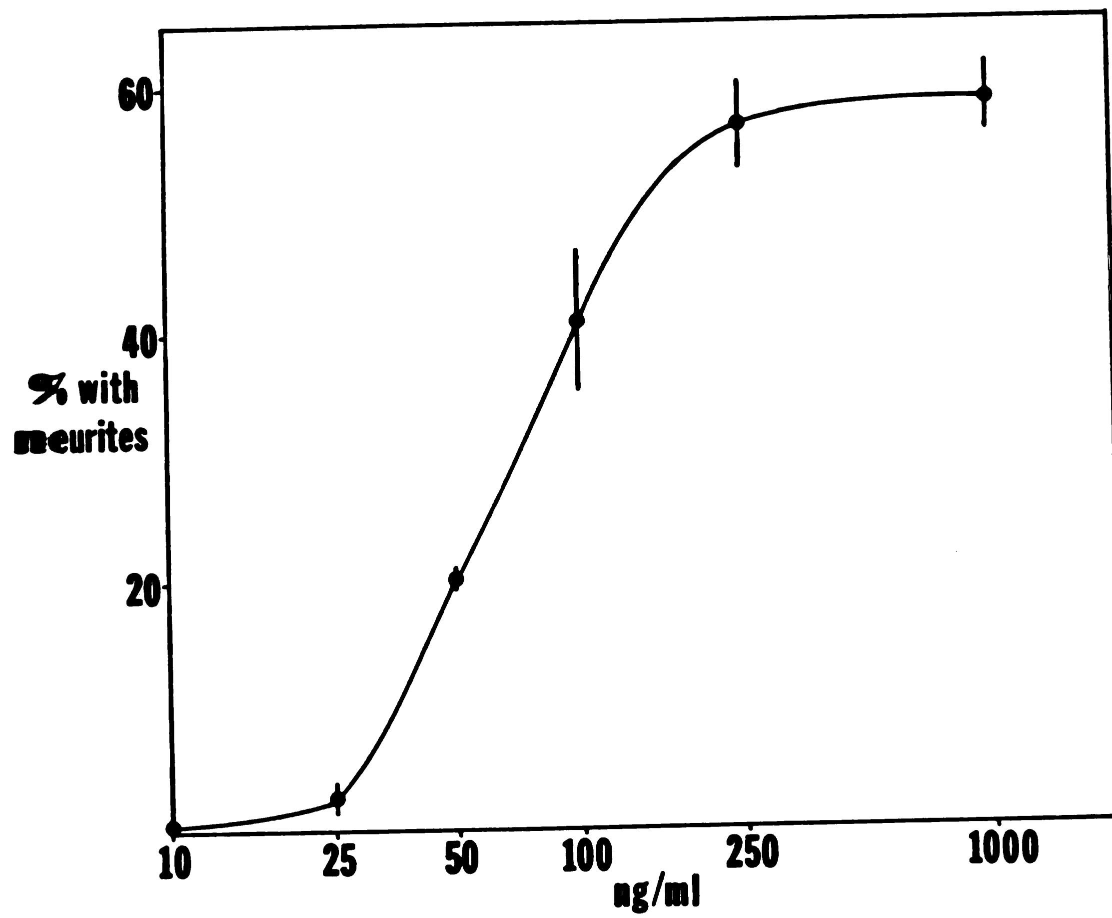

60
40
20
% with
neurites
10
25
50
100
250
1000
ng/ml

TABLE 3.2. Effect of anti-laminin antiserum on the neurite outgrowth-promoting activity of laminin-treated and CM-treated substrata.

	Neurite outgrowth (% with neurites) after antibody treatment of substratum		
Substratum	No antibody	Anti-laminin applied, washed off before culture	Anti-laminin present in culture
Laminin 5 ug/ml	77 ± 1.2	6 ± 0.7	2 ± 0.5
BCE-CM	65 ± 3.8	72 ± 5.4	64 ± 6.5
PC12-CM	53 ± 0.4	50 ± 2.4	51 ± 2.6
RN22-CM	49 ± 1.0	52 ± 0.5	47 ± 1.4
C_2-CM	31 ± 2.9	35 ± 1.7	32 ± 1.6
Chick Muscle CM	73 ± 3.4	75 ± 7.9	76 ± 2.4
PFHR9-CM	59 ± 0.6	32 ± 1.0	14 ± 1.9
Control: untreated polylysine	<2	<2	<2

before addition of neurons. However, when the same experiments were performed using substrata treated with BCE-CM, and CM's from PC12 rat pheochromocytoma cells, RN22 rat Schwannoma cells, C_2 mouse myotubes, primary chick muscle cultures, and PFHR9 endodermally-differentiated mouse teratocarcinoma cells, very different results were seen (Table 3.2). With the exception of PFHR9-CM, the neurite outgrowth-promoting activity of the other CM's was not affected by treatment of the substratum with anti-laminin antibodies or by inclusion of anti-laminin antibodies during neuronal culture. For PFHR9-CM, neurite outgrowth-promoting was diminished somewhat by anti-laminin antibodies, but was not completely blocked. The amount of anti-laminin serum used in these experiments was sufficient to block the activity of saturating amounts of laminin, so it is unlikely that the results in Table 3.2 merely reflect an insufficiency of antibody. Instead, it must be concluded that the "neurite outgrowth-promoting factors" in BCE-CM and at least four of five other CM's tested are immunochemically distinct from mouse laminin. Whether this implies that laminin is not related to or involved in the activity of the "neurite outgrowth-promoting factors" in these CM's will be discussed below.

DISCUSSION

Neurite Outgrowth-Promoting Activity of Laminin

The experiments reported in this chapter indicate that laminin, a basement membrane glycoprotein, can adsorb to polylysine-coated substrata and promote neurite outgrowth in a manner much like the "neurite outgrowth-promoting factor" in BCE-CM and other CM's. These results extend the observations of Baron-Van Evercooren et al. (1982) who reported that substratum-bound laminin enhanced the neuritic growth of cultured human fetal sensory neurons. These results are also corroborated by the work of Manthorpe et al. (1983), who reported that laminin-treated polyornithine-coated substrata promoted neurite outgrowth by several classes of chick, mouse and rat neurons, with half-maximal activity when a concentration of 50 ng/ml was applied to the substratum (cf. Figure 3.1).

The observation that many other ECM molecules do not promote neurite outgrowth (Table 3.1) is also in agreement with other reports. For example, Manthorpe et al. (1983) confirmed that heparin and hyaluronic acid have no neurite outgrowth-promoting activity when tested on chick parasympathetic neurons. In addition, they screened two proteoglycans, a chondroitin sulfate proteoglycan from a rat yolk sac tumor, and a heparan sulfate proteoglycan from a rat hepatoma, and found that these also lacked neurite outgrowth-promoting activity.

In contrast to the results reported in this chapter, several groups have reported that substratum-bound fibronectin has some neurite outgrowth-promoting activity (e.g. Akers et al. 1981; Baron-Van Evercooren et al., 1982; Rogers et al., 1983; Manthorpe et al., 1983).

However, certain observations distinguish the effects of fibronectin from those of laminin and the "neurite outgrowth-promoting factors" in CM's. For example, whereas laminin appears to promote outgrowth from all of the types of neurons that respond to CM-treated substrata, the actions of fibronectin are limited to neurons of the peripheral nervous system (e.g. Rogers et al. 1983). Whereas laminin elicits a response from the same percentage of neurons in a culture as do the "neurite outgrowth-promoting factors" in CM's, fibronectin has a much weaker effect (e.g. Baron-Van Evercooren et al., 1982; Manthorpe et al., 1983). Indeed, the similarity in the actions of laminin and the CM-derived "neurite outgrowth-promoting factors" has been observed to be extremely close, with both agents promoting the early outgrowth of similar numbers of neurites per cell, of similar length and morphology (Davis et al., 1984a). These data lend support to the hypothesis that the "neurite outgrowth-promoting factors" in CM's are related in some way to laminin. It is thus worth reviewing what is known about the biochemical and functional properties of laminin.

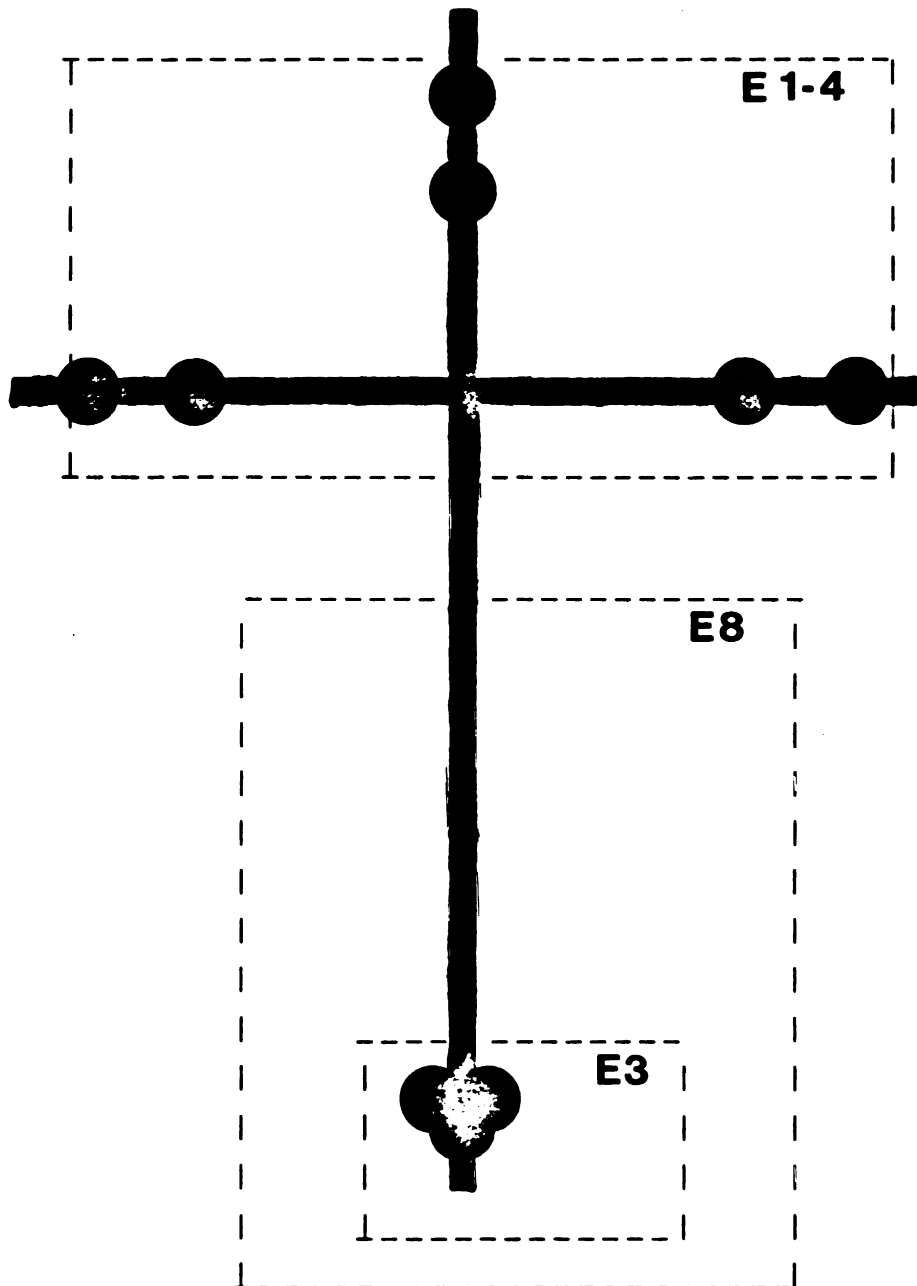
Structure and Function of Laminin

Laminin is a high molecular weight glycoprotein that appears to be a major component of basement membranes in vivo (Timpl et al., 1982). It was first isolated as a major product of certain mouse tumor cells that form basement membrane-like structures (Timpl et al., 1979; Chung et al., 1979). One of these tumors, the Engelbreth-Holm-Swarm (EHS) sarcoma, produces such a large quantity of easily purified laminin that the characterization of this glycoprotein has been able to proceed at a remarkable pace.

Laminin from the EHS sarcoma has a molecular weight somewhere between 850,000 and 1,000,000 Da, and, when subjected to electrophoresis under reducing conditions, it appears to contain two kinds of polypeptide chains: a heavy or "A" chain (forming a diffuse band at about 400,000 Da) and a light or "B" chain (forming a diffuse band at about 200,000 Da) (Timpl et al., 1979). The ratio of A to B chain suggests that laminin is composed of one A chain and either two (Howe and Dietzschold, 1983) or three (Engel et al., 1981) B chains. It is clear, however, from biosynthetic studies with cultured teratocarcinoma cells--the laminin from which runs less diffusely on SDS-gels--that at least two, and probably three, different light chain polypeptides exist. These have been tentatively termed B1a, B1b and B2, and they have molecular weights of 205,000, 200,000 and 185,000 respectively (Barlow et al., 1984). It is not known whether laminin molecules contain one each of the different B chains, or whether laminin molecules normally contain only one type or another.

The structure of the laminin molecule is one of its most intriguing features. Electron microscopic observations of rotary shadowed or negatively stained laminin molecules reveals an asymmetric cross-like structure, with three short arms and one long arm. A schematic diagram of this structure is shown in Figure 3.2. At least seven globular domains have been identified on this cross. Because each of the three short arms appear similar in size and shape, and because the long arm is about twice the length of each short arm, it initially seemed likely that the three short arms represented three B chains, and the long arm a single A chain. While some studies with proteolytic fragments of laminin supported this assignment (e.g. Rao

Figure 3.2. Schematic representation of a laminin molecule. The regions from which fragments E1-4, E3, and E8 are derived are indicated (after Ott _et al._, 1982; Timpl _et al._ 1983; and Paulsson _et al._, 1985)


E 1-4
E8
E3

et al., 1982), it now appears unlikely to be correct. It seems, for example, that at least one of the short arms is different in protease sensitivity from the other two (Timpl *et al.*, 1983). In addition, one fragment has been isolated from the long arm and clearly shown, by immunochemical analysis and sequence comparison, to be derived from a B1 chain (Paulsson *et al.*, 1985). At present, there is no generally accepted model for the polypeptide arrangement in the laminin cross, but it is hoped that further work with laminin fragments and laminin cDNA's (e.g. Barlow *et al.*, 1984; Paulsson *et al.*, 1985) will resolve this question.

Despite the confusion about the correct arrangement of A and B chains in the laminin molecule, much progress has been made toward identifying functions of laminin, and in assigning these functions to domains within the molecule. In particular, laminin has been shown to bind type IV collagen, heparin and heparan sulfate, and a 68,000 Da high-affinity receptor present on the surfaces of epithelial, endothelial and muscle cells (Sakashita *et al.*, 1980; Del Rosso *et al.*, 1981; Bächinger *et al.*, 1982; Terranova *et al.*, 1983; Brown *et al.*, 1983). While the major cell surface- and collagen-binding domains appear to be located within the short arms of the laminin molecule, the major heparin-binding domain appears to be located at the distal end of the long arm (Ott *et al.*, 1982). It may be recovered in active form in the 50,000 Da elastase fragment E3, which consists of the globular region at the end of the long arm, or in the 280,000 Da limited elastase fragment E8, which contains E3 as well as much of the rest of the long arm (see Figure 3.2). Another, although possibly weaker, heparin-binding domain also appears to be present in the elastase

fragment E1-4 (see Figure 3.2), which consists of most of the conjoined short arms of laminin (Ott et al., 1982; Timpl et al., 1983).

Recently, the domains of laminin involved in its neurite outgrowth-promoting activity have been investigated. Edgar et al. (1984) showed that the neurite outgrowth-promoting activity of laminin could be blocked not only by antibodies directed against the entire laminin molecule (as in Table 3.2), but also by antibodies directed only against fragment E3, the 50,000 Da fragment of the long arm that includes a heparin binding domain. Antibodies directed against various other fragments of laminin--including the collagen IV-binding and major cell-binding domains-were not found to be effective in blocking its neurite outgrowth-promoting activity. Although these results suggest that fragment E3 represents the part of the laminin molecule that is responsible for neurite outgrowth-promoting activity, Edgar et al. were not able to demonstrate any ability of the isolated fragment E3 to promote neurite outgrowth, although some promotion of outgrowth could be achieved with the 280,000 Da, E3-containing fragment E8. Oddly, this group found that fragment E1-4 also possessed neurite outgrowth-promoting activity. Since the activity of this fragment was not suppressed by any of the antisera tested--including antisera directed agains whole laminin, which completely blocked the outgrowth-promoting activity of intact laminin--it was concluded that the neurite outgrowth-promoting domain within fragment E1-4 is either unmasked or activated by the proteolytic cleavage that generates the fragment (Edgar et al., 1984). Unmasking or activation of functional domains by proteolysis has been observed with other molecules, including the ECM glycoprotein fibronectin (Sekiguchi et al., 1983; Johansson and

Höök, 1984).

Laminin and "Neurite Outgrowth-Promoting Factors": Similarities and Differences

It has already been pointed out that the ability of substratum-bound laminin to mimic closely the neurite outgrowth-promoting effects of CM-treated substrata suggests that laminin and the "neurite outgrowth-promoting factors" in CM's may be related. Indeed, there are other similarities. For example, CM-treated substrata have been found not only to promote neurite outgrowth by chick sympathetic neurons, but also to alter their requirements for Nerve Growth Factor (NGF), so that more neurons survive in the presence of less NGF (Edgar and Thoenen, 1982). Laminin-treated substrata also mimic this effect of CM-treated substrata (Edgar *et al.*, 1984). In fact, the same domains of laminin that have been implicated in its neurite outgrowth-promoting activity also appear to mediate this potentiation of NGF action (Edgar *et al.* 1984).

Certain other features of laminin coincide with some of the observations made on "neurite outgrowth-promoting factors" in CM's. For example, laminin is an unusually large molecule (cf. Figure 2.2), laminin is produced by many cells types (cf. Table 1.3), and although laminin is not a heparan sulfate proteoglycan, laminin binds heparan sulfate (Sakashita *et al.*, 1980; Del Rosso *et al.*, 1981) and might very well associate with heparan sulfate proteoglycans in CM's (cf. Chapter 2, discussion).

Despite these similarities, the antibody blocking experiments summarized in Table 3.2 indicate a clear difference between laminin

and the "neurite outgrowth-promoting factors" in several CM's. These data have been confirmed by several groups (Manthorpe et al., 1983; Edgar et al., 1984; Coughlin, M.D., personal communication). One group has shown not only that anti-laminin antibodies do not block the activity of the "neurite outgrowth-promoting factor" in one CM, but also that antibodies raised against a partially purified "neurite outgrowth-promoting factor" from that CM block the activity of that "neurite outgrowth-promoting factor" but not the activity of laminin (Edgar et al., 1984).

These results have generally been interpreted as implying that laminin is not involved in the neurite outgrowth-promoting activity of CM-treated substrata (e.g. Manthorpe et al., 1983; Edgar et al., 1984). It seems premature, however, to base such a conclusion on these immunochemical data alone. Antisera that block a biological activity may do so because they contain antibodies that bind a few crucial epitopes on the active molecule. Antibody blockade could therefore be circumvented either by slight alterations in the structure of these few epitopes, or by any process that sterically hinders the access of antibodies to these epitopes. That likelihood that such phenomena could occur with laminin molecules is discussed at length in Chapter 5. Before that, however, the data obtained from the purification of the "neurite outgrowth-promoting factor" in BCE-CM need to be considered. These data are presented in the following chapter.

C H A P T E R 4

THE "NEURITE OUTGROWTH-PROMOTING FACTOR" IN BCE-CM IS COMPOSED OF LAMININ, ENTACTIN, AND A HEPARAN SULFATE PROTEOGLYCAN

SUMMARY

In this chapter, the purification of the "neurite outgrowth-promoting factor" from BCE-CM is described. The "factor" is shown to be composed of the glycoprotein laminin, and two associated laminin-binding molecules: a sulfated protein known as entactin, and a large heparan sulfate proteoglycan. Of these molecules, only laminin was found to be present, throughout the purification, in all fractions possessing neurite outgrowth-promoting activity and absent from all fractions lacking activity. Importantly, removal of laminin from BCE-CM by immunoprecipitation with anti-laminin antibodies resulted in the disappearance of neurite outgrowth-promoting activity. Although the results presented and discussed in this chapter imply that laminin is primarily responsible for the biological activity of the "neurite outgrowth-promoting factor", evidence is also presented that the association of a proteoglycan with laminin is responsible for efficient attachment the "factor" to polycationic substrata, particularly in the presence of competing molecules.

RESULTS

Purification of the "Neurite Outgrowth-Promoting Factor" from BCE-Conditioned Medium

To aid in the detection of proteins present at low concentrations, purification was carried out using BCE-CM that had been metabolically labeled with [^{35}S]methionine. In the first step of purification (Table 4.1), BCE-CM was depleted of fibronectin by absorption with gelatin-Sepharose (Ruoslahti et al., 1982). Neurite outgrowth-promoting activity was detected only in the fraction not bound to the affinity-matrix (Table 4.1, line 2). To this fraction was added ammonium sulfate to 48% of saturation, the minimum concentration required to precipitate all outgrowth-promoting activity (Table 4.1, line 3). The ammonium sulfate precipitate was collected, redissolved, dialyzed, and applied to DEAE-cellulose. Nearly all of the neurite outgrowth-promoting material bound to the matrix, while > 85% of the applied [^{35}S]methionine did not (Table 4.1, line 4). The active material was eluted from the DEAE-cellulose with a step of 1.0 M NaCl; salt gradient elution was not used because the active material was found to elute in a very broad peak between 0.1 M and 0.8 M NaCl. The DEAE eluate was further fractionated by gel filtration on a column of Sepharose CL4B (Table 4.1, line 5). As shown in Figure 4.1, neurite outgrowth-promoting activity appeared in a single peak just after the void volume, with a shoulder trailing off into more included fractions. The majority of applied [^{35}S]methionine was found in later fractions not associated with outgrowth-promoting activity. This pattern of fractionation was observed in all preparations, although

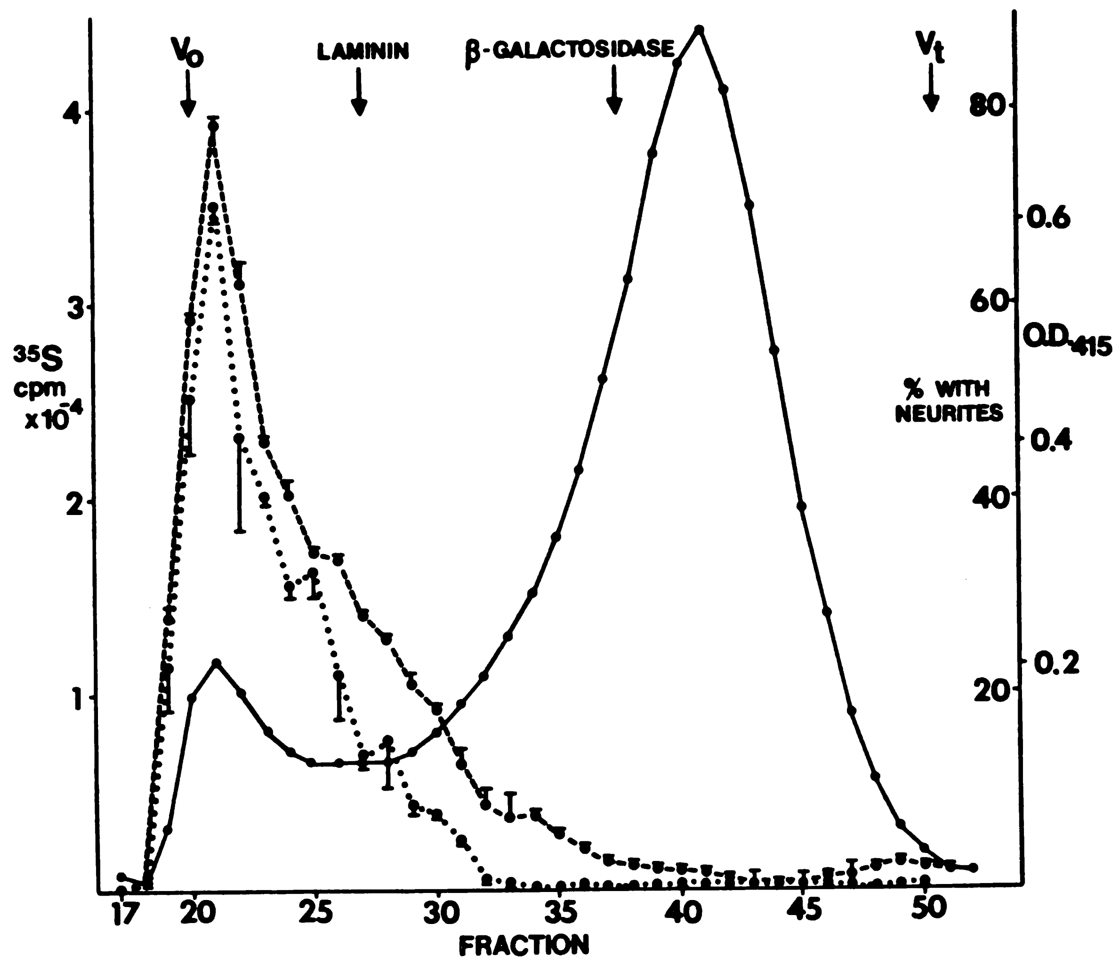
Legend for Table 4.1

Methionine-labeled BCE-CM was fractionated as described (Methods). For fractions containing unincorporated [^{35}S]methionine (i.e. prior to dialysis of ammonium sulfate pellet) the data shown refer to trichloroacetic acid-precipitable material (removal of free methionine by dialysis gave similar results). For subsequent fractions total ^{35}S content is shown. Neurite outgrowth-promoting units are defined in Methods. Where neurite outgrowth-promoting activity was not detectable, activity is expressed as less than the greatest number of units that, given the sample volume, would have just reached the limit of detection (roughly 0.25 units/ml). Explanation for apparent increase in the total number of units during the purification are discussed in the text. Given this behavior, an accurate assessment of the purification factor is not possible, and yields must be expressed as "apparent". The fraction numbers shown for the gel filtration and velocity sedimentation steps refer to figures 1 and 2, respectively.

TABLE 4.1 Purification of Neurite Outgrowth-Promoting Activity from BCE-CM

	Neurite Outgrowth-Promoting Activity (units)	Incorporated $[^{35}S]$methionine (cpm x 10^{-6})	Specific Activity (units ÷ cpm x 10^{-6})	Apparent Yield (%)
1. BCE-CM	155	1480	0.105	(100)
2. Gelatin-Sepharose: not bound	170	1224	0.139	(109)
bound, eluted with 4M urea	< 20	139		
3. Ammonium sulfate: pellet	213	586	0.363	(137)
supernatant	< 50	651		
4. DEAE-cellulose: bound, eluted	200	71.1	2.81	(129)
not bound	< 20	520		
5. Sepharose CL4B: fractions 19-28	166	9.13	18.2	(107)
6. Velocity Sedimentation: fractions 5-14	140	4.14	33.8	(90)

Figure 4.1. Gel Filtration on Sepharose CL4B. The presence of [^{35}S]methionine label was assayed in 10 ul samples of each fraction (________________) Dilutions of each fraction (1:12 in TBS) were applied to polylysine-treated wells for determination of neurite outgrowth-promoting activity (••••••••••). and laminin immunoreactivity (- - - - - - -) as described. Chromatographic positions of standards (see Methods) are shown (arrows).

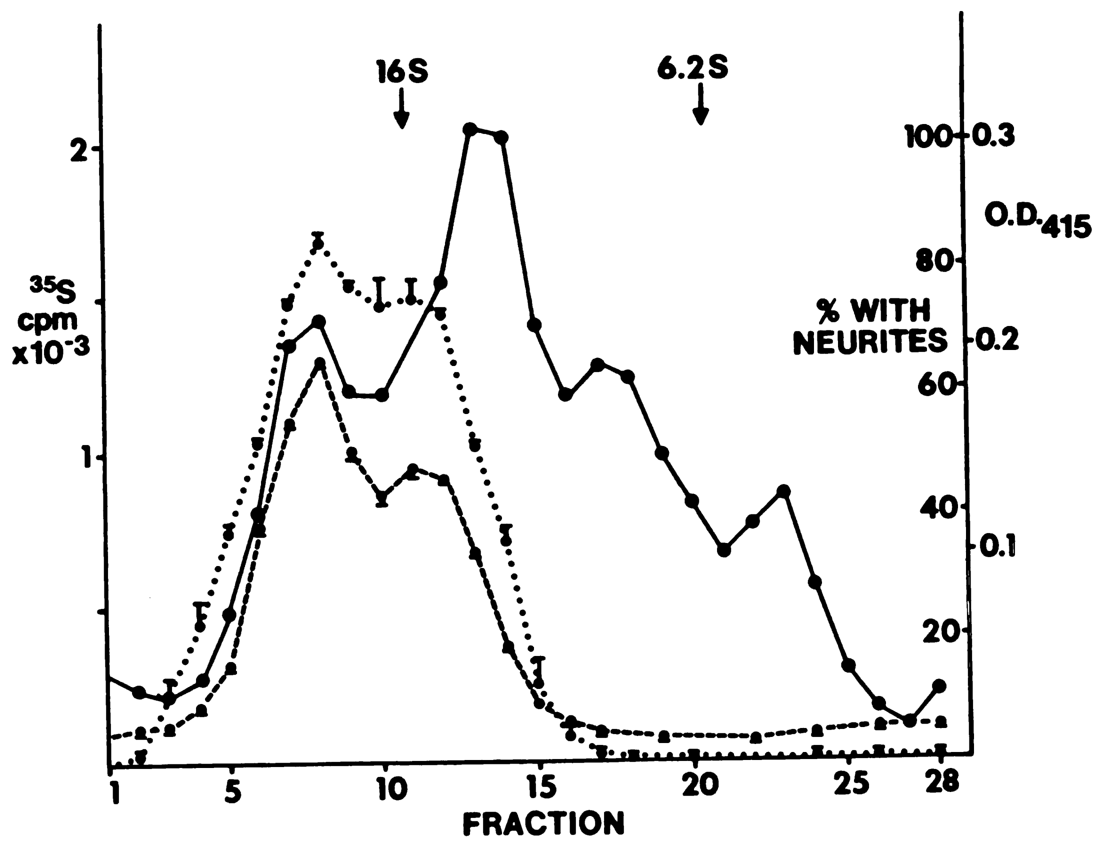


Vo
LAMININ
β-GALACTOSIDASE
Vt
35S cpm x10-4
O.D.415
% WITH NEURITES
FRACTION

the relative proportions of the peak and shoulder varied. As a final step of purification, pooled fractions from gel filtration were concentrated, dialyzed, and sedimented through a 5-20% sucrose gradient. The labeled material fractionated into four peaks (Figure 4.2). The most mobile peak ($\sim$19S) was associated with neurite outgrowth-promoting activity, as was the leading edge (i.e., $\sim$ 16S) of the second most mobile peak (centered at $\sim$ 13S). The two peaks of lowest mobility ($\sim$9S and $\sim$4S) lacked biological activity. This pattern was highly reproducible. The immunoassay results shown in Figures 4.1 and 4.2 will be discussed below.

As shown in Table 4.1, each purification step resulted in an increase in specific activity. This increase was approximately 2.6 fold after ammonium sulfate precipitation, a further 7.8 fold after ion-exchange chromatography, 6.5 fold following gel filtration, and 1.9 fold following velocity sedimentation. The net increase in specific activity was 322-fold. This number cannot be considered an accurate estimate of fold purification, because, as Table 4.1 shows, the total quantity of neurite outgrowth-promoting activity markedly increased after each of the first two purification steps. Results such as these usually indicate that substances present in the starting material inhibit activity or interfere with the assay, artifically depressing the quantity of activity measured. For this reason, the actual amount of neurite outgrowth-promoting activity initially present is not known, but must be at least as great as the amount measured after the second purification step, and most likely even greater (since some loss probably occurred during the first two purification steps). Thus, the purification factor can be estimated as less than 235-fold.

Figure 4.2. Velocity Sedimentation through 5-20% sucrose. The presence of [^{35}S]methionine label was assayed in 10 ul samples of each fraction (___________). Dilutions of each fraction (1:8 in TBS) were applied to polylysine-treated wells for determination of neurite outgrowth-promoting activity (·············) and laminin immunoreactivity (- - - - - -) as described. Positions of sedimentation standards (β-galactosidase, 16S, and intestinal alkaline phosphatase, 6.2S) are shown (arrows).



16S
6.2S
^{35}S cpm x10^{-3}
2
1
% WITH NEURITES
100
80
60
40
20
0.3
0.2
0.1
O.D.415
1
5
10
15
20
25
28
FRACTION

Composition of the "Neurite Outgrowth-Promoting Factor"

To identify the molecules purified by the above procedure, fractions from different stages of purification were analyzed by denaturing polyacrylamide gel electrophoresis (SDS-PAGE) in 2.9-15% exponential gradient gels under non-reducing conditions, and labeled molecules were visualized by fluorography (Figure 4.3). Comparison of lanes 1 and 2 illustrates that absorption with gelatin-Sepharose removes a major protein band of 440,000 Da, representing fibronectin, from the starting material. Subsequent purification by ammonium sulfate precipitation (lane 3) and ion-exchange chromatography (lane 4) removes many additional protein bands. Lane 5 shows the labeled molecules present in the active peak of the gel filtration profile (fraction 21 of Figure 4.1) and lane 6 is from the "shoulder" (fraction 25 of Figure 4.1). While lane 5 contains some bands not present in lane 6, only two bands are present in both lanes, one that comigrates with a laminin standard (850,000- 10^6 Da), marker 'a', and one that comigrates with a mouse IgG standard (150,000 Da), marker 'c'. The unusually high molecular weight of the first band allows it to be visualized even in crude CM (lane 1). By following it through the steps of purification, examining side fractions as well (not shown), it can be seen that each purification step enriches for this protein. Also present in lanes 4, 5 and 6 is labeled material that does not band sharply, but appears as a diffuse blur beginning at the top of the separating gel, and extending beyond the location of the laminin standard. It is particularly apparent in lane 5.

Figure 4.4 illustrates in greater detail the fractionation of labeled molecules during gel filtration. Alternate fractions from the

Figure 4.3. Analysis of purification by SDS-PAGE. Samples at different stages of purification were analyzed by SDS-PAGE under non-reducing conditions (see Methods). The samples in each lane and the amount (in cpm x 10^{-3}) applied were: Lane 1, BCE-CM (191); Lane 2, Material not adsorbed to gelatin-Sepharose (142); Lane 3, Ammonium sulfate pellet (309); Lane 4, material bound to DEAE-cellulose and eluted with 1 M NaCl (33). Lane 5, Gel filtration, fraction 21 (see Figure 4.1) (5.9); Lane 6, gel filtration, fraction 25 (see Figure 4.1). (3.3) Standards: a) laminin (850,000- 10^6 Da) b) cellular fibronectin (440,000 Da) c) mouse IgG (150,000 Da).

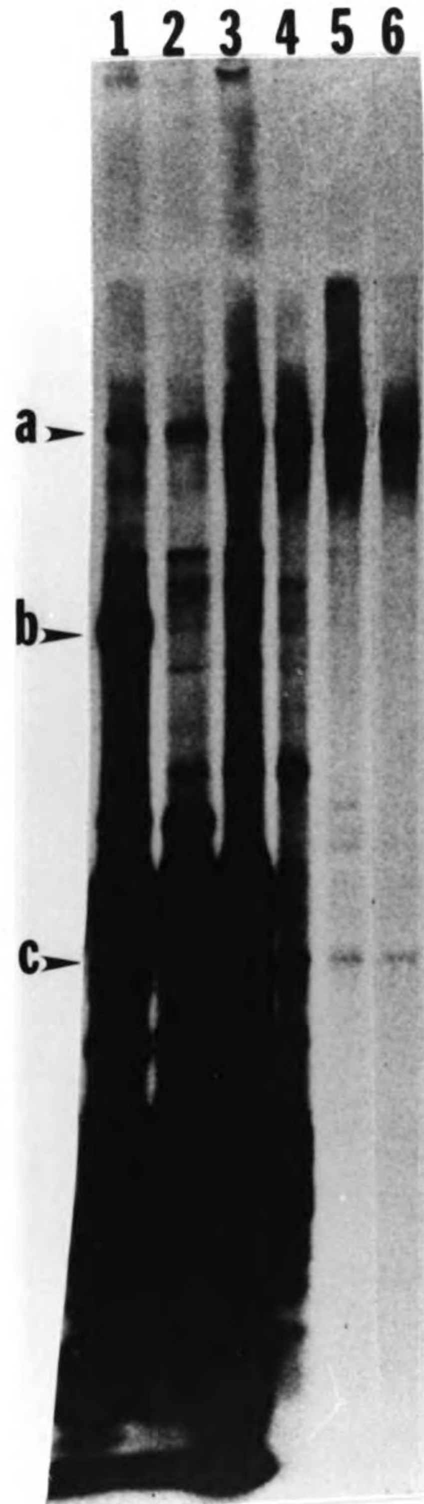
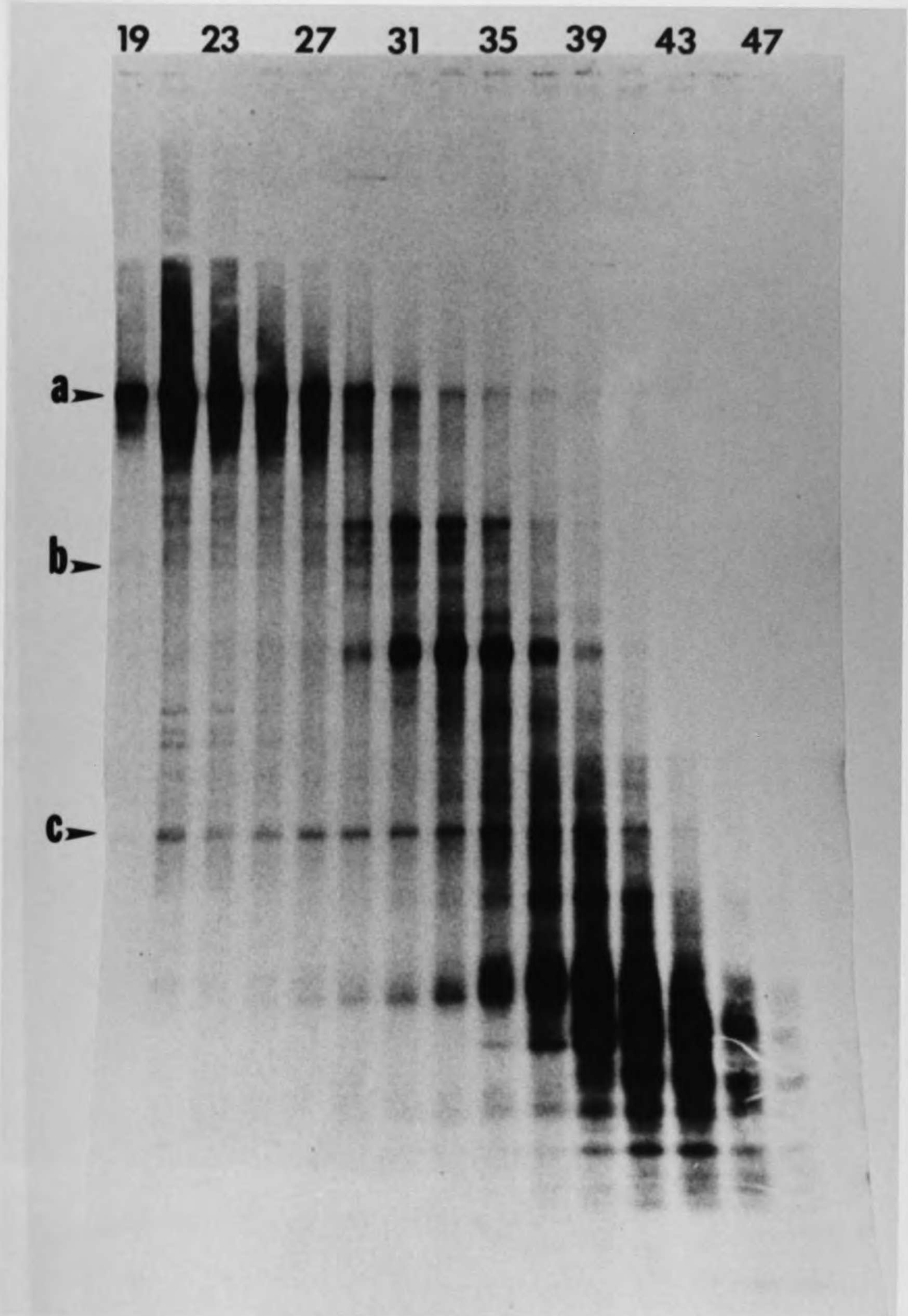

1 2 3 4 5 6
a
b
c

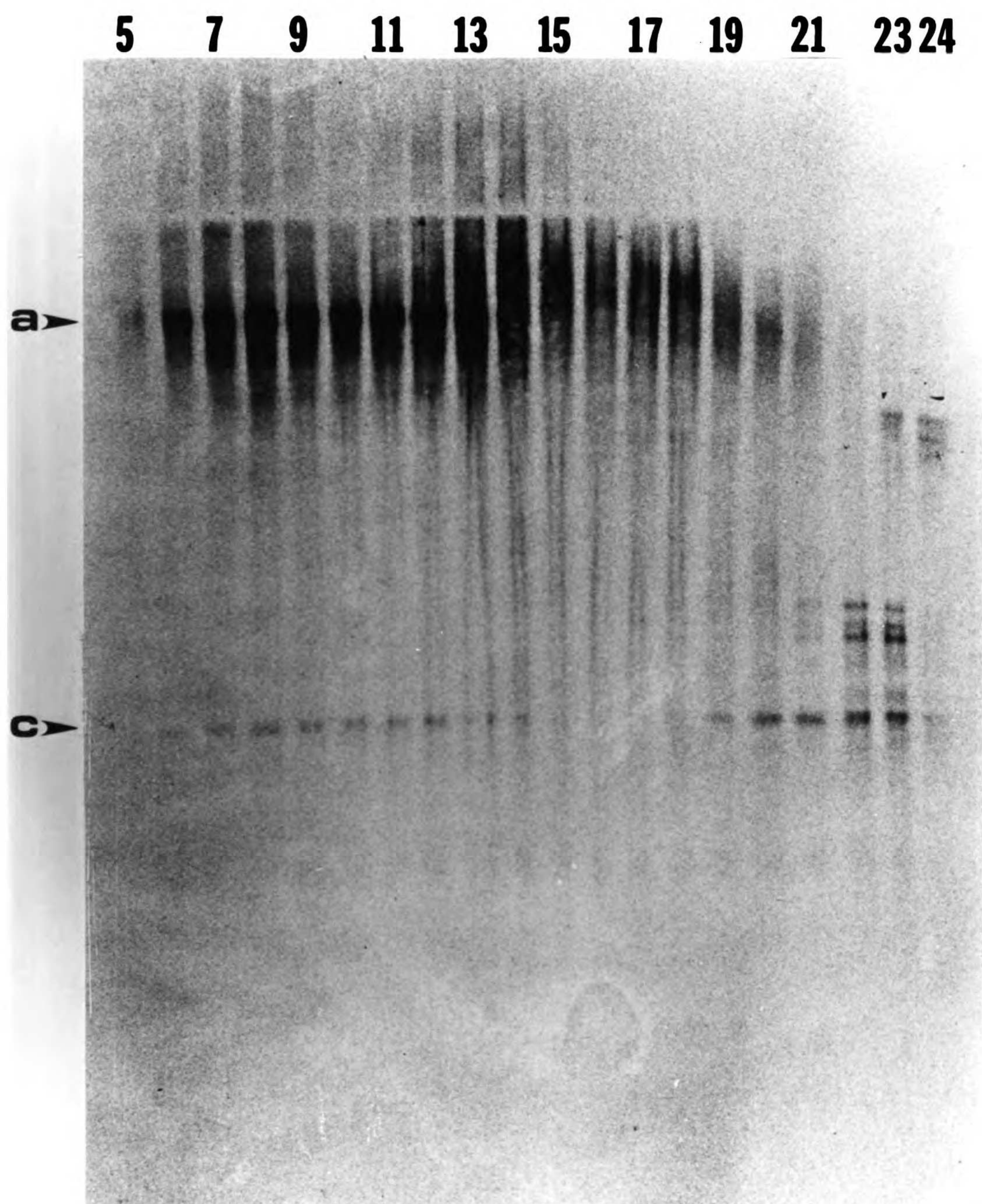
Figure 4.4. Gel Filtration on Sepharose CL4B: Analysis by SDS-PAGE. Equal volumes of odd-numbered fractions from the material shown in Figure 4.1 were analyzed by non-reducing SDS-PAGE. Fractions are identified by numbers above lanes. Standards: a) laminin (850,000-10^6 Da) b) fibronectin (440,000 Da) c) mouse IgG (150,000 Da).


19
23
27
31
35
39
43
47
a
b
c

experiment shown in Figure 4.1 were analyzed by SDS-PAGE under non-reducing conditions. The distribution and amount of fluorographic exposure of the band that comigrates with a laminin standard (marker 'a') correlates well with the distribution and amount of neurite outgrowth-promoting activity (Figure 4.1, fractions 19-31). Also broadly distributed over these same fractions is the "high molecular weight blur" described in lanes 4-6 of Figure 4.3. A few other molecules are present in neurite outgrowth-promoting fractions, however one of these, represented by a 150,000 Da band (marker 'c'), is also distributed among fractions that lack neurite outgrowth-promoting activity (i.e. fractions 33-41), and the others, represented by a group of bands between markers 'b' and 'c', are present in the void volume peak (fractions 19-23) of outgrowth-promoting activity, but absent from the adjacent "shoulder" (fractions 25-31) of activity. Thus, the molecules that best correlate with neurite outgrowth-promoting activity are those represented by the band that comigrates with laminin (marker 'a') and the "high molecular weight blur".

To determine how the labeled molecules purified by gel filtration behaved when subjected to velocity sedimentation, fractions from the sucrose gradient shown in Figure 4.2 were analyzed by SDS-PAGE under non-reducing conditions. As shown in Figure 4.5, the labeled molecules applied to the sucrose gradient--i.e. the band that comigrates with laminin (marker 'a'), the 150,000 Da band (marker 'c'), the minor bands present in the "peak" but not the "shoulder" of the gel filtration profile, and the "high molecular weight blur"--can all be accounted for. The most mobile peak ($\sim$19S; fractions 5-10) contains the band that comigrates with laminin, the 150,000 Da band, and the "high mole-

Figure 4.5. Velocity sedimentation through 5-20% sucrose: Analysis by SDS-PAGE. Equal volumes of fractions 5-24 from the material shown in Figure 4.2 were analyzed by non-reducing SDS-PAGE. Fractions are identified by numbers above lanes. Standards: a) laminin (850,000-10^6 Da) c) mouse IgG (150,000 Da).


5
7
9
11
13
15
17
19
21
23
24
a
c

cular weight blur". This peak of radioactivity is associated with a peak of neurite outgrowth-promoting activity (Figure 4.2). The second peak (~13S; fractions 11-16) contains the same three molecules in its leading fractions (fractions 11-13), but contains only the "high molecular weight blur" in its trailing fractions (fractions 14-16). Only the leading fractions of this peak are associated with neurite outgrowth-promoting activity (Figure 4.2). The third peak (~9S; fractions 17-20) contains only the "high molecular weight blur", and the fourth (~4S; fractions 21-24) contains much of the 150,000 Da band, as well as the other minor bands noted in Figure 4.4, fractions 19-23. Neither of these peaks is associated with outgrowth-promoting activity.

The data in Figures 4.4 and 4.5 demonstrate that all neurite outgrowth-promoting fractions contain three different labeled molecules: the band that comigrates with laminin, the 150,000 Da band, and the "high molecular weight blur". The identity of these molecules, and evidence that they physically associate with one another, are presented below. Figures 4.4 and 4.5 also demonstrate that, whereas the 150,000 Da band and the high molecular weight blur may be found in fractions that lack neurite outgrowth-promoting activity, the band that comigrates with laminin is only found in fractions possessing this activity. In fact, the profile of neurite outgrowth-promoting activity in Figures 4.1 and 4.2 correlates well with the distribution of the molecule represented by this band, as judged by the intensity of fluorographic exposure, in Figures 4.4 and 4.5, respectively.

Laminin and Entactin

It seemed likely that the labeled molecule in Figures 4.4 and 4.5

that correlates with the presence of neurite outgrowth-promoting activity and comigrates with a laminin standard was, in fact, laminin, given that laminin is known to promote neurite outgrowth in this assay system (see Chapter 3). To test this hypothesis, fractions from gel filtration were assayed for laminin by enzyme-linked immunoassay (EIA) using affinity-purified antibodies directed against laminin from the EHS sarcoma. The results, shown in Figure 4.1, indicate a very good correlation between laminin immunoreactivity, neurite outgrowth-promoting activity, and the band that comigrates with a laminin standard (Figure 4.4, marker 'a'). Similar analysis was carried out on fractions that had been subjected to velocity sedimentation. Figure 4.2 shows that the profile of anti-laminin binding again matches the profile of neurite outgrowth-promoting activity, as well as that of the band that comigrates with the laminin standard (Figure 4.5, marker 'a').

The fact that neurite outgrowth-promoting activity correlates well with laminin immunoreactivity and with the presence of a band that comigrates with laminin is not sufficient to prove that laminin is associated with the "neurite outgrowth-promoting factor". To establish such an association, the following experiments were done. First, BCE-CM was subjected to immunoprecipitation with affinity-purified anti-laminin antibodies. After removal of the precipitate, the remaining CM was assayed for neurite outgrowth-promoting activity. As shown in Table 4.2, this procedure completely removed the "neurite outgrowth-promoting factor" from the CM, while control precipitations, performed without anti-laminin, did not.

To establish that immunoprecipitation of neurite outgrowth-

TABLE 4.2

Removal of Neurite Outgrowth-Promoting Activity from BCE-CM

by Immunoprecipitation with Anti-Laminin Antibodies

Sample	Neurite outgrowth (%)
1. BCE-CM (untreated)	84 $\pm$ 2.3
2. BCE-CM immunoprecipitated with anti-laminin	5 $\pm$ 1.4
3. BCE-CM immunoprecipitation control	85 $\pm$ 1.6

Samples of BCE-CM were subjected to immunoprecipitation as described in Methods. Sample 2 received 9 ug of affinity-purified anti-laminin per ml of CM. Sample 3 received no anti-laminin, but was otherwise processed identically. After removal of the immunoprecipitates from samples 2 and 3 the supernatants were assayed for neurite outgrowth-promoting activity.

promoting activity under these circumstances was indeed related to the removal of a band that comigrates with laminin, [^{35}S]methionine-labeled BCE-CM was subjected to immunoprecipitation with affinity-purified anti-laminin in various amounts. The supernatants were not only assayed for neurite outgrowth-promoting activity, but were also analyzed by SDS-PAGE. As Figure 4.6 shows, increasing amounts of anti-laminin antibody resulted in a progressive loss of neurite outgrowth-promoting activity, and this was paralleled by a progressive disappearance of a band that comigrated with a laminin standard (Figure 4.6 insert, arrow).

To obtain more complete information about the molecules removed from labeled BCE-CM by immunoprecipitation, the pelleted material from anti-laminin and control immunoprecipitations was also analyzed by SDS-PAGE (Figure 4.7) In order that all immunoprecipitated molecules be detected, precipitates were not extensively washed, and therefore contained some non-specifically precipitated molecules. These non-specifically precipitated molecules could be distinguished as bands present in precipitates prepared both with (Figure 4.7, lane 1) and without (Figure 4.7, lane 2) anti-laminin. The major molecule that was specifically precipitated (i.e. present in lane 1 but not lane 2) appeared as a band that comigrated with a laminin standard (marker 'a') strongly supporting the identification of this band as authentic laminin. Interestingly, another band at 150,000 Da (marker 'c') was also immunoprecipitated by anti-laminin antibodies. It is especially apparent in lane 3 (a longer exposure of lane 1) and its absence in lane 4 (a longer exposure of lane 2) establishes that it was specifically immunoprecipitated. This result implies either that the 150,000 Da band represents a molecule recognized by anti-laminin anti-

Figure 4.6. Concomitant removal of laminin and outgrowth-promoting activity from BCE-CM by immunoprecipitation. [^{35}S]methionine-labeled BCE-CM was subjected to immunoprecipitation with varying amounts of affinity purified anti-laminin (abcissa: amount of antibody added per ml of CM) After removal of the precipitate, the supernatants were assayed for neurite outgrowth-promoting activity at a 1:16 dilution (chosen within a range where the neuronal response would be roughly linear with concentration of the active factor). The same samples were also analyzed by electrophoresis under non-reducing conditions. The inset shows, from left to right, supernatants of precipitates performed using 0, 0.12, 0.33, 0.67, 1.5, and 3.0 ug of anti-laminin per ml of CM. In each lane, the density of fluorographic exposure of the band that comigrates with a laminin standard (arrow) was measured (Suissa, 1983). These data are plotted (■) together with the neurite outgrowth determinations (percent of neurons with neurites)(●). Loss of neurite outgrowth-promoting activity coincides with depletion of laminin.

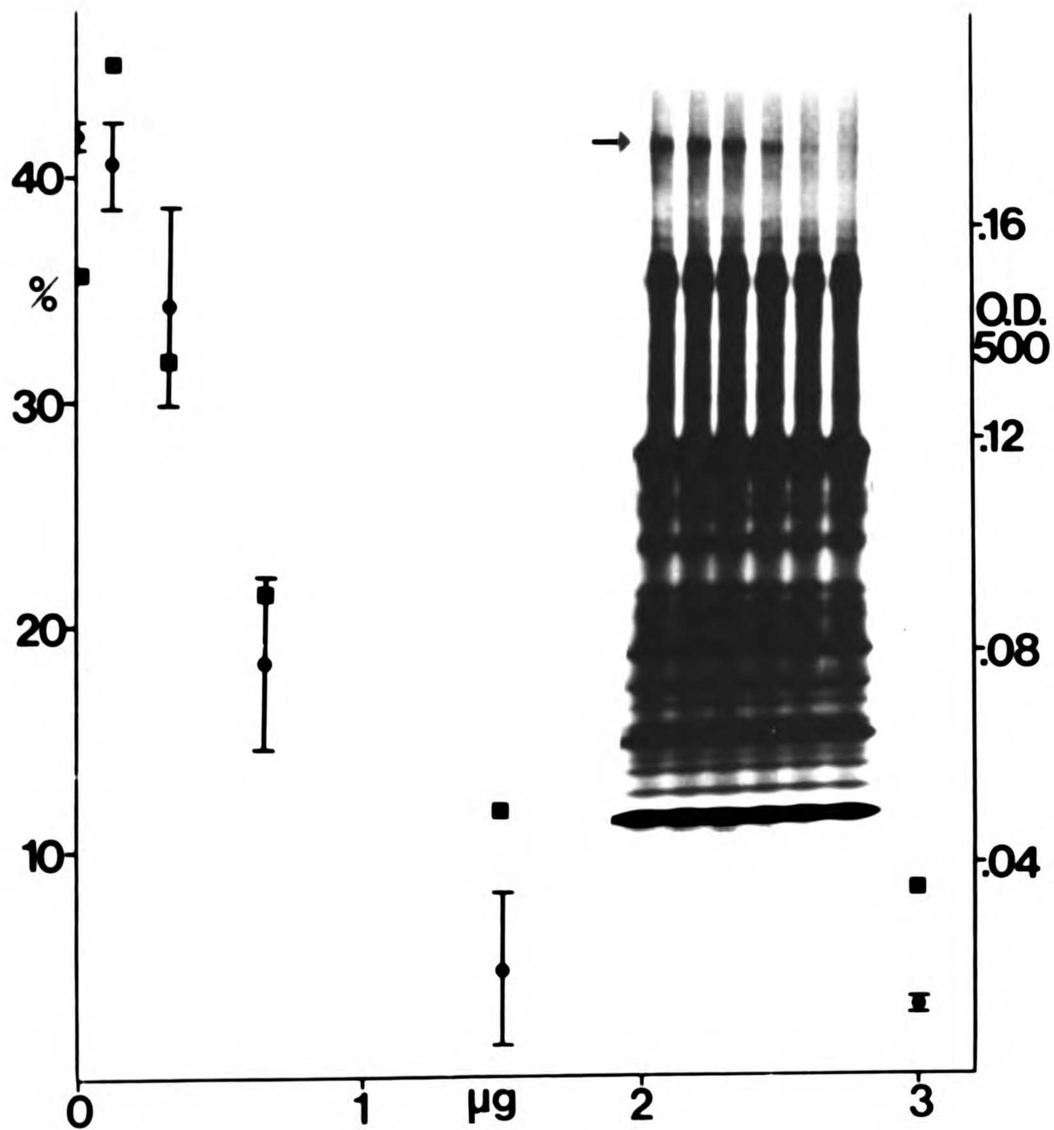
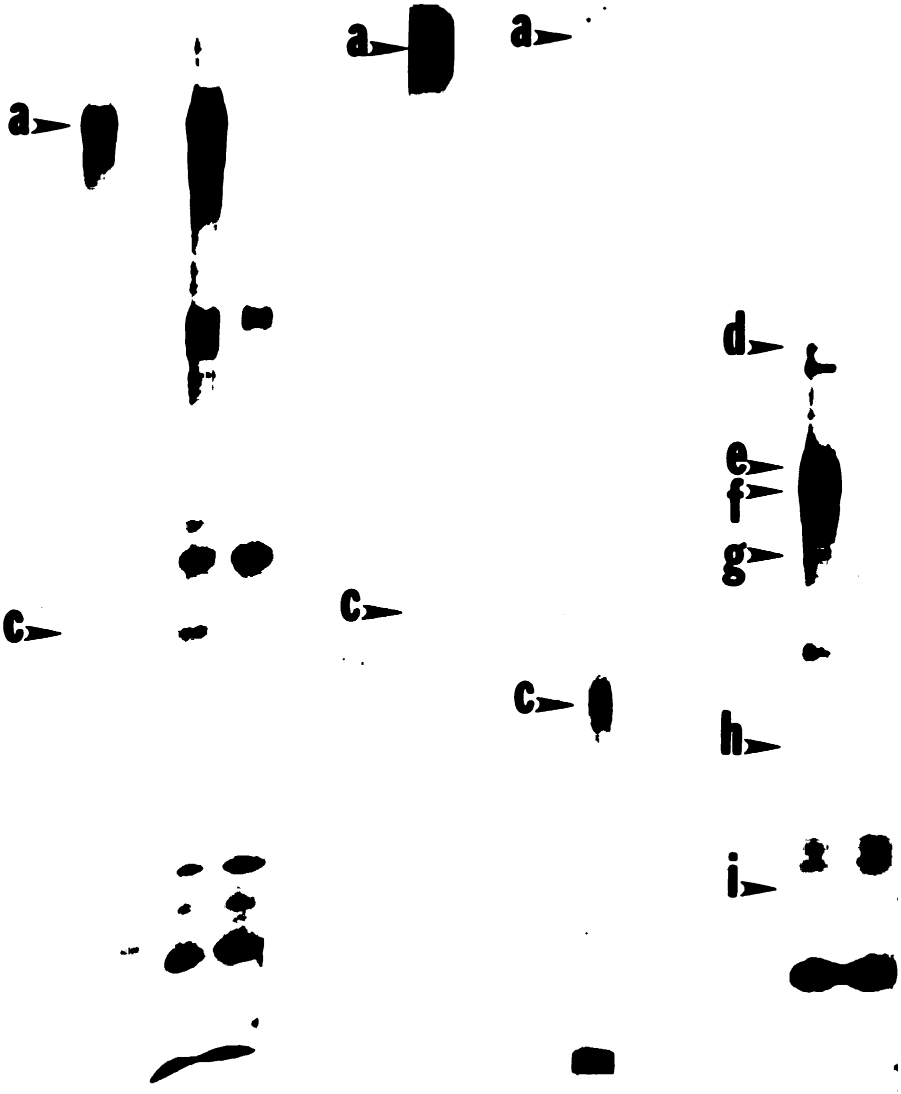

40
%
30
20
10
0
1
μg
2
3
.16
O.D.
500
.12
.08
.04

Figure 4.7. Analysis of BCE-CM by immunoprecipitation and immunoblotting.

Lanes 1-4, 7-8: analysis by immunoprecipitation. [^{35}S]methionine-labeled BCE-CM was subjected to immunoprecipitation with affinity-purified anti-laminin, as described, and the pellet was analyzed by non-reducing SDS-PAGE. Lanes 1 and 3, experimental; Lanes 2 and 4, control (no anti-laminin used). Lanes 3 and 4 were exposed four times longer than lanes 1 & 2. Note the protein band at 150,000 Da that specifically co-precipitates (i.e. present in lanes 1 & 3, but not 2 & 4) with laminin. Anti-laminin immunoprecipitates analyzed in lanes 1-4 were also prepared in sample buffer containing 5% 2-mercaptoethanol for analysis by SDS-PAGE under reducing conditions. Lane 7, experimental (same sample as lanes 1 and 3); Lane 8, control (same sample as lanes 2 and 4). Standards: d) laminin, heavy chain (400,000 Da), e) cellular fibronectin (220,000 Da), f) laminin, light chain (200,000-220,000 Da), g) myosin (200,000 Da), h) β -galactosidase (116,000 Da), i) bovine serum albumin (68,000 Da). Note the 150,000 Da band found between standards 'g' and 'h' in lane 7.

Lanes 5 and 6: analysis by immunoblotting. Unlabeled BCE-CM was subjected to non-reducing SDS-PAGE, electrophoretically transferred to nitrocellulose and probed with affinity-purified rabbit anti-laminin (lane 5) and rabbit anti-entactin serum (lane 6) (see Methods). Standards: a) laminin (850,000-10^6 Da) c) mouse IgG (150,000 Da).


1 2 3 4
5
6
7 8
a
a
a
c
c
c
d
e
f
g
h
i

bodies, or it represents a molecule that co-immunoprecipitates with laminin because it associates with laminin. To distinguish between these possibilities, BCE-CM was subjected to non-reducing SDS-PAGE, electrophoretically blotted onto nitrocellulose, and probed with anti-laminin antibodies (Figure 4.7, lane 5). These antibodies bound to a band comigrating with a laminin standard (marker 'a'), but did not recognize any band at or near 150,000 Da. Thus, there appears to be a 150,000 Da protein in BCE-CM that is associated with laminin. This observation explains why, during purification of the "neurite outgrowth-promoting factor" from BCE-CM a 150,000 Da band was consistently seen in all fractions that contained the laminin band (cf. Figures 4.3-4.5).

The identity of this 150,000 Da band was strongly suggested by the literature. Several groups have reported that a laminin-associated protein of 150,000 Da is present in a number of rat and mouse CM's and tissues (Cooper et al., 1981; Kühl et al., 1982; Cornbrooks et al., 1983) and this protein has been purified and named entactin (Carlin et al., 1983) (also known as glycoprotein C (Hogan et al., 1982)). To confirm that the laminin-associated 150,000 Da protein present in BCE-CM is (bovine) entactin, immunoblots were probed with an antiserum specific for entactin (generously provided by Dr. Hynda Kleinman). As shown in lane 6 of Figure 4.7, this antiserum recognized the 150,000 Da band in BCE-CM.

As further proof that the molecules immunoprecipitated from BCE-CM by anti-laminin antibodies were indeed laminin and entactin, the immunoprecipitates analyzed by non-reducing SDS-PAGE in lanes 1-4 of Figure 4.7 were further analyzed under reducing conditions (lanes 7-8). As expected, the band that comigrated with non-reduced laminin

was replaced by bands comigrating with the light and heavy chains of reduced laminin. The light chain appeared as a diffuse band (see marker 'f'), and instead of a single heavy chain, a doublet was observed (marker 'd'), although the doublet was not a consistent finding in all batches of CM (splitting of the chains of laminin into doublets has been reported by others, e.g. Timpl et al., 1982). Under reducing conditions, there appeared to be no significant change in the apparent molecular weight of the 150,000 Da band, entirely consistent with the fact that entactin consists of a single polypeptide chain (Carlin et al., 1983). Additional evidence that this protein is entactin comes from [^{35}S]-sulfate labeling, described below.

Proteoglycan

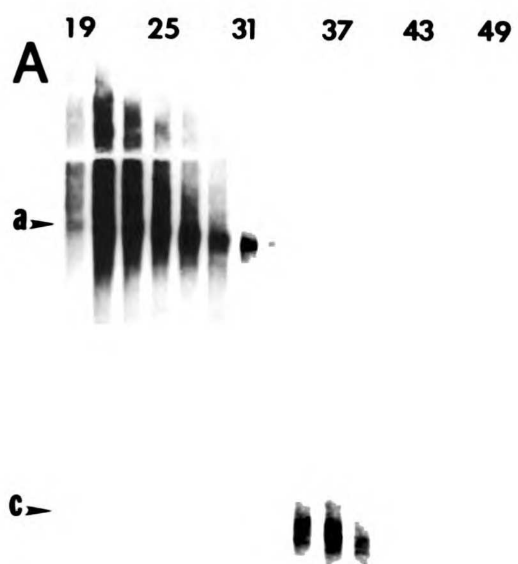
The only other labeled material detected in all neurite outgrowth-promoting fractions in Figures 4.4 and 4.5 is the "high molecular weight blur" that, like the 150,000 Da molecule identified as entactin, is also found in some fractions that do not promote outgrowth (e.g. Figure 4.5, fractions 15-20). For several reasons, it was suspected that this material represents a heparan sulfate proteoglycan. First, the evidence presented in Chapter 2 suggests that "neurite outgrowth-promoting factors" in BCE-CM and other CM's are associated with proteoglycans of the heparan sulfate class. Second, laminin is known to bind heparan sulfate (Sakashita et al., 1980; Del Rosso et al., 1981), which in CM's is found as part of heparan sulfate proteoglycans (e.g. Oohira et al., 1983; Robinson and Gospodarowicz, 1983; Matthew et al., 1985); it would not, therefore, be unexpected to find a heparan sulfate proteoglycan co-purifying with the laminin in BCE-CM (e.g. in Figures

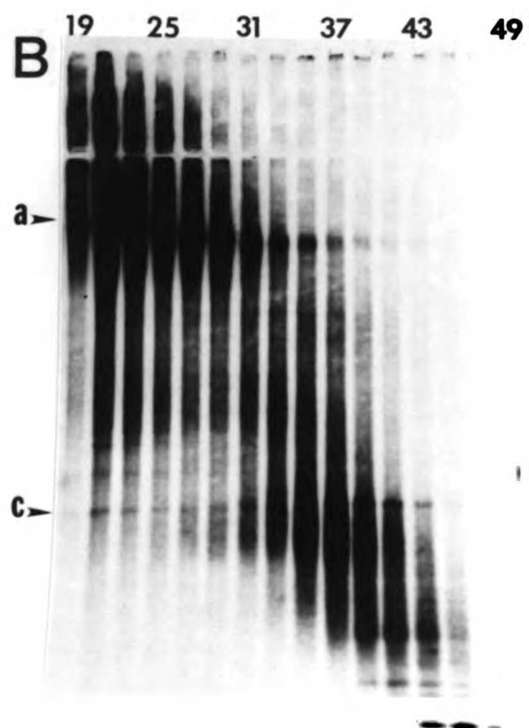
4.3-4.5). Third, when proteoglycans are subjected to SDS-PAGE, they often migrate as diffuse "blurs" (e.g. Hedman _et al._, 1982). In order to establish that the "high molecular weight blur" in Figures 4.3-4.5 represents a heparan sulfate proteoglycan, several experiments were done.

First, BCE-CM was prepared from cells grown in [^{35}S]sulfate, which labels the glycosaminoglycan side chains of most classes of proteoglycans (cf. Chapter 2). Sulfate-labeled material was taken through all purification steps described in Table 4.1, except for the final step of velocity sedimentation. Fractions from gel filtration were then analyzed by SDS-PAGE (Figure 4.8A). The major sulfated molecular species appears as a blur in the very high molecular weight region of the gel. This material is present in the completely excluded fractions and trails off into included fractions. When Figure 4.8A is compared with Figure 4.4, it can be seen that methionine (Fig. 4.4) and sulfate (Fig. 4.8A) are both incorporated into high molecular weight blurs with the same electrophoretic and gel filtration behavior. This result suggests that methionine and sulfate are incorporated into the same molecule, as would be expected for a proteoglycan.

Very few protein bands seen in Figure 4.4 are also seen in Figure 4.8A or 4.8B (a longer exposure of 4.8A), reflecting the fact that sulfated (glyco)proteins are relatively rare. One band, however, that clearly appears in Figure 4.8B is a band corresponding to the 150,000 Da protein described above. Since entactin has been shown to be sulfated (Carlin _et al._, 1981; Hogan _et al._, 1982) this result further corroborates the identification of the 150,000 Da band as bovine entactin.

Figure 4.8. (A) Gel filtration of [^{35}S]sulfate-labeled BCE-CM. Samples were purified and analyzed as in Figure 4.4, except [^{35}S]sulfate-labeled, BCE-CM was used. Every odd fraction from gel filtration is shown in sequence, so the pattern may validly be compared with that in Figure 4.4. (B) The same gel, exposed seven times longer. Fractions are identified above lanes. Standards: a) laminin (850,000-10^6 Da) c) mouse IgG (150,000 Da). Note the presence of a sulfated band at 150,000 Da.


19
25
31
37
43
49
A
a
c


19
25
31
37
43
49
B
a
c

The sulfate and methionine labeling of the high molecular weight blur in Figures 4.4 and 4.8 provides suggestive evidence that this blur represents a proteoglycan. To obtain conclusive evidence, [^{35}S]methionine-labeled samples that had been purified and subjected to velocity sedimentation (i.e. the fractions from Figure 4.2) were digested with purified heparitinase, an enzyme that specifically degrades heparan sulfate side chains (Linker and Hovingh, 1972). Digested samples were analyzed by SDS-PAGE, as shown in Figure 4.9. Comparison of this Figure with Figure 4.5--SDS-PAGE of the same samples before heparitinase digestion--shows clearly that the effect of digestion with heparitinase was to convert all of the "high molecular weight blur" to a single band of approximately 400,000 Da. Because this enzyme was not contaminated with chondroitinase or protease activity, this result indicates that the original "blur" represented a heparan sulfate proteoglycan, whose protein core has a molecular weight of about 400,000 Da. When subjected to SDS-PAGE under reducing conditions, the core protein again migrated as a single band of approximately 400,000 Da, indicating that it consists of a single polypeptide chain. These data are shown in Figure 4.10. The other, smaller bands in Figure 4.10 represent the two chains of laminin (markers 'd' and 'f') and entactin (between markers 'g' and 'h').

With the aid of Figure 4.9, it is possible to identify those molecules contributing to the different peaks of total labeled protein at the last step of purification (Figure 4.2). The peak at $\sim$19S (fractions 5-10), which possesses neurite outgrowth-promoting activity, contains laminin, proteoglycan and entactin. The peak at $\sim$13S (fractions 11-16), which possesses neurite outgrowth-promoting activity in

Figure 4.9. Heparitinase digestion of samples from velocity sedimentation. Those samples that were analyzed by SDS-PAGE in Figure 4.5 were digested with heparitinase (see methods) and then analyzed by non-reducing SDS-PAGE. Fractions are identified above lanes, and may be validly compared with the same fractions in Figure 4.5. Standards: a) laminin (850,000-10^6 Da) b) cellular fibronectin (440,000 Da) and c) mouse IgG (150,000 Da). Note the dispppearance of the high molecular weight blur present in figure 4.5, and the appearance of a core protein band of about 400,000 Da.

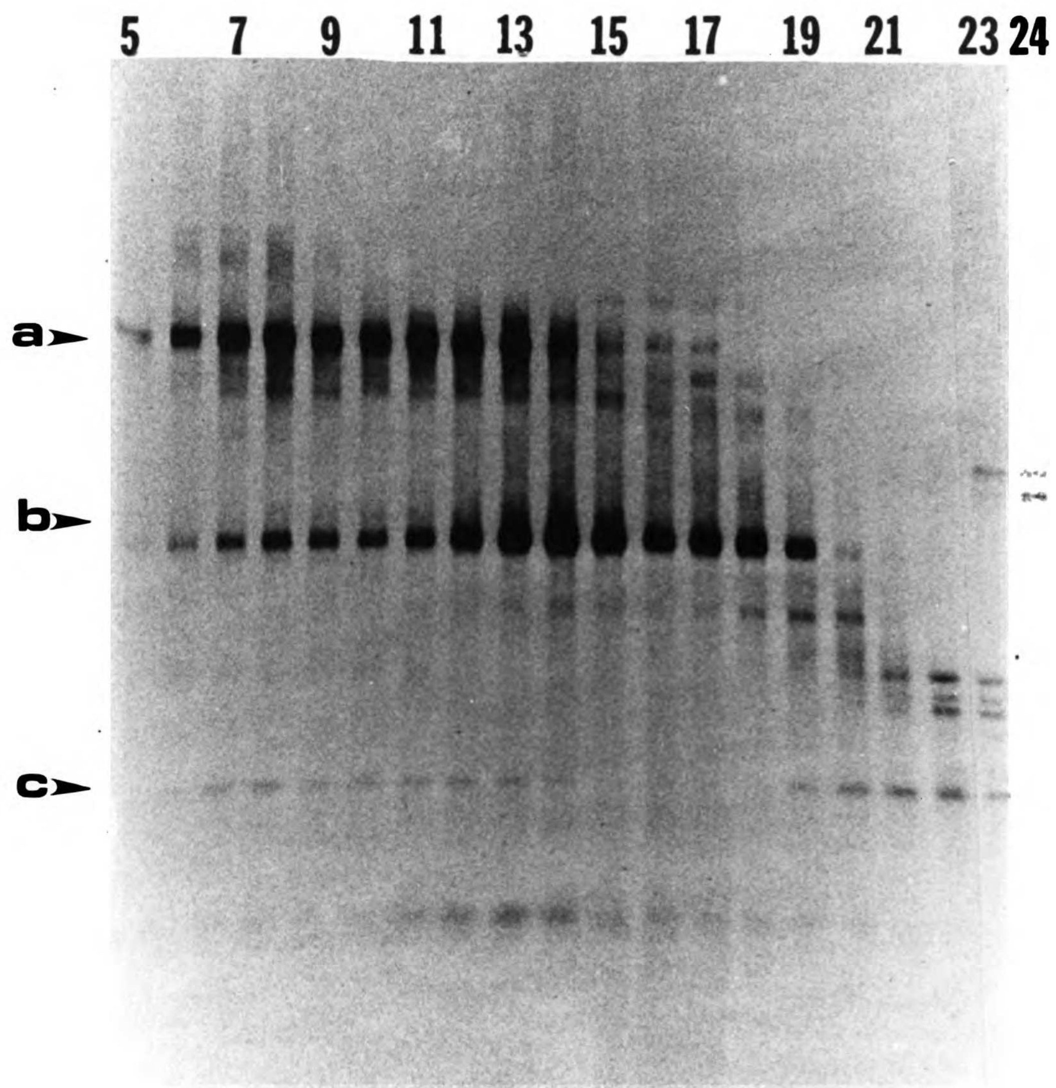

5 7 9 11 13 15 17 19 21 23 24
a
b
c

Figure 4.10. Analysis of heparitinase-digested "neurite outgrowth-promoting factor" by reducing SDS-PAGE. Material purified from [^{35}S]methionine-labeled BCE-CM through the gel filtration step was digested with heparitinase as described, mixed with sample buffer containing 5% 2-mercaptoethanol and analyzed by SDS-PAGE. Standards: d) laminin, heavy chain (400,000 Da), e) cellular fibronectin (220,000 Da), f) laminin, light chain (200,000-220,000 Da), g) myosin (200,000 Da), h) β-galactosidase (116,000 Da), i) bovine serum albumin (68,000 Da). Arrow: proteoglycan core protein. Entactin is found between standards 'g' and 'h'.

d
e
f
g
h
i

its leading fractions (fractions 11-13), contains laminin, proteoglycan and entactin in its leading fractions, but only proteoglycan in its trailing fractions. The peak at $\sim$ 9S (fractions 17-20) contains proteoglycan only, and the peak at $\sim$4S (fractions 21-24) contains entactin and other molecules not yet identified.

The appearance of entactin, both in the laminin-containing fractions and in the 4S peak, presumably reflects the fact that a portion of the entactin is bound to laminin and a portion is free. Likewise, the appearance of the proteoglycan in all laminin-containing fractions, as well as in the 9S peak and trailing edge of the 13S peak suggests that a portion of the proteoglycan is bound to laminin and a portion is free. Evidence that some of the proteoglycan is indeed physically associated with laminin was obtained by showing that immunoprecipitation of one molecule brings down the other, the same approach that was taken to demonstrate the association of entactin with laminin (cf. Figure 4.7). It was necessary to modify the precedure slightly over that used in Figure 4.7, in order to enhance the visibility of the proteoglycan in SDS-gels. This was done by performing anti-laminin immunoprecipitations with [^{35}S]sulfate-labeled BCE-CM, rather than [^{35}S]methionine-labeled BCE-CM. As shown earlier (Figure 4.8), [^{35}S]sulfate strongly labels the proteoglycan and does not label laminin; this was helpful, given that laminin and the proteoglycan normally migrate to somewhat overlapping positions in SDS-gels (e.g. Figures 4.3-4.5). The results of these immunoprecipitations are shown in Figure 4.11. The characteristic "blur" of the proteoglycan is clearly present in the anti-laminin immunoprecipitate (lane 1) but not the control (lane 2). Precipitation of the proteoglycan with anti-

Figure 4.11. Immunoprecipitation of [^{35}S]sulfate-labeled BCE-CM with anti-laminin. Sulfate-labeled CM was subjected to immunoprecipitation as described, and as in figure 4.7. The pellet was analyzed by non-reducing SDS-PAGE. Lane 1, experimental; lane 2, control (no anti-laminin used). Standards a) laminin (850,000-10^6 Da) b) cellular fibronectin (440,000 Da) c) mouse IgG (150,000 Da).

1 2

a

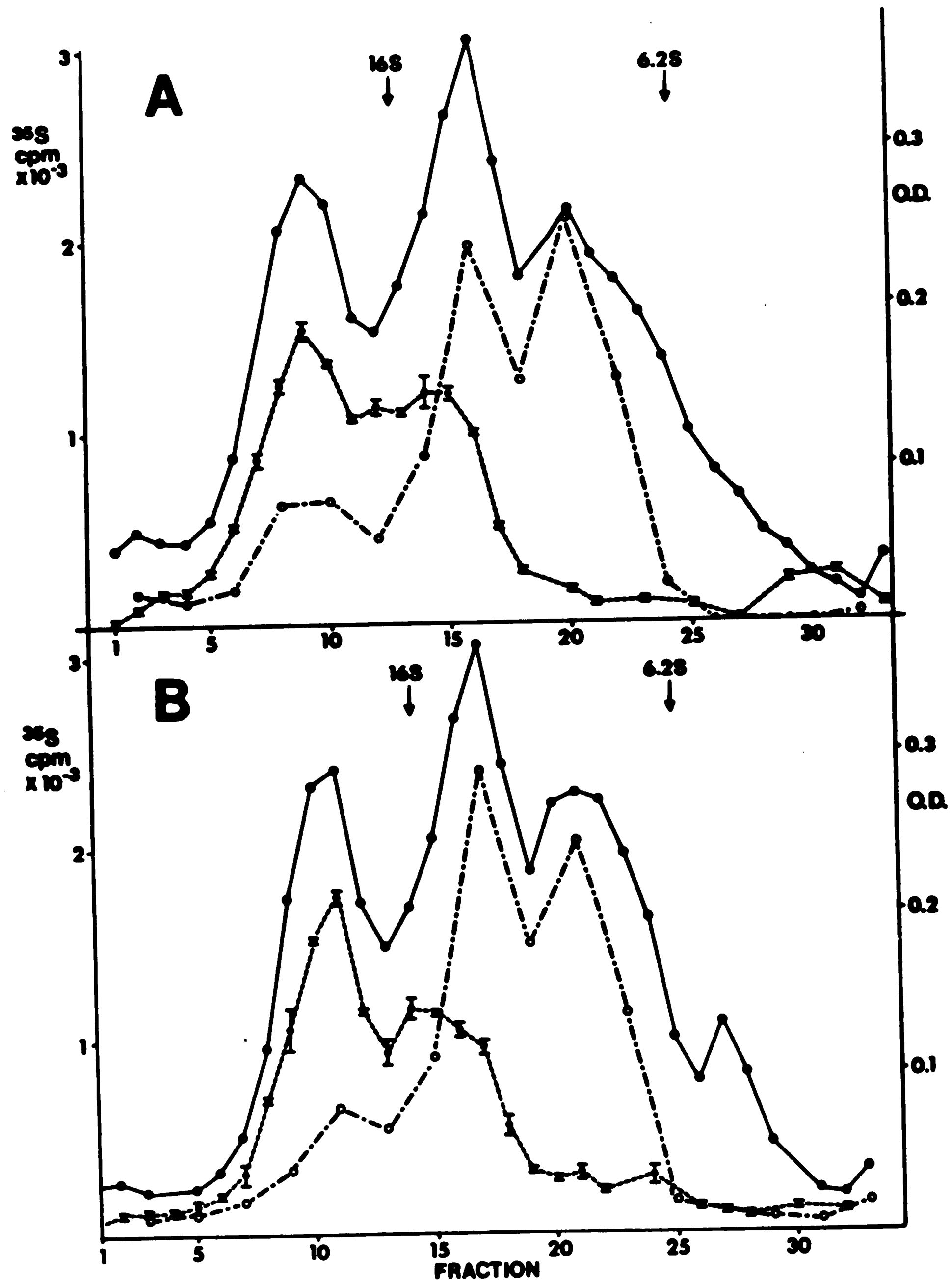
b

c

laminin antibodies could not have been due to reactivity of the antibodies towards the proteoglycan, because examination of Figures 4.2, 4.5, and 4.9 reveals that anti-laminin antibodies show no binding to sucrose gradient fractions that contain the proteoglycan but lack laminin (e.g. fractions 17-19). In addition, the proteoglycan "blur" was not recognized by anti-laminin on immunoblots (Figure 4.7, lane 5). Thus, some of the heparan sulfate proteoglycan in BCE-CM must be physically associated with laminin.

It is known that purified laminin (from the EHS sarcoma) will bind glycosaminoglycans, particularly heparin and heparan sulfate, and that the binding of heparin and heparan sulfate to EHS-sarcoma laminin can be blocked by high (0.5 M) salt (Sakashita _et al._, 1980; Del Rosso _et al._, 1981; Ott _et al._, 1982). To see whether this interaction accounted for binding of the heparan sulfate proteoglycan to laminin in BCE-CM, [^{35}S]methionine-labeled BCE-CM was purified, as described above, through the gel filtration step (cf. Figures 4.1 and 4.4), and fractions with neurite outgrowth-promoting activity were pooled and divided in equal aliquots, one of which was sedimented through a sucrose gradient in 0.1 M NaCl, the other through a sucrose gradient in 0.5 M NaCl. The profile of total labeled protein in the 0.1M NaCl gradient (Figure 4.12a) was nearly identical to that in the 0.5 M NaCl gradient (Figure 4.12b). The distribution of laminin, assessed by EIA, also appeared the same in the two gradients. The relative amounts of proteoglycan in alternate fractions were assessed by heparitinase digestion, SDS-PAGE of the digests, and quantification of core protein by measurement of the density of fluorographic exposure. Again, the profiles in in the two gradients were not significantly different. In

Figure 4.12. Effect of increased salt concentration on laminin-proteoglycan association. [^{35}S]methionine-labeled BCE-CM was purified through the gel filtration step, then divided into two aliquots. One, shown in "A", was sedimented through 5-20% sucrose in TBS, as described (see Figure 2). The other, shown in "B" was adjusted to 0.5 M in NaCl by the addition of 4 M NaCl, and sedimented in the same manner through 5-20% sucrose in 0.5 M NaCl, 0.05 M Tris HCl, 0.1% Triton X-100, pH 7.4. [^{35}S]methionine-label was assayed in 10 ul aliquots of each fraction (————————), and dilutions of each fraction (1:9 in TBS) were applied to polylysine-treated wells for determination of laminin immunoreactivity by EIA (- - - - - - - - -), as described. Aliquots of selected fractions were also digested with heparitinase and analyzed by nonreducing SDS-PAGE, in order to produce fluorographs, such as the one shown in Figure 4.9, in which the [^{35}S]methionine incorporated into proteoglycan runs as an easily identifiable core protein band. The density of exposure of this band in each lane, measured as described in <u>Methods</u>, was plotted (—— - —— - —— - ——) and indicates the proteoglycan content of different fractions. Sedimentation standards are shown as in Figure 2. As a rough measurement of the amount of proteoglycan that was associated with laminin in each gradient, the measured amount of core protein sedimenting between 13S-23S in each gradient was divided by the total amount of core protein in that gradient. The 13S-23S region corresponded to fractions 4-16 in "A" and 7-17 in "B" and was chosen because within it was found nearly all of the laminin in each gradients. The ratio thus obtained was 0.49 for "A" and 0.48 for "B".


A
16S
6.2S
35S cpm x10-3
O.D.
3
2
1
0.3
0.2
0.1
1
5
10
15
20
25
30
B
16S
6.2S
35S cpm x10-3
O.D.
3
2
1
0.3
0.2
0.1
1
5
10
15
20
25
30
FRACTION

particular, the proportion of the total quantity of applied proteoglycan that was found in laminin-containing fractions was the same for the two gradients (see legend to Figure 4.12). It appears, therefore, that the interaction between the heparan sulfate proteoglycan and laminin from BCE-CM is less salt-labile than has been reported for heparan sulfate and purified mouse laminin. These findings may explain why, in Chapter 2, the neurite outgrowth-promoting activity in BCE-CM remained associated with [^{35}S]sulfate-labeled material during the course of centrifugation through 3M CsCl.

Partial Purification of the "Neurite Outgrowth-Promoting Factor" using an Alternate Method

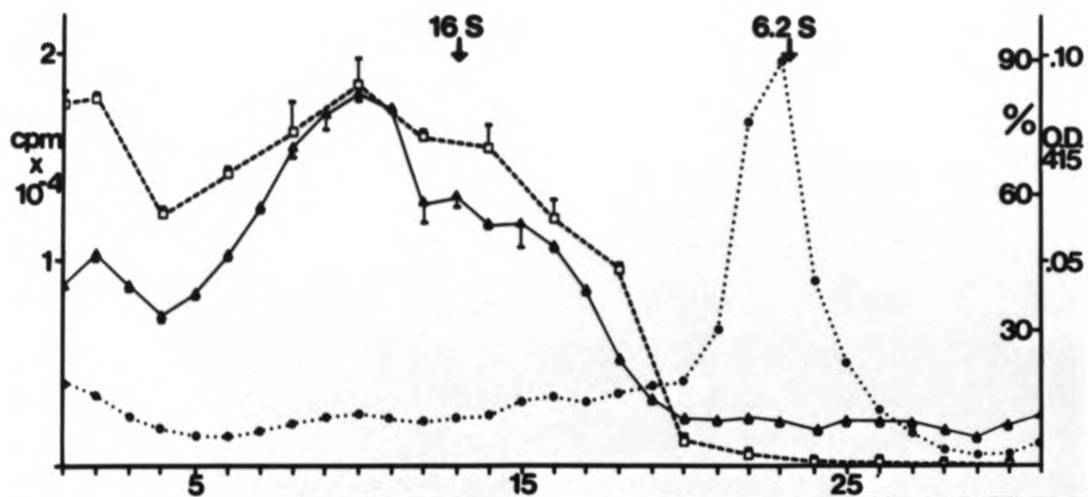
The purification and characterization described above indicates that the "neurite outgrowth-promoting factor" in BCE-CM consists of laminin and two associated molecules, entactin and a heparan sulfate proteoglycan. An alternate method of purification was devised to corroborate this conclusion. The method was designed to involve only very mild conditions. It is rapid, and lacks the ammonium sulfate and high ionic strength buffers used in the purification already described, and therefore might be considered less likely to dissociate molecules that are associated with each other, or to favor the establishment of spurious molecular associations. Briefly, [^{35}S]methionine-labeled BCE-CM was first depleted of fibronectin by absorption with gelatin-Sepharose, and the "neurite outgrowth-promoting factor" was enriched by two rounds of ultracentrifugation, employing forces sufficient to pellet molecules with a sedimentation coefficient > 14 S. The resulting material, enriched about 30 fold in neurite outgrowth-

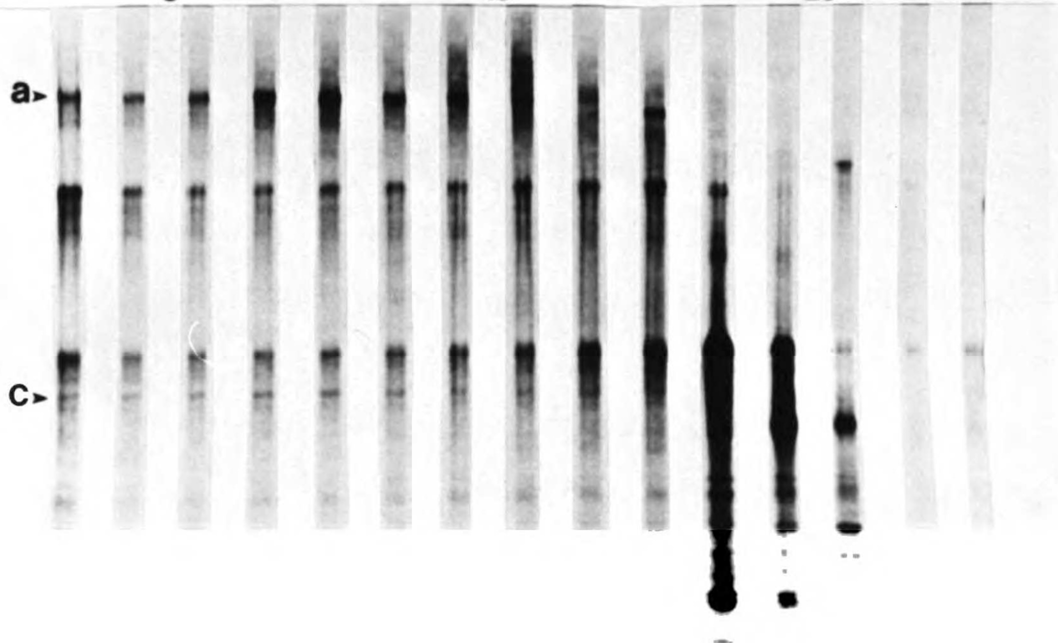
promoting activity (not shown) was then analyzed by sedimentation through a 5-20% sucrose gradient (Figure 4.13 A). Neurite outgrowth-promoting activity was found to be broadly distributed over many of the fastest sedimenting fractions, following a profile not unlike that in Figure 4.2. As expected, the profile of laminin-immunoreactivity followed the profile of neurite outgrowth-promoting activity reasonably well, and when alternate fractions were analyzed by SDS-PAGE (Figure 4.13 B), the laminin band (marker 'a') was appropriately distributed to account for laminin-immunoreactivity. Significantly, both entactin (marker 'c') and the proteoglycan (the "high molecular weight blur"; see especially fractions 14-20) can be identified in Figure 4.13 B, and are clearly present in the laminin-containing fractions. Other protein bands are also present in the neurite outgrowth-promoting fractions; these appear to represent proteins of $\leq$ 10 S that, possibly due to aggregation, "smear out" into more rapidly sedimenting fractions. Thus, this procedure does not constitute a complete purification of the "neurite outgrowth-promoting factor". It does, however, demonstrate the association of laminin, entactin, and the heparan sulfate proteoglycan in a sample of BCE-CM that was exposed only to gelatin-Sepharose and ultracentrifugation. In fact, the gelatin-Sepharose step is only necessary to optimize the yield of the procedure (for reasons that will be discussed below), and can be omitted without significantly changing the results of Figure 4.13 (see Appendix).

Contribution of the Heparan Sulfate Proteoglycan to Neurite Outgrowth-Promoting Activity

Although laminin, entactin and the heparan sulfate proteoglycan

Figure 4.13. Results of alternate method for partial purification of the "neurite outgrowth-promoting factor" from BCE-CM. [^{35}S]methionine-labeled BCE-CM was absorbed with gelatin-Sepharose, enriched in neurite outgrowth-promoting activity by two rounds of ultracentrifugation, and sedimented through a 5-20% sucrose gradient. (A) Eluted fractions were assayed for radioactivity (··········), neurite outgrowth-promoting activity (- - - - - - -), and laminin-immunoreactivity (————). The positions of sedimentation standards (β-galactosidase, 16S, and alkaline phosphatase, 6.2S) are marked (arrows). (B) Aliquots of alternate fractions were electrophoretically analyzed under non-reducing conditions. Lanes have been aligned under appropriate fractions in (A). Molecular weight markers were (a) laminin and (c) mouse IgG. Entactin comigrates with marker 'c'.


16 S
6.2 S
cpm
x
10^{-4}
2
1
90
60
30
%
.10
.05
O.D.
415
5
15
25


a
c

may be considered components of the "neurite outgrowth-promoting factor" in BCE-CM, it is unlikely that all three molecules contribute to its neurite outgrowth-promoting activity. Indeed, there is ample evidence that laminin is most directly involved in promoting neurite outgrowth; this will be reviewed in the discussion. In chapter 2, however, indirect evidence was presented that a heparan sulfate proteoglycan also contributed to the neurite outgrowth-promoting activity of BCE-CM. To obtain a definitive answer to this question, the "neurite outgrowth-promoting factor", purified as described above, was digested with purified heparitinase to completion, as judged by SDS-PAGE, and neurite outgrowth-promoting activity was assayed under three different conditions (Table 4.3).

First, the digested and control (undigested) samples were diluted in an equal volume of buffer (TBS) and assayed (Table 4.3, line 1). Both samples promoted neurite outgrowth equally well, indicating that the proteoglycan, or at least its heparan sulfate side chains, is not essential for neurite outgrowth-promoting activity. In the second part of the experiment, the digested and control samples were diluted not with TBS, but with 20% fetal calf serum in TBS (Table 4.3, line 2). When mixed with this amount of serum, the digested sample showed no neurite outgrowth-promoting activity at all, while the control sample remained as active as before. In the third part of the experiment, the digested and control samples were diluted with "laminin-depleted CM_{SF}", which was BCE-CM_{SF} that had been depleted of laminin, and neurite outgrowth-promoting activity, by immunoprecipitation with anti-laminin antibodies (cf. Table 4.2). Dilution with this "laminin-depleted CM_{SF}" (Table 4.3, line 3) also markedly inhibited the activity

TABLE 4.3. Role of Heparan Sulfate Proteoglycan in Neurite Outgrowth-Promoting Activity of Material Purified from BCE-CM: Effect of Heparitinase Digestion on Ability to Withstand Addition of Other Substances.

	Neurite Outgrowth (%)	
Sample Mixed With	Heparitinase-Digested Sample	Control (Undigested) Sample
Tris-buffered Saline (TBS)	71 ± 1.7	67 ± 0.8
20% Fetal Calf Serum in TBS	0	70 ± 2.9
Laminin-depleted CM_{SF}	27 ± 6.3	75 ± 5.3

Neurite outgrowth-promoting material from BCE-CM was purified through the gel filtration step (see text), and an aliquot was digested with heparitinase as described (Methods). Aliquots of digested and undigested (control) material were mixed with equal volumes of the indicated solutions, and applied to polylysine-treated substrata for determination of neurite outgrowth-promoting activity, which is expressed as the percent of plated neurons bearing neurites. "Laminin-depleted CM_{SF}" refers to BCE-CM_{SF} that was depleted of laminin by immunoprecipitation with anti-laminin antibodies (see Methods). Neurite outgrowth on wells treated with laminin-depleted CM_{SF} alone was ≈ 9%, as compared with ≈ 75% prior to depletion.

of the digested sample, but not the control sample.

Thus, despite the ability of crude heparinase to inactivate the "neurite outgrowth-promoting factor" in BCE-CM, as described in Chapter 2, it appears that the purified "factor" does not absolutely require intact heparan sulfate for biological activity. Instead, it is only after the purified "neurite outgrowth-promoting factor" is made "impure", by the addition of molecules found in serum or in $BCE-CM_{SF}$, that the presence of an intact proteoglycan becomes important. This difference could not have been appreciated in Chapter 2, when an already impure source of "neurite outgrowth-promoting factor" was treated with a crude enzyme, heparinase, which contributed 1 mg/ml of additional protein impurities. In the following discussion, it will be argued that impurities contaminating the "neurite outgrowth-promoting factor" have the potential to interfere with its attachment to the culture substratum, and that the intact heparan sulfate proteoglycan protects against this interference.

DISCUSSION

Purification of the "Neurite Outgrowth-Promoting Factor"

The results presented in this chapter demonstrate that purification of the "neurite outgrowth-promoting factor" from BCE-CM yields laminin, entactin, and a heparan sulfate proteoglycan. Because only [^{35}S]methionine-labeled protein--not total protein--was monitored during the purification, it is theoretically possible that other proteins also contribute to the composition of the "neurite outgrowth-promoting factor", but escape detection because they are unlabeled. This seems very unlikely for two reasons. First, BCE cells were labeled for a very long time (4 days) so that all methionine-containing proteins they secrete should be labeled. Second, the only other source of unlabeled proteins in BCE-CM is the 0.5% fetal calf serum in the labeling medium. Since the "neurite outgrowth-promoting factor" is produced by BCE cells in normal amounts in the absence of serum and can promote outgrowth in the absence of serum (Chapter 1), it is very unlikely that serum molecules are associated in any functionally significant way with the "factor".

The scheme devised for purification of the "neurite outgrowth-promoting factor" consists largely of common fractionation methods such as ammonium sulfate precipitation, ion exchange chromatography and gel filtration. Certain points, however, merit discussion. For example, the purification begins with absorption of CM with gelatin-Sepharose, to remove fibronectin. Very poor yields were consistently obtained when BCE-CM was not thus treated, because, in the presence of fibronectin, concentration of the activity at any step, e.g. by am-

monium sulfate precipitation, absorption to DEAE, or ultracentrifugation, resulted in the appearance of insoluble material in the concentrate and losses of 40-90% of the "neurite outgrowth-promoting factor", apparently due to trapping in the insoluble fraction. Analysis of the insoluble material by SDS-PAGE indicated that its major component was fibronectin (not shown). BCE cells produce a large amount of fibronectin (Gospodarowicz et al., 1981a), and cellular fibronectin, when concentrated, is capable of spontaneous covalent polymerization (Hynes, 1981).

Several precautions were taken during purification to minimize non-specific losses. Triton X-100 was used in all buffers following ammonium sulfate precipitation. To minimize adsorption to Sepharose during gel filtration, the column buffer also contained high salt (0.5 M) and a pure protein carrier (hemoglobin). Inclusion of hemoglobin also made it possible to concentrate active fractions from gel filtration without major losses, by loading them into dialysis bags and dehydrating against dry Sephadex. Hemoglobin was later separated from the "neurite outgrowth-promoting factor" during velocity sedimentation (in Figure 4.2, the hemoglobin peak is fraction 25). Precautions were also taken during purification to minimize proteolysis. Protease inhibitors were added to the CM just after collection and included in all buffers. Despite this, one fractionation step--precipitation with polyethylene glycol--that had been used in early attempts at purification (see Appendix) was found to greatly accelerate the degradation of many proteins in BCE-CM, including laminin; this step was abandoned.

Table 1 summarizes the results of a typical purification. As dis-

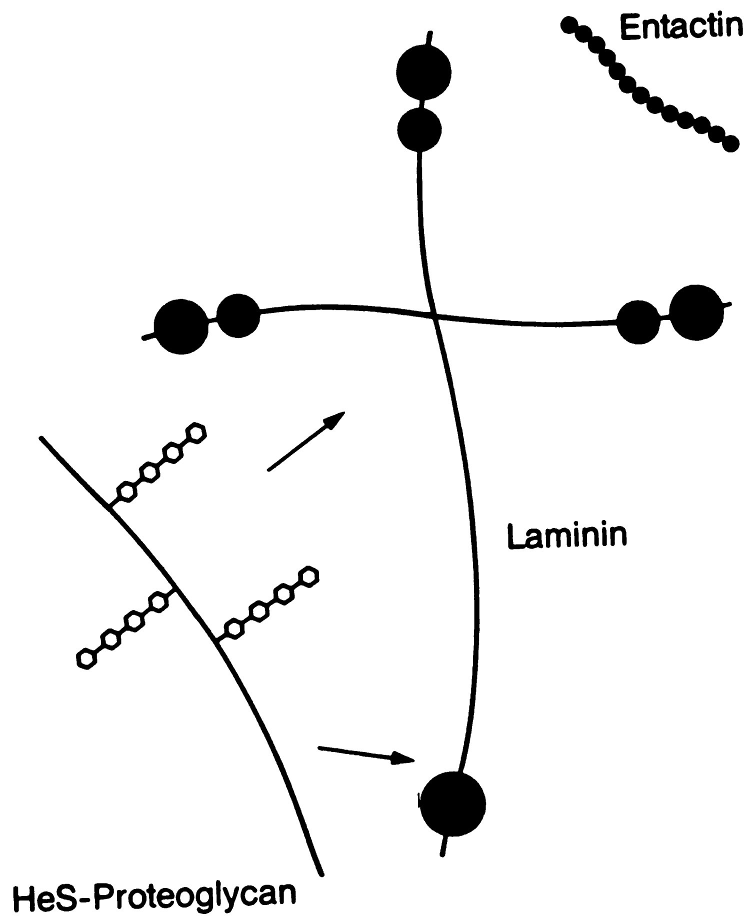
cussed above, the fact that the total amount of neurite outgrowth-promoting activity increased 37% after the first two steps of purification suggests that these steps removed "inhibitors" from the CM. These "inhibitors" need not be molecules that block neurite outgrowth-promoting activity per se, but may simply be molecules in BCE-CM that bind polylysine, and can thereby compete with the "neurite outgrowth-promoting factor" for attachment to the culture substratum. The presence of "inhibitors" in BCE-CM causes fold purification to be overestimated when calculated from specific activities. As already stated, the degree of purification of the neurite outgrowth-promoting factor is therefore less than or equal to 235-fold. Consistent with this estimate, when the laminin-band seen on SDS-PAGE at each step of purification was quantified (using Figure 4.3 and results from other batches of BCE-CM), it was found to be enriched 75-200 fold, depending on the batch of CM (calculations not shown).

Composition of the "Neurite Outgrowth-Promoting Factor"

In the most purified fractions of the "neurite outgrowth-promoting factor", three molecules--laminin, entactin and a heparan sulfate proteoglycan--have been identified. These three molecules appear to associate physically with each other (illustrated in Figure 4.14), although no claim is made that they exist in a stoichiometric complex.

Laminin is identifiable as an [^{35}S]methionine-labeled band that comigrates, by non-reducing SDS-PAGE, with a laminin standard (Figures 4.3-4.5 and 4.13), is specifically precipitated from BCE-CM by antibodies to mouse laminin and is recognized by these antibodies on immunoblots of BCE-CM (Figures 4.6 and 4.7). During gel filtration

Figure 4.14. Components of the "neurite outgrowth-promoting factor". The drawing schematically depicts the molecules that associate with laminin in BCE-CM. The structure of laminin is based on electron microscopic data (Engel *et al.*, 1981). The structures of the heparan sulfate proteoglycan and entactin are speculative. The interaction of the heparan sulfate proteoglycan with heparin-binding domains of laminin (Timpl *et al.*, 1983) is indicated by arrows. The binding site for entactin is not known.


Entactin
Laminin
HeS-Proteoglycan

and velocity sedimentation, the profile of anti-laminin immunoreactivity correlates with the amounts of the laminin band present in each fraction (compare Figures 4.1 with 4.4, 4.2 with 4.5, 4.13A with 4.13B). Removal of the laminin band from BCE-CM by immunoprecipitation with laminin-specific antibodies results in the loss of neurite outgrowth-promoting activity (Table 4.2 and Figure 4.6). The polypeptide composition of the laminin in BCE-CM indicates that heavy and light chains are present and similar in molecular weight to the heavy and light chains of mouse laminin (Figures 4.7 and 4.10). The stoichiometry of these chains cannot be determined from [^{35}S]methionine-labeled material without information about methionine content. However, assuming that the proportion of methionine in the two chains is not too different, the relative amount of heavy chain in Figure 6 appears to be lower than expected, given the 1:1.6 heavy:light chain weight ratio reported for mouse laminin from the EHS sarcoma (Engel _et al._, 1981). Furthermore, the relative amount of heavy chain seen by SDS-PAGE varied among preparations (compare Figures 6 and 9), and often decreased during the course of purification. In certain instances, the purified "neurite outgrowth-promoting factor", though fully active, appeared by reducing electrophoresis to lack the heavy chain altogether (unpublished observations). The fact that decreases occurred during purification suggests that proteolysis of the heavy chain was taking place, and is consistent with the observation that the heavy chain of mouse laminin is very sensitive to proteolysis, while the light chain is relatively stable (Ott _et al._, 1982). Because losses of heavy chain seen by reducing electrophoresis were never accompanied by a change in the non-reduced molecular weight of the

laminin, it seems likely that proteolysis of the heavy chain was limited to regions of the polypeptide cross-linked by intrachain disulfide bonds, so that fragmentation could occur only after reduction.

Entactin, a sulfated laminin-binding protein (Carlin et al., 1981; Hogan et al., 1982; Carlin et al., 1983), is also a component of the purified "neurite outgrowth-promoting factor". The 150,000 Da protein present in Figures 4.3-4.5, 4.7, and 4.13 can be identified as entactin, because it is of the correct molecular weight before (Figures 4.3-4.5, 4.7, and 4.13) and after (Figures 4.7 and 4.10) reduction, is sulfated (Figure 4.8), and is recognized by anti-entactin antibodies (Figure 4.7). It appears to be physically associated with the "neurite outgrowth-promoting factor" because it is precipitated with laminin by anti-laminin antibodies (Figure 4.7). Entactin is known to be a laminin-binding protein, which probably accounts for this association. While entactin is present in every fraction with neurite outgrowth-promoting activity (Figures 4.1, 4.2, 4.4, 4.5, and 4.13) it is not possible to calculate its stoichiometry without knowing its methionine content.

The third constituent of the purified "neurite outgrowth-promoting factor" is a heparan sulfate proteoglycan. When analyzed by SDS-PAGE, it appears as a high molecular weight blur, which is characteristic of proteoglycans (e.g. Hedman et al., 1982). As expected for a heparan sulfate proteoglycan, it can be labeled with [^{35}S]sulfate (Figure 4.8). Purified heparitinase, which digests heparan sulfate but not other classes of glycosaminoglycans, changes the mobility of this molecule to that of a single polypeptide of about 400,000 Da (Figures 4.9 and 4.10). This proteoglycan appears to be physically associated

with the "neurite outgrowth-promoting factor" because it is precipitated, along with laminin, by anti-laminin antibodies (Figure 4.10). The ability of laminin to bind to heparan sulfate (Sakashita et al., 1980; Del Rosso et al., 1981) could account for such an association. Interestingly, a heparan sulfate proteoglycan with a 400,000 Da core protein has been reported by others to be a component of murine basement membranes (Hassel et al., 1984). The proteoglycan identified in this report may be the bovine form of this molecule.

Physico-chemical Properties of the "Neurite Outgrowth-Promoting Factor"

Most of the behavior of the "neurite outgrowth-promoting factor" in BCE-CM during purification can be understood in terms of the known physico-chemical properties of laminin and the effects that bound proteoglycan and entactin might have on these properties. For example, the strong binding of the "neurite outgrowth-promoting factor" to DEAE-cellulose (requiring 1M NaCl for complete elution) likely reflects the presence of the heparan sulfate proteoglycan, a polyanion. Fractionation of the "neurite outgrowth-promoting factor" on Sepharose CL4B (Figure 4.1) indicates that most of it behaves as a larger entity than purified murine laminin (note laminin standard in Figure 4.1). This could reflect bound proteoglycan and entactin, or it could reflect self-aggregation of laminin (Engel et al., 1981). Some of the active factor in Figure 4.1 does, however, migrate appropriately for pure laminin (fractions 25-31) forming a shoulder off the main peak (fractions 19-23). Significantly, these shoulder fractions appear to contain less of the proteoglycan than the peak fractions, as judged by the amount of "high molecular weight blur" seen in Figure 4.4.

In sucrose gradients, the "neurite outgrowth-promoting factor" sediments more rapidly than purified laminin. In Figure 4.2, neurite outgrowth-promoting activity is associated with peaks at $\sim$ 19S and $\sim$ 16S (the leading edge of the peak at $\sim$ 13S), while purified laminin sediments at 11.5S (Engel et al., 1981). These results also suggest that molecules are bound to laminin, but self-aggregation of laminin could account for some of this behavior.

One unexpected result, shown in Figure 4.12, is that the purified "neurite outgrowth-promoting factor" is stable to a salt concentration that should disrupt the binding of heparan sulfate or heparin to laminin (Sakashita et al., 1980; Del Rosso et al., 1981; Ott et al., 1982). It is possible that binding of the heparan sulfate proteoglycan to laminin is stabilized by entactin, or by the protein core of the proteoglycan. There is some evidence that the core proteins of proteoglycans influence their binding to laminin. For example, a fragment of the heparan sulfate proteoglycan present in the EHS sarcoma, which contains all of the heparan sulfate chains of the intact molecule but lacks much of the core protein, shows reduced binding to laminin (Laurie et al., 1984). It is also possible that there are modifications of the heparan sulfate chains of the proteoglycan in BCE-CM that increase its effectiveness as a ligand. Finally, if there is more than one heparan sulfate-binding site on laminin (cf. Ott et al., 1982), a very large heparan sulfate proteoglycan such as the one in BCE-CM may be able to bind to both sites simultaneously, giving rise to an interaction of high avidity. Although it is not yet clear why laminin's interaction with the proteoglycan appears more stable than its interaction with glycosaminoglycans, it is interesting to note that similar

observations have been made regarding the interaction of another extracellular matrix glycoprotein, fibronectin, with heparan sulfate proteoglycans (Hedman et al., 1982).

In some ways, the physico-chemical properties of the "neurite outgrowth-promoting factor" are reminiscent of those of "adherons". Adherons are also macromolecular complexes that are found in CM's and can mediate cell-substratum interactions (Schubert et al., 1983a). The best understood adherons are those produced by myoblasts. Like the "neurite outgrowth-promoting factor" in BCE-CM, myoblast adherons are composed of ECM molecules, however their composition is quite unique: Myoblast adherons contain fibronectin, collagen, hyaluronic acid, and several classes of proteoglycans, but apparently no laminin (Schubert et al., 1983a). Furthermore, whereas myoblast adherons characteristically aggregate in the presence of millimolar amounts of calcium, calcium has no effect on the sedimentation behavior of the "neurite outgrowth-promoting factor" in BCE-CM. Adherons have also been described in CM produced by retinal cells, however, retinal cell adherons have only been purified to a very rudimentary degree (Schubert et al., 1983b), and it is still unclear whether they are homogeneous complexes or a heterogeneous population of macromolecular aggregates. Interestingly, retinal adherons are not aggregated by calcium, and the biological activity of retinal adherons seems to depend on a heparan sulfate proteoglycan (Cole et al., 1985). Unlike the "neurite outgrowth-promoting factor" in BCE-CM, however, retinal adherons seem to require for their activity a shed cell surface molecule of 170,000 Da (Cole and Glaser, 1984). Further comparisons between "neurite outgrowth-promoting factors" and retinal adherons will have to await further purification and charac-

terization of the components of these adherons.

Functional roles of the components of the "neurite outgrowth-promoting factor"

Because purification of the "neurite outgrowth-promoting factor" yields three molecules--laminin, entactin and a heparan sulfate proteoglycan--it is important to determine which of the three are involved in the promotion of neurite outgrowth. The data presented here suggest that laminin is primarily responsible for this activity. Laminin was purified from BCE-CM to approximately the same extent as the "neurite outgrowth-promoting factor" (75-200 fold, as discussed above). During the purification, all fractions containing laminin promoted neurite outgrowth; all fractions lacking laminin did not. Furthermore, the profiles of neurite outgrowth-promoting activity after gel filtration (Figure 4.1) and velocity sedimentation (Figure 4.2 and 4.13A) are closely paralleled by the profiles of laminin immunoreactivity (Figures 4.1, 4.2, and 4.13A) and by the electrophoretic band representing laminin (Figures 4.4, 4.5 and 4.13B). Importantly, when laminin is specifically removed from BCE-CM by immunoprecipitation, laminin and neurite outgrowth-promoting activity disappear concomitantly (Table 4.2 and Figure 4.6).

The hypothesis that laminin is responsible for the activity of the "neurite outgrowth-promoting factor" in BCE-CM is also supported by the observation that pure murine laminin derived from the EHS sarcoma is capable of promoting neurite outgrowth (Chapter 3). The specific activity of EHS sarcoma-laminin is between 10 and 38 neurite outgrowth-promoting units per ug (see Chapter 3 and Manthorpe _et al._, 1983). By

silver staining SDS-polyacrylamide gels on which anti-laminin immunoprecipates of BCE-CM were run alongside known amounts of EHS sarcoma-laminin, it has been possible to estimate the amount of laminin in BCE-CM (ca. 0.2 - 0.7 ug per ml in different batches of CM, data not shown). Assuming the same specific activity as EHS sarcoma-laminin, this quantity is sufficient to account for the approximately 12 neurite outgrowth-promoting units found in each ml of BCE-CM (Chapter 2, Figure 2.1).

Neither of the two other components of the "neurite outgrowth-promoting factor", entactin and the heparan sulfate proteoglycan, appear able to promote neurite outgrowth, since both are frequently found in fractions that lack this activity (cf. Figures 4.1, 4.2, 4.4 and 4.5). It is also unlikely that these molecules must necessarily be present along with laminin for neurite outgrowth-promoting activity to be seen, for the following reasons: First, much of the entactin associated with the "neurite outgrowth-promoting factor" in BCE-CM can be removed by velocity sedimentation in 2M urea, without reducing the factor's activity (data not shown). Furthermore, as will be shown in the next chapter, the "neurite outgrowth-promoting factors" in some other CM's apparently lack entactin entirely, yet are fully active. Second, the heparan sulfate proteoglycan associated with the "neurite outgrowth-promoting factor" in BCE-CM can be digested with heparitinase (which removes heparan sulfate) without a reduction in activity (Table 4.3).

Although the heparan sulfate proteoglycan is not absolutely required for the activity of the "neurite outgrowth-promoting factor" in BCE-CM, the data in Table 4.3 suggest that it plays an important role.

It seems likely that this role is to mediate the attachment of laminin to polylysine substrata, and is achieved through the interaction of polyanionic heparan sulfate chains with polylysine, a polycation. Such a role would be important, despite the fact that laminin by itself can adsorb to polycationic substrata (e.g. Manthorpe _et_ _al._, 1983), because laminin is only a minor constituent of CM's, being outnumbered $>$ 100 fold by other secreted proteins in BCE-CM and up to 10,000 fold by serum proteins, depending on the serum content of the CM. The role of heparan sulfate proteoglycan in making attachment of laminin favorable under these circumstances is clearly reflected in the marked ability of serum, and molecules secreted by BCE cells, to inhibit the heparitinase-digested "neurite outgrowth-promoting factor". The fact that serum is routinely present throughout the neurite outgrowth assay (as part of the neuronal culture medium), yet only inhibits the digested "factor" when it is present at the time of application of the "factor" to the substratum, is compelling evidence that the inhibitory effect of serum on the digested "factor" is the result of interference with attachment to the substratum, not interference with neurite outgrowth-promoting activity per se. Interestingly, these results suggest that, were it not for the heparan sulfate proteoglycan, the amount of neurite outgrowth-promoting activity detectable in BCE-CM_{SF} would be low, and in BCE-CM with $\geq$ 10% serum, (c.f. Chapter 1), none would be detected at all. The results also suggest an explanation for the observation, reported in Chapter 2, that _crude_ heparinase completely inactivated the "neurite outgrowth-promoting factor" in BCE-CM_{SF}: Since crude heparinase contains not only the enzyme heparinase and heparitinase but also nearly 1 mg/ml of additional protein, these

added impurities were probably sufficiant to block the attachment of the digested "neurite outgrowth-promoting factor" to the culture substratum.

Antibody blockade of "Neurite Outgrowth-Promoting Factors"

Although the results of this chapter point to laminin as the component of the "neurite outgrowth-promoting factor" in BCE-CM that is primarily responsible for neurite outgrowth-promoting activity, it was demonstrated in Chapter 3 that anti-laminin antibodies block the neurite outgrowth-promoting activity of purified laminin, but not the neurite outgrowth promoting activity of BCE-CM (Table 3.2). However, since the laminin used in these studies was purified from a mouse tissue (the EHS sarcoma), and the antibodies were generated against it, not against the bovine laminin that should be found in BCE-CM, it is possible that cross-reactivity between the laminin from the two different species is not sufficiently good, causing blockade of neurite outgrowth-promoting activity in BCE-CM to fail, even though immunoprecipitation of neurite outgrowth-promoting activity succeeded (Table 4.2 and Figures 4.6 and 4.7). This could occur because successful immunoprecipitation requires antibody binding to any epitopes on laminin, while blockade of activity presumably requires antibody binding to particular important epitopes.

If this explanation is correct, it should be possible to demonstrate that antibodies prepared against bovine laminin will block the neurite outgrowth-promoting activity of BCE-CM. Unfortunately, there is not, at present, any suitable source from which bovine laminin may be purified. Yet, although this seems to be a plausible hypothesis,

there are already good reasons to suspect that the real explanation is considerably more complicated. For example, it was shown in Chapter 3 (Table 3.2), that the neurite outgrowth-promoting activity of several CM's was not blocked by anti-laminin, including three murine CM's (one mouse and two rat CM's). Furthermore, some workers have obtained antibodies that block the neurite outgrowth-promoting activity of some murine CM's, but fail to block the activity of purified EHS sarcoma-laminin (see discussion, Chapter 3). To some researchers, these results have been taken as evidence that the "neurite outgrowth-promoting factors" in certain CM's must not involve laminin (e.g. Manthorpe et al., 1983; Edgar et al., 1984). However, as the following chapter will make clear, there is good reason to believe that the "neurite outgrowth-promoting factors" in most CM's do involve laminin, in much the same way as the "factor" in BCE-CM. How the failure of anti-laminin antibodies to block the activity of these other CM's might be explained is one of the issues discussed in Chapter 5.

C H A P T E R 5

LAMININ IS ASSOCIATED WITH THE "NEURITE OUTGROWTH-PROMOTING FACTORS" IN SEVERAL CONDITIONED MEDIA

SUMMARY

BCE-CM is only one of many CM's that contain a substratum-binding "neurite outgrowth-promoting factor" (see Chapter 1). As discussed in Chapter 2, certain of the characteristics of the "neurite outgrowth-promoting factors" in other CM's suggest that the active substance in them, and in BCE-CM, is the same. In this chapter, this hypothesis is tested. Laminin is shown to be associated with the "neurite outgrowth-promoting factors" in five CM's other than BCE-CM. In some, but not all CM's, there also appears to be entactin associated with the laminin. Interestingly, anti-laminin antibodies fail to block the neurite outgrowth-promoting activity of all but one of the CM's tested (Chapter 3). The implications of these results are discussed.

RESULTS

The "Neurite Outgrowth-Promoting Factors" in Other CM's Contain Laminin

The experiments described in the previous chapter demonstrate that laminin is a component of the "neurite outgrowth-promoting factor" in BCE-CM. To determine whether this is also the case for the "neurite outgrowth-promoting factors" in other CM's, CM's prepared from five other cell types--a rat Schwannoma line (RN22), a rat pheochromocytoma line (PC12), an endodermally differentiated mouse teratocarcinoma cell line (PFHR9), a mouse myoblast line (C_2) and primary embryonic chicken muscle cells--were subjected to immunoprecipitation with affinity purified anti-laminin antibodies. As shown in Table 5.1, neurite outgrowth-promoting activity was specifically removed from all CM's tested, implying that laminin immunoreactivity is associated with the active factor in these CM's.

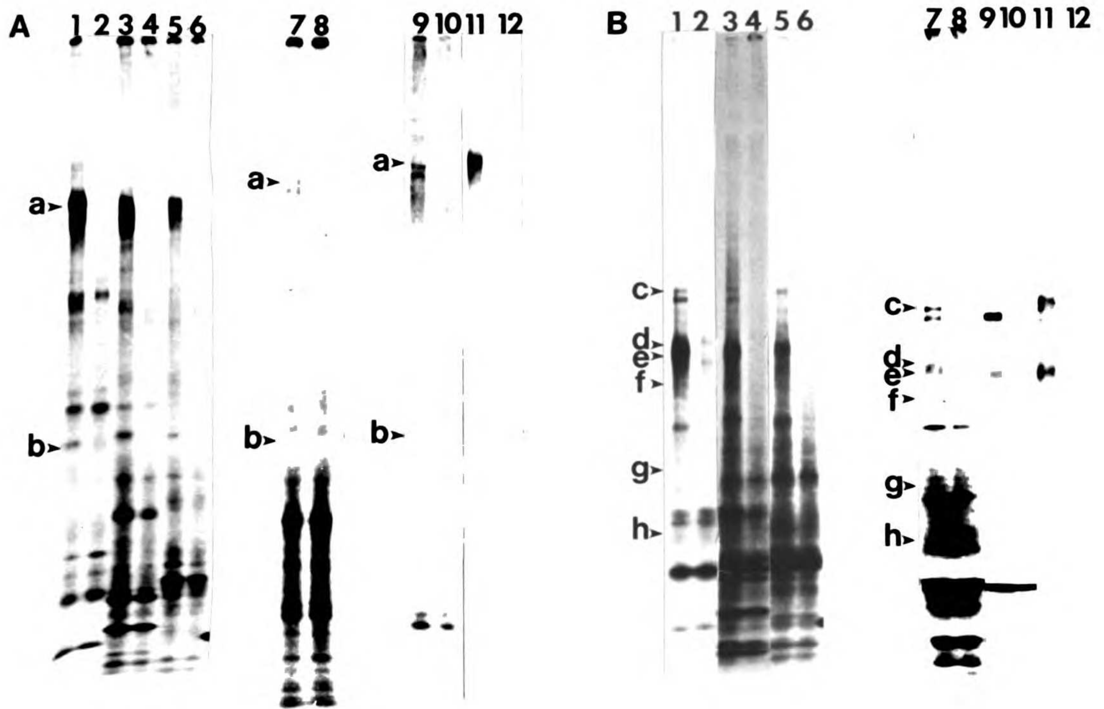
To demonstrate that the immunoreactive molecule in each CM was indeed laminin, identical immunoprecipitations were performed using [^{35}S]methionine-labeled CM's. A sample of [^{35}S]methionine-labeled BCE-CM was also included for comparison. Precipitates, washed only once in 0.1 M NaCl, 0.05 M Tris, pH 7.4, were subjected to electrophoresis under non-reducing conditions (Figure 5.1a). A band that comigrates with a laminin standard was found to be specifically immunoprecipitated from each CM. (in chick muscle CM, this band has a slightly lower apparent molecular weight). (In some cases (lanes 7,9), the laminin band appears as a doublet, but this was not a consistent finding, and probably reflects some proteolytic degradation). Upon reduction (Figure 5.1b), the laminin band was replaced, in each case,

TABLE 5.1

Removal of neurite outgrowth-promoting activity from CM's by immunoprecipitation with affinity-purified anti-laminin antibodies. The amount of antibody used is in micrograms of antibody per milliliter of CM.

CM	Amount of anti-laminin	Neurite outgrowth (%)
BCE	0	85 ± 1.6
	9	5 ± 1.4
PC12	0	60 ± 2.1
	9	2 ± 1.2
RN22	0	28 ± 2.1
	9	1 ± 0.6
C_2	0	35 ± 4.1
	9	2 ± 1.5
Chick Muscle	0	82 ± 1.4
	9	49 ± 0.9
	27	14 ± 1.4
PFHR9	0	60 ± 2.3
	9	21 ± 3.8
	27	4 ± 1.5

Figure 5.1. Immunoprecipitation of [^{35}S]methionine-labeled CM's with affinity-purified anti-laminin. Immunoprecipitates, prepared as described, were washed once in TBS and resuspended in 9M urea. After 1-12 hours, samples were diluted in electrophoresis buffer prepared in 9M urea, and lacking 2-mercaptoethanol. Samples were heated (100°C) for 10 min. Aliquots were subjected to electrophoresis under non-reducing conditions (A), or were made 5% in 2-mercaptoethanol for electrophoresis under reducing conditions (B). The CM's, and the amount of anti-laminin used (per ml of CM), were as follows (for both (A) and (B)): BCE-CM, 9 ug (lane 1) and 0 ug (lane 2); RN22-CM, 9 ug (lane 3) and 0 ug (lane 4); C_2-CM, 9 ug (lane 5) and 0 ug (lane 6); PC12-CM, 9 ug (lane 7) and 0 ug (lane 8); Chick muscle CM, 27 ug (lane 9) and 0 ug (lane 10); PFHR9-CM, 27 ug (lane 11) and 0 ug (lane 12). Fluorographic exposure was 4 d for lanes 1-6 and 9-10, 7 d for lanes 7-8, and 2.5 hr for lanes 11-12. Unreduced molecular weight standards were a) purified laminin and b) mouse IgG. Reduced molecular weight standards were c) laminin, large subunit, d) cellular fibronectin, e) laminin, small subunit, f) myosin, g) β -galactosidase, h) bovine serum albumin. Note the 150 kDa polypeptide in lanes 1,3 and 5 of (A) and (B).


A
1 2 3 4 5 6
7 8
9 10 11 12
a
b
B
1 2 3 4 5 6
7 8 9 10 11 12
c
d
e
f
g
h

by bands comigrating with the heavy and light chains of a laminin standard (the chick muscle CM bands are of slightly lower apparent molecular weight). In some CM's, a doublet, rather than a single band, corresponded to the high molecular weight chain of laminin (also described by Timpl et al., 1982).

Interestingly, in Figure 5.1b, the relative intensity of labeling of heavy and light chains is considerably different among CM's. The true stoichiometry of the chains cannot be deduced without information about methionine content. However the data suggest that some CM's have a lower than expected amount of heavy chain for the amount of light chain seen. A possible explanation for this phenomenon, already discussed with regard to similar observations with BCE-CM (Chapter 4, Discussion), is that in some CM's, limited proteolysis occurs within disulfide-bridged regions of the very protease-sensitive heavy chain. The laminin molecule would then remain in one piece prior to reduction, but after reduction, much of the heavy chain would fragment into smaller pieces that would most likely not be detected.

Nearly all of the other bands in Figure 5.1 represent proteins that are non-specifically precipitated (i.e. are present in control lanes 2,4,6,8,10,12). The one exception is a band of ca. 150,000 Da (unchanged by reduction) that is specifically immunoprecipitated from BCE-CM, RN22-CM and C_2-CM. As described in Chapter 4, in BCE-CM this 150,000 Da protein can be identified as entactin. It is very likely that entactin is also responsible for the 150,000 Da band immunoprecipitated from RN22-CM and C_2-CM.

Whether heparan sulfate proteoglycans were also specifically immunoprecipitated from some or all of the CM's is not apparent from

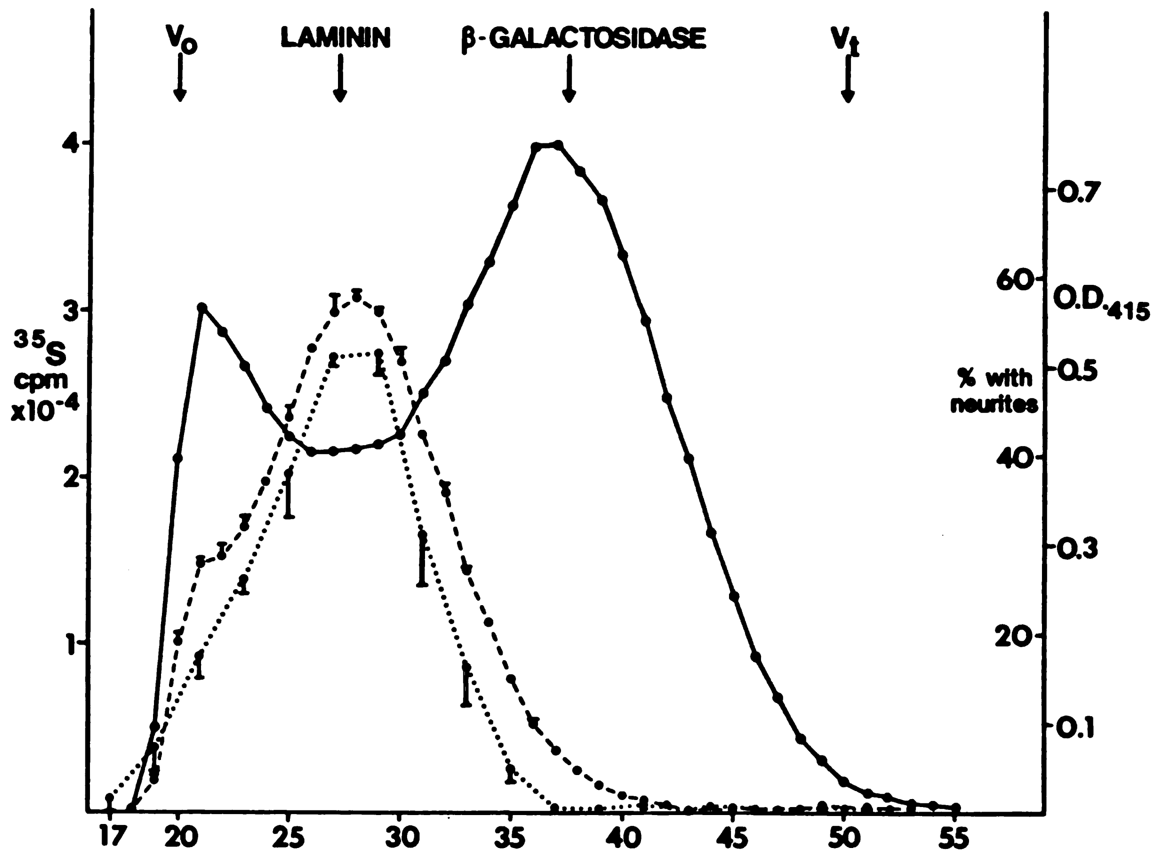
Figure 5.1. Experiments designed to answer this question, such as immunoprecipitation of [^{35}S]sulfate-labeled CM's or digestion with heparitinase, have not yet been carried out. However, indirect evidence that the "neurite outgrowth-promoting factors" in these CM's are associated with heparan sulfate proteoglycans was mentioned in Chapter 2, and will be reviewed below.

Laminin Immunoreactivity and Neurite Outgrowth-Promoting Activity Comigrate During Gel Filtration

As further confirmation that laminin is associated with the "neurite outgrowth-promoting factors" produced by these different cell types, [^{35}S]methionine-labeled CM's from C_2 and RN22 cells were fractionated by the procedures developed to purify the "neurite outgrowth-promoting factor" in BCE-CM (Chapter 4). Specifically, freshly labeled C_2-CM and RN22-CM were treated with protease inhibitors and subjected to absorption with gelatin-Sepharose, ammonium sulfate precipitation, DEAE-cellulose chromatography, and gel filtration on Sepharose CL4B, as described in the Methods section. Fractions from gel filtration were then assayed for [^{35}S]methionine content, neurite outgrowth-promoting activity, and laminin immunoreactivity, as already described. The results for C_2-CM are shown in Figure 5.2, and those for RN22-CM in Figure 5.3. As observed with BCE-CM (Chapter 4, Figure 4.1), there was a good correspondence between the profile of neurite outgrowth-promoting activity and the profile of laminin immunoreactivity in both C_2- and RN22-CM.

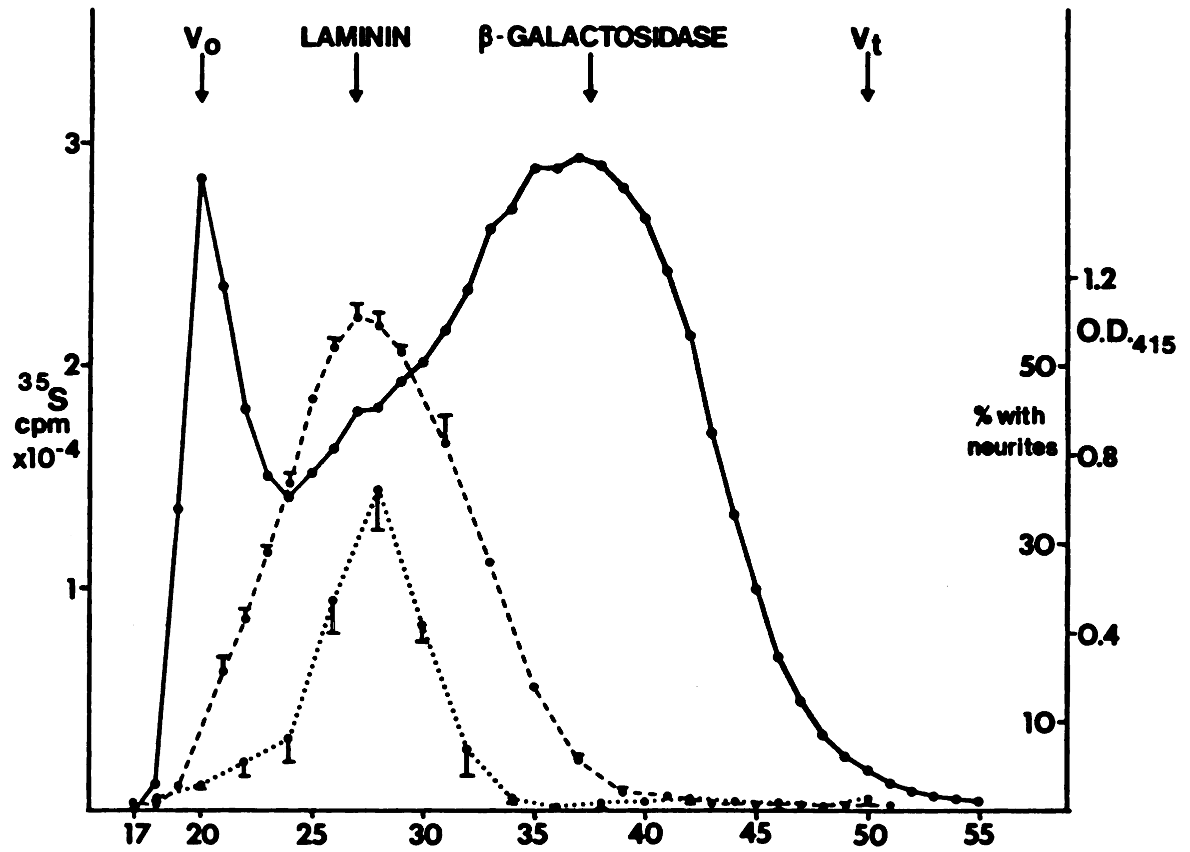
Interestingly, when Figures 5.2, 5.3 and 4.1 are compared, it can be seen that the shape of the peak of laminin immunoreactivity (or,

Figure 5.2. Gel filtration of the "neurite outgrowth-promoting factor" from C_2-CM on Sepharose CL4B. The profiles of [^{35}S]methionine-labeling (________________), neurite outgrowth-promoting activity (···············), and laminin immunoreactivity (- - - - - - - - - -) are shown. Chromatographic positions of standards are indicated by arrows.



V_o
LAMININ
β-GALACTOSIDASE
V_t
^{35}S cpm x10^{-4}
% with neurites
O.D.$_{415}$
4
3
2
1
60
40
20
0.7
0.5
0.3
0.1
17
20
25
30
35
40
45
50
55

Figure 5.3. Gel filtration of the "neurite outgrowth-promoting factor" from RN22-CM on Sepharose CL4B. The profiles of [^{35}S]methionine-labeling (________________), neurite outgrowth-promoting activity (•••••••••••••••), and laminin immunoreactivity (- - - - - - - - - - -) are shown. Chromatographic positions of standards are indicated by arrows.



Vo
LAMININ
β-GALACTOSIDASE
Vt
35S cpm x10-4
3
2
1
1.2
O.D.415
50
% with neurites
0.8
30
0.4
10
17
20
25
30
35
40
45
50
55

equivalently, neurite outgrowth-promoting activity) was not the same among the three different CM's. One way these differences may be understood is the following: The profile of laminin immunoreactivity in each CM appears to consist of two components, a peak of completely excluded material, centered about V_o, and a peak of partially included material, centered approximately about fraction 27. In BCE-CM (Figure 4.1), most laminin immunoreactivity is in the excluded peak (fractions 19-23). Only a small amount is present in the more included peak, and it appears only as a "shoulder" off the larger void volume peak (fractions 24-32). In C_2-CM, however, the proportions are reversed, and the partially included peak (fractions 24-32) is much larger than the excluded peak, which forms only a small shoulder (fractions 19-23). RN22-CM is an even more extreme example, with nearly all of the laminin immunoreactivity present in the partially included peak centered about fraction 27. Significantly, when purified EHS-sarcoma laminin was chromatographed as a standard on this gel filtration column, it migrated as a single partially included peak centered about fraction 27. Thus, of the two components of the profile of laminin immunoreactivity seen in Figures 4.1, 5.2 and 5.3, the peak centered about fraction 27 appears to represent laminin that is displaying an appropriate molecular size for single laminin molecules, while the void volume peak appears to represent laminin that is effectively larger. As already discussed for BCE-CM, such an increase in effective size could be a reflection of the fact that much of the laminin in BCE-CM is associated with a large heparan sulfate-proteoglycan, or it could reflect self-aggregation of laminin molecules (cf. Engel _et al._, 1981). If so, the results in Figures 5.2 and 5.3 may be taken to indicate that, compared

with BCE-CM, less of the laminin in C_2-CM, and very little of the laminin in RN22-CM, is involved in such molecular associations. This could be because such associations are weaker in these two CM's, at least under the conditions employed during chromatography, or it could reflect a relative lack of a laminin-binding molecule, such as a heparan sulfate proteoglycan, in these two CM's. This issue will be discussed further below.

DISCUSSION

Association of Laminin with "Neurite Outgrowth-Promoting Factors"

The experiments in this chapter investigated the composition of the "neurite outgrowth-promoting factors" in five CM's other than BCE-CM. As with the "neurite outgrowth-promoting factor" in BCE-CM, laminin appeared to be a component of the "neurite outgrowth-promoting factors" in these other CM's, since its removal by immunoprecipitation resulted in the disappearance of neurite outgrowth-promoting activity (Figure 5.1, Table 5.1). In addition, fractionation of C_2-CM and RN22-CM, according the the procedures outlined in Chapter 4, revealed a good correspondence between neurite outgrowth-promoting activity and laminin-immunoreactivity. Similar gel-filtration results have also been reported for embryonic chicken muscle CM (Calof and Reichardt, 1985).

Some recent findings by other groups support the conclusions of this chapter. For example, an abstract by Davis _et al._ (1984b) reported that the "neurite outgrowth-promoting factor" in RN22-CM is associated with a laminin-immunoreactive polypeptide of approximately 200,000 Da (the same size as a laminin light chain). No laminin heavy chain was observed by this group; however, it is possible that partial proteolysis of the heavy chain prevented it from being detected after gel electrophoresis under reducing conditions, a phenomenon sometimes seen with the laminin in BCE-CM (see Chaper 4, discussion). Indeed, the recent evidence of this group suggests that this explanation is correct (Davis _et al._, 1985). Interestingly, when Collins and Lee (1984) analyzed a partially purified "neurite outgrowth-

promoting factor" from chick heart cell-CM by reducing SDS-PAGE, the major polypeptide band seen was also about the size of a laminin light chain.

Other Components of "Neurite Outgrowth-Promoting Factors"

As shown in Chapter 4, the "neurite outgrowth-promoting factor" in BCE-CM is composed of laminin, entactin and a heparan sulfate proteoglycan. While laminin also appears to be a component of the "neurite outgrowth-promoting factors" in other CM's, it is less clear what other molecules, if any, are associated with these "factors". Entactin, for example, appears in Figure 5.1 to be associated with laminin in some CM's, but not all. At present, the evidence that heparan sulfate proteoglycans are associated with the "neurite outgrowth-promoting factors" in other CM's is still indirect, consisting of several observations. For example, the "neurite outgrowth-promoting factors" in several CM's have a density in CsCl similar to that observed with BCE-CM (Lander _et al._, 1983). In addition, the "neurite outgrowth-promoting factors" in most CM's are readily assayed in CM's containing large amounts of serum (e.g. Adler _et al._, 1981), suggesting that the laminin in these CM's is extremely efficient at competing for binding sites on polylysine substrata. Since this competitive advantage seems to be conferred upon the "neurite outgrowth-promoting factor" in BCE-CM by a heparan sulfate proteoglycan (see Chapter 4, Table 4.3, and Discussion), it would be reasonable to expect that the same is the case for other CM's. Consistent with this, the neurite outgrowth-promoting activity of some CM's has been shown to be affected by crude heparinase (Lander _et al._, 1983; V. Nurcombe, personal com-

munication; M. Gurney, personal communication) or purified heparitinase and heparinase (Calof and Reichardt, 1984). For one cell type (PC12), monoclonal antibodies have been obtained that recognize a heparan sulfate proteoglycan released into CM; these antibodies have been shown to be able to immunoprecipitate the "neurite outgrowth-promoting factor" from this CM (Matthew et al., 1985).

A recent additional piece of evidence comes from the purification of the "neurite outgrowth-promoting factor" in RN22-CM by Davis et al. (1985). Although this group had not detected a proteoglycan in SDS-gels of their purified material, when they examined it by electron microscopy, they observed molecules resembling somewhat degraded laminin apparently bound to molecules that contained many side chains and looked strikingly like proteoglycans that have previously been described. Their experience illustrates how proteoglycans, because of their tendency to run as "blurs" by SDS-PAGE, may be missed when analyzed in this manner, unless [^{35}S]sulfate-labeling is used to highlight them, or digestion of glycosaminoglycan chains is employed, to produce a polypeptide that forms an identifiable band (cf. Chapter 4).

Although it seems likely, for the reasons just discussed, that most or all of the "neurite outgrowth-promoting factors" in CM's involve both laminin and a heparan sulfate proteoglycan, it seems unlikely that the same heparan sulfate proteoglycan is involved in each CM. For example, the "neurite outgrowth-promoting factor"-associated heparan sulfate proteoglycan in PC12-CM (Matthew et al., 1985) is structurally quite different from the heparan sulfate proteoglycan identified in BCE-CM. The PC12 proteoglycan appears to have a much smaller core protein (Matthew et al., 1985), and is recognized by five

monoclonal antibodies that do not react with any component of BCE-CM (unpublished observations). Ultimately, what heparan sulfate proteoglycan associates with the laminin in a CM may depend both on what heparan sulfate proteoglycans are present in the CM, and on the affinity of different heparan sulfate proteoglycans towards laminin (cf. Laurie et al., 1984). Interestingly, this variability could explain differences observed among the gel filtration profiles of the "neurite outgrowth-promoting factors" in BCE-CM, C_2-CM and RN22-CM (Figures 4.1, 5.2 and 5.3). For example, if heparan sulfate proteoglycans in C_2- and RN22-CM's are unlike the proteoglycan in BCE-CM, and bind laminin only as strongly as heparan sulfate binds laminin (cf. Figure 4.12), then laminin-proteoglycan complexes in C_2- and RN22-CM's might be expected to dissociate in 0.5 M NaCl (Sakashita et al., 1980; Del Rosso et al., 1981). Since gel filtration of these CM's was carried out in a buffer containing 0.5 M NaCl, this could explain why much of the laminin in C_2-CM, and most of the laminin in RN22-CM, had an apparent size roughly equal to that of single laminin molecules (Figures 5.2 and 5.3).

Failure of Anti-Laminin Antibodies to Block the Effects of "Neurite Outgrowth-Promoting Factors"

In Chapter 3, it was reported that anti-laminin antibodies that blocked the neurite outgrowth-promoting activity of laminin-treated substrata failed to block the neurite outgrowth-promoting activity of substrata treated with BCE-CM, C_2-CM, RN22-CM, PC12-CM, primary chicken muscle cell CM (Table 3.2), and CM's tested by other groups (Manthorpe et al., 1983; Edgar et al., 1984). Since the evidence

presented in this chapter and in Chapter 4 indicates that laminin is an essential component of the "neurite outgrowth-promoting factors" in all of these CM's, the failure of antibody blockade requires an explanation (although the explanation need not be the same for every CM).

One explanation was already suggested in Chapter 4. Since the anti-laminin antibodies used in these experiments were generated against murine laminin, cross-reactivity with the bovine laminin in BCE-CM might be insufficient to permit blockade of activity. The same could easily be said for the chicken laminin in chicken muscle CM. It would be difficult, however, to extend this explanation to cover the rat laminin in RN22-CM and PC12-CM, given that there is very good immunological cross-reactivity between rat and mouse laminin (Engvall et al., 1983) and that anti-rat laminin has been shown to block the neurite outgrowth-promoting activity of mouse laminin (Manthorpe et al., 1983). Finally, for C_2-CM, a mouse CM, this explanation cannot be invoked at all. (Similarly, Manthorpe et al. (1983) have reported that anti-rat laminin fails to block the neurite outgrowth-promoting activity of RN22-CM, a rat CM).

In considering the alternatives, it must be pointed out that the "neurite outgrowth-promoting factors" in the rat and mouse CM's described in this chapter (RN22-CM, PC12-CM and C_2-CM) have not been as fully characterized as the "neurite outgrowth-promoting factor" in BCE-CM, for which the strongest case is made that laminin is responsible for neurite outgrowth-promoting activity. Thus, the possibility that the "neurite outgrowth-promoting factors" in the rat and mouse CM's contain laminin but derive their neurite outgrowth-promoting activity from some other molecule cannot be entirely excluded. Such an

hypothesis however, would create more problems than it would solve: Why, for example, is the activity of the "neurite outgrowth-promoting factors" in these CM's so similar to the activity of both laminin and the "neurite outgrowth-promoting factor" in BCE-CM? Why, for C_2- and RN22-CM's, does the profile of laminin-immunoreactivity match the profile of neurite outgrowth-promoting activity, and why does the peak of this activity migrate appropriately for laminin molecules (Figures 5.2 and 5.3)? It seems much more reasonable to retain the hypothesis that laminin is the active component of the "neurite outgrowth-promoting factors" in all of the CM's looked at in this study, and conclude that, for some reason, the laminin in most of the "neurite outgrowth-promoting factors" resists antibody blockade. Fortunately, there are several plausible ways in which this could occur.

First, molecules that interact with the laminin in CM's could interfere with the binding of blocking antibodies. This hypothesis is attractive, since it is clear that the "neurite outgrowth-promoting factors" in CM's are composed of laminin that has molecules bound to it. In fact, one of the molecules that seems to be associated with laminin--namely, heparan sulfate proteoglycan--ought to bind to the heparin-binding site at or near the E3 portion of the laminin molecule--the very part of the laminin molecule that "blocking" antibodies recognize (Edgar _et al._, 1984). Interference with antibody binding could thus be purely steric, or could involve conformational changes induced by the binding of a proteoglycan molecule. If this explanation is correct, it should be possible to isolate the "neurite outgrowth-promoting factor" from a rat or mouse CM, remove all bound proteoglycan molecules, and thereby restore sensitivity to blockade by anti-laminin

antibodies. These experiments have not yet been done.

Alternatively, the laminin in CM's may itself be resistant to antibody blockade because it is structurally different from "standard" EHS sarcoma-laminin. The occurence of isoforms of laminin would not be a surprising finding. Fibronectin, an ECM glycoprotein similar to laminin in many of its functions, has at least three isoforms, generated from a single gene by differences in RNA processing and by post-translational modifications (e.g. Schwarzbauer et al., 1983; Paul and Hynes, 1984; Sekiguchi et al., 1985). It has already been noted (Chapter 3) that the light chains of laminin come in at least three different forms: B1a, B1b, and B2. The mRNA's for the B1a and B1b chains show sequence homology with each other (and therefore could arise from alternate RNA splices) but not with the B2 chains (Barlow et al., 1984). Because of the existence of three types of light chains, either laminin molecules all have the structure AB1aB1bB2, or laminin isoforms must necessarily exist. At present, it is not known which is the case, but the fact that, in at least two cell types, B2 mRNA's are considerably more prevalent than B1 mRNA's argues mildly against the AB1aB1bB2 structure (Carlin et al., 1983; Barlow et al., 1984). It should also be kept in mind that post-translational modifications may also create isoforms. While little is known about the carbohydrate structures added to laminin, it is significant that the few species of laminin for which carbohydrate composition is known differ greatly in this regard (Chung et al., 1979; Shibata et al., 1982; Engvall, et al., 1983).

Yet a third possibility exists to explain the failure of anti-laminin antibodies to block the activity of the laminin in the "neurite

outgrowth-promoting factors" in CM's. In Chapter 3, it was noted that Edgar et al. (1984) discovered two non-overlapping fragments of EHS sarcoma-laminin that had some neurite outgrowth-promoting activity. One of these (fragment E1-4) apparently owed its neurite outgrowth-promoting activity to a site that did not function in the intact laminin molecule, since the activity of this fragment was not blocked by antibodies that completely inactivated intact laminin. It is puzzling why such a "latent" neurite outgrowth-promoting site should exist, unless it is meant to be "activated" under some conditions. Possibly, the tumor-derived EHS sarcoma-laminin is aberrant in this regard, and more physiologically relevant laminin makes use of this site even when it has not been proteolyzed. Alternatively, the activation of this site could be triggered by the binding of some other molecule to laminin. Finally, it could be that naturally-occuring proteolysis is normally responsible for activating this site. Whatever the reason, it is easy to imagine how this site might already be active in the laminin in CM's. CM-laminin, unlike EHS sarcoma-laminin, already has molecules bound to it, and it is likely to have already undergone some amount of proteolysis (described above, and in Chapter 4).

It appears, therefore, that there are several possible ways in which laminin could be responsible for the activity of "neurite outgrowth-promoting factors" in CM's yet resist blockade by anti-EHS sarcoma-laminin. Whether any of these correctly explains the observations reported and discussed here is unknown. It is hoped that insight into this question will be gained by closer examination of PFHR9-CM, the one CM whose neurite outgrowth-promoting activity was partially blocked by anti-laminin antibodies (Table 3.2) If laminin isoforms exist, then

perhaps the isoform(s) found in PFHR9-CM is the same as the one(s) found in the EHS sarcoma. In support of this possibility, it should be noted that laminin polypeptides from PFHR9-CM, seen after reducing SDS-PAGE, do seem to line up more closely with the heavy and light chains of the EHS sarcoma-laminin standard than do the laminin polypeptides from other CM's (Figure 5.1). Alternatively, it may turn out that the laminin in PFHR9-CM differs from the laminin in other CM's by being relatively free of laminin-binding molecules that might interfere with the binding of blocking antibodies. In support of this hypothesis, it has been noted that PFHR9-CM contains so much more laminin than other CM's that at least one laminin-binding molecule that PFHR9 cells secrete--namely, entactin (Hogan _et al._, 1982)--appears to be in relatively short supply (unpublished observations).

In addition to further study of PFHR9-CM, other approaches will no doubt be of great value in elucidating the real immunochemical difference between EHS sarcoma-laminin and the laminin in the "neurite outgrowth-promoting factors" in CM's. For example, a monoclonal antibody has been isolated that blocks the activity of the "neurite outgrowth-promoting factor" in PC12-CM (Matthew and Patterson, 1983), but fails to block the neurite outgrowth promoting activity of EHS sarcoma-laminin (W.D. Matthew, personal communication). Although this antibody apparently recognizes a laminin-containing complex in PC12-CM, it does not even bind to purified EHS-sarcoma laminin, and may, in fact, recognize an epitope generated by the binding of a heparan sulfate proteoglycan to laminin (W.D. Matthew, personal communication). Further characterization of this epitope will undoubtedly provide important new information. In addition, recent results from cloning and

sequencing of laminin mRNA's make possible the generation of antibodies directed against epitopes specific for different laminin B chains, providing one direct way to test for the existence of laminin isoforms directly.

Thus, it seems likely that the reasons behind the failure of anti-laminin antibodies to block the activity of "neurite outgrowth-promoting factors" in CM's will be uncovered in the near future. This will be important not simply because it will resolve an issue involving "factors" that act on neurons in cell culture, but more because it will significantly affect the interpretability of certain experiments--such as antibody blocking experiments--that now need to be performed *in vivo*. These will be discussed in the following section.

C O N C L U S I O N S

Summary of Results

This report describes the detection, purification and characterization of a factor that promotes neurite outgrowth.

The existence of such a factor was suspected when rat sympathetic neurons cultured on BCE-ECM were observed to initiate rapid, extensive neurite outgrowth, even in the absence of NGF. Similar results were obtained with rat sympathetic neurons plated on polylysine-coated substrata that had been exposed to BCE-CM or $-CM_{SF}$. These observations were extended to other classes of neurons, and to substrata treated with CM's produced by a variety of different cell types. These results are described in Chapter 1.

Preliminary information about the biochemical nature of the "neurite outgrowth-promoting factor" in BCE-CM was then gathered. Evidence was obtained that both protein and the glycosaminoglycan heparan sulfate are involved in its activity. The hypothesis that the "neurite outgrowth-promoting factor" is either a heparan sulfate proteoglycan or a macromolecular complex containing a heparan sulfate proteoglycan was formulated. These results are described in Chapter 2.

Several ECM molecules were screened, to determine whether any could promote neurite outgrowth, and laminin, a basement membrane glycoprotein, was found to mimic closely the activity of the "neurite outgrowth-promoting factor" in BCE-CM. Although it seemed reasonable to propose that the "neurite outgrowth-promoting factor" involves laminin or a molecule similar to laminin, it was found that anti-laminin antibodies block only the neurite outgrowth-promoting activity of laminin-treated substrata, and not the neurite outgrowth-promoting

activity of substrata treated with BCE-CM or several other CM's. These results are described in Chapter 3.

In order to determine the composition of the "neurite outgrowth-promoting factor" in BCE-CM directly, a five step purification was devised. Analysis of the material purified in this way made use of metabolic labeling with [^{35}S]methionine and [^{35}S]sulfate, enzymatic digestion with heparitinase, immunochemical techniques such as solid phase immunoassays, immunoprecipitation and immunoblotting, velocity sedimentation, and SDS-PAGE performed under reducing and non-reducing conditions. This analysis indicated that the "neurite outgrowth-promoting factor" in BCE-CM consists of laminin that is associated with a large heparan sulfate proteoglycan, and with entactin, a sulfated ECM glycoprotein. Evidence was obtained that neurite outgrowth-promoting activity is mediated primarily by laminin, but that the proteoglycan is important in ensuring the attachment of the "neurite outgrowth-promoting factor" to polycationic substrata. These results are described in Chapter 4.

Some of the procedures used to isolate and analyze the "neurite outgrowth-promoting factor" in BCE-CM were then applied to five other CM's. Laminin was found to be a component of the "neurite outgrowth-promoting factor" in all of these CM's. Attempts were made to generate plausible hypotheses that explain why anti-laminin antibodies had not been able to block the activity of the "neurite outgrowth-promoting factors" in four of these five CM's. These results are described in Chapter 5.

The aim of the research summarized above was to identify and characterize a substance that dramatically affects the behavior of

neurons *in vitro*. The achievement of this goal has little meaning, however, unless it leads to a better understanding of events that occur *in vivo*. To this end, it is important to review relevant questions raised and hypotheses suggested by this work. In particular, three areas will be discussed: the biochemistry of laminin, the biology of neurite outgrowth, and the role of laminin in neuronal development, regeneration and disease. Directions for future research in these areas will be suggested.

The Biochemistry of Laminin

Much of what is known about the biochemistry of laminin comes from studies of laminin purified from the EHS sarcoma, a particularly abundant source (e.g. Timpl *et al.*, 1979; Engel *et al.*, 1981). Other tumor cells have also been used for biochemical and molecular biological studies of laminin (e.g. Chung *et al.*, 1979; Howe and Dietzschold, 1983; Engvall *et al.*, 1983; Carlin *et al.*, 1983; Barlow *et al.*, 1984). Less direct information is available, however, on the laminin produced by the classes of cells that normally elaborate it *in vivo* (e.g. Cooper *et al.*, 1981; Cooper and MacQueen, 1983; Alitalo *et al.*, 1980a). One of the questions raised by the study of "neurite outgrowth-promoting factors" reported here is whether isoforms of laminin exist. Laminin isoforms, it was argued, could explain immunochemical differences between EHS sarcoma-laminin and the "neurite outgrowth-promoting factors" in CM's. If such isoforms do exist, it will be important to determine whether they differ functionally, as is the case for isoforms of fibronectin (Yamada, 1983; Sekiguchi *et al.*, 1985). If so, it will be of great importance to determine when and

where different laminin isoforms are expressed *in vivo*.

The "neurite outgrowth-promoting factors" in CM's may prove valuable in answering these questions. For example, a monoclonal antibody, such as the one isolated by Matthew and Patterson (1983), that blocks the activity of a "neurite outgrowth-promoting factor" but fails even to bind EHS sarcoma-laminin, is a candidate for an isoform-specific antibody. It remains to be seen, however, whether this is the case for the antibody described by Matthew and Patterson, or whether, as some preliminary observations suggest (W.D. Matthew, personal communication), the antibody recognizes a determinant generated by the binding of a heparan sulfate proteoglycan to laminin.

Other potentially isoform-specific monoclonal antibodies have been described by Wan *et al.* (1984). In their study, a polyclonal anti-EHS sarcoma-laminin serum was used to define, immunohistochemically, structures that contain laminin during mouse development. Two monoclonal antibodies that recognized distinct laminin B-chain epitopes were then shown, at particular stages of development, to recognize only subsets of the laminin-containing structures. Unfortunately, these authors could not eliminate the possibility that the absence of certain epitopes in some structures *in vivo* was caused by the binding of other ECM molecules to laminin, rather than by the absence of particular laminin isoforms. It might prove worthwhile to screen these monoclonal antibodies against "neurite outgrowth-promoting factors", since differentiating between an epitope that is absent and an epitope that is blocked by a bound molecule might be accomplished more easily for the laminin in "neurite outgrowth-promoting factors" that for the laminin found *in vivo*.

Ultimately, the most direct way to search for laminin isoforms may be to use cDNA sequence information for the B1a, B1b and B2 chains of laminin (Barlow _et al._, 1984), and for other chains as this information becomes available, to predict immunogenic regions of amino acid sequence that distinguish among different chains, and generate chain-specific antibodies. It would then be possible to search directly for laminin molecules that differ in their representation of these chains. Again, "neurite outgrowth-promoting factors" would be a good starting point for screening. The most significant limitation of this technique is that it would not detect differences among laminin molecules conferred by any of the 38 or more carbohydrate chains that account for 10-15% of the laminin molecule (Howe and Dietzschold, 1983).

The existence of isoforms is not the only question about laminin structure and function raised by the study of "neurite outgrowth-promoting factors". For example, Edgar _et al._ (1984), in an attempt to define the domain(s) of laminin responsible for promoting neurite outgrowth, found that antibodies directed against fragment E3, a 50,000 Da heparin-binding region of laminin's long arm, blocked the neurite outgrowth-promoting activity of EHS sarcoma-laminin, and that neurite outgrowth-promoting activity could be recovered in a 280,000 Da fragment that contained E3. Unexpectedly, they found that an entirely unrelated large fragment (E1-4) also possessed neurite outgrowth-promoting activity, and that this activity, unlike that of intact laminin, was not blocked by anti-E3 antibodies (see Discussion, Chapter 3). It remains to be determined what part of fragment E1-4 mediates its neurite outgrowth-promoting activity, why this activity first appears after proteolysis of the laminin molecule, and whether mechanisms other

than proteolysis could "activate" this site. Since it has been suggested that "neurite outgrowth-promoting factors" in CM's might differ from EHS sarcoma-laminin by having this site already "activated", further study of "neurite outgrowth-promoting factors" may prove a useful way to approach these questions.

Another issue raised by the study of the "neurite outgrowth-promoting factor" in BCE-CM involves the affinity of laminin for heparan sulfate proteolgycans. It was shown in Chapter 4 that 0.5 M NaCl, which is sufficient to displace heparin or heparan sulfate from EHS sarcoma-laminin, is not sufficient to dissociate heparan sulfate proteoglycan from the laminin in BCE-CM. Further evaluation of this phenomenon might reveal whether it is due to unexplored binding sites on the laminin molecule, contributions by the proteoglycan core protein to binding, stabilizing influences of entactin, or other reasons (cf. Lark and Culp, 1984; Laurie _et al._, 1984). This information would likely aid the understanding of how basement membranes are assembled _in vivo_.

The Biology of Neurite Outgrowth

The ability to extend neurites is one of the many extraordinary features of neurons. Observations, especially those using time lapse cinematography and electron microscopy, have provided a great deal of descriptive information about neurite outgrowth (reviewed by Yamada _et al._, 1971; Johnston and Wessels, 1980; Bray, 1982), and even suggest that the activity of growth cones, the motile organelles at the ends of neurites, may be analogous to the "fibroblastic" motility exhibited by many cell types (Solomon, 1981). Nevertheless, little is known

about the cellular mechanisms responsible for the processes of recognition, adhesion, force generation, membrane insertion, and detachment which take part in the extension of a neurite.

It seems likely that further study of the effects of laminin, and the "neurite outgrowth-promoting factors" in CM's, on neurons will yield new insights into the mechanism of neurite outgrowth. It can be inferred that laminin can exert a local effect on growth cones, since growth cones can be observed actively choosing CM-treated or laminin-treated zones or stripes arranged on a polycationic substratum (Collins and Garret, 1980; Hammarback et al., 1985). These observations may indicate that laminin increases substratum adhesiveness, since growth cones have similarly been observed to choose among various artificial substrata on the basis of what appears to be differential adhesiveness (Letourneau, 1975a,b). It seems unlikely, however, that adhesiveness alone accounts for the neurite outgrowth-promoting activity of laminin, since many highly adhesive substrata, including substrata treated with antibodies that bind neurons, do not specifically promote neurite outgrowth (reviewed in Chapter 3). It may be that laminin participates in or facilitates some receptor mediated event at the neuronal cell surface. One possibility is that neurons possess a specific laminin receptor, which, when bound by laminin, stimulates the normal mechanisms of neurite outgrowth. If this is correct, it suggests that neurons are specialized to respond to laminin, and probably do so in vivo.

A second possibility is that the normal mechanisms of neurite outgrowth involve, as a rate-limiting step, the secretion by neurons of a substratum-binding molecule that then binds to the neuronal cell

surface, and that laminin is able to substitute for this molecule, and thereby accelerate neurite outgrowth. If this possibility is correct, it will remain to be seen whether the ability of laminin to substitute for this molecule is fortuitous, or of _in vivo_ significance.

A third possibility exists which is a combination of the first two: neurons may possess a laminin-specific receptor because neurite outgrowth normally involves the secretion and recognition of laminin by neurons. According to this model, the rate of laminin secretion by neurons would limit the rate of neurite outgrowth, explaining why outgrowth is so much more rapid and extensive on laminin-treated substrata.

The hypothesis that neurons require laminin to extend neurites _in vitro_, but can synthesize it themselves, is worth considering briefly. It is known that PC12 cells and neuroblastoma cells, both of which can extend neurites in culture, produce laminin (Chapter 5, and Alitalo _et al._, 1980b). It has also been observed that neurites will grow rapidly, and in an NGF-independent manner, along regions of a substratum on which neurites had previously been maintained, but then removed by lysis with distilled water (Campenot, 1981). Finally, if it is the case that axons encounter exogenous laminin _in vivo_ (a question that will be discussed below), then it might be expected that neurons placed into culture would not already be synthesizing laminin, and would require a significant period of time for the induction of laminin synthesis before neurite outgrowth could commence, unless the neurons were plated onto a substratum already containing laminin, in which case neurite outgrowth could begin much sooner. In fact, this describes very well the behavior of rat sympathetic neurons on different

substrata (cf. Chapter 1).

Although seemingly straightforward, it is difficult to determine whether neurons synthesize laminin. Since all of the non-neuronal cells that are present in primary neuronal cultures produce laminin--i.e., Schwann cells (Cornbrooks _et al._, 1983), astrocytes (Liesi _et al._, 1983) and fibroblasts (Couchman _et al._, 1983)--it would be necessary to obtain an extremely pure culture of neurons before it would be meaningful to look for laminin released in neuronal CM. More important is the fact that laminin is so effective at promoting neurite outgrowth that, if neuronal secretion of laminin is assumed to be limiting--this is the most that may be assumed if exogenous laminin is to have any effect on neurons--then the amount of laminin secreted by neurons would have to be extremely small. In particular, using the concentration of laminin that elicits half maximal neurite outgrowth-promoting activity (Chapter 3), assuming that all of it adheres to the culture substratum (an overestimate, especially since it ignores the walls of the culture well), and assuming a molecular weight of 900,000 Da for laminin, it is possible to calculate that neurite outgrowth-promoting activity requires at most 100 laminin molecule for every 1-2 μ^2 of substratum[3]. Assuming an idealized growth cone of 10 μ x 10 μ, it would be possible for a neuron to promote the extention of a 100 μ long neurite with the directed secretion of $\leq 10^5$ laminin molecules or less. The point of this demonstration is not to support the

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[3] (50-100 ng/ml) x (50 μl per culture well) ÷ (900,000 g/mole) x (6.02×10^{23} molecules/mole) ÷ (0.32 cm^2/culture well) = 50 - 100 molecules per μ^2.

notion that neurons produce laminin. That hypothesis is just one of several, and the evidence supporting it is, at best, suggestive. However, these calculations clearly indicate that this hypothesis cannot be ruled out unless very sensitive techniques are used. So far, immunocytochemistry of monensin-treated neurons with anti-laminin antibodies (cf. Liesi _et al._, 1983) has failed to demonstrate the presence of laminin in them (J. Winter, unpublished observations), but the sensitivity of this technique is not known. Perhaps _in situ_ hybridization with laminin cDNA's will help answer this question. This technique could take advantage to the fact that a neuron that is extending multiple neurites would still have all of its laminin mRNA restricted to a single location, the soma. _In situ_ hybridization can also be used to determine whether neuronal synthesis of laminin occurs _in vivo_, a question of more direct relevance.

Regardless of whether neurons make their own laminin, make a molecule which laminin substitutes for, or are simply specialized to respond to exogenous laminin, a great deal about neurite outgrowth can probably be learned by studying how laminin promotes it. As a first step, it will be important to determine whether a neuronal receptor for laminin exists. It is known that certain non-neuronal cells possess a 68,000 Da high-affinity laminin receptor (Terranova _et al._, 1983; Malinoff and Wicha, 1983; Lesot _et al._, 1983; Rao _et al._, 1983; Sugrue 1984.). However, several pieces of information indicate that this receptor is not involved in mediating the neurite outgrowth-promoting effects of laminin. The most persuasive argument comes from the results of Edgar _et al._ (1984) which indicate that the neurite outgrowth-promoting activity of laminin requires a 50,000 Da region on

laminin's long arm, and can be mimicked by a 280,000 Da fragment derived from the long arm. In contrast, the laminin receptor that has been found on non-neuronal cells binds to domains on the short arms of laminin (Terranova et al., 1983).

Numerous approaches exist for identifying a cell surface receptor, and they will not be reviewed here. However, the search for a neuronal laminin receptor poses certain unusual potential problems. First, neurites do not simply adhere to laminin, they migrate across it; such a transient interaction might be mediated by a low-affinity receptor, the existence of which could be difficult to demonstrate. Second, none of the laminin fragments generated by Edgar et al. (1984) promoted neurite outgrowth as effectively as undigested laminin, raising the possibility that more than one site on the laminin molecule is required for its neurite outgrowth-promoting activity. Thus, there may be two laminin receptors on neurons. Third, the region of laminin proposed by Edgar et al. (1984) to mediate neurite outgrowth promoting activity (i.e. fragment E3) is a region that contains the major heparin-binding domain of laminin, and the fragment of laminin reported by Edgar et al. to possess a "latent" neurite outgrowth-promoting domain (i.e. fragment E1-4) also possesses a heparin-binding domain that could be termed "latent" (since the heparin-binding of undigested laminin can apparently be accounted for by E3 alone)(Ott et al., 1982). These considerations suggest that the neuronal laminin receptor may be a cell surface heparan sulfate proteoglycan. If so, the interaction of laminin with neurons must involve more than simply binding this proteoglycan, since other molecules that bind heparan sulfate (polylysine, fibronectin, platelet factor 4, specific monoclonal antibodies) do

not promote neurite outgrowth (Chapter 3), and even fragment E3, which retains heparin-binding activity (Ott et al., 1982) fails to promote neurite outgrowth (Edgar et al., 1984).

Despite these potential difficulties, some encouraging progress has been made. It has been found that, under appropriate conditions, PC12 cells will extend neurites in response to laminin-treated substrata, a response that may be analogous to that of primary neurons (Tomaselli and Reichardt, 1984). PC12 cells could serve as a source from which a neuronal laminin receptor could be purified. Mutants could also be obtained that fail to respond to laminin, allowing the actions of laminin to be analyzed by genetic means.

Recently, monoclonal antibodies have been described that disrupt the cell-substratum adhesion of several types of non-neuronal cells (Neff et al., 1982; Chapman, 1984). Some exciting new evidence suggests that a complex of cell surface glycoproteins recognized by these antibodies (Chen et al., 1985) may also be present on neurons, and involved in the growth of neurites on laminin-treated substrata (Bozyczko et al., 1984; K. Tomaselli, unpublished observations). It remains to be seen whether these molecules are specifically involved in the response of neurons to laminin, or participate in neurite outgrowth in some other way.

Perhaps some insight into the mechanism of action of laminin on neurons can be gained by considering the fact that laminin not only promotes neurite outgrowth, but also appears, at least for some neurons, to decrease neuronal requirements for the trophic factor NGF (Edgar and Thoenen, 1982; Edgar et al., 1984; Discussed in Chapter 3). Importantly, this activity of laminin seems to involve the same domains that

are responsible for promoting neurite outgrowth (Edgar *et al.*, 1984). One possible explanation of this phenomenon is that the binding of neurons to a laminin-treated substratum increases the affinity or number of NGF receptors they express. This could occur because laminin has a direct action on NGF receptors, because laminin induces the synthesis of NGF receptors, or merely because laminin, by promoting neurite outgrowth, induces an increase in neuronal surface area, which indirectly leads to the insertion of a greater number of NGF receptors per neuron. A second possibility is that laminin does not alter the number or affinity of NGF receptors, but instead elicits an intracellular response that, in some way, lowers the cell's requirement for NGF. These hypotheses may be tested by measuring the affinity and number of NGF receptors directly, or by comparing the intracellular actions of laminin (e.g. effects on cyclic nucleotides, phosphorylation, etc.) with those of NGF. The first hypothesis may also be distinguished from the second by the fact that it predicts that laminin should not only alter the NGF dose-response curve for neuronal survival, but it should shift it to an equal degree for all other actions of NGF. In particular, the induction of ornithine decarboxylase could be looked at, since it is one of the earliest effects of NGF (e.g. MacDonnell *et al.*, 1977). Furthermore, since the effects of laminin on NGF requirements can be demonstrated using PC12 cells (Tomaselli and Reichardt, 1984), it would be convenient to carry out such experiments with these cells, since they induce a large amount of ornithine decarboxylase in response to NGF (Greene and McGuire, 1978).

Role of Laminin in Neuronal Development, Regeneration and Disease

Observations on the effects of laminin and "neurite outgrowth-promoting factors" *in vitro* suggest that laminin could play a role in the growth or guidance of axons *in vivo*. While such a role remains to be demonstrated, certain observations are encouraging. Laminin is abundant in the endoneurial sheaths of peripheral nerves (Palm and Furcht, 1983; Cornbrooks *et al.*, 1983), and during nerve regeneration, axonal profiles can be found in contact with these laminin-containing structures (Bignami *et al.*, 1984). During the initial outgrowth of axons from the chick spinal cord, laminin appears to be present along the ventral root pathways that these axons follow (Rogers *et al.*, 1984). Laminin is also present in muscle basal laminae, over which motor axons arborize, and although its distribution includes the sites of nerve-muscle synapses, it is not restricted to these sites (Sanes, 1982). Thus, a strong case can be made that laminin is appropriately localized to influence the growth of peripheral nerves and nerve terminals.

In contrast, little evidence exists to support a role for laminin in the central nervous system. Even though laminin is produced by astrocytes *in vitro*, immunocytochemical studies have failed to detect laminin in the brain, except in association with blood vessel walls (K. Valentino, unpublished observations). If laminin is indeed absent from the central nervous system, its absence may have something to do with the poor regenerative efforts that most central axons exhibit. It is probably too early, however, to rule out a role for laminin in the central nervous system, since, as has already been pointed out, the quantity of laminin required to promote outgrowth is very small, and could be missed by immunochemical techniques.

It is likely that direct experiments designed to determine whether laminin plays a role in axonal growth or guidance *in vivo* will be attempted in the future. The most straightforward of these will involve the introduction of blocking antibodies into animals. Antibodies could be used systemically, as they were in the experiments that first supported a role for NGF in development (Levi-Montalcini and Booker, 1960), or applied locally to structures in which axons grow (e.g. developing iris, regenerating peripheral nerve). Although it might seem simplest to use antibodies directed against EHS sarcoma-laminin in such studies, experience with the "neurite outgrowth-promoting factors" in CM's suggests that these experiments be interpreted with caution. In particular, if the laminin *in vivo* resembles the laminin in "neurite outgrowth-promoting factors" (e.g. because of isoform differences, or as a result of bound molecules), it probably will not be possible to block its neurite outgrowth-promoting activity with anti-EHS sarcoma-laminin antibodies. Instead, antibodies directed against one of the "neurite outgrowth-promoting factors" would be required. As evidence that this may indeed be the case, the outgrowth of neurites *in vitro* on thin slices of regenerating peripheral nerve has been found to be blocked by a monoclonal antibody specific for the "neurite outgrowth promoting factor" in PC12-CM (Matthew and Patterson, 1983, and W.D. Matthew, personal communication).

As the mechanism of laminin's neurite outgrowth-promoting activity becomes better known, it may be possible to perform additional studies using antibodies directed against neuronal laminin receptors. Ultimately, such experiments may be cleaner than experiments involving the use of antibodies recognizing laminin, since the latter anti-

bodies might interfere with the actions of laminin in the development of non-neuronal tissues as well.

In addition to investigating the potential role of laminin in normal neuronal development and regeneration, it is also worth considering whether laminin plays a role in the genesis of abnormal neural structures, such as may be associated with injury or disease. For example, the regeneration of injured peripheral nerves is often hampered by the formation of a neuroma, a tangled mass of hopelessly misdirected nerve fibers, that, if it contains certain sensory fibers, may become exceedingly painful (e.g. Devor and Wall, 1976). If laminin is involved in stimulating normal regeneration, it may also play a role in the development of neuromas. Similarly, in certain disease states such as neurofibromatosis, laminin may be involved in the genesis of cutaneous and nerve-associated tumors that also contain masses of abnormally regenerating nerve fibers (Schoene, 1979). If laminin can be shown to play a role in the formation of either of these structures, it may be possible to use this information toward the development of specific therapies.

It may also be possible to use laminin itself in a clinical situation: The cut ends of a severed peripheral nerve often become separated by a gap too large to regenerate across. At present, the only techniques for reestablishing continuity involve nerve grafting, using either allografts--which suffer from the problem of severe immunological rejection--or autografts--which are not always available and often poorly viable (Sunderland, 1978). In future, however, it may become possible to bridge such a gap with a laminin-impregnated matrix. It may even be possible to avoid immunological destruction of such a

matrix by impregnating it not with EHS sarcoma-laminin, but with a "neurite outgrowth-promoting factor" easily derived from cultures of cells from the injured patient.

B I B L I O G R A P H Y

Adler, R., Manthorpe, M., Skaper, S. and Varon, S. (1981) Polyornithine-attached neurite-promoting factors (PNPFs). Culture sources and responsive neurons. Brain Res. 206, 129-144.

Adler, R. and Varon, S. (1980) Cholinergic neuronotrophic factors V. Segregation of survival- and neurite-promoting activities in heart-conditioned medium. Brain Res. 188, 437-448.

Akers, R.M., Mosher, D.F. and Lilien, J.E. (1981) Promotion of retinal neurite outgrowth by substratum-bound fibronectin. Dev. Biol. 86, 179-187.

Alitalo, K., Kurkinen, M., Vaheri, A., Krieg, T. and Timpl, R. (1980a) Extracellular matrix components synthesized by human amniotic epithelial cells in culture. Cell 19, 1053-1062.

Alitalo, K., Kurkinen, M., Vaheri, A., Virtanen, I., Rohde, H. & Timpl, R. (1980b) Basal lamina glycoproteins are produced by neuroblastoma cells. Nature 287, 465-466.

Amano, T., Richelson, E. and Nirenberg, M. (1972) Neurotransmitter synthesis by neuroblastoma clones. Proc. Natl. Acad. Sci. U.S.A. 69, 258-263.

Bächinger, H.P., Fessler, L.I. and Fessler, J.H. (1982) Mouse procollagen IV. Characterization and supramolecular association. J. Biol. Chem. 257, 9796-9803.

Barlow, D.P., Green, N.M., Kurkinen, M. and Hogan, B.L.M. (1984) Sequencing of laminin B chain cDNAs reveals C-terminal regions of coiled-coil alpha-helix. EMBO J. 3, 2355-2362.

Baron-Van Evercooren, A., Kleinman, H.K., Ohno, S., Marangos, P., Schwartz, J.P. and Dubois-Dalcq, M.E. (1982) Nerve growth factor, laminin and fibronectin promote nerve growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8, 179-183.

Batteiger, B., Newhall W.J.,V, and Jones, R.B.(1982) The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. J. Immunol. Methods 55, 297-307.

Bentley, D. and Caudy, M. (1983) Navigational substrates for peripheral pioneer growth cones: limb-axis polarity cues, limb-segment boundaries, and guidepost neurons. Cold Spring Harbor Symp. Quant. Biol. 48, 573-587.

Berg, D.K. (1984) New neuronal growth factors. Ann. Rev. Neurosci 7, 149-170.

Bignami, A., Chi, N.H. and Dahl, D. (1984) Laminin in rat sciatic nerve undergoing Wallerian degeneration. Immunofluorescence study with laminin and neurofilament antisera. J. Neuropathol. Expl. Neurol. 43, 94-103.

Bitter, T. and Muir, H.M. (1962) A modified uronic acid carbazole reaction. Anal. Biochem. 4, 330-334.

Bonner, W.M. and Laskey, R.A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46, 83-88.

Boss, B. (1984) An improved in vitro immunization procedure for the production of monoclonal antibodies against neural and other antigens. Brain Res. 291, 193-196.

Bottenstein, J.E. and Sato, G.H. (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. U.S.A. 76, 514-517.

Boucaut, J.-C., Darribere, T., Poole, T.J., Aoyama, H., Yamada, K., and Thierry, J.P. (1984) Biologically active synthetic peptides as probes of embryonic development: a competitive peptide inhibitor of fibronectin function inhibits gastrulation in amphibian embryos and neural crest migration in avian embryos. J. Cell Biol. 99, 1822-1830.

Bozyczko, D., Decker, C and Horwitz, A. (1984) A membrane antigen involved in neuronal-substratum adhesion. J. Cell Biol. 99, 117a.

Brandford-White, C.J. (1980) Sulphated glycoproteins in synaptosomes. Neurosci. Lett. 16, 307-311.

Bray, D. (1982) Filopodial contraction and growth cone guidance. In: Cell Behavior, R. Bellairs, A. Curtis and G. Dunn, editors. Cambridge University Press, Cambridge. 299-317.

Brown, S.S., Malinoff, H.L. and Wicha, M.S. (1983) Connectin: cell surface protein that binds both laminin and actin. Proc. Natl. Acad. Sci. U.S.A. 80, 5927-5930.

Calof, A.L. and Reichardt, L.F. (1984) Motoneurons purified by cell sorting respond to two distinct activities in myotube conditioned medium. Dev. Biol. 106: 194-210.

Calof, A.L. and Reichardt, L.F. (1985) Response of purified chick motoneurons to myotube conditioned medium: laminin is essential for the substratum-binding neurite outgrowth-promoting activity. Neurosci. Lett., In press.

Campenot, R.B. (1981) Regeneration of neurites in long-term cultures of sympathetic neurons deprived of nerve growth factor. Science 214, 579-581.

Cantor, C.R. and Schimmel, P.R. (1980) Biophysical chemistry. Part II: techniques for the study of biological structure and function. W.H. Freeman and Co., San Francisco, 612-616.

Carbonetto, S., Gruver, M.M. and Turner, D.C. (1983) Nerve fiber growth in culture on fibronectin, collagen and glycosaminoglycan substrates. J. Neurosci. 3, 2324-2335.

Carlin, B., Jaffe, R., Bender, B. and Chung, A.E. (1981) Entactin, a novel basal lamina-associated sulfated glycoprotein. J. Biol. Chem. 256, 5209-5214.

Carlin, B.E., Durkin, M.E., Bender, B., Jaffe, R. and Chung, A.E. (1983) Synthesis of laminin and entactin by F9 cells induced with retinoic acid and dibutyryl cyclic AMP. J. Biol. Chem. 258, 7729-7737.

Castellot, J.J.Jr., Addonizio, M.L., Rosenberg, R. and Karnovsky, M.J. (1981) Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. J. Cell Biol. 90, 372-379.

Chapman, A.E. (1984) Characterization of a 140 kD cell surface glycoprotein involved in myoblast adhesion. J. Cell. Biochem. 25, 109-121.

Chen, W.-T., Hasegawa, E., Hasegawa, T., Weinstock, C. and Yamada, K.M. (1985) Development of cell surface linkage complexes in cultured fibroblasts. J. Cell Biol. 100, 1103-1114.

Chiarugi, V.P. and Vannucchi, S. (1976) Surface heparan sulphate as a control element in eukaryotic cells: a working model. J. Theor. Biol. 61, 459-475.

Chun, L.Y. and Patterson, P.H. (1977) Role of nerve growth factor in the development of rat sympathetic neurons in vitro. I. Survival, growth, and differentiation of catecholamine production. J. Cell Biol. 75, 694-704.

Chung, A.E., Jaffe, R., Freeman, I.L., Vergnes J.-P., Braginski, J.E. and Carlin, B. (1979) Properties of a basement membrane-related glycoprotein synthesized in culture by a mouse embryonal carcinoma-derived cell line. Cell 16, 277-287.

Clarke, S. (1975) The size and detergent binding of membrane proteins. J. Biol. Chem. 250, 5459-5469.

Cohn, R.H., Cassiman, J.-J. and Bernfield, M.H. (1976) Relationship of transformation, cell density and growth control to the cellular distribution of newly synthesized glycoaminoglycan. J. Cell Biol. 71, 280-294.

Cole, G.J. and Glaser, L. (1984) Cell-substratum adhesion in embryonic chick central nervous system is mediated by a 170,000-mol-wt neural-specific polypeptide. J. Cell Biol. 99, 1605-1612.

Cole, G.J., Schubert, D. and Glaser, L. (1985) Cell-substratum adhesion in chick neural retina depends upon protein-heparan sulfate interactions. J. Cell Biol. 100, 1192-1199.

Collins, F. (1978a) Axon initiation by ciliary neurons in culture. Dev. Biol. 65, 50-57.

Collins, F. (1978b) Induction of neurite outgrowth by a conditioned-medium factor bound to the culture substratum. Proc. Natl. Acad. Sci. USA 75, 5210-5213.

Collins, F. (1980) Neurite outgrowth induced by the substrate associated material from nonneuronal cells. Dev. Biol. 79 247-252.

Collins, F. and Garret, J.E. Jr. (1980) Elongating nerve fibers are guided by a pathway of material released from embryonic nonneuronal cells. Proc. Natl. Acad. Sci. U.S.A. 77, 6226-6228.

Collins, F. and Lee, H.R. (1984) The spatial control of ganglionic neurite growth by the substrate associated material from conditioned medium: an experimental model of haptotaxis. J. Neurosci. 4: 2823-2829.

Cooper, A.R., Kurkinen, M., Taylor, A. and Hogan, B.L.M. (1981) Studies of the biosynthesis of laminin by murine parietal endoderm cells. Eur. J. Biochem. 119, 189-197.

Cooper, A.R. and MacQueen, H.A. (1983) Subunits of laminin are differentially synthesized in mouse eggs and early embryos. Dev. Biol. 96, 467-471.

Cornbrooks, C.J., Carey D.J., McDonald, J.A., Timpl, R. and Bunge, R.P. (1983) In vivo and in vitro observations on laminin production by Schwann cells. Proc. Natl. Acad. Sci. USA 80, 3850-3854.

Couchman, J.R., Höök, M., Rees, D.A. and Timpl, R. (1983) Adhesion, growth and matrix production by fibroblasts on laminin substrates. J. Cell Biol. 96, 177-183.

Coughlin, M.D., Bloom, E.M. and Black, I.B. (1981) Characterization of a neuronal growth factor from mouse heart cell-conditioned medium. Dev. Biol. 82, 56-68.

Coughlin, M.D. and Kessler, J.A. (1982) Antiserum to a new neuronal growth factor: Effects on neurite outgrowth. J. Neurosci. Res. 8, 298-302.

Craven, G.R., E. Steers and C.B. Anfinsen (1965) Purification, composition and molecular weight of the β-galactosidase of Escherichia coli. J. Biol. Chem. 240, 2468-2477.

Culp, L.A. (1978) Biochemical determinants of cell adhesion. Curr. Top. Membr. Transp. 11, 327-396.

Culp, L.A., Ansbacher, R. and Domen, C. (1980) Adhesion sites of neural tumor cells: biochemical composition. Biochemistry, 19, 5899-5907.

Culp, L.A., Rollins, B.J., Buniel, J. and Hitri, S. (1978) Two functionally distinct pools of glycosaminoglycan in the substrate adhesion site of murine cells. J. Cell Biol. 79, 788-801.

Davis, G.E., Manthorpe, M. and Varon, S. (1984a) Parameters of neuritic growth for ciliary ganglion neurons. Transactions of the Am. Soc. Neurochem. 15, 176.

Davis, G.E., Manthorpe, M. and Varon, S. (1984b) Purification of rat Schwannoma neurite-promoting factor. Soc. Neurosci. Abstr. 10, 38.

Davis, G.E., Manthorpe, M., Engvall, E. and Varon, S. (1985) J. Neurosci., in press.

Del Rosso, M., Cappelletti, R., Viti, M., Vannucchi, S. and Chiarugi, V. (1981) Binding of the basement-membrane glycoprotein laminin to glycosaminoglycans. Biochem. J. 199, 699-704.

Devor, M. and Wall P.D. (1976) Type of sensory nerve fibre sprouting to form a neuroma. Nature 262, 705-708.

Edgar, D. and Thoenen, H. (1982) Modulation of NGF-induced survival of chick sympathetic neurons by contact with a conditioned medium factor bound to the culture substrate. Dev. Brain Res. 5, 89-92.

Edgar, D., Timpl, R. and Thoenen, H. (1984) The heparin-binding domain of laminin is responsible for its effects on neurite outgrowth and neuronal survival. EMBO J. 3: 1463-1468.

Engel, J., Odermatt, E., Engel, A., Madri, J.A., Furthmayr, H., Rohde, H. and Timpl, R.(1981) Shapes, domain organizations and flexibility of laminin and fibronectin, two multifunctional proteins of the extracellular matrix. J. Mol. Biol. 150, 97-120.

Engvall, E., Krusius, T., Wewer, U. & Ruoslahti, E. (1983) Laminin from rat yolk sac tumor: Isolation, partial characterization and comparison with mouse laminin. Arch. Biochem. Biophys. 222, 649-656.

Fazekas de St. Groth, S. and Scheidegger, D. (1980) Production of monoclonal antibodies: strategies and tactics. J. Immunol. Methods 35, 1-21.

Fields, K.L., Brockes, J.P., Mirsky, R. and Wendon, L.M.B. (1978) Cell surface markers for distinguishing different types of rat dorsal root ganglion cells in culture. Cell. 14, 43-51.

Fraser, S.E. and Hunt, R.K. (1980) Retinotectal specificity: models and experiments in search of a mapping function. Ann. Rev. Neurosci. 3, 319-352.

Fritze, L.M.S., Reilly, C.F. and Rosenberg, R.D. (1985) An antiproliferative heparan sulfate species produced by postconfluent smooth muscle cells. J. Cell Biol. 100, 1041-1049.

Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., and Parks, W.P. (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer Inst. 51, 1417-1423.

Gospodarowicz, D., Bialecki, H. and Greenberg, G. (1978) Purification of the fibroblast growth factor activity from bovine brain. J. Biol. Chem. 253, 3736-3744.

Gospodarowicz, D., Delgado, D. and Vlodavsky, I. (1980) Control of cell proliferation in vitro by the extracellular matrix. Proc. Natl. Acad. Sci. U.S.A. 77, 4094-4098.

Gospodarowicz, D. and Greenburg, G. (1978) The coating of bovine and rabbit corneas denuded of their endothelium with bovine corneal endothelial cells. Exp. Eye Res 28, 249-265.

Gospodarowicz, D., Greenburg, G., Foidart, J.M. and Savion, N. (1981a) The production and localization of laminin in cultured vascular and corneal endothelial cells. J. Cell Physiol. 107, 171-183.

Gospodarowicz, D., Hirabayashi, K., Giguere, L., and Tauber, J.-P. (1981b) Factors controling the proliferative rate, final cell density, and life span of bovine vascular smooth muscle cells in culture. J. Cell Biol. 89, 568-578.

Gospodarowicz, D. and Ill, C. (1980) Extracellular matrix and control of proliferation of vascular endothelial cells. J. Clin. Invest. 65, 1351-1364.

Gospodarowicz, D., Ill, C., Hornsby, P.J., and Gill, G.N. (1977a) Control of bovine adrenal cortical cell proliferation by fibroblast growth factor. Lack of effect of epidermal growth fator. Endocrinol. 100, 1080-1089.

Gospodarowicz, D., Mescher, A.L. and Birdwell, C.R. (1977b) Stimulation of corneal endothelial cell proliferation in vitro by fibroblast and epidermal growth factors. Exp. Eye Res. 25, 75-87.

Gospodarowicz, D., Moran, J., and Braun, D. (1977c) Control of proliferation of bovine vascular endothelial cells. J. Cell Physiol. 91, 377-385.

Gospodarowicz, D., Moran, J., Braun, D., and Birdwell, C.R. (1976) Clonal growth of bovine endothelial cells in tissue culture: fibroblast growth factor as a survival agent. Proc. Natl. Acad. Sci. U.S.A. 73, 4120-4124.

Greene, L.A. and McGuire, J.C. (1978) Induction of ornithine decarboxylase by nerve growth factor dissociated from effects on survival and neurite outgrowth. Nature 276, 191-194.

Hall, H.G., Farson, D.A., Chin, S. and Bissell, M.J. (1982) Extracellular matrix and morphogenesis: collagen overlay induces lumen formation by epithelial cell lines. In: Extracellular Matrix, S. Hawkes and J.L. Wang, editors. Academic Press, San Francisco. pp. 233-238.

Hammarback, J.A., Palm, S.L., Furcht, L.T. and Letourneau, P.C. (1985) Guidance of neurite outgrowth by pathways of substratum-adsorbed laminin. J. Neurosci. Res. 13, 231-220.

Harrington, W.F. and Kegeles, G. (1973) Pressure effects in ultracentrifugation of interacting systems. Methods Enzymol. 27, 307-345.

Harrison, R.G. (1907) Observations on the living developing nerve fiber. Anat. Rec. 1, 116-118.

Hassel, J.R., Ledbetter, S., Leyshon, W., Tyree, B., Laurie, G.W. and Kleinman, H.K. (1984) Basement membrane proteoglycans. J. Cellular Biochem, Supplement 8B, 273.

Hassel, J.R., Robey, P.G., Barrach, H.-J., Wilczek, J., Rennard, S.I., and Martin, G.R. (1980) Isolation of a heparan sulfate-containing proteoglycan from basement membrane. Proc. Natl. Acad. Sci. USA 77, 4494-4498.

Hawrot, E. (1980) Cultured sympathetic neurons: effects of cell-derived and synthetic substrata on survival and development. Dev. Biol. 74, 136-151.

Hawrot, E. and Patterson, P.H. (1979) Long-term culture of dissociated sympathetic neurons. Methods Enzymol. 58, 574-584.

Hay, E. (1981a) Extracellular Matrix. J. Cell Biol. 91, 205s-223s

Hay, E. (1981b) Collagen and embryonic development. In: Cell Biology of the extracellular matrix. E. Hay, Editor. Plenum Press, New York. 379-410.

Hedman, K., Johansson, S., Vartio, T., Kjellen, L., Vaheri, A. and Höök, M. (1982) Structure of the pericellular matrix: association of heparan and chondroitin sulfates with fibronectin-procollagen fibers. Cell 28, 663-671.

Helfand, S.L., Riopelle, R.J. and Wessells, N.K. (1978) Non-equivalence of conditioned medium and nerve growth factor for sympathetic, parasympathetic, and sensory neurons. Exp. Cell Res. 113, 39-45.

Hogan, B.L.M., Taylor, A., Kurkinen, M. and Couchman, J.R. (1982) Synthesis and localization of two sulphated glycoproteins associated with basement membranes and the extracellular matrix. J. Cell Biol. 95, 197-204.

Höök, M., Kjellén, L., Johansson, S. and Robinson, J. (1984) Cell-Surface Glycosaminoglycans. Ann. Rev. Biochem. 53, 847-869.

Howe, C.C. and Dietzschold, B. (1983) Structrual analysis of three sub-units of laminin from teratocarcinoma-derived parietal endoderm cells. Dev. Biol. 98, 383-391.

Hudson, L. and Hay, F.C. (1980) Practical Immunology, 2nd Ed. Blackwell Scientific Publications, Oxford.

Hurst, R.E., Parmley, R.T., Nakamura, S., West, S. and Denys, F.R. (1981) Heparan sulfate of AH-130 ascites hepatoma cells: a cell-surface glycosaminoglycan not displaced by heparin. J. Histochem. Cytochem. 29, 731-737.

Hynes, R.O. (1981) Fibronectin and its relation to cellular structure and behavior. In Cell Biology of the Extracellular Matrix E. Hay, Editor. Plenum Press, New York. 295-333.

Jacobson, M. (1978) Developmental neurobiology Plenum Press, New York, passim.

Johansson, S. and Höök, M (1984) Substrate adhesion of rat hepatocytes: on the mechanism of attachment to fibronectin. J. Cell Biol. 98, 810-817.

Johnston, R.N. and Wessels, N.K. (1980) Regulation of the elongating nerve fiber. Curr. Top. Dev. Biol. 16, 165-206.

Kanwar, Y.S. and Farquahar, M.G. (1979) Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. Proc. Nat. Acad. Sci. USA 76 4493-4497.

Katz, M.J. and Lasek, R.J. (1979) Substrate pathways which guide growing axons in Xenopus embryos. J. Comp. Neurol. 183, 817-832.

Katz, M.J. and Lasek, R.J. (1981) Substrate pathways demonstrated by transplanted Mauthner axons. J. Comp. Neurol. 195, 627-641.

Kjellén, L., Oldberg, A. and Höök, M. (1980) Cell surface heparan sulfate. Mechanisms of proteoglycan-cell association. J. Biol. Chem. 255, 10407-10413.

Kjellén, L., Petersson, I. and Höök, M. (1981) Cell-surface heparan sulfate: an intercalated membrane proteoglycan. Proc. Natl. Acad. Sci. U.S.A. 78, 5371-5375.

Kleinman, H.K., McGarvey, M.L., Liotta, L.A., Robey, P.G., Tryggvason, K. and Martin, G.R. (1982) Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteglycan from the EHS sarcoma. Biochemistry 21, 6188-6193.

Kosher, R.A. and Searls, R.L. (1973) Sulfated mucopolysaccharide synthesis during the development of Rana pipiens. Dev. Biol. 32, 50-68.

Kraemer, P.M. and Tobey, R.A. (1972) Cell cycle dependent desquamation of heparan sulfate from the cell surface. J. Cell Biol. 55, 713-717.

Kühl, U., Timpl, R. and von der Mark, K. (1982) Synthesis of type IV collagen and laminin in cultures of skeletal muscle cells and their assembly on the surface of myotubes. Dev. Biol. 93, 344-354.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Lance-Jones, C. and Landmesser, L. (1980) Motoneurone projection patterns in the chick hind limb following early partial reversals of the spinal cord. J. Physiol. 302, 581-602.

Lander, A.D., Fujii, D.K., Gospodarowicz, D. and Reichardt, L.F. (1982) Characterization of a factor that promotes neurite outgrowth: evidence linking activity to a heparan sulfate proteoglycan. J. Cell Biol. 94, 574-585.

Lander, A.D., Fujii, D.K. and Reichardt, L.F. (1985a) Laminin is associated with the "neurite outgrowth-promoting factors" found in conditioned medium. Proc. Natl. Acad Sci. USA. 82, 2183-2187.

Lander, A.D., Fujii, D.K. and Reichardt, L.F. (1985b) Purification of a factor that promotes neurite outgrowth: isolation of laminin and associated molecules. J. Cell Biol. 101, In press.

Lander, A.D., Tomaselli, K., Calof, A.L. and Reichardt, L.F. (1983) Studies on extracellular matrix components that promote neurite outgrowth. C.S.H. Symp. Quant. Biol. 48, 611-623.

Lark, M.W. and Culp, L.A. (1983) Modification of proteoglycans during the maturation of fibroblast substratum adhesion sites. Biochemistry 22, 2289-2296.

Lark, M.W. and Culp, L.A. (1983) Multiple classes of heparan sulfate proteoglycans from fibroblast substratum adhesion sites. Affinity fractionation on columns of platelet factor 4, plasma fibronectin and octyl-Sepharose. J. Biol. Chem. 259, 6773-6782.

Laskey, R.A. and Mills, A.D. (1975) Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur. J. Biochem. 56, 335-341.

Laterra, J., Gilbert, J.E. and Culp, L.A. (1983) Cell surface heparan sulfate mediates some adhesive responses to glycosaminoglycan-binding matrices, including fibronectin. J. Cell Biol. 96, 112-123.

Laurie, G.W., Hassell, J.R., Kleinman, H.K. and Martin, G.R. (1984) Ultrastructure and interactions of heparan sulfate proteoglycan from basement membrane. J. Cell Biol 99, 78a.

Lesot, H., Kühl, U. and von der Mark, K. (1983) Isolation of a laminin-binding protein from muscle cell membranes. EMBO J. 2, 861-865.

Letourneau, P.C. (1975a) Possible roles for cell-to-substratum adhesion in neuronal morphogenesis. Dev. Biol. 44, 77-91.

Letourneau, P.C. (1975b) Cell to substratum adhesion and guidance of axonal elongation. Dev. Biol. 44, 92-101.

Levi-Montalcini, R. and Angeletti, P.U. (1963) Essential role of the nerve growth factor in the survival and maintenance of dissociated sensory and sympathetic nerve cells in vitro. Dev. Biol 7, 653-659.

Levi-Montalcini, R. and Booker, B. (1960) Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. Proc. Natl. Acad. Sci. U.S.A. 46, 384-391.

Levine, S.P. and Wohl, H. (1976) Human platelet factor 4: purification and characterization by affinity chromatography. J. Biol. Chem. 251, 324-328.

Liesi, P., Dahl, D. and Vaheri, A. (1983) Laminin is produced by early rat astrocytes in primary culture. J. Cell Biol. 96, 920-924.

Lindahl, U. and Höök, M (1978) Glycosaminoglycans and their binding to biological macromolecules. Ann. Rev. Biochem. 47, 385-417.

Lindahl, U., Höök, M., Backström, G., Jacobsson, I., Risenfield, J., Malmstrom, A. Roden, L. and Feingold, D.S. (1977) Structure and biosynthesis of heparin-like polysaccharides. Fed. Proc. 36, 19-23.

Linker, A. and Hovingh, P. (1972) Heparinase and heparitinase from flavobacteria. Methods Enzymol. 28, 902-910.

Linker, A. and Hovingh, P. (1977) The uses of degradative enzymes as tools for identification and structural analysis of glycosamino-glycans. Fed. Proc. 36, 43-46.

Luben, R.A. and Mohler, M.A. (1980) In vitro immunization as an adjunct to the production of hybridomas producing antibodies against the lymphokine osteoclast activating factor. Mol. Immunol. 17, 635-639.

MacDonnell, P.C., Nagaiah, K., Lakshmanan, J. and Guroff, G. (1977) Nerve growth factor increases activity of ornithine decarboxylase in superior cervical ganglia of young rats. Proc. Natl. Acad. Sci. U.S.A. 74, 4681-4684.

Malinoff, H.L. and Wicha, M.S. (1983) Isolation of a cell surface receptor protein for laminin from murine fibrosarcoma cells. J. Cell Biol. 96, 1475-1479.

Manthorpe, M., Engvall, E., Ruoslahti, E., Longo, F.M., Davis, G.E. and Varon, S. (1983) Laminin promotes neuritic regeneration from cultured peripheral and central neurons. J. Cell Biol. 97, 1882-1890.

Manthorpe, M., Varon, S. and Adler, R. (1981) Neurite-promoting factor in conditioned medium from RN22 Schwannoma cultures: bioassay, fractionation, and properties. J. Neurochem. 37, 759-767.

March, S.C., Parikh, I. and Cuatrecasas, P. (1974) A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem 60, 149-152.

Margolis, R.U., Margolis, R.K., Chang, L.B. and Preti, C. (1975) Glycosaminoglycans of brain during development. Biochemistry, 14, 85-88.

Matthew, W.D., Greenspan, R.J., Lander, A.D. and Reichardt, L.F. (1985) Immunopurification and characterization of a neuronal heparan sulfate proteoglycan. J. Neurosci 5, 1842-1850.

Matthew, W.D. and Patterson, P.H. (1983) The production of a monoclonal antibody that blocks the action of a neurite outgrowth promoting factor. Cold Spring Harbor Symp. Quant. Biol. 48, 625-631.

Morris, D.G. (1978) Development of functional motor innervation in supernumerary hindlimbs of the chick embryo. J. Neurophys. 41, 1450-1465.

Neff, N.T., Lowrey, C., Decker, C., Tovar, A., Damsky, C., Buck, C. and Horwitz, A.F. (1982) A monoclonal antibody detaches embryonic skeletal muscle from extracellular matrices. J. Cell Biol. 95, 654-666.

Nitkin, R.M., Wallace, B.G., Spira, M.E., Godfrey, E.W. and McMahan, U.J. (1983) Molecular components of the synaptic basal lamina that direct differentiation of regenerating neuromuscular junctions. Cold Spring Harbor Symp. Quant. Biol. 48, 653-666.

Oldberg, A., Kjellen, L. and Höök, M. (1979) Cell surface heparan sulfate. Isolation and characterization of a proteoglycan from rat liver membranes. J. Biol. Chem. 254, 8505-8510.

Oohira, A., Wight, T.N. and Bornstein, P. (1983) Sulfated proteoglycans synthesized by vascular endothelial cells in culture. J. Biol. Chem 258, 2014-2021.

Ott, U., Odermatt, E., Engel, J., Furthmayr, H. and Timpl, R. (1982) Protease resistance and conformation of laminin. Eur. J. Biochem 123, 63-72.

Palm, S.L. and Furcht, L.T. (1983) Production of laminin and fibronectin by Schwannoma cells: Cell-protein interactions in vitro and protein localization in peripheral nerve in vivo. J. Cell Biol. 96, 1218-1226.

Pardue, R.L., Brady, R.C., Perry, G.W. and Dedman, J.R. (1983) Production of monoclonal antibodies against calmodulin by in vitro immunization of spleen cells. J. Cell Biol. 96, 1149-1154.

Paul, J.I. and Hynes, R.O. (1984) Multiple fibronectin subunits and their post-translational modifications. J. Biol. Chem. 259, 13477-13487.

Paulsson, M., Deutzmann, R., Timpl, R., Dalzoppo, D., Odermatt, E. and Engel, J. (1985) Evidence for coiled-coil alpha-helical regions in the long arm of laminin. EMBO J. 4, 309-316.

Radhakdrishnamurthy, B., Smart, F., Dalferes, E.R. Jr. and Berenson, G.S. (1980) Isolation and characterization of proteoglycans from bovine lung. J. Biol. Chem. 255, 7575-7582.

Rao, C.N., Marguilies, I.M.K., Goldfarb, R.H., Madri, J.A., Woodley, D.T. and Liotta, L.A. (1982) Differential proteolytic susceptability of laminin alpha and beta subunits. Arch. Biochem. Biophys. 219, 65-70.

Rao, C.N., Barsky, S.H., Terranova, V.P. and Liotta, L.A. (1983) Isolation of a tumor cell laminin receptro. Biochem. Biophys. Res. Comm. 111, 804-808.

Raper, J.A., Bastiani, M.J. and Goodman, C.S. (1983) Guidance of neuronal growth cones: selective fasciculation in the grasshopper embryo. Cold Spring Harbor Symp. Quant. Biol. 48, 587-598.

Reading, C.L. (1982) Theory and methods for immunization in culture and monoclonal antibody production. J. Immunol. Methods 53, 261-291.

Reddi, A.H. (1984) Extracellular matrix and development. In: Extracellular matrix biochemistry, K.A. Piez and A.H. Reddi, editors. Elsevier, New York. 375-412.

Robinson, J. and Gospodarowicz, D. (1983) Glycosaminoglycans synthesized by cultured bovine corneal endothelial cells. J. Cell Physiol. 368-376.

Rogers, S.L., Letourneau, P.C., Palm, S.L., McCarthy, J. and Furcht, L.T. (1983) Neurite extension by peripheral and central nervous system neurons in response to substratum-bound fibronectin and laminin. Dev. Biol. 98, 212-220.

Rogers, S.L., McLoon, S.C. and Letourneau, P.C. (1984) Distribution of laminin and fibronectin during early axonal growth in the chick PNS. Soc. Neurosci. Abstr. 10, 39.

Rohde, H., Bächinger, H.P. and Timpl, R. (1980) Characterization of pepsin fragments of laminin in a tumor basement membrane: Evidence for the existence of related proteins. Hoppe-Seyler's Z. Physiol. Chem. 361, 1651-1660.

Ruoslahti, E., Hayman, E.G., Pierschbacher, M. and Engvall, E. (1982) Fibronectin: purification, immunochemical properties, and biological activities. Methods Enzymol. 82, 803-831.

Sajdera, S.W. and Hascall, V.C. (1969) Proteinpolysaccharide complex from bovine nasal cartilage. A comparison of low and high shear extraction procedures. J. Biol. Chem. 244, 77-87.

Sakashita, S., Engvall, E. and Ruoslahti, E. (1980) Basement membrane protein laminin binds to heparin. FEBS Letters 116, 243-246.

Sanes, J.R. (1982) Laminin, fibronectin and collagen in synaptic and extrasynaptic portions of muscle fiber basement membrane. J Cell Biol. 93, 442-451.

Sanes, J.R. and Chiu, A.Y. (1983) The basal lamina of the neuromuscular junction. Cold Spring Harbor Symp. Quant. Biol. 48, 667-678.

Schlesinger, M.J. and Barret, K. (1965) The reversible dissociation of the alkaline phosphatase of Escherichia coli. J. Biol. Chem. 240, 4284-4292.

Schoene, W.C. (1979) "Tumors of the nervous system" In: Pathologic Basis of Disease, Robbins, S.L. and Cotran, R.S., Editors. W.B. Saunders Co., Philadelphia. pp. 1564-1578.

Schubert, D., LaCorbiere, M., Klier, F.G. and Birdwell, C. (1983a) The structure and function of myoblast adherons. Cold Spring Harbor Symp. Quant Biol. 48, 539-549.

Schubert, D., LaCorbiere, M., Klier, F.G. and Birdwell, C. (1983b) A role for adherons in neural retina cell adhesion. J. Cell Biol. 96, 990-998.

Schwarzbauer, J.E., Tamkun, J.W., Lemischka, I.R. and Hynes, R.O. (1983) Three fibronectin messenger RNA species arise by alternative splicing within the coding region. Cell 35: 421-431.

Sekiguchi, K., Hakomori, S., Funahashi, M., Matsumoto, I. and Seno, N. (1983) Binding of fibronectin and its proteolytic fragments to glycosaminoglycans: exposure of cryptic glycosaminoglycan-binding domains upon limited proteolysis. J. Biol. Chem. 258, 14359-14365.

Sekiguchi, K., Siri, A., Zardi, L. and Hakomori, S. (1985) Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. Studies with domain-specific antibodies. J. Biol. Chem. 260, 5105-5114.

Shibata, S., Peters, B.P., Roberts, D.D., Goldstein, I.J. and Liotta, L.A. (1982) Isolation of laminin by affinity chromatography on immobilized Griffonia simplicifolia I lectin. FEBS Lett. 142, 194-198.

Solomon, F. (1981) Organizing the cytoplasm for motility. Cold Spring Harbor Symp. Quant. Biol. 46, 17-22.

Sotelo, J., Gibbs, C.J. Jr., Gajdusek, D.C., Toh, B.H., and Wurth, M. (1980) Method for preparing cultures of central neurons: cytochemical and immunochemical studies. Proc. Natl. Acad. Sci. U.S.A. 77, 653-657.

Sugrue, S.P. (1984) The identification of a cell surface binding protein for laminin from the embryonic corneal epithelia. J. Cell Biol. 99, 165a.

Suissa, M. (1983) Spectrophotometric quantitation of silver grains eluted from autoradiograms. Anal. Biochem 133, 511-514.

Sundberg, L., and Porath, J. (1974) Preparation of adsorbents for biospecific affinity chromatography. I. Attachment of group-containing ligands to insoluble polymers by means of bifunctional oxiranes. J. Chromatography 90, 87-98.

Sunderland, S. (1978) Nerves and nerve injuries, 2nd edition. Churchill Livingstone, New York.

Swanson, G.J. and Lewis, J. (1982) The timetable of innervation and its control in the chick wing bud. J. Embryol. Exp. Morphol. 71, 121-137.

Terranova V.P., Rao, C.N., Kalebic, T., Margulies, I.M. and Liotta, L.A. (1983) Laminin receptor on human breast carcinoma cells. Proc. Natl. Acad. Sci. USA 80, 444-448.

Thanos, S., Bonhoeffer, F. and Rutishauser, U. (1984) Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. Proc. Natl. Acad. Sci. U.S.A. 81, 1906-1910.

Thiery, J.P., Duband, J.L. and Delouvee, A. (1982) Pathways and mechanisms of avian trunk neural crest cell migration and localization. Dev. Biol. 93, 324-343.

Timpl, R., Johansson, S., vanDelden, V., Oberbäumer, I. and Höök, M. (1983) Characterization of protease-resistant fragments of laminin mediating attachment and spreading of rat hepatocytes. J. Biol. Chem. 258, 8922-8927.

Timpl, R., Rohde, H., Risteli, L., Ott, U., Robey, P.G., and Martin, G.R. (1982) Laminin. Methods Enzymol. 82, 831-838.

Timpl, R., Rohde, H., Robey, P.G., Rennard, S.I., Foidart, J.-M. and Martin, G.R. (1979) Laminin--a glycoprotein from basement membranes. J. Biol. Chem. 254, 9933-9937.

Toledo, O.M.S. and Dietrich, C.P. (1977) The specific distribution of sulfated mucopolysaccharides in mammals. Biochim. Biophys. Acta. 498, 114-122.

Tomaselli, K. and Reichardt, L.F. (1984) Laminin substrates enhance nerve growth factor (NGF) induced neuritogenesis and regeneration in PC12 cells. Soc. Neurosci. Abstr. 10, 40.

Toole, B.P. (1976) Morphogenetic role of glycosaminoglycans (acid mucopolysaccharides) in brain and other tissues. In: Neuronal recognition, S. Barondes, editor. Plenum, New York. 275-328.

Toole, B.P. (1981) Glycosaminoglycans in morphogenesis. In: Cell biology of the extracellular matrix, E. Hay, editor. Plenum, New York. 259-294.

Valentino, K.L., Winter, J. and Reichardt, L.F. (1985) Applications of monoclonal antibodies to neuroscience research. Ann. Rev. Neurosci. 8, 199-232.

Varon, S. and Raiborn, C. (1971) Excitability and conduction in neurons of dissociated ganglionic cell cultures. Brain Res. 30, 83-98.

Varon, S. and Raiborn, C. (1972) Dissociation, fractionation and culture of chick embryo sympathetic ganglion cells. J. Neurocytol. 1, 211-221.

Wakshull, E., Johnson, M.I., and Burton, H. (1979) Postnatal rat sympathetic neurons in culture. I. A comparison with embryonic neurons. J. Neurophys. 42, 1410-1425.

Wan, Y.J., Wu, T.C., Chung, A.E. and Damjanov, I. (1984) Monoclonal antibodies to laminin reveal the heterogeneity of basement membranes in the developing and adult mouse tissues. J. Cell Biol. 98, 971-979.

Weiss, P. (1955) Nervous system (neurogenesis). In Analysis of Development B.H. Willier, P.A. Weiss and V. Hamburger, Ed. Saunders, Philadelphia, PA. pp. 346-401.

Wyman, R.J. and Thomas, J.B. (1983) What genes are necessary to make an identified synapse? Cold Spring Harbor Symp Quant. Biol. 48, 641-652.

Yaffe, D. and Saxel, O. (1977) Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270, 725-727.

Yamada, K.M. (1983) Cell surface interactions with extracellular materials. Ann. Rev. Biochem. 52, 761-799.

Yamada, K.M., Kennedy, D.W., Kimata, K, and Pratt, R.M. (1980) Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. J. Biol. Chem. 255, 6055-6063.

Yamada, K., Spooner, B. and Wessels, N. (1971) Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol. 49, 614-635.

A P P E N D I X

SUPPLEMENT TO THE PURIFICATION OF THE "NEURITE OUTGROWTH-PROMOTING FACTOR" FROM BCE-CM

Not all of the approaches taken to purify and characterize the "neurite outgrowth-promoting factor" in BCE-CM were discussed in the chapters above. Some attempted purification steps proved unreliable or unnecessary. Certain experiments designed to answer particular question were abandoned, when other experiments yielding the same answers were found to be more feasible. Although the temptation to forget unsuccessful experiments and unusable data is strong, such information can be of practical value in corroborating other results, in demonstrating the limitations of methods, and in suggesting directions to take--and not to take--in the future. For this reason, certain observations are reported and discussed below. In the interest of brevity, results already presented in the main body of this dissertation will be referred to, but not reiterated.

Development of a Purfication Scheme: Cesium Chloride Gradients

Some early attempts at purifying the "neurite outgrowth-promoting factor" in BCE-CM incorporated steps that were eventually omitted. One of these, isopycnic centrifugation in CsCl, was tried because, in the pilot studies reported in Chapter 2, it appeared to separate the "neurite outgrowth-promoting factor" from most of the proteins in BCE-CM (Figure 2.3). While certain practical considerations contributed to the eventual abandonment of the technique--it was time consuming, it had a low total capacity for protein, its resolution was not optimal and could not be improved, it introduced significant nonspecific losses--the main difficulty was encountered when the "neurite outgrowth-promoting factor" in many experiments was found to band not in one, but

in two distinct peaks in CsCl. In particular, this was seen whenever the material applied to the CsCl gradient was a sample of "neurite outgrowth-promoting factor" that had already been partially purified by some other means (e.g. by two rounds of ultracentrifugation of BCE-CM, as described in Chapter 4). In these cases, a peak of neurite outgrowth-promoting activity was found not only at a density of 1.37 (as in Figure 2.3), but also at 1.32. Since some of these experiments were done with BCE-CM that had been metabolically labeled with [^{3}H]leucine and [^{35}S]sulfate, it was further noted that, while the peak at 1.37 contained both leucine and sulfate (as in Figure 2.3), the "new" peak at 1.32 contained leucine only.

The explanation for these puzzling findings can, in retrospect, be made clear, now that it is known that the "neurite outgrowth-promoting factor" contains both laminin and a heparan sulfate proteoglycan. Because laminin and the heparan sulfate proteoglycan are interacting molecules of different densities (the density of laminin should be lower than that of a proteoglycan), they are subject to certain peculiar effects during isopycnic centrifugation. These effects, which have been well characterized on experimental and theoretical grounds (Harrington and Kegeles, 1973), arise out of the following situation: During ultracentrifugation, a steep gradient of pressure occurs accross the length of a sample tube. Pressure, consequently, has an effect on most binding equilibria, which, for biological macromolecules, can be very large (Harrington and Kegeles, 1973). As a result, the equilibrium constant for any interaction may vary continuously along the length of an ultracentrifuge tube. Thus, in a CsCl gradient, while buoyant forces will always favor the dissociation of interacting molecules of

different density, the pressure effect may favor the association of interacting molecules--and more so in some parts of the gradient than others. The net result predicted for most such systems is the establishment, at equilibrium, of two peaks. One should consist purely of the complex of the two bound molecules and band at a position appropriate for that complex. The other peak should contain whichever single molecule is in excess, and should band at a position appropriate for that molecule (Harrington and Kegeles, 1973).

The two peaks of neurite outgrowth-promoting activity observed when the partially purified "neurite outgrowth-promoting factor" was subjected to isopycnic centrifugation may thus be identified: The peak at 1.37, which contains both sulfate and leucine, must represent the complex of laminin and proteoglycan (and possibly entactin as well). The peak at 1.32, which lacks sulfate, must represent laminin alone, or laminin with only entactin bound to it.

What remains to be explained is why the peak of neurite outgrowth-promoting activity at 1.32 was only observed in experiments where a partially purified "neurite outgrowth-promoting factor" was fractionated, and not in experiments where crude BCE-CM or $-CM_{SF}$ was used (e.g. Figure 2.3). In fact, this result also makes sense in retrospect. As the results of heparitinase digestion showed (Chapter 4, Table 4.3), removal of the proteoglycan from the "neurite outgrowth-promoting factor" leaves laminin that is poorly equipped to compete with other molecules for binding to the culture substratum. In Figure 2.3, there probably _is_ a laminin peak at 1.32, but, because nearly every other protein in BCE-CM had been concentrated into a peak at at about 1.32, the proteoglycan-free laminin could not compete for substratum attach-

ment, and was therefore never detected in the neurite outgrowth assay. Only when partially purified samples of "neurite outgrowth-promoting factor", containing fewer competing proteins, were fractionated on CsCl gradients, did the laminin peak at 1.32 become detectable[4].

Development of a Purification Scheme: Use of Mixed Leucine- and Sulfate-Labeled BCE-CM

In Chapter 2, analysis of a mixture of [^{3}H]leucine- and [^{35}S]sulfate-labeled BCE-CM proved valuable in implicating a proteoglycan as a component of the "neurite outgrowth-promoting factor". Ultimately, however, purification and analysis of the "neurite outgrowth-promoting factor" was carried out mostly using [^{35}S]methionine-labeled BCE-CM, mainly because fluorography of gels containing [^{35}S]-labeled protein requires about ten fold less time than gels containing an equal amount

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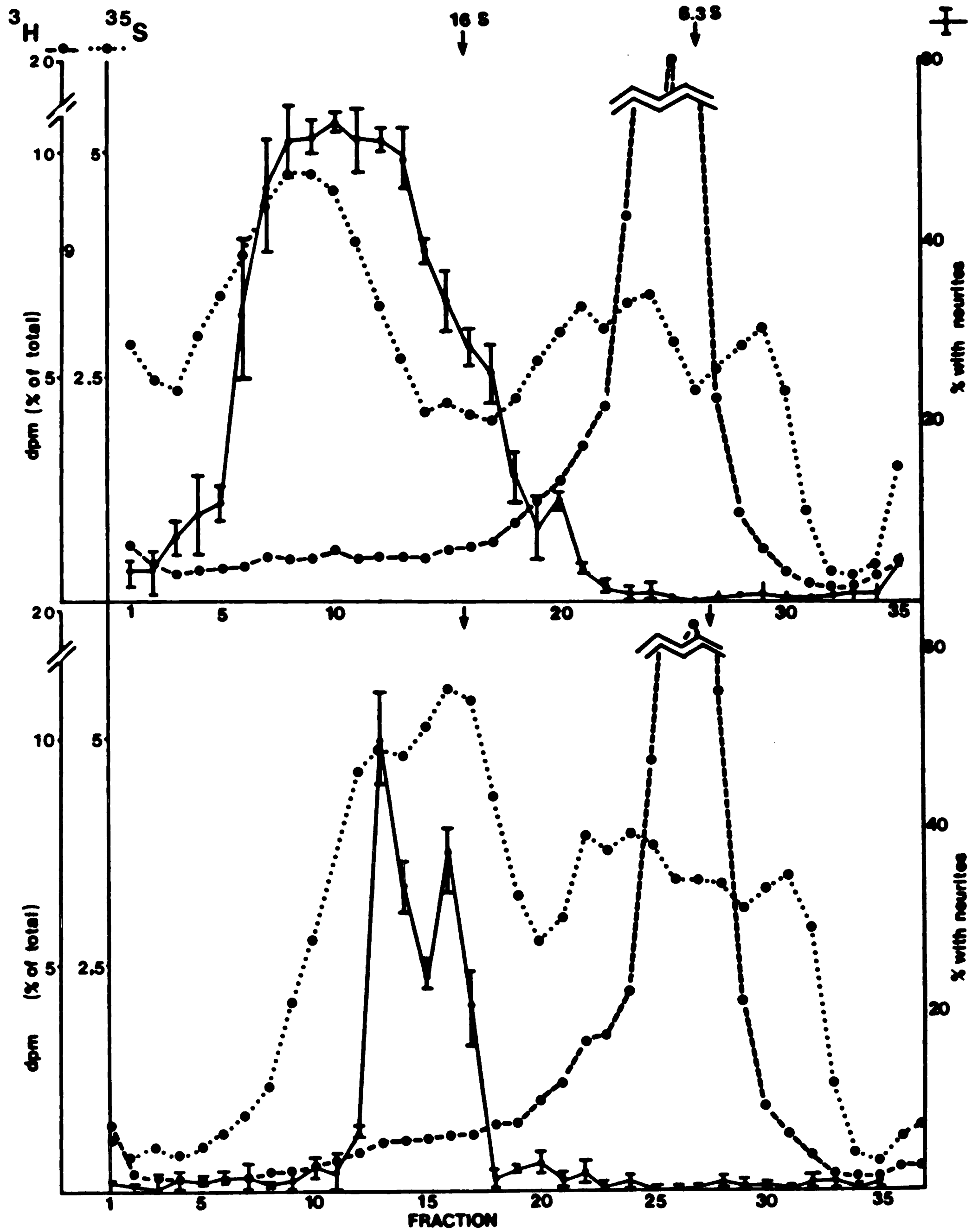
[4] In aggreement with this, it was found for CsCl gradients of crude BCE-CM$_{SF}$ that, on occasion, some neurite outgrowth-promoting activity could be detected at densities near 1.32, provided that samples were tested at extreme dilution. With regard to the binding of molecules to a substratum, it might be expected that extreme dilution would favor equilibrium, rather than kinetic, effects, and thus might improve the chances of a very large molecule like laminin, that diffuses slowly, but probably adsorbs tightly (due to multi-site attachment). It should also be added that the double peaks at 1.37 and 1.32 are not unique to the "neurite outgrowth-promoting factor in BCE-CM, but appeared during CsCl gradient fractionation of other CM's. Some of these results are illustrated in a publication (Lander et al., 1983).

of [^{3}H]-labeled protein (Bonner and Laskey, 1974). Despite this, some preliminary studies were carried out using mixed [^{3}H]leucine/ [^{35}S]sulfate-labeled material, and the data provided by these experiments help motivate the approaches used in Chapter 4.

For example, one of the first methods devised for partially purifying the "neurite outgrowth-promoting factor" was the series of ultracentrifugation steps that later became known as the "alternate purification method" (Chapter 4). Figure A.1 shows the results of subjecting "double-labeled" BCE-CM to two rounds of ultracentrifugation, followed by sucrose gradient fractionation (as described for the "alternate purification" in Methods). In the top graph, a sample containing a substantial quantity of neurite outgrowth-promoting activity was applied to the sucrose gradient. In the bottom graph, the same material was applied to an identical gradient, but at a significant dilution, containing only one-twelfth as much neurite outgrowth-promoting activity. The profiles of [^{3}H]leucine, [^{35}S]sulfate, and neurite outgrowth-promoting activity have been plotted.

The results in figure A.1 confirm the continuing association of sulfate and leucine during purification of the "neurite outgrowth-promoting factor". They also compare favorably with the results of velocity sedimentation presented in Figures 4.5 and 4.13. However, what is most interesting about Figure A.1 is the apparent concentration dependence of the profile of neurite outgrowth-promoting activity, and of the labeled material associated with it. Although it is well known that the concentration of a macromolecule can affect its sedimentation behavior, this is usually reflected in a decrease in sedimentation velocity at high concentration (Cantor and Schimmel, 1980). In contrast,

Figure A.1. Concentration-dependent behavior of the partially-purified "neurite outgrowth-promoting factor". BCE-CM labeled with [^{3}H]leucine and [^{35}S]sulfate was prepared and concentrated by two rounds of ultracentrifugation, and fractionated by sedimentation through a 5-20% sucrose gradient (as described in Methods). The material applied to the gradient shown in the bottom graph was a 1/12 dilution of the material applied to the gradient shown in the top graph. Equal volumes were applied to the two gradients, and they were centrifuged simultaneously. Plotted for each gradient are the content of [^{3}H]leucine (- - - - - - - - -), [^{35}S]sulfate (················), and neurite outgrowth-promoting activity (__________________). Positions of sedimentation standards (β -galactosidase, 16S, and bacterial alkaline phosphatase, 6.3S) are shown.

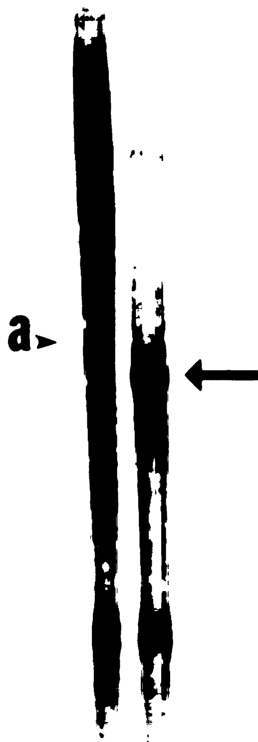


3H
35S
16 S
6.3 S
dpm (% of total)
% with neurites
FRACTION

the "neurite outgrowth-promoting factor" appeared to sediment further at higher concentration (Figure A.1). These were among the earliest results to suggest strongly that neurite outgrowth-promoting activity was associated with molecules that formed aggregates or complexes.

The "double labeled" material shown in Figure A.1 was also used to pilot the analysis of the "neurite outgrowth-promoting factor" by gel electrophoresis. When material pooled from the active fractions in the top graph of Figure A.1 was subjected to non-reducing SDS-PAGE, the pattern seen in lane 1 of Figure A.2 was observed. Although very high molecular weight standards were not included, the gel was prepared and run in the same manner as the gels shown in Chapters 4 and 5, and the positions of labeled molecules may be roughly compared. The most striking feature of Figure A.2, lane 1, is the heavily labeled high molecular weight blur it contains. In retrospect, it is clear that this "blur" represents the heparan sulfate proteoglycan, and it is so heavily labeled because it is rich in [^{35}S]sulfate, which fluorographs so much more efficiently that [^{3}H]. At the time, however, it was necessary to determine this, which was done by subjecting a sample of the material analyzed on this gel to heparitinase digestion, and comparing its electrophoretic pattern. The result, shown in lane 2, indicates that the high molecular weight blur is completely eliminated by heparitinase digestion. This was also accompanied by a conversion of nearly all of the [^{35}S] in the sample to small molecules not precipitable with trichloroacetic acid or cetylpyridinium chloride (not shown). Thus, not only must the high molecular weight blur represent a heparan sulfate proteoglycan, but the bands remaining in lane 2 of Figure A.2 must represent proteins containing little or no [^{35}S].

Figure A.2. Analysis by non-reducing SDS-PAGE of the [^{3}H]leucine- and [^{35}S]sulfate-labeled, partially purified "neurite outgrowth-promoting factor" from BCE-CM. Pooled active fractions from the sucrose gradient shown in Figure A.1, top, were analyzed by SDS-PAGE before (lane 1) or after (lane 2) digestion with heparitinase (as described in Methods). Standards: a) nonreduced myosin (495,000 Da), b) β-galactosidase (116,000 Da), c) phosphorylase a (94,000 Da), d) bovine serum albumin (68,000 Da). The proteoglycan core protein that is generated by heparitinase digestion is indicated with an arrow.


1 2
a
b
c
d

In fact, the protein bands in Figure A.2, lane 2, are also easily identifiable, in retrospect. The faint very high molecular weight band that is unmasked by digestion of the proteoglycan is undoubtedly laminin. The darker band below it was clearly generated by the heparitinase digestion, and must be the 400,000 Da core protein of the proteoglycan (cf. Chapter 4). It probably is so darkly labeled because a few residual [^{35}S]sulfate-containing oligosaccharides remain attached to it. Below it, a doublet can be seen at about 170,000 Da. This material is a contaminant consisting of a major 7S protein found in BCE-CM (cf. Figure A.1) that tends to aggregate and "smear out" across sucrose gradients (it can also been seen doing this in Figure 4.13). The remaining band in Figure A.2 is at about 150,000 Da. It no doubt represents entactin, and the fact that entactin contains a small amount of sulfate (cf. Figure 4.8) probably enhances its visualization.

<u>Development of a purification scheme: problems with proteolysis</u>

One of the most important conceptual steps in the purification and characterization of the "neurite outgrowth-promoting factor" in BCE-CM was overcoming the prejudice that laminin could not be involved. While the discussions in Chapters 3-5 imply that the failure of anti-laminin antibodies to block the neurite outgrowth-promoting activity of BCE-CM was the main grounds for this prejudice, historically this is an oversimplification. In fact, a host of facts and artifacts conspired simultaneously to refute the suggestion that laminin played a role in the activity of "neurite outgrowth-promoting factors".

For example, the "neurite outgrowth-promoting factor" in BCE-CM was found to be sensitive to concentrations of urea and other chao-

tropes that did not affect the activity of purified laminin (not shown). The "neurite outgrowth-promoting factor" in BCE-CM had different sedimentation behavior from laminin (cf. Engel _et al._, 1981). The amount of laminin in BCE-CM had previously been estimated by Gospodarowicz _et al._ (1981) and this estimate was much too low for laminin to be able to account for any significant amount of neurite outgrowth-promoting activity. Furthermore, based on the _in vivo_ localization of laminin, the types of cells originally believed capable of synthesizing laminin were not as varied as the types of cells that make "neurite outgrowth-promoting factors" (cf. Timpl _et al._, 1982).

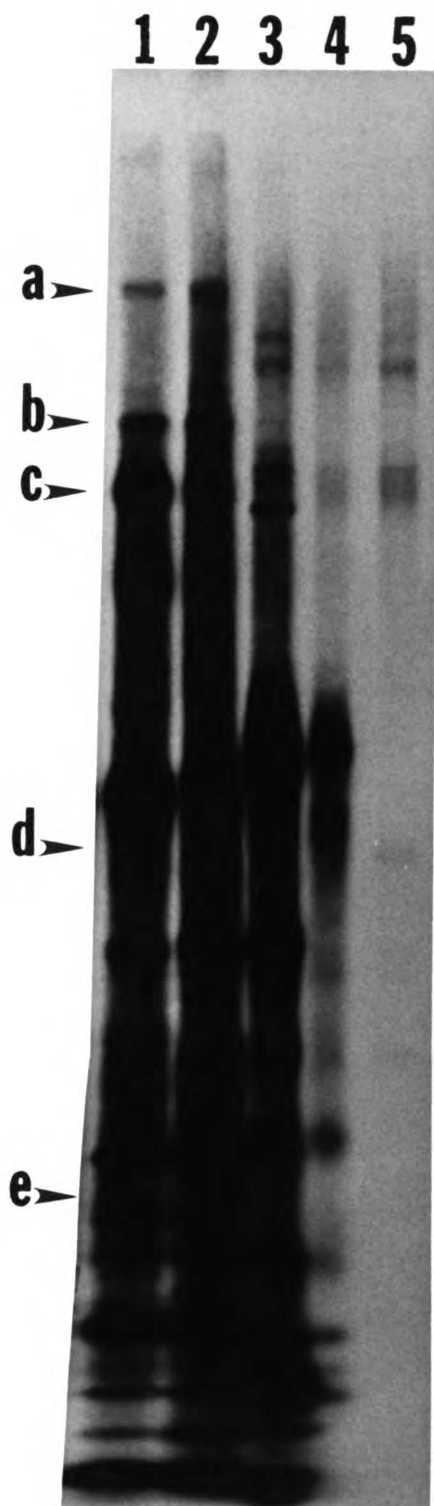
In the end, the sensitivity of the "neurite outgrowth-promoting factor" in BCE-CM to chaotropes disappeared after a few steps of purification, and probably had something to do with displacement of the proteoglycan from laminin and the effects of molecules that competed for substratum attachment. The sedimentation behavior of the "neurite outgrowth-promoting factor" probably also reflected the association of the proteoglycan with laminin. The estimates of laminin synthesis by Gospdarowicz _et al._ (1981) were re-checked, and found to be two orders of magnitude too low. And with time, it became clear that many cell types, even those not associated with detectable laminin _in vivo_, could synthesize laminin _in vitro_ (e.g. Liesi _et al._, 1983; Couchman _et al._, 1983).

The most serious challenge to a role for laminin in "neurite outgrowth-promoting factors" came, however, from artifacts introduced by proteolysis, causing the polypeptide composition of the "neurite outgrowth-promoting factor" in BCE-CM to appear different from that of laminin. The greatest problems with proteolysis occured in association

with a single purification step, polyethylene glycol (PEG) precipitation. Briefly, ammonium sulfate precipitates of BCE-CM were redissolved, brought to 12.5% in PEG-6000 (Sigma), and neurite outgrowth-promoting activity was recovered in the precipitate that formed. This procedure was introduced to solve a particular problem. When ammonium sulfate precipitates of BCE-CM were simply dissolved and dialyzed against TBS or PBS, a large sticky precipitate slowly but invariably formed in the dialysis bag, trapping much of the "neurite outgrowth-promoting factor". However, if PEG precipitation were performed at once, before dialysis of the ammonium sulfate precipitate, the recovered material no longer tended to form a sticky mass, suggesting that the offending substance had been purified away from the "neurite outgrowth-promoting factor".

Much later it was learned that the "offending substance" was cellular fibronectin, which tends to polymerize at high concentration (Hynes, 1981). The reason that PEG precipitation circumvented this problem was not because it separated the "neurite outgrowth-promoting factor" from fibronectin, but because, it was later learned, the PEG procedure accelerated the proteolytic degradation of molecules in BCE-CM, and fibronectin, being quite protease sensitive, was one of the first to be destroyed. Surprisingly, this occured even in the presence of high levels of protease inhibitors. Were proteolysis limited to fibronectin alone, it would not have mattered for the purposes of the purification of the "neurite outgrowth-promoting factor". Unfortunately, laminin--particularly its A chain--is also protease sensitive (Ott _et al._, 1982), and it apparently did not come through the PEG procedure unscathed. Evidence for this is presented in Figure A.3.

Figure A.3. Purification of the "neurite outgrowth-promoting factor" from [^{35}S]methionine-labeled BCE-CM: effect of polyethylene glycol precipitation. Samples at each stage of purification were analyzed by SDS-PAGE under non-reducing conditions. The samples in each lane, and the amount (in cpm x 10^{-3}) applied were: Lane 1, BCE-CM (137); lane 2, ammonium sulfate pellet (276); lane 3, polyethylene glycol precipitate (138); lane 4, material bound to DEAE-cellulose and eluted with 1 M NaCl (20); lane 5, pooled active material from gel filtration on Sepharose CL4B (2). Standards: a)laminin (850,000 - 1,000,000 Da), b) myosin (495,000 Da), c)cellular fibronectin (440,000 Da), d) mouse immunoglobulin G (150,000 Da), e) bovine serum albumin (68,000). Note the disappearance of intact laminin and fibronectin following the polyethylene glycol step.


1
2
3
4
5
a
b
c
d
e

On this non-reducing SDS-gel of [^{35}S]methionine-labeled material, the purification of the "neurite outgrowth-promoting factor" is followed through several steps: ammonium sulfate precipitation, PEG precipitation, DEAE-cellulose chromatography, and gel filtration on Sepharose CL4B. In the most purified material, no band is seen that comigrates with a laminin standard (marker 'a'). Instead, a doublet is seen (between markers 'a' and 'b') that is about 200,000 Da smaller than laminin. Looking over the sequence of purification steps, it becomes clear that this doublet first appears following PEG precipitation, which is exactly when a band of the right molecular weight for laminin disappears, suggesting that the smaller doublet represents a fragment of laminin. When this material was further analyzed by SDS-PAGE under reducing conditions, it was converted to three bands of about 200,000, 220,000 and 240,000 Da. Since the first two of these bands comigrated with the B chains of laminin, the third band probably represents a fragment of the A chain (data not shown). Figure A.3 also illustrates the disappearance of fibronectin (marker 'c') during the PEG procedure.

It is not known why PEG precipitation led to the degradation of these molecules. PEG lowers the activity of water, and may have thereby enhanced the enzymatic activity of proteases. PEG also generates a "polymer phase" in solution, and may have allowed protease inhibitors to partition into this phase, and away from proteases. It is also possible that it was not the PEG itself, but a contaminant in it, that was responsible for the effect, e.g. metal ions that could stimulate proteases. Whatever the explanation, it was fortunate that a less destructive way of eliminating fibronectin, i.e. gelatin-Sepharose ad-

sorption, was found, so that PEG precipitation could be abandoned.

Attempts to Generate Monoclonal Antibodies that Recognize the "Neurite Outgrowth-Promoting Factor" in BCE-CM

As a means of identifying molecules expressed by particular cell types, in particular locations *in vivo*, or associated with particular functions, the monoclonal antibody technique has been of great value to neurobiologists. In addition to helping to identify molecules, monoclonal antibodies can also be useful in purifying them, blocking their functions, or studying their expression *in vivo* (reviewed by Valentino *et al.*, 1985). During the characterization and purification of the "neurite outgrowth-promoting factor" in BCE-CM, various observations suggested that attempts to generate monoclonal antibodies would be appropriate, and could potentially yield important information.

For example, early characterization of the "neurite outgrowth-promoting factor" suggested that it was part of some type of complex, but biological activity could not be recovered following dissociative treatments (Chapter 2). Monospecific antibodies could distinguish among components of such a complex, prove that they were associated with each other, and determine which components were required for activity. Monospecific antibodies could also determine whether "neurite outgrowth-promoting factors" were present *in vivo*, and were associated with ECM.

Especially before the purification of the "neurite outgrowth-promoting factor" from BCE-CM was fully worked out, the advantages of monoclonal antibodies over polyclonal antisera, as sources of monospecific antibodies, seemed particularly great. Monoclonal antibodies could be

generated using an impure immunogen. Also, monoclonal antibodies could be generated using less immunogen than would be needed to generate polyclonal antisera. This latter advantage was particularly important, because early attempts at purifying the "neurite outgrowth-promoting factor" had very low yields. In fact, the small amount of material available for immunization was one reason that the method of *in vitro* immunization (Reading, 1982) was chosen for the production of monoclonal antibody-producing cells. The second reason for chosing *in vitro* immunization was that Matthew and Patterson (1983) had used the technique with great success to obtain a monoclonal antiboy that not only recognized the "neurite outgrowth-promoting factor in PC12-CM, but also blocked its effects *in vitro*[5].

In vitro immunization, while not a new technique, has only recently been used for the production of monoclonal antibodies (reviewed by Reading, 1982). Briefly, mouse spleen cells are exposed to an immunogen *in vitro* for several days, to promote activation of specific B-cells, and then fused with cells from a myeloma line, to produce antibody-secreting hybridoma cells. As little as 50-200 ng of an antigen may be used for a single immunization (Luben and Mohler, 1980; Pardue *et al.*, 1983). The procedures used in attempts to generate, by this method, monoclonal antibodies directed against the "neurite outgrowth-promoting factor" in BCE-CM are summarized briefly:

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[5] Had the antibody described by Matthew and Patterson also recognized the "neurite outgrowth-promoting factor" in BCE-CM, it might not have been necessary to look for other antibodies. Unfortunately, no such cross-reactivity was observed (unpublished observations).

To obtain an immunogen, BCE-CM was fractionated by ammonium sulfate precipitation, PEG precipitation (its effects on proteolysis had not been discovered at the time) and DEAE cellulose chromatography. Three immunizations were performed. In one trial, the material prepared by these three steps of purification (estimated 1-2 ug protein) was used as the immuogen. In a second trial, the immunogen was further purified by sucrose gradient fractionation (yielding about 0.2-1 ug protein). In the third trial, the immunogen was prepared as in the second trial, except that the sample was subjected to heparitinase digestion before sucrose gradient fractionation. All purification steps were performed without the use of detergents or protease inhibitors. Before use, each immunogen was brought to 10% in fetal calf serum and dialyzed against the culture medium to be used for immunization.

Immunization was carried out _in vitro_ as described by Reading (1982), with the following modifications: Instead of rabbit serum, fetal calf serum was used at 20%. The lots of serum used (Hyclone, Logan, UT) were pre-screened for the ability to support clonal growth of myeloma cells, and were reported to possess low levels of non-specific B-cell mitogen activity. To the immunization medium was also added the adjuvant peptide, N-acetylmuramyl-_L_-alanyl-_D_-isoglutamine (Sigma) at 25 ug/ml (Matthew and Patterson, 1983; Boss, 1984)

Immunization proceeded for 4-4.5 days, at which point splenocyte cultures were fused to Sp2/0 myeloma cells as described (Reading, 1982). Each fusion was distributed among 600 wells of 96-well microtiter plates containing normal BALB/c mouse peritoneal macrophages (Fazekas de St. Groth and Scheidegger, 1980) in hypoxanthine-amino-

pterin-thymidine selection medium (Reading, 1982). Proliferating hybridoma cells eventually appeared in 20-60% of the wells, depending on the particular fusion.

Hybridoma supernatants were initially screened in two ways. First, they were screened by enzyme-linked immunoassay (EIA) for binding to a partially purified fraction of "neurite outgrowth-promoting factor" from BCE-CM, using the EIA methods already described (except that the enzyme-conjugated second antibody was rabbit anti-mouse IgG). During this screen, hybridoma supernatants were also tested for binding to serum components present in the EIA buffer, and all serum-binding supernatants were eliminated from further testing. Second, all supernatants were screened for the ability to block the neurite outgrowth-promoting activity of BCE-CM. Specifically, polylysine culture wells were exposed to BCE-CM overnight, washed, and exposed to 50 ul aliquots of sterile hybridoma supernatants overnight. Rat sympathetic neurons and neuronal culture medium were added directly to these wells (without removing the hybridoma supernatants), and cultured for 11 hours. Neurite outgrowth was then scored.

From three fusions, 49 supernatants were positive for binding to the partially purified "neurite outgrowth-promoting factor" (and not to serum), but none were able to block neurite outgrowth-promoting activity. Further screening of positive supernatants for binding that was stronger to the partially purified "neurite outgrowth-promoting factor" than to crude BCE-CM resulted in the recovery of 15 hybridoma lines, that were then cloned. These were further screened in the following way:

A sample of the partially purified "neurite outgrowth-promoting

factor" was fractionated, by chromatography on Sepharose CL4B, into six fractions. Each fraction was tested for neurite outgrowth-promoting activity, and for binding by supernatants from each of the 15 hybridomas. The results are shown in Table A.1. As expected, neurite outgrowth-promoting activity was found in the leading fractions (cf. Figure 4.1.)[6]. The pattern of antibody binding differed among the hybridomas. Two of them (5E3 and 26A9) bound in a pattern resembling the pattern of neurite outgrowth-promoting activity. One other (31G1) showed very weak binding, but bound most strongly to the fraction with the most neurite outgrowth-promoting activity. The binding of the remaining 12 did not coincide with the profile of neurite outgrowth-promoting activity. Some of these (6E8, 7E10, 21B3, 21F5, 23F8), however, appeared to recognize an antigen running slightly behind the "neurite outgrowth-promoting factor" (e.g. most concentrated in fraction "2"), which may have represented the heparan sulfate proteoglycan[7].

To test whether any of these antibodies recognized the "neurite

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[6] The pattern of laminin immunoreactivity, determined at a later date, matched this pattern, as expected.

[7] This possibility was considered because, in this CL4B column, the heparan sulfate proteoglycan did appear to run slightly behind, though overlapping with, the peak of "neurite outgrowth-promoting activity" (as judged by sulfate-labeling). This behavior is slightly different from what is reported in Chapter 4, and reflects the fact that the material used in Table A.1 had been subjected to PEG precipitation, and had undergone a certain amount of proteolysis, which altered the mobilities of both laminin and the proteoglycan (data not shown).

Table A.1. Binding of Hybridoma Supernatants to Fractions Containing the "Neurite Outgrowth-Promoting Factor"

	Fraction from Gel Filtration					
	1	2	3	4	5	6
<u>Neurite outgrowth-promoting activity</u>						
(% with neurites)	75	54	12	1	0	2
<u>Monoclonal antibody binding</u>						
4E5	+	++	++	+	-	-
5E3	+++	++	±	-	-	-
6E8	+	+++	+	-	-	-
7E10	-	+	±	-	-	-
21B3	+	+++	+	-	-	-
21F5	+	+++	+	-	-	-
21H7	+	+++	++	-	-	-
23F2	-	++	+++	+	-	-
23F8	+	+++	+	-	-	-
24D2	-	-	+	+++	+++	+
26A9	+++	++	±	-	-	-
26G7	+	++	++	+	-	-
31G1	+	-	-	-	-	-
35F6	+	++	++	±	-	-

A partially purified "neurite outgrowth-promoting factor" from BCE-CM was fractionated on Sepharose CL4B into six fractions, as described, so that the binding of 15 monoclonal antibodies (measured qualitatively by EIA) could be compared with the profile of neurite outgrowth-promoting activity in the different fractions. +++ = strongest binding.

outgrowth-promoting factor", BCE-CM was subjected to immunoprecipitation with each of them. Equivalence immunoprecipitation, using a second antibody, was employed essentially as described in Methods, except that the carrier IgG was from mouse, and the precipitating antibody was rabbit anti-mouse Ig. Unhappily, none of the antibodies tested were able to remove the "neurite outgrowth-promoting factor" from BCE-CM by immunoprecipitation. Indeed, when immunoprecipitates of [^{35}S]methionine-labeled BCE-CM were analyzed by SDS-PAGE, none of the antibodies appeared to precipitate any labeled material.

It remains possible that some of these antibodies do recognize the "neurite outgrowth-promoting factor" in BCE-CM[8]. They may bind it with low affinity, or only recognize it on a substratum, not in solution. Generation of low-affinity antibodies is a significant risk when using primary *in vitro* immunization, since the procedure of antigen "boosting" *in vivo*, which selects for high-affinity antibodies, is omitted. Similarly, when the initial screen for antibodies involves solid phase assays only, the risk that antibodies failing to bind in solution will be obtained is introduced.

Other experiments to determine whether these antibodies recognize the "neurite outgrowth-promoting factor", such as immunoblotting or immunohistochemistry, have not been done. At the time these hybridomas were generated, monoclonal antibodies that blocked neurite outgrowth-promoting activity, or could be used for immunoprecipitation or immunopurification, were what was primarily desired, and the antibodies that

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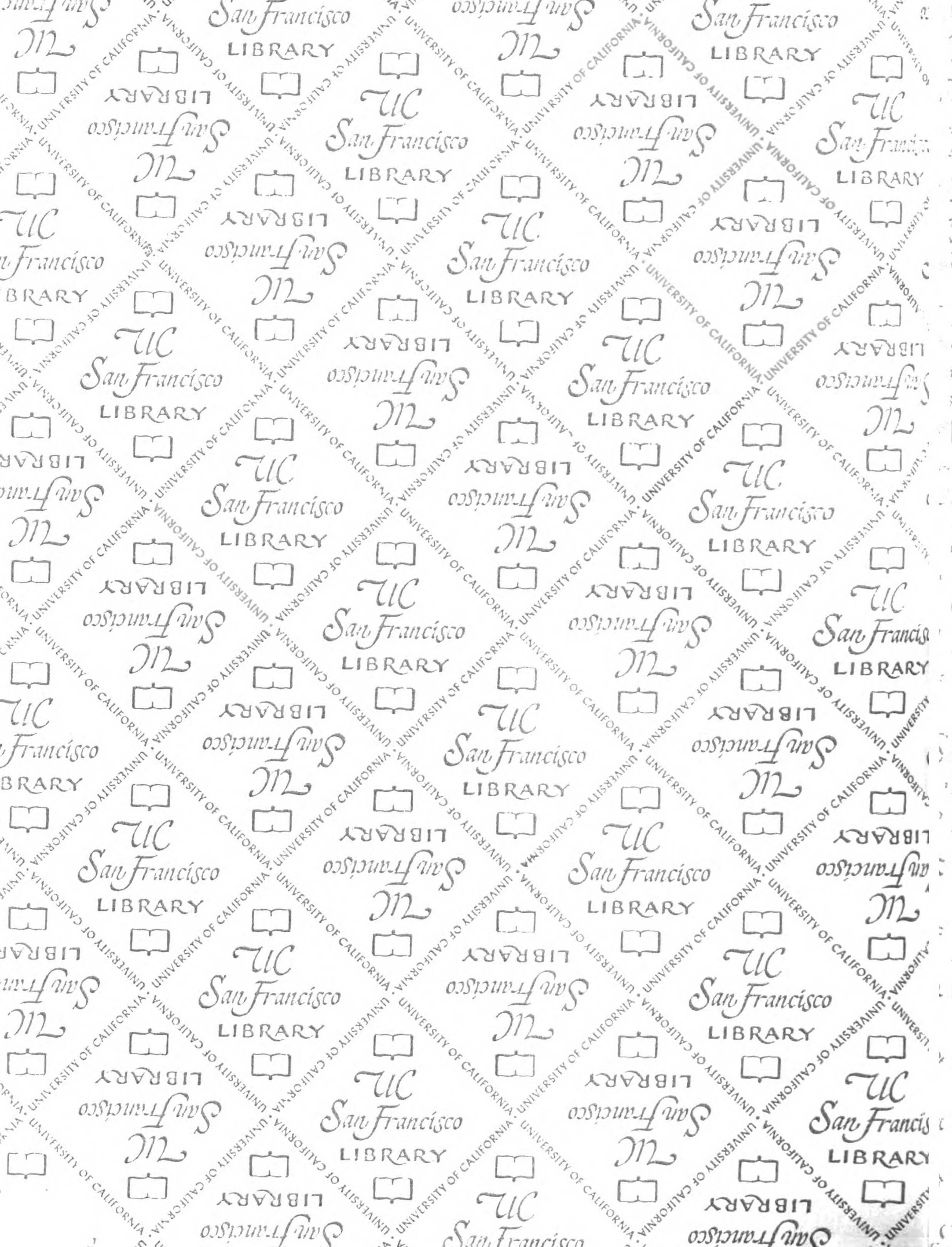
[8] Encouragingly, one of them (31G1) apparently binds, though weakly, to purified laminin from the EHS sarcoma.

were obtained were apparently unsuitable for these purposes. Fortunately, completion of the purification of the "neurite outgrowth-promoting factor" from BCE-CM, and the discovery that laminin is a component of it, obviated much of the need for monoclonal antibodies. Nevertheless, it remains possible that some of the monoclonal antibodies that were generated may be useful in the future, particularly if "neurite outgrowth-promoting factors" are found to contain an isoform of laminin different from that present in the EHS sarcoma.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