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To Arthur

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Last, but never least, I thank my mother for her love and support.

IN VITRO STUDIES OF SPINAL MOTONEURON DEVELOPMENT

Anne Leighton Calof

ABSTRACT

Spinal motoneurons from chick embryos were purified by fluorescence-activated cell sorting after having been labelled by retrograde axonal transport of a lectin-fluorochrome conjugate. When these motoneurons were plated onto polylysine-coated dishes that had been exposed to medium conditioned over cultures of embryonic chick myotubes (MCM), they rapidly extended neurites. The neurite outgrowth-promoting activity in MCM was initially characterized by enzymatic digestions and sedimentation in associative cesium chloride gradients: These experiments suggested that both protein and heparan sulfate were important in the activity of the unfractionated conditioned medium. To further characterize the neurite outgrowth-promoting activity, MCM was metabolically labelled with ^{35}S-methionine, and the activity was partially purified by salt precipitation and ion exchange chromatography and fractionated on Sepharose CL-4B. The peak of neurite outgrowth-promoting activity corresponded to the presence, in nonreducing SDS-polyacrylamide gels, of a protein band that comigrated with a laminin standard; it also corresponded to a peak of laminin immunoreactivity. Antibodies to laminin could immunoprecipitate all neurite outgrowth-promoting activity from MCM, and the specifically immunoprecipitated material comigrated with a laminin standard in both reducing and nonreducing gels. These data

suggested that laminin in MCM is responsible for the ability of this conditioned medium to promote motoneuron neurite outgrowth.

MCM in the culture medium of motoneurons also enhanced their survival over periods greater than two days in culture. This enhancement of survival could not be explained by MCM providng motoneurons with a continuous supply of the neurite outgrowth-promoting activity. The motoneuron survival-enhancing activity was sensitive to trypsin and to boiling, suggesting that a protein or proteins in MCM is responsible for this activity. In ultrafiltration experiments, most of the survival-enhancing activity passed through a filter with a nominal retention limit of 25,000 Da.

Purified fractions of two putative neuronal trophic factors were tested for their ability to enhance motoneuron survival in culture. A 56,000 Da protein, purified on the basis of its binding to antibodies that block motor nerve terminal sprouting, had no effect on motoneuron survival. The "Ciliary Neuronotrophic Factor" (CNTF), a 20,000 Da protein which supports the survival of ciliary ganglion neurons in culture, enhanced motoneuron survival to some extent. Further experiments are needed to determine whether CNTF is responsible for some or all of the motoneuron survival-enhancing activity in MCM.

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CHAPTER 1.

INTRODUCTION

CHAPTER 1.

INTRODUCTION

A. NATURALLY-OCCURRING NEURONAL CELL DEATH

What processes are the architects of the mature form of the vertebrate nervous system? Nervous system development (after determination of the neuroectoderm) proceeds in three sequential phases: cell proliferation, cell migration, and, finally, the differentiation of cells into their mature forms and the formation of specific interconnections among them. There are at least two examples of changes in this final stage of nervous system development that involve a reduction in the number of neuronal elements present in a given neuron-target system. One of these changes is a gradual decline in the number of axons that contact target cells. It has been postulated that this process of synapse elimination ensures a quantitatively appropriate distribution of synapses among target cells (e.g., Purves and Lichtman, 1980). The second change is the reduction in size of neuronal populations by the degeneration of differentiated post-mitotic neurons. This phenomenon is referred to as naturally-occurring neuronal cell death. Because many of the experiments devoted to the study of naturally-occurring neuronal cell death have examined the development of neurons with cellular processes in peripheral tissues, this discussion will focus on these systems.

The first evidence for the existence of naturally-occurring neuronal cell death was reported by Hamburger and Levi-Montalcini

(1949), who showed that a massive loss of neurons in spinal sensory ganglia occurred during normal development. Since that time, naturally-occurring neuronal cell death has been observed in many populations of central and peripheral neurons (reviewed in Oppenheim, 1981a). Naturally-occurring neuronal cell death is widespread among different vertebrate species and different developing neuronal populations within these species. The number of neurons that dies can be very large in a given population; for example, about 60% of the neurons in the chick isthmo-optic nucleus degenerate during the course of normal development (Cowan and Clarke, 1976). Thus, naturally-occurring neuronal cell death appears to be a major regulatory mechanism in vertebrate development, and the factors that regulate it are of great interest. Many populations of neurons have been studied in an attempt to understand the regulation of naturally-occurring neuronal cell death, and by far the best-studied among these are the spinal motoneurons of the chick embryo.

During embryogenesis, the chick nervous system generates about twice as many motoneurons as the adult organism will possess (Hamburger, 1975). This "excess" of motoneurons is eliminated during the period of naturally-occurring motoneuron death, whose onset coincides with the time at which characteristic motoneuron projection patterns are established (Landmesser, 1978). The period of greatest cell death lasts for about three to four days, between days 6 and 10 <u>in ovo</u> (Hamburger, 1975). The precise factors governing naturally-occurring motoneuron death have yet to be established. It could be that a percentage of motoneurons is

genetically programmed to die, but several observations argue that naturally-occurring motoneuron death is regulated to a great extent by interactions of the spinal motoneuron with its peripheral target tissue, skeletal muscle. For example, motoneurons that will eventually die have sent axons to the periphery prior to their deaths (Chu-Wang and Oppenheim, 1978b). Reduction or enlargement in the amount of target tissue available for innervation -- generally by ablation or implantation of limb tissue during embryonic development -- can increase or decrease, respectively, the extent of motoneuron death (Hamburger, 1958; Chu-Wang and Oppenheim, 1978a; Hollyday and Hamburger, 1976). Furthermore, a functional neuromuscular interaction must somehow be involved, since paralysis of embryos by pharmacological agents during the period of cell death can drastically decrease the number of motoneurons that die (Pittman and Oppenheim, 1979). Thus, the evidence supports the idea that epigenetic events occurring as a consequence of motoneuron-muscle contact may mediate much of naturally-occurring motoneuron death.

In addition to target-tissue effects on naturally-occurring motoneuron death, a recent study indicates a contribution of afferent inputs to this phenomenon. Okado and Oppenheim (1984) have shown that elimination of supraspinal or sensory inputs to the lumbar spinal cord does not affect the extent of motoneuron death by day 10 *in ovo*, but appears to cause an overall increase in motoneuron death by day 16 *in ovo*. The extent of this increase in motoneuron death was greater if both deafferentation procedures were performed, but the maximum increase in motoneuron death that could result from

these procedures was never as great as that resulting from limb ablation. Naturally-occurring motoneuron death may therefore be regulated both by motoneuron-muscle interactions, and by afferent-motoneuron interactions.

B. INFLUENCES OF SURROUNDING TISSUES ON NEURONAL DEVELOPMENT

1. POSSIBLE REGULATION OF NATURALLY-OCCURRING NEURONAL CELL DEATH BY TROPHIC FACTORS.

The means by which muscle might influence the survival of motoneurons during embryogenesis has not yet been established. It is possible that the neuromuscular interaction regulating naturally-occurring motoneuron death involves a diffusible substance, or substances, produced by muscle. Such a substance could conceivably regulate motoneuron survival if it were produced in limiting amounts by muscle, and if motoneurons were dependent on it for their survival. The notion that such substances (now generally referred to as "trophic factors") are important in the regulation of neuronal cell death was first proposed by Hamburger and Levi-Montalcini (1949) as one possible explanation for the naturally-occurring and experimentally-induced death that they observed in spinal ganglion neurons. This model for the biological basis of naturally-occurring motoneuron death is supported, albeit indirectly, by the existence of Nerve Growth Factor and the knowledge of its effects on induced and naturally-occurring cell death in sympathetic and sensory ganglia.

a. NERVE GROWTH FACTOR AS A MODEL

Nerve Growth Factor (NGF) is, to date, the only macromolecule identified that appears to fulfill the requirements for a trophic substance regulating naturally-occurring neuronal cell death. The effects of NGF were first demonstrated *in vivo*. In a landmark series of studies, Levi-Montalcini and Hamburger demonstrated that the transplantation into embryos of certain mouse sarcomas caused hyperplasia of the sympathetic and spinal sensory ganglia (Levi-Montalcini and Hamburger, 1951). The source of the hyperplasia proved to be a diffusible factor released from the sarcoma tissue (Levi-Montalcini, 1952; Levi-Montalcini and Hamburger, 1953). This factor was subsequently purified from a variety of sources and shown to be a protein which was called Nerve Growth Factor (reviewed in Thoenen and Barde, 1980, and Yanker and Shooter, 1982).[1]

The claim for a physiological role for NGF -- in the regulation of naturally-occurring cell death in sympathetic and sensory ganglia -- rests primarily on two lines of evidence. First, NGF treatment of fetal and neonatal animals has been shown to reduce both experimentally-induced and naturally-occurring cell death in these tissues (Levi-Montalcini et al., 1975; Hendry and Campbell, 1976; Kessler et al., 1979; Hamburger et al., 1981). Second, chronic administration of anti-NGF antibodies to fetal and neonatal animals can cause destruction of sensory and sympathetic ganglia

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[1] The various forms in which NGF can be isolated, the number and structures of the subunits of these forms, and the chemistry of NGF, are all discussed in exhaustive detail in these reviews; no such discussion will be attempted here.

(e.g., Levi-Montalcini and Booker, 1960; Gorin and Johnson, 1979). It is also significant that degeneration of sympathetic neurons resulting from surgical or chemical axotomy of sympathetic ganglia can be prevented by systemic administration of NGF (Angletti and Levi-Montalcini, 1970; Hendry, 1975; Levi-Montalcini et al., 1975). These studies suggest that retrograde axonal transport of NGF from target tissues may be the means by which sympathetic neurons obtain the trophic support necessary for their survival during development, and it has been shown that radiolabelled NGF can be retrogradely transported by sympathetic neurons (Hendry et al., 1974; Dumas et al., 1979). Only recently, however, have the presence of NGF and the expression of the NGF gene been demonstrated in the target tissues of sympathetic and sensory neurons (Ebendal et al., 1980; Korsching and Thoenen, 1983; Shelton and Reichardt, 1984). It is not yet known which cells in these tissues produce the NGF that has been detected in these studies.

b. IN VITRO BIOASSAYS FACILITATE THE PURIFICATION OF OTHER PUTATIVE NEURONAL TROPHIC FACTORS

The development of an in vitro bioassay, which allowed the effects of tissue extracts and purified fractions to be investigated under controlled experimental conditions, was essential to the purification of NGF (Levi-Montalcini et al., 1954; Cohen et al., 1954; Cohen, 1960). This now-standard bioassay is the outgrowth of neurites from cultured chick sensory ganglia in response to NGF or NGF-like activity. Because of this success in the purification of

NGF, the use of similar tissue culture systems has become commonplace in the search for new neuronal growth factors that might play a role in the regulation of naturally-occurring neuronal cell death in systems other than the sympathetic and sensory ganglia (reviewed in Barde et al., 1983; Berg, 1984).

The purification of the "Ciliary Neuronotrophic Factor" (CNTF) is a successful case of the use of *in vitro* bioassay systems for the isolation of a putative neuronal growth factor (Barbin et al., 1984). The neurons of the parasympathetic ciliary ganglion undergo a period of naturally-occurring cell death, during which about half of the neurons die (Landmesser and Pilar, 1974); however, all of the neurons can survive in dissociated cell culture if they are taken from embryos prior to the onset of this period, and if they are grown in the presence of muscle cells or muscle cell conditioned medium (Helfand et al., 1976; Nishi and Berg, 1977; Nishi and Berg, 1979). NGF is not the active factor, because the survival of ciliary ganglion neurons is not supported by NGF alone, and NGF does not enhance the ability of conditioned medium to support ciliary neuron survival in culture (Helfand et al., 1976). Muscle cells of the eye are the targets of ciliary neurons *in vivo*, and a survey of extracts of different embryonic tissues showed that the survival of ciliary neurons *in vitro* was best supported by extracts of embryonic eye tissue (Adler et al., 1979). The survival activity present in eye tissue extracts was shown to increase during the period of naturally-occurring ciliary neuron death (Landa et al., 1980), and the dependence of cultured ciliary neurons on CNTF has

been shown to appear and disappear over roughly the same time course (Manthorpe et al., 1981; Adler and Varon, 1982). Thus, CNTF is a reasonable candidate for a diffusible, target tissue-derived trophic substance that may play a role in regulating the naturally-occurring death of ciliary neurons.[2]

The presence of NGF enhances the survival *in vitro* and *in vivo* of some, but not all, of the neurons from the spinal sensory ganglia (also known as dorsal root ganglion, or DRG, neurons) (Levi-Montalcini and Angeletti, 1968; Weiss, 1971; Greene, 1977). Barde and his colleagues first observed the effects of a putative second trophic factor for DRG neurons, when they noticed that medium conditioned over cultures of C6 glioma cells could support the survival of DRG neurons in the absence of exogenous NGF (Barde et al., 1978). Survival of and neurite extension by DRG neurons grown in glioma conditioned medium (GCM) were not as great as those of neurons grown in the presence of NGF. However, while antibodies to NGF abolished the effect of NGF on DRG neurons, these antibodies did not affect the survival of DRG neurons in GCM, suggesting that the effects of GCM and NGF were mediated by different substances (Barde et al., 1978). Because the survival-enhancing effect of GCM on DRG neurons was sensitive to protease treatment, and in view of the results of the antibody blocking experiments, the

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[2] Nishi and Berg (1981) have devised culture conditions under which ciliary neurons can survive for periods of over two weeks in the absence of tissue extracts or conditioned medium. However, under these conditions, a component of eye extract that is probably identical to CNTF has been shown to stimulate the *growth* of ciliary neurons, as shown by the increase in levels of a cytoplasmic enzyme (Berg, 1984).

authors concluded that a protein -- distinct from NGF and released by cells of central nervous system origin -- might support the survival of sensory neurons during their phase of naturally-occurring cell death (Barde et al., 1978). They subsequently demonstrated that the survival requirements of cultured embryonic chick DRG neurons changed if the neurons were taken from embryos of different ages: NGF supported the survival of neurons taken from embryos 8 to 12 days old, but GCM enhanced survival of neurons from older embryos (12 to 16 days), when these neurons are declining in their responsiveness to NGF (Barde et al., 1980). It was noted at this time that the survival-enhancing activity of GCM could be mimicked by extracts of mammalian brain, and it was from this source -- readily available in great quantity -- that a protein with the ability to support sensory neuron survival in culture was purified (Barde et al., 1980; Barde et al., 1982). This protein has been proposed as a second trophic factor for sensory ganglion neurons (Barde et al., 1982).

The discovery of the mammalian brain-derived factor that acts on chick DRG neurons, and studies of the "Ciliary Neuronotrophic Factor" (CNTF), which acts on chick ciliary neurons, have raised an important issue that bears on the use of in vitro bioassay systems in the study of neuronal trophic factors. It is apparent that, in vitro at least, DRG neurons respond to at least two identified substances that fulfill several of the requirements for a role as diffusible trophic substances regulating naturally-occurring neuronal cell death: NGF and the brain-derived survival factor. In fact,

there has been one report that DRG neurons can also respond to CNTF (Barbin et al., 1984). There are two possible explanations, which are not necessarily exclusive of one another, for these results. First of all, there may be many substances which act as diffusible trophic factors in the mediation of naturally-occurring neuronal cell death for a given neuronal cell type, at least in the peripheral nervous system. Since naturally-occurring cell death in these systems has not yet been shown to be prevented completely by administration of any one of these putative trophic agents, this hypothesis remains plausible. Such a dependence on multiple trophic substances may be reflected in the changing trophic requirements exhibited by neurons cultured from embryos of different ages, as discussed above. Second, it is possible that trophic factors have, in vitro, a range of effectiveness that extends beyond their normal range in vivo. This expansion of effectiveness in supporting neuronal survival could derive from changes in neuronal properties when the neurons are removed from the embryo and placed into culture. Alternatively, in vivo, neurons could be restricted to utilizing only one among many possible trophic substances because of physical restrictions on the accessibility of those substances.

Whether one or both of the two explanations for the wide range of neuronal responsiveness to these putative neuronal trophic factors is correct, is not known. It is clear, however, that while the brain-derived DRG survival factor and the "Ciliary Neuronotrophic Factor" are reasonable candidates for diffusible trophic substances that might mediate naturally-occurring neuronal

cell death, their role in this developmental process is by no means proved. For neither of these proteins have the *in vivo* studies, which would support the notion of a role for these substances in normal development, been performed. Such studies, involving the inactivation of these factors by antibodies or the supplementation of their endogenous levels by local or systemic administration, would help to resolve some of the issues that have arisen as the number of putative neuronal trophic factors increases.

Despite these cautionary remarks, however, it must be remembered that the isolation and purification of putative growth factors using *in vitro* bioassays is probably the most direct, and certainly the easiest, method for beginning such studies. The success in the purification of NGF still stands as an example in this regard.

2. ENVIRONMENTAL INFLUENCES ON OTHER ASPECTS OF NEURONAL DEVELOPMENT

In addition to their effects on neuronal survival, the tissues in the periphery affect neuronal development in other ways. The peripheral tissues through which neuronal processes grow appear to direct the pattern of innervation in developing limbs. This control of nerve pattern by the periphery is sufficiently great to ensure a normal pattern of peripheral nerves even in supernumerary or rotated limbs, when these limb surgeries have been performed prior to the first outgrowth of neuronal processes (e.g., Morris, 1978; Summerbell and Stirling, 1981). The rate of nerve outgrowth can also be controlled to some extent by the periphery, since innervation of

grafted limbs from embryos of different ages occurs with the time course of innervation appropriate to the age of the graft, not the age of the host (Swanson and Lewis, 1982). The notion that the information provided by the limb tissues may reside in their extracellular matrix gains support from studies in which reinnervation of previously-innervated sites occurs when underlying muscle cells have been disrupted or killed, but the extracellular matrix remains intact (Marshall et al., 1977; Sanes et al., 1978).

a. EFFECTS OF THE SUBSTRATUM ON NEURITE OUTGROWTH *IN VITRO*

In an attempt to determine likely candidates for molecules that might direct the patterns of neuronal process outgrowth *in vivo*, many investigators have used *in vitro* bioassay systems to isolate substances that affect the extension of cellular processes (neurites) from cultured neurons. Two major approaches have been used with these *in vitro* assays: The effects on neurite outgrowth of substrata treated with conditioned medium or tissue extracts have been assessed, in order to identify molecules from these sources that might be important in neuronal process extension. Alternatively, identified molecules -- primarily derived from the extracellular matrix -- have been tested for their ability to promote neurite outgrowth from different types of neurons.

Conditioned medium from a large number of cell types contains a substance which can adsorb to a polycationic culture substratum and promote rapid, extensive growth of processes from neurons grown on that subtratum (Adler et al., 1981; Lander et al., 1982). This

neurite outgrowth-promoting factor, first demonstrated by Collins (1978) to be present in heart cell conditioned medium and to act on chick ciliary ganglion neurons, has since been shown to act on a variety of neuronal types. These include sympathetic neurons, dorsal root ganglion neurons, embryonic retinal neurons, and spinal cord neurons (Coughlin et al., 1981; Adler et al., 1981; Lander et al., 1982). The similarities in range and mode of action, and in physical and chemical characteristics, have led to the consensus that the "neurite outgrowth-promoting factors" from all of these sources are probably the same (Manthorpe et al., 1981; Lander et al., 1982; Coughlin and Kessler, 1982). Recent biochemical and immunochemical studies have identified the active factor as a large glycoprotein, laminin (Lander et al., 1985a,b). In conditioned medium, this laminin appears to be found in association with heparan sulfate proteoglycan. In addition, in conditioned medium from several mammalian cell types, a glycoprotein identified as entactin has been found complexed with laminin and the proteoglycan (Lander et al., 1985a).

Laminin is a major constituent of extracellular matrices (Timpl et al., 1979). Thus, the finding that the active factor from conditioned medium is laminin is consistent with a role for components of the extracellular matrix in directing neuronal process outgrowth. In addition, laminin has been identified in regions of neuronal process outgrowth, such as the spinal cord ventral roots, during periods in embryonic development in which this outgrowth is commencing (Rogers et al., 1984); this result is consistent with

the possibility that laminin may provide guidance cues along early axonal pathways.

Purified laminin has been shown to promote rapid and profuse neurite outgrowth by many types of neurons; of other extracellular matrix components tested, only fibronectin has been shown to promote neurite outgrowth when applied to tissue culture substrata (Akers et al., 1981; Manthorpe et al., 1983; Rogers et al., 1983; Baron-Van Evercooren et al., 1982; Lander et al., 1983, 1985a). However, while the effects on neurite outgrowth of laminin-treated substrata are indistinguishable from the effects of substrata treated with conditioned medium (Davis et al., 1984), the neurite outgrowth-promoting effects of fibronectin are both quantitatively and qualitatively different from the effects of laminin and conditioned medium (Baron-Van Evercooren et al., 1982; Manthorpe et al., 1983; Rogers et al., 1983). In all of the studies cited above, fibronectin-treated substrata have been shown to support less extensive outgrowth, from fewer classes of neurons, than either laminin- or conditioned medium-treated substrata.

b. EFFECTS OF THE SUBSTRATUM ON NEURONAL RESPONSIVENESS TO TROPHIC SUBSTANCES.

The substratum on which a neuron grows may affect not only the rate and extent of neuronal process outgrowth; it may also affect neuronal requirements for diffusible trophic substances, such as those discussed earlier in this chapter. Because of the dramatic effects on neuronal morphology of substrata treated with conditioned

medium factors and isolated extracellular matrix molecules, investigators have been prompted to examine the ability of these substrata to support neuronal survival in vitro.

In several cases, it has been demonstrated that certain classes of neurons, which normally display an absolute requirement for a trophic substance in order to survive in vitro, can survive for a short time in the absence of such trophic support if they are cultured on appropriate substrata. For example, rat sympathetic neurons must normally be grown in the presence of NGF in order to survive for even one day in culture (Chun and Patterson, 1977). However, Lander and colleagues have shown that these neurons can survive for more than two days when grown on substrata treated with the neurite outgrowth-promoting factor from bovine corneal endothelial cell conditioned medium, or on the extracellular matrix deposited by these cells (Lander et al., 1982). Similarly, ciliary ganglion neurons normally require the presence of conditioned medium or the "Ciliary Neuronotrophic Factor" in order to survive in culture (Helfand et al., 1979; Varon et al., 1979; Nishi and Berg, 1979; Barbin et al., 1984). However, if these neurons are grown on hypotonically-lysed fibroblasts, they can survive for five days in culture in the absence of CNTF or any other medium supplements (Nishi and Berg, 1981). In both cases described above, the neurons eventually die unless the medium is supplemented (Lander et al., 1982; Nishi and Berg, 1981). However, for a short time, the presence of an appropriate substratum alone appears to be able to sustain the growth of these neurons.

The survival of sympathetic neurons in culture has been shown to be modulated in another way by the substratum on which they are grown. If these neurons are grown on substrata treated with heart cell conditioned medium, the percentage of neurons that survives in the presence of maximal concentrations of NGF is increased, suggesting that an additional population of sympathetic neurons is surviving under these culture conditions (Edgar and Thoenen, 1982). In addition, examination of neuronal responsiveness at varying levels of NGF showed that the effective dose of NGF required for half-maximal survival was reduced ten-fold when sympathetic neurons were grown on substrata treated with heart cell conditioned medium (Edgar and Thoenen, 1982). Subsequent studies by this group have demonstrated that purified laminin can substitute for heart cell conditioned medium in modulating the effects of NGF on sympathetic neuron survival (Edgar et al., 1984). Thus, it may be the case that peripheral tissues can not only direct the pattern of innervation by outgrowing neurons; they may also affect the survival of neurons by modulating their responsiveness to diffusible trophic substances.

c. OTHER FACTORS AFFECTING NEURONAL SURVIVAL AND DEVELOPMENT

It is clear that neurons require more than target-specific trophic substances and an appropriate substratum in order to survive in culture. Development of defined media that support neuronal growth <u>in vitro</u> has demonstrated that polypeptide hormones such as insulin are essential (Bottenstein et al., 1980). In addition, several laboratories have recently identified protease-resistant,

low molecular weight substances that are present in conditioned medium and can enhance the survial of certain classes of central neurons (e.g., Muller et al., 1984; Varon et al., 1984). These low molecular weight substances are found in medium conditioned over cultures of glial cells (Muller et al., 1984; Varon et al., 1984). It is probably the case that several kinds of trophic support, including that provided by the nonneuronal cells surrounding neuronal cell bodies, that provided by the tissues through which neuronal processes extend, and that provided by the circulation, are necessary for neuronal growth and development. The knowledge that the *in vitro* growth of neurons can be affected by many such substances requires that the effects of putative specific "neuronal trophic factors" be interpreted with caution. An *in vitro* bioassay is merely the first step in evaluating a role for putative trophic substances *in vivo*.

C. AN *IN VITRO* SYSTEM FOR STUDYING ENVIRONMENTAL EFFECTS ON MOTONEURON DEVELOPMENT

The evidence that the presence of limb tissue is critical for the survival of motoneurons during embryogenesis was discussed earlier in this chapter. The possibility that muscle might influence motoneuron survival by providing motoneurons with an essential trophic substance was mentioned, and a model for the biological basis of naturally-occurring motoneuron death -- based on the effects of Nerve Growth Factor on sympathetic and sensory neurons -- was suggested. Have any candidates for a "motoneuron trophic factor"

been identified?

The presence of muscle cells, muscle cell conditioned medium, or muscle extracts, has been shown to enhance survival (Bennett et al., 1980; Longo et al., 1982; Slack and Pockett, 1982; Tanaka and Obata, 1982; Tanaka et al., 1982; Eagleson and Bennett, 1983; Nurcombe et al., 1984; Doherty et al., 1985) and neurite outgrowth (Dribin and Barrett, 1980, 1982 a,b; Obata and Tanaka, 1980; Henderson et al., 1981, 1983, 1984; Pollack et al., 1981; Hsu et al., 1982; Smith and Appel, 1983; Nurcombe and Bennett, 1983; Nurcombe et al., 1984; Doherty et al., 1985) by cultured spinal cord explants and dissociates. However, in studies using whole spinal cord cultures, it is impossible to distinguish effects on motoneurons from effects on other classes of spinal cord cells, which vastly outnumber motoneurons in these cultures.[3] Although it is possible to identify some of the motoneurons present in mixed cultures (e.g., Bennett et al., 1980), the motoneurons must, nevertheless, be maintained in the company of other cell types. Under these circumstances it is difficult to determine whether the effects of putative trophic agents on motoneurons are direct, or are instead secondary to effects on the other cells in the culture.

Until recently, it was impossible to obtain identified motoneurons, separated from other spinal cord cells, and culture them.

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[3] Estimates for the spinal cord of 4- to 5-day chick embryos (based on a calculated number of about 5×10^4 motoneurons and $1\text{-}1.5 \times 10^6$ total cells per spinal cord) indicate that motoneurons make up about 5% of the total cells in the embryonic cord at this age. This is the age at which spinal cords are usually taken for cell culture (cf. Hollyday and Hamburger, 1977; Barald and Berg, 1979; Oppenheim, 1981b).

This has been remedied by the development of a technique for purifying avian motoneurons, which utilizes the motoneurons' capacity to retrogradely transport a lectin-fluorochrome conjugate that has been injected into limb muscles *in ovo* (McPheeters and Okun, 1980; Okun, 1981). A fluorescence-activated cell sorter is used to separate the fluorescent motoneurons from the other cells of dissociated spinal cords, yielding purified populations of motoneurons for study.

Identification of substances affecting the survival and development of spinal motoneuron *in vitro* can be accomplished, now that pure populations of motoneurons are available for study. In the work described in this dissertation, a modification of the method of McPheeters and Okun (1980) has been used to obtain purified motoneurons from avian embryos at the stage just before they undergo their phase of naturally-occurring cell death. The method is described in detail in Chapter 2. The first goal has been to clarify the growth conditions for purified motoneurons, and these experiments have focused on the effects of conditioned medium, obtained from cultures of embryonic chick myotubes, on motoneuron survival and neurite outgrowth. Two separable actions of myotube conditioned medium will be described. The first, a neurite outgrowth-promoting activity, is discussed in Chapter 3. The second action of myotube conditioned medium is to enhance the survival of motoneurons over longer periods in culture. This survival-enhancing activity is discussed in Chapter 4. Chapters 5 and 6 contain descriptions of tests of the ability of two purified proteins -- both putative

"neuronal trophic factors" -- to enhance motoneuron survival.

A summary of the work presented in the previous chapters, conclusions that can be drawn from this work, and directions for future research, are discussed in Chapter 7.

CHAPTER 2.

PURIFICATION OF EMBRYONIC SPINAL MOTONEURONS

CHAPTER 2.

PURIFICATION OF EMBRYONIC SPINAL MOTONEURONS

Previous investigators have attempted to enrich spinal cord cultures with motoneurons by velocity sedimentation in Ficoll (Berg and Fischbach, 1978), separation using iso-osmotic metrizamide density gradients (Schnaar and Schaffner, 1981), or the use of mitotic inhibitors in cultures obtained from very young embryos (Masuko et al., 1979). Although all of these studies have shown that the resulting cultures are enriched for the presence of cholinergic cells and/or neurons that will synapse upon skeletal muscle, none has provided direct evidence that the cells in these cultures are ventral horn motoneurons. For the present study, the technique of McPheeters and Okun (1980) was used, since it is the only method thus far devised for obtaining identified motoneurons separated from other spinal cord cells. This chapter describes in detail a modification of the method of McPheeters and Okun (1980), which has been used to obtain purified motoneurons from avian embryos at the stage just before they undergo their phase of naturally-occurring cell death.

A. MATERIALS

Fertile White Leghorn chicken eggs (Feather Hill Farm, Petaluma, California) were incubated at 99-101°F in a humidified, forced-draft incubator. Lucifer Yellow VS for pilot studies was the kind gift of Dr. Walter Stewart (National Institutes of Health, Bethesda,

Maryland). Later, it was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Cell culture supplies were purchased from the U.C.S.F. Cell Culture Facility. All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Missouri) unless specifically noted.

B. PREPARATION OF FLUORESCENT CONJUGATE

To 20 mg of wheat germ agglutinin in 2 ml of buffer (150 mM Na borate, pH 9.0) was added 20 mg of Lucifer Yellow VS. The mixture was incubated for 1 hour at 37°C (Okun, 1981). The fluorescent conjugate was separated from unbound dye on a column (7 cm x 1.5 cm) of Biogel P-2 (200-400 mesh wet; BioRad Corporation, Richmond, California) that had been equilibrated in 145 mM ammonium bicarbonate, 5 mM Tris-HCl, pH 7.8. Colored fractions eluting in the void volume of the column were pooled, divided into aliquots, lyophilized on a Speed Vac Concentrator (Savant Instruments, Hicksville, New York), and stored at -20°C. Immediately before use, the conjugate was reconstituted to 20-25 mg/ml protein in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4).

C. INJECTION AND TRANSPORT

Six day chick embryos (stages 28-29; Hamburger and Hamilton, 1951) were removed from the egg, decapitated, eviscerated, and pinned out in Sylgard-coated (Dow Corning Corp., Midland, Michigan)

60 mm petri dishes containing Leibovitz L-15 medium supplemented with penicillin (100 U/ml) and streptomycin (100 ug/ml). Muscle masses in all four limbs were injected with the fluorescent conjugate (approximately 1 ul/limb), and the embryos were washed twice in L-15 and placed in 3.5 ml per dish of organ culture medium (see below). At this point, a partial ventral laminectomy was performed to ensure proper oxygenation of the cord during incubation: Several segments of vertebral cartilage were removed from the region between the cervical and lumbar enlargements, and cephalad to the cervical enlargement, by careful dissection. Organ culture medium consisted of Dulbecco's Modified Eagle's medium (DME) containing 0.45% glucose, with sodium chloride and sodium bicarbonate concentrations adjusted for pH 7.2 at equilibrium with 5% CO_2 (1.85 g/l $NaHCO_3$; 7.68 g/l NaCl). This DME was supplemented with 10% horse serum (Kansas City Biologicals), 2.5% chick embryo extract (Gibco), 2 mM glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin. The embryos were incubated for 20-24 hours at 30°C on a rocking platform in a humidified chamber equilibrated with 95% O_2-5% CO_2. Embryos used for negative controls were prepared exactly as described above, except that their muscles were not injected.

D. DISSOCIATION AND SORTING OF MOTONEURONS

Spinal cords were dissected out of the embryos, freed of meninges and dorsal root ganglia, and placed in calcium- and magnesium-free Dulbecco's Phosphate-Buffered Saline (CMF-PBS) supplemented with 0.6% glucose, penicillin (100 U/ml), and strep-

tomycin (100 ug/ml). The cords were minced and incubated for 11 minutes at 37°C in 0.2% trypsin (Sigma Type III-S). The digestion was stopped by the addition of 4 ml of 5.5 mg/ml soybean trypsin inhibitor (Sigma Type I-S) and the cord fragments were collected and washed into BSA-L15 holding medium (L-15 supplemented with 0.025% bovine serum albumin [BSA; Fraction V, Sigma], 0.6% glucose, 2mM glutamine, and antibiotics at above concentrations). Cords were dissociated into single cells by gentle trituration through a pasteur pipette and filtered through monofilament nylon mesh with 37 um mesh openings (Small Parts, Inc., Miami, Florida). Single-cell suspensions of 7 ml were underlaid with 2 ml of a sterile solution of 4% BSA in L-15 and centrifuged in a 15 ml conical centrifuge tube (Corning) for 10 minutes at 100 x g to remove small debris. Pelleted cells were resuspended in BSA-L15 holding medium and re-filtered through the 37 um mesh prior to sorting.

Cell suspensions were sorted using a Becton-Dickenson FACS IV with 18 watt argon ion laser illumination (Spectra Physics, Mountain View, California) and a logarithmic amplifier (Becton-Dickenson, Sunnyvale, California). The 458 nm emission line of the laser (280 mW) was used for excitation. Fluorescence emission from cells was passed through a 475 nm long-pass filter and detected with a photomultiplier (EN-330KC, EMI-Gencom Inc., Planview, New York) operated at 380 V.

Since all cells may autofluoresce weakly in this range, each sort was calibrated using spinal cord cells from uninjected embryos as a negative control. The negative offset of the logarithmic

amplifier was adjusted so that less than 0.5% of light scatter events in the negative control showed fluorescence above a standard intensity (e.g., Channel 55 of 255). The positive sample was then analyzed with the logarithmic amplifier calibrated according to this standard. The positive sample normally contained 3-9% of scatter events with fluorescence intensities above the negative level (i.e., from channel 55 to channel 255). This fraction of the positive sample was sorted as labelled motoneurons. The sorter profile from a typical experiment is shown in Figure 1. Samples from each sort were independently analyzed for purity by counting numbers of labelled cells in the fluorescence microscope (Zeiss Blue Violet filter set 487705: BP 400-440 nm, LP 470 nm).

E. PURITY OF SORTED MOTONEURONS

The rationale of the motoneuron sorting technique requires that motoneurons are the only cells within the spinal cord that take up and accumulate the fluorescent conjugate when it is injected into limb muscles. In the present study, the extent of labelling in the spinal cords of injected embryos was evaluated by examining cryostat sections of formaldehyde-fixed spinal cords in the fluorescence microscope. A comparison of fluorescence and phase micrographs of such a section (Figure 2) shows that the fluorescent conjugate is found only in large cells of the ventral horn, presumed to be motoneurons. Although labelling of spinal cord interneurons by retrograde transsynaptic transport from motoneurons has been reported (Harrison et al., 1984), no evidence for such labelling

FIGURE 1.

CELL SORTER PROFILES OF SPINAL CORD DISSOCIATES

Spinal cords were dissociated into single cells and analyzed with a Becton Dickenson FACS IV cell sorter equipped with logarithmic amplification, as described in the text. This figure shows superimposed profiles of dissociates from negative (uninjected) embryos and positive (injected) embryos from one experiment. A. Light scattering profiles. Approximately 70% of all light scattering events in each sample were contained within the broad peak, which represents the cells. The light scattering profiles of positive and negative samples are essentially identical and appear superimposed. B. Fluorescence profiles. a. Positive sample from injected embryos. b. Negative sample from uninjected embryos. In this experiment, the positive sample (a.) had 8% of its light scattering events with fluorescence intensities above that designated by channel 55. The negative sample (b.) had 0.5% of its light scattering events with fluorescence in this range.

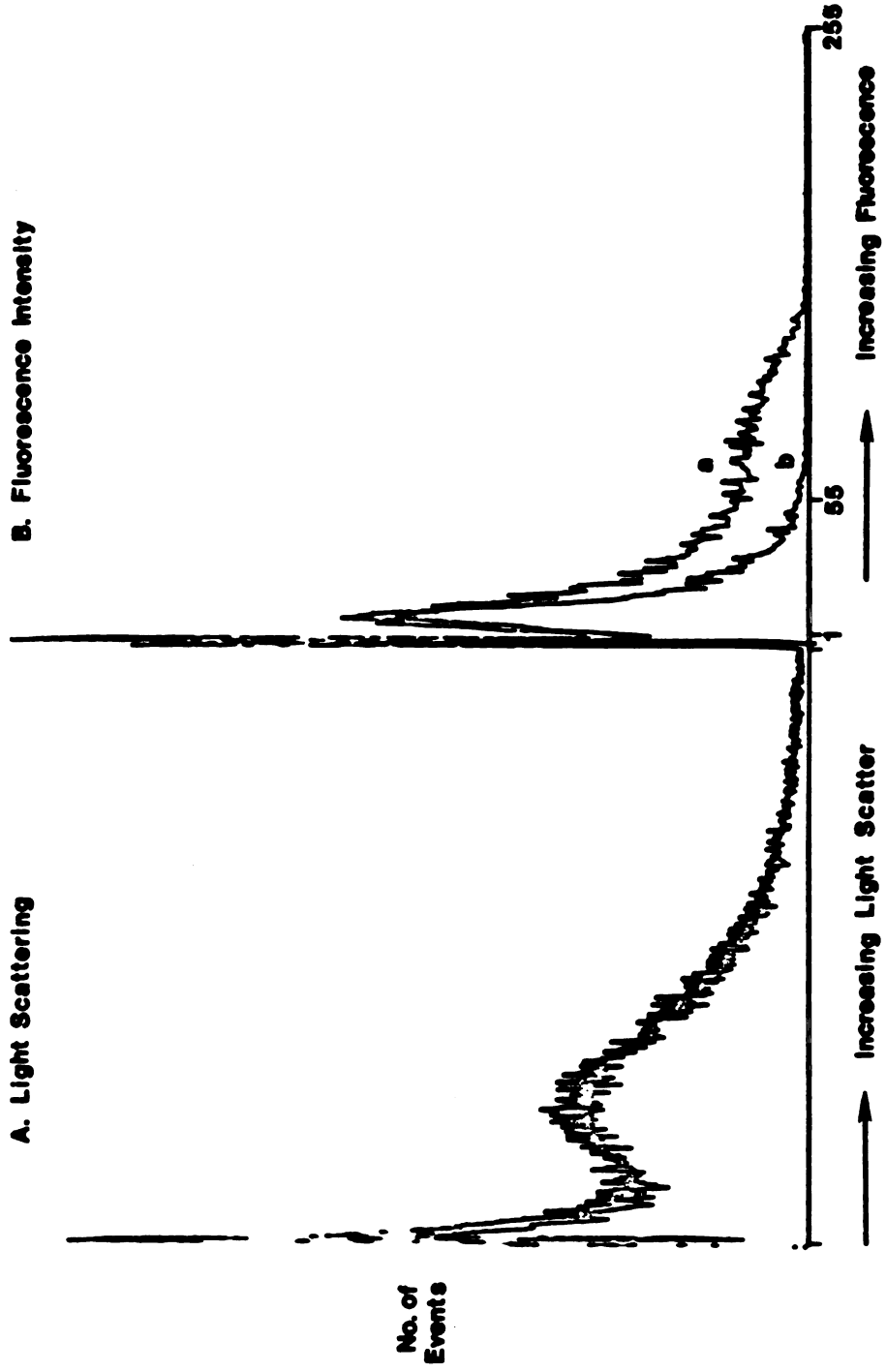
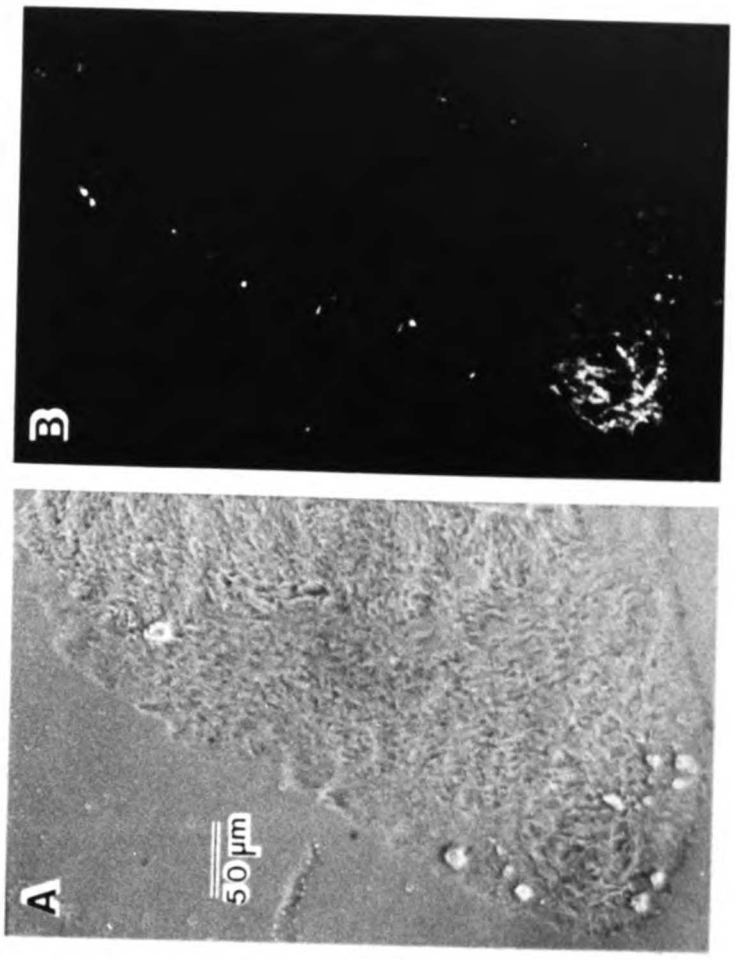

A. Light Scattering
B. Fluorescence Intensity
No. of Events
Increasing Light Scatter
Increasing Fluorescence
1
65
256
a
b

FIGURE 2.

SECTION OF EMBRYONIC CHICK SPINAL CORD LABELLED BY RETROGRADE TRANSPORT

A stage 29 chick embryo was prepared for organ culture and its limb muscles were injected with fluorescent conjugate as described in the text. After 24 hours, the spinal cord was dissected out, fixed in 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.4, and sectioned on a cryostat. A. Phase contrast picture of spinal cord hemisection. B. Fluorescence micrograph of same section.


B
A
50 µm

was found in the present experiments. The extensive transsynaptic labelling of interneurons reported by Harrison et al. (1984) required several days transport time at 37°C; the organ culture conditions used in the present experiments (Section 2.C.) probably did not permit such labelling to occur. Examination of sections from whole embryos has shown that some cells of the dorsal root ganglia are labelled (data not shown), but these cells are not of concern because spinal cords are carefully stripped of meninges and dorsal root ganglia prior to dissociation and sorting. During the course of dissociation and sorting, the Lucifer Yellow-wheat germ agglutinin conjugate remains within the motoneurons, and does not appear to be transferred to other cells (McPheeters and Okun, 1980; McPheeters, 1982).

For the reasons described above, the presence of three or more brightly-fluorescing granules inside a sorted cell qualified that cell as a motoneuron in the present study. Motoneurons retained the fluorescent label for at least 24 hours after dissociation and plating into cultures, and it did not appear to prevent their survival and development of neurites in culture. Figure 3 shows phase and fluorescence micrographs of two motoneurons grown for 24 hours in culture.

The purity of each sorted cell sample was evaluated by examining at least 200 cells in the fluorescence microscope. Before sorting, only a few of the cells in a suspension were labelled (Figure 4). After sorting, however, almost of the cells in the sorted motoneuron fraction were labelled. The purity of the sorted motoneuron fraction depended upon the sorting criteria and the inten-

FIGURE 3.

SORTED MOTONEURONS GROWN IN CULTURE

Motoneurons were purified by retrograde transport and fluorescence-activitated cell sorting as described in the text. Sorted motoneurons were plated onto coverslips upon which spinal cord dividing cells (see Chapter 3, Section A.2.c.) had been grown to confluence and hypotonically lysed. Motoneurons were grown in Dulbecco's Modified Eagle's Medium containing 0.45% glucose (DME). Serum-free myotube conditioned medium (see Chapter 3, Section A.2.a. for details of preparation) was added to 50% of the final volume, and this growth medium was supplemented with 3% horse serum, glutamine (2mM), penicillin (100 U/ml), streptomycin (100 ug/ml), ovotransferrin (25 ug/ml), insulin (5 ug/ml), putrescine dihydrochloride (100 uM), progesterone (20 nM), and selenium (30 nM) (for details of motoneuron growth conditions, see Chapter 3, Section A.3.). All cells were grown in a water-saturated atmosphere at 8% CO_2. After 24 hours in culture, cells were fixed in 10% formalin in PBS and photographed. A. Phase contrast. B. Fluorescence. Note bright fluorescent granules in cell bodies of motoneurons.


B

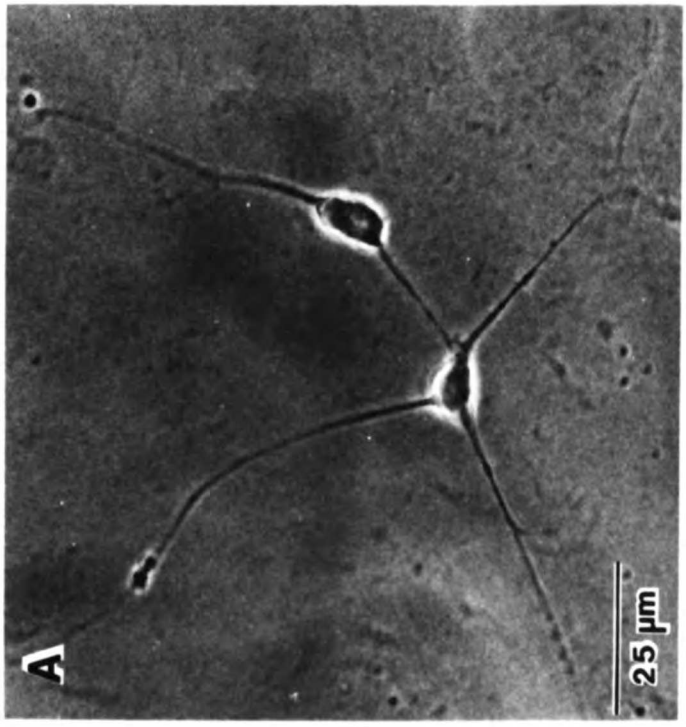
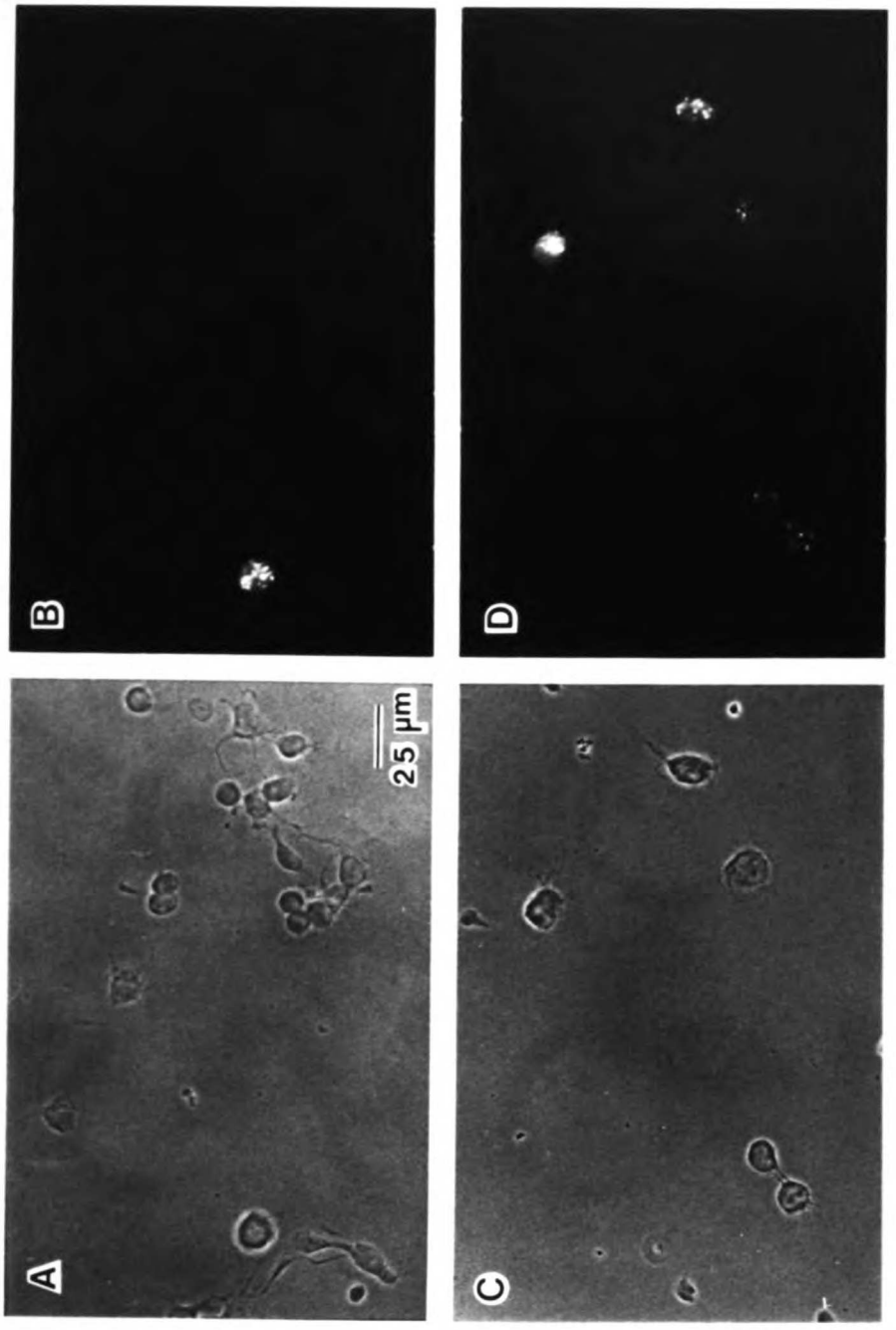

A
25 µm

FIGURE 4.

SPINAL CORD CELLS BEFORE AND AFTER CELL SORTING

Dissociated spinal cord cells were prepared from injected embryos as described in the text. Aliquots of cell suspensions, before and after sorting, were plated onto polylysine-coated glass coverslips, allowed to settle, and fixed in absolute methanol at -20°C. A. Phase contrast picture of cells before sorting. B. Fluorescence micrograph of same field. Very few cells are labelled. C. Phase contrast of cells after sorting. D. Fluorescence of same field. All cells are labelled.


A
B
C
D
25 µm

sity of label in a given experiment. The sorting criteria described in this chapter routinely gave purities of greater than 80%. With stricter criteria, higher purities -- but poorer yields -- could be obtained.

About 1.5-2 x 10^6 cells were obtained from each dissected spinal cord by the initial dissociation. Approximately 10% of these cells were lost during the BSA sedimentation that preceded cell sorting. Sorter analysis of cell samples, after the BSA sedimentation step, indicated that about 70-80% of all light scattering events were in one peak, corresponding to the cells. Of the scatter events within this peak, from 3-9% showed fluorescence above negative control levels. This number agreed well with the extent of labelling seen in unsorted samples evaluated with the fluorescence microscope. A maximum of 14 injected embryos could be prepared for a given experiment, and sorter yields were approximately 20,000-50,000 positive scatter events per embryo. For example, the yield from a typical experiment was 810,000 positive fluorescent scatter events obtained after sorting a total of 22 x 10^6 scatter events. Only about half the number of positive fluorescent scatter events was recovered as cells. It was assumed that this apparent loss was due mainly to two factors: 1) Sorting of erroneous light scattering events. For example, pieces of cells or cell processes, from labelled cells that were broken during the process of dissociation and sorting, could fall within the "cell" range of light scattering events and be sorted as positively-labelled cells. 2) Failure to recover cells from the sorter. Problems of this sort include sticking of cells to the sides of tubes in which they are recovered,

and loss of cells due to misdirection of the cell-containing fluid stream as it is deflected during the sorting process.

CHAPTER 3.

CONDITIONED MEDIUM EFFECTS ON MOTONEURONS: ANALYSIS OF SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY

CHAPTER 3.

CONDITIONED MEDIUM EFFECTS ON MOTONEURONS: ANALYSIS OF SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY

The experiments in this and the following chapter describe the effects of myotube conditioned medium on motoneuron growth and survival. Two separable actions of myotube conditioned medium will be discussed. The first, a neurite outgrowth-promoting activity, acts by adsorbing to the culture substratum and is similar to a neurite outgrowth-promoting factor that is known to be produced by many cell types. The second action of myotube conditioned medium is the enhancement of motoneuron survival over the course of a week in culture. The survival-enhancing activity in myotube conditioned medium will be discussed in Chapter 4.

A. PRELIMINARY CHARACTERIZATION OF THE SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY

1. MATERIALS

Laminin from the Engelbreth-Holm-Swarm sarcoma was purified by the method of Timpl et al. (1979). Antisera to Nerve Growth Factor and laminin were prepared in this laboratory. Heparinase and heparitinase from Flavobacterium heparinum were purified and separated from each other and from chondroitinase on hydroxylapatite (Linker and Hovingh, 1972). Trypsin (Type III), chondroitinase ABC, neuraminidase (Type V), collagenase (Type VII), and soybean

trypsin inhibitor (Type I-S), were purchased from the Sigma Chemical Co. (St. Louis, Missouri). Cell culture supplies were purchased from the U.C.S.F Cell Culture Facility.

Iron-saturated ovotransferrin ("crude Otf fraction") was prepared according to Kimura et al. (1982). Briefly, egg whites were extracted in an equal volume of ice-cold acetone in a Waring blender (2 minutes, top speed). Precipitated material was removed by centrifugation, resuspended in acetone, and extracted again in the blender. The extraction procedure was performed 4 times, and the final precipitate was dried at room temperature for 4 days. The dried egg white residue was then ground to powder using a mortar and pestle. To 60 gm of powder were added 900 ml of buffer (50 mM Tris-HCl, pH 8.0), and the mixture was stirred at 4°C. When the powder was thoroughly suspended in the buffer, 9 ml of 20 mM $FeCl_3$ were added. The mixture was stirred continuously for 4 hours at 4°C, after which the salmon-pink supernatant was decanted and filtered through cheesecloth to remove debris. Iron-saturated ovotransferrin was precipitated from this pink supernatant with an ammonium sulfate cut from 60 to 75% saturation. Precipitated material from the 75% saturation step was removed by centrifugation (20,000 x g for 1 hour); all colored material was removed from solution by this step. The precipitated material was resuspended in water, dialyzed exhaustively against buffer (20 mM Hepes, 150 mM NaCl, pH 7.4), and stored at -20°C until use. Purity and ovotransferrin concentration were assessed by SDS gel electrophoresis: Serial dilutions of the ovotransferrin preparation were compared

with a known quantity of a human transferrin standard.

2. CULTURE OF NONNEURONAL CELLS

a. MYOTUBES

Cultures of chick myotubes that were substantially free of fibroblasts were prepared by a modification of the method of Konieczny et al. (1982). Leg and pectoral muscles of 11 day embryos were dissected, cleaned of skin and connective tissue, minced, and dissociated into single cells by trituration through a pasteur pipette. Cells were diluted to 2 x 10^5 cells/ml in plating medium (see below), preplated for 1 hour at 37°C in tissue culture flasks to remove adherent fibroblasts, and finally decanted and plated as 25 ml per collagen-coated 100 mm-diameter, tissue culture dish. Collagen was applied as a 1:4 dilution, in distilled water, of rat tail tendon extract (Hawrot, 1980). A thin film was air-dried and sterilized by UV irradiation. Myoblast plating medium consisted of Dulbecco's Modified Eagle's Medium containing 0.45% glucose (DME), supplemented with 10% horse serum (HS; Kansas City Biologicals), 2% chick embryo extract (CEE; Gibco), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 ug/ml), Fungizone (2.5 ug/ml), and ovotransferrin (Otf; 25 ug/ml). These, and all dissociated cell cultures, were incubated in 8% CO_2 at 37°C.

After 2 days in culture, myoblasts were washed in calcium- and magnesium-free Dulbecco's Phosphate Buffered Saline (CMF-PBS) and placed in fusion inhibition medium (Konieczny et al., 1982), consis-

ting of calcium-magnesium-free (CMF) DME, supplemented with 10% CMF-horse serum, 2.5% CMF-chick embryo extract, 25 uM $CaCl_2$, 25 ug/ml Otf, glutamine (2mM), penicillin (100 U/ml), and streptomycin (100 ug/ml). After 2 days in fusion inhibition medium, most non-myoblast cells had detached from the dish and were removed by rinsing the cultures twice in PBS. Cultures were then placed in growth medium for 3 days, to allow fusion and maturation of myotubes, before myotube conditioned medium was prepared (see below). Myotube growth medium consisted of DME with 10% horse serum, 25 ug/ml Otf, glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 ug/ml). Myotube cultures prepared in this way had few, if any, fibroblasts visible upon microscopic inspection at this stage. To maintain myotube cultures at this low level of fibroblast contamination during the several weeks for which they were maintained, cultures were fed with myotube growth medium containing 10 uM cytosine-1-beta-D-arabinofuranoside (Ara-C; Sigma) for 2 to 3 days between periods of conditioned medium preparation. After Ara-C treatment, cultures were maintained for 12-24 hours in plain myotube growth medium before another batch of conditioned medium was prepared from them.

b. FIBROBLASTS

Chick fibroblast cultures were prepared from the adherent cells obtained during the preplating step of myotube culture preparation. Adherent cells in 75 cm^2 tissue culture flasks were grown to 50% confluence in fibroblast growth medium consisting of DME supplemented

with 10% fetal calf serum (FCS; Hyclone-Sterile Systems, Logan, Utah), glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 ug/ml). Cultures obtained in this way were passaged at least once, to remove any remaining myoblasts, and grown to near confluence prior to their use in conditioned medium preparation (see below).

c. SPINAL CORD DIVIDING CELLS

Spinal cords from 6 day chick embryos (Stage 28-29; Hamburger and Hamilton, 1951) were dissected and freed of meninges and dorsal root ganglia. The cords were minced and incubated for 11 minutes at 37°C in 4 ml of CMF-PBS containing 0.2% trypsin. Digestion was stopped by the addition of 4 ml of 5.5 mg/ml soybean trypsin inhibitor. The spinal cord fragments were washed into fibroblast growth medium (see above), and dissociated into single cells by trituration through a pasteur pipette. Cells were grown to 50% confluence in fibroblast growth medium, passaged at least once, and grown to near confluence in 75 cm^2 tissue culture flasks prior to their use in conditioned medium preparation (see below).

3. MOTONEURON CELL CULTURE

Motoneurons were cultured in multiwell plates (96-well clusters, Costar). Culture surfaces were exposed to poly-D-lysine (Sigma; 1 mg/ml in 0.1 M sodium borate, pH 8.4) overnight, washed with de-ionized distilled water, and sterilized under ultraviolet light. Cells were either plated directly onto these surfaces, or onto

polylysine-coated surfaces that had been further treated with solutions to be tested for neurite outgrowth-promoting activity. These solutions were applied for 8-16 hours at 4°C, after which the culture surfaces were washed 3-4 times in DME. Culture medium was added shortly before the cells were plated. Cells were plated at a density of 3,000-5,000 cells (6,000 to 10,000 scatter counts) per well of 96-well plates, as estimated by hemacytometer counts. Counts of living cell cultures were made to determine the total number of cells present in each well at 24 hours after plating. Approximately 90% (range 86-93%) of the cells estimated to have been put into a well could be accounted for at 24 hours.

Motoneurons were usually grown in serum-free growth medium: DME supplemented with Otf (25 ug/ml), insulin (5 ug/ml), putrescine dihydrochloride (100 uM), progesterone (20 nM), and selenium (30 nM) (modified from Bottenstein and Sato, 1979). Glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 ug/ml) were also added. For some experiments, 3% horse serum was added to this medium. When growth medium was supplemented with conditioned medium, the final mixture (50% conditioned medium, 50% fresh DME) was brought to the listed concentrations of supplements.

4. EVALUATING NEURITE OUTGROWTH

For cell counting and photography, cultures were fixed in PBS containing 2.5% glutaraldehyde and 5% sucrose, and washed into 0.9% NaCl prior to counting. To determine how many cells were lost during fixation and washing, counts of the total number of cells

per well were made before and after fixation (24 hour cultures were used for this determination). About 12% (range 6-17%) of cells were lost during these procedures. Therefore, because most cells remained attached to the dish, the cell counts reported here should serve as reasonable estimates of the behavior of the entire cell sample plated.

At least 200 cells in a minimum of 10 random fields were counted for each culture. Two cultures were counted for each time point in all reported tests. Cells were counted as having neurites only if they possessed 1 or more processes greater than 2 cell diameters in length. Cells with obvious fibroblast-like morphology were not counted. All other cells, including dead cells, were counted. Thus, the reported percentage of cells with neurites reflects the number of intact motoneurons, alive and bearing processes, at the recorded time in culture. Using these criteria, reliable and reproducible results were obtained.

5. PREPARATION AND FRACTIONATION OF CONDITIONED MEDIA

a. PREPARATION OF CONDITIONED MEDIA

Cultures of myotubes, fibroblasts or spinal cord cells were washed twice in plain DME. Serum-free conditioned media (MCM_{SF}, FCM_{SF}, and $SCCM_{SF}$, respectively) were prepared by incubating washed cultures in 7-8 ml of DME supplemented with 25 ug/ml Otf, 10 ug/ml insulin, 0.5 mg/ml polyvinyl pyrollidone (PVP-360; Sigma), and glutamine and antibiotics as with fibroblast growth medium (adapted

from Konigsberg, 1979). Serum-containing myotube conditioned medium (MCM) was prepared by incubating washed cultures in DME supplemented with 1% horse serum, 25 ug/ml ovotransferrin, and glutamine and antibiotics as with fibroblast growth medium. After 3-5 days of incubation, conditioned media were collected, cleared by centrifugation, and stored at -20°C until used. They were filtered through a 0.22 um filter prior to use.

b. ENZYMATIC TREATMENTS OF MYOTUBE CONDITIONED MEDIUM

Serum-free myotube conditioned medium (MCM_{SF}) was digested with 100 ug/ml trypsin for 2.5 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. Collagenase at 75 U/ml, neuraminidase at 2.5 U/ml, and choidroitinase ABC at 5 U/ml were all used to digest MCM_{SF} for 6 hours at 37°C. Heparatinase was used at 35 ug/ml for 6 hours at 43°C. As a control, MCM_{SF} without added enzyme was heated at 43°C for 6 hours. Heparinase was used at 20 ug/ml for 6 hours at 30°C.

c. ISOPYCNIC CENTRIFUGATION

60 ml of MCM_{SF} was concentrated on 3.5 ml of DEAE cellulose (DE52, Whatman, Ltd.). The column was washed in 2 column volumes of 100 mM NaCl/50 mM Tris HCl, pH 7.4, and eluted with 9 ml of 36.9% CsCl (wt/wt) in 50 mM Tris HCl, pH 7.4. The density of the eluate was measured by weighing known volumes in preweighed micropipettes,

and the CsCl concentration was corrected to a final concentration of 37% (wt/wt). Guanidinium chloride (GuHCl) was added to a final concentration of 0.4 M. These conditions constitute the associative gradient conditions of Sajdera and Hascall (1969). The samples were centrifuged as 5°C to equilibrium (approximately 48 hours) at 40,000 rpm using a SW50.1 rotor (DuPont Instruments, Newton, Connecticut). Gradients were eluted and the density of fractions was determined by weighing volumes in preweighed 10 ul micropipets. Fractions were dialyzed exhaustively against 100 mM NaCl, 50 mM Tris HCl, pH 7.4.

d. SALT FRACTIONATION AND GEL FILTRATION OF MYOTUBE CONDITIONED MEDIUM

Saturated ammonium sulfate was added to 25 ml of MCM (containing 1% horse serum) to 45% saturation. The solution was centrifuged, and the pellet was resuspended in 1.3 ml of column buffer (200 mM NaCl, 20 mM Hepes, pH 7.4, 0.2 mg/ml hemoglobin [Sigma Type IV]). A column (1.5 x 8.5 cm) of Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated in column buffer at 4°C. The void volume was measured using blue dextran 2000 (Pharmacia). The 1.3 ml sample was applied and 1 ml fractions were collected.

6. RESULTS

a. EFFECT OF MYOTUBE CONDITIONED MEDIUM ON MOTONEURON NEURITE OUTGROWTH

Factors that bind to polyornithine- or polylysine-treated surfaces and enhance neurite outgrowth are known to be produced by many cell types, including the mouse skeletal muscle cell line C_2 (Adler et al., 1981; Lander et al., 1982). It was suspected that the same kind of factor might be produced by primary cultures of chick myotubes. A test was performed to see whether myotube conditioned medium could exert a neurite outgrowth-promoting effect when applied to a polylysine substratum. Serum-free medium was conditioned by 4 days of exposure to cultures of chick myotubes. Polylysine-coated tissue culture dishes were exposed overnight to this conditioned medium (MCM_{SF}), and washed thoroughly. Motoneurons plated onto MCM_{SF}-treated substrata and examined after 24 hours in culture had extended long neurites, but motoneurons plated onto polylysine alone had few or no processes (Figure 5). This indicated that MCM_{SF} could exert a short-term, neurite outgrowth-promoting effect on motoneurons when applied to the substratum alone; it did not have to be present in the cell culture medium to exert this effect.

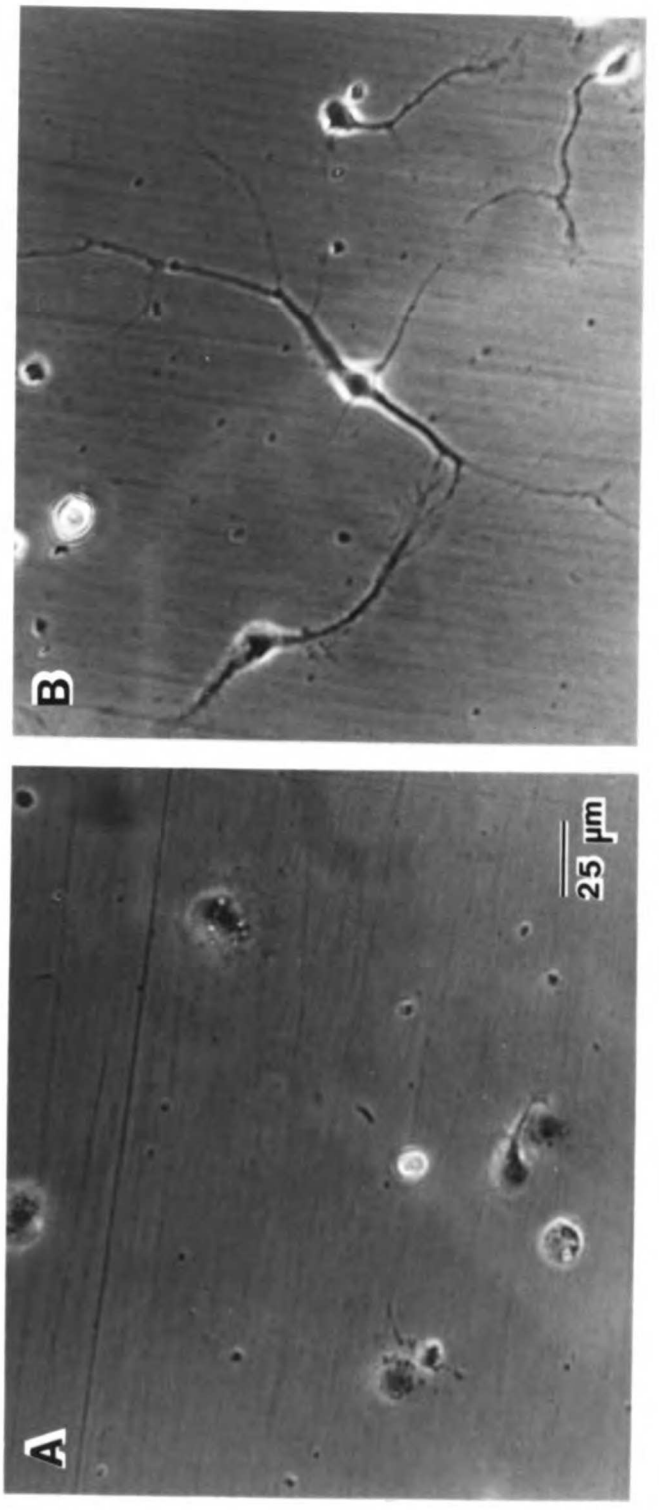
Sympathetic neurons grown on a polylysine substratum that has been treated with medium conditioned by bovine corneal endothelial cells (BCE-CM) respond by rapidly extending neurites. The factor in BCE-CM that was responsible for promoting neurite outgrowth by sympathetic neurons had been characterized by Lander and colleagues (Lander et al., 1982), and observations detailed below confirmed the similarity of the motoneuron neurite outgrowth-promoting activity from MCM_{SF}, with the BCE-CM factor.

MCM_{SF} promoted neurite outgrowth from sympathetic neurons (data

FIGURE 5.

EFFECT OF MYOTUBE CONDITIONED MEDIUM ON MOTONEURON NEURITE OUTGROWTH

Sorted motoneurons were grown for 24 hours in serum-free growth medium, either on polylysine substrata, or on polylysine substrata that had been exposed to myotube conditioned medium and then washed. A. Motoneurons on polylysine substratum. B. Motoneurons on substratum treated with conditioned medium.


A
25 µm
B

not shown), and the BCE factor promoted neurite outgrowth from motoneurons. Thus, both factors promoted neurite outgrowth from both neuronal types. Figure 6 shows results from motoneurons grown in serum-free culture conditions either on polylysine alone, or on polylysine treated with various conditioned media. After being grown for 24 hours on substrata treated with MCM_{SF}, over 50% of cells have extended one or more neurites greater than 2 cell diameters in length. The number of responding cells is less on substrata treated with BCE conditioned medium or with chick fibroblast conditioned medium, but still 5 or 6 times greater than that of cells plated on polylysine alone.

b. ENZYMATIC AND PHYSICAL CHARACTERIZATIONS OF THE NEURITE OUTGROWTH-PROMOTING FACTOR FROM MYOTUBE CONDITIONED MEDIUM

The substratum-binding, neurite outgrowth-promoting activities of MCM_{SF} and the BCE factor were also similar in their susceptibilities to enzymes. Table 1 lists the responses of motoneurons plated onto polylysine substrata treated with MCM_{SF} that had been subjected to a variety of enzymatic treatments. The neurite outgrowth-promoting activity of MCM_{SF} was sensitive to trypsin, heparitinase, and heparinase, but insensitive to collagenase, neuraminidase, and chondroitinase ABC. This suggested that both protein and heparan sulfate were important in the substratum-binding, neurite outgrowth-promoting activity of MCM_{SF}. This characterization corresponded to that of the BCE factor, which -- based on similar

FIGURE 6.

MOTONEURON NEURITE OUTGROWTH ON SUBSTRATA TREATED WITH DIFFERENT CONDITIONED MEDIA

Serum-free conditioned media were applied to polylysine substrata for 8-16 hours at 4°C, and then the substrata were washed and plated with sorted motoneurons. Motoneurons were grown for 24 hours in serum-free growth medium, fixed, and cells with neurites were counted in duplicate cultures. Error bars show mean $\pm$ the deviation.

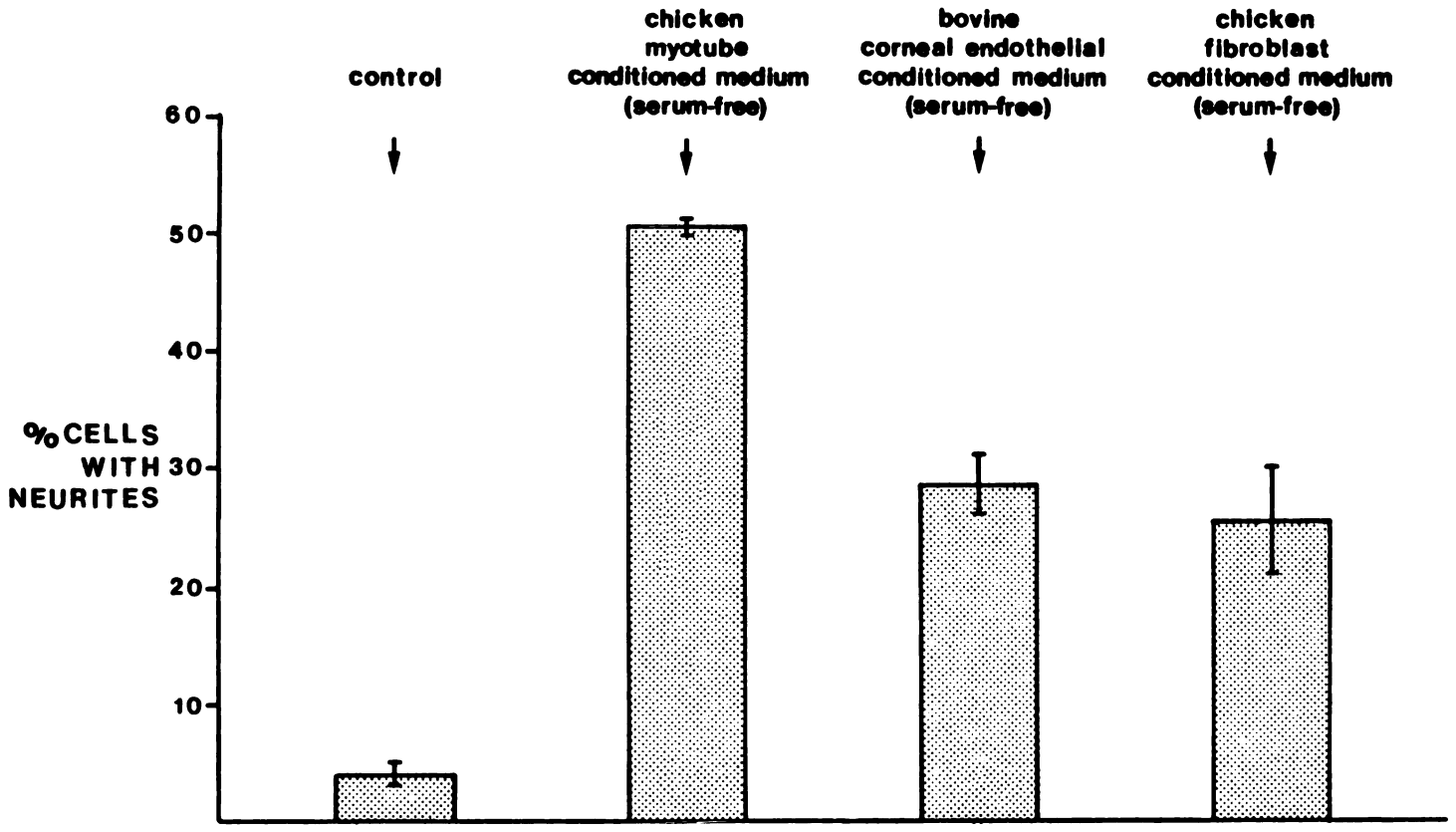

control
chicken myotube conditioned medium (serum-free)
bovine corneal endothelial conditioned medium (serum-free)
chicken fibroblast conditioned medium (serum-free)
60
50
40
30
20
10
% CELLS WITH NEURITES

TABLE 1.

SENSITIVITY OF SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY TO ENZYMATIC DIGESTION

Enzymatic Treatment	% Cells with Neurites (% of Control)	
Trypsin	1 ± 0	(3%)
Heparitinase	10 ± 1	(32%)
Heparinase	7 ± 1	(23%)
Soybean Trypsin Inhibitor + Trypsin	28 ± 2	(90%)
Collagenase	24 ± 1	(77%)
Neuraminidase	25 ± 3	(81%)
Chondroitinase ABC	25 ± 1	(81%)
Untreated	31 ± 1	(100%)
43°C for 6 hours	25 ± 3	(81%)

MCM_{SF} was subjected to enzymatic treatments as described in the text. Treated MCM_{SF} (or control, untreated MCM_{SF}) was applied to polylysine substrata for 12 hours at 4°C, and the substrata were then washed and plated with sorted motoneurons. Motoneurons were grown for 24 hours in serum-free growth medium, fixed, and cells with neurites were counted in duplicate cultures. Numbers in second column show mean ± the deviation. Numbers in parentheses indicate neurite outgrowth expressed as percent of control reponse on untreated MCM_{SF}.

tests -- was thought to contain protein and a heparan sulfate proteoglycan (Lander et al., 1982).

The substratum-binding, neurite outgrowth-promoting activity in MCM_{SF} has a buoyant density similar to that of the BCE factor. This is illustrated in Figure 7. MCM_{SF} was concentrated on DEAE cellulose and sedimented in a cesium chloride gradient containing 0.4 M guanidinium chloride (the "associative" conditions ofSajdera and Hascall (1969)). Fractions from the gradient were dialyzed and applied to polylysine substrata. The treated substrata were washed, plated with motoneurons, and analyzed for their ability to promote neurite outgrowth. The peak of neurite outgrowth-promoting activity eluted at a density of about 1.34, between the densities of pure proteins and polysaccharides, and close to the density of the BCE factor (Lander et al., 1982). This was consistent with the presence of glycosaminoglycan as well as protein in the MCM_{SF} neurite outgrowth-promoting factor, as was indicated by enzymatic tests.

Virtually all of the neurite outgrowth-promoting activity from MCM could be precipitated in 45% saturating ammonium sulfate. When the supernatant from such a precipitation was concentrated to less than the original volume of the starting material (15 ml versus 25 ml), dialyzed against PBS, applied to a polylysine substratum, and plated with motoneurons, it proved to be negative in the neurite outgrowth test (Figure 8). The precipitate was dissolved in column buffer (Section 3.A.5.d.) and fractionated on a column of Sepharose CL-6B. The peak of neurite outgrowth-promoting activity eluted

FIGURE 7.

ISOPYCNIC SEDIMENTATION IN ASSOCIATIVE CESIUM CHLORIDE GRADIENT

MCM_{SF} was concentrated and centrifuged in CsCl containing 0.4 M Gu HCl, as described. The densities of fractions were measured, and fractions were dialyzed and applied to polylysine substrata. Treated surfaces were washed and plated with motoneurons. After 24 hours, duplicate cultures were fixed and cells with neurites were counted. Error bars show mean $\pm$ the deviation.

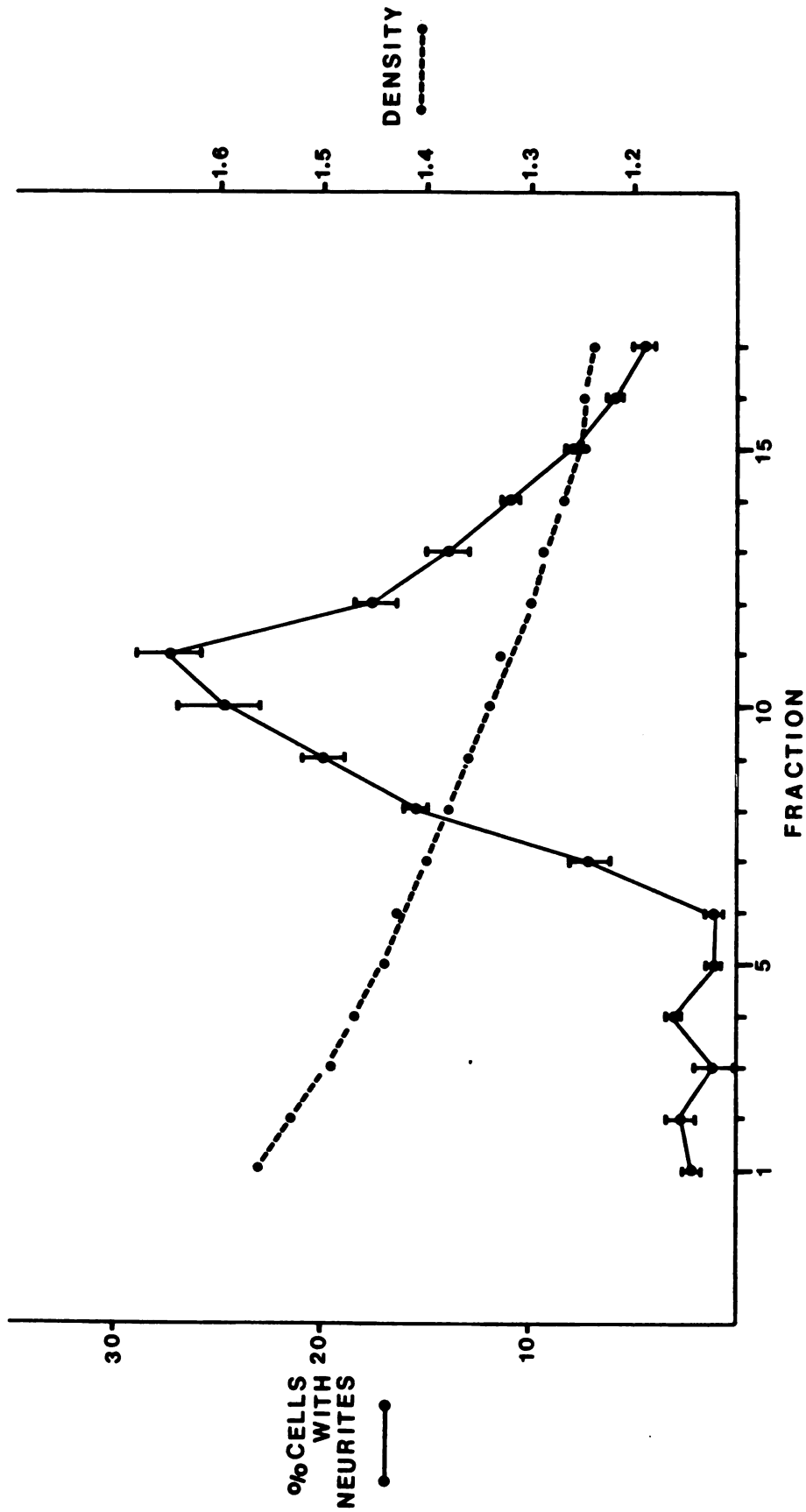
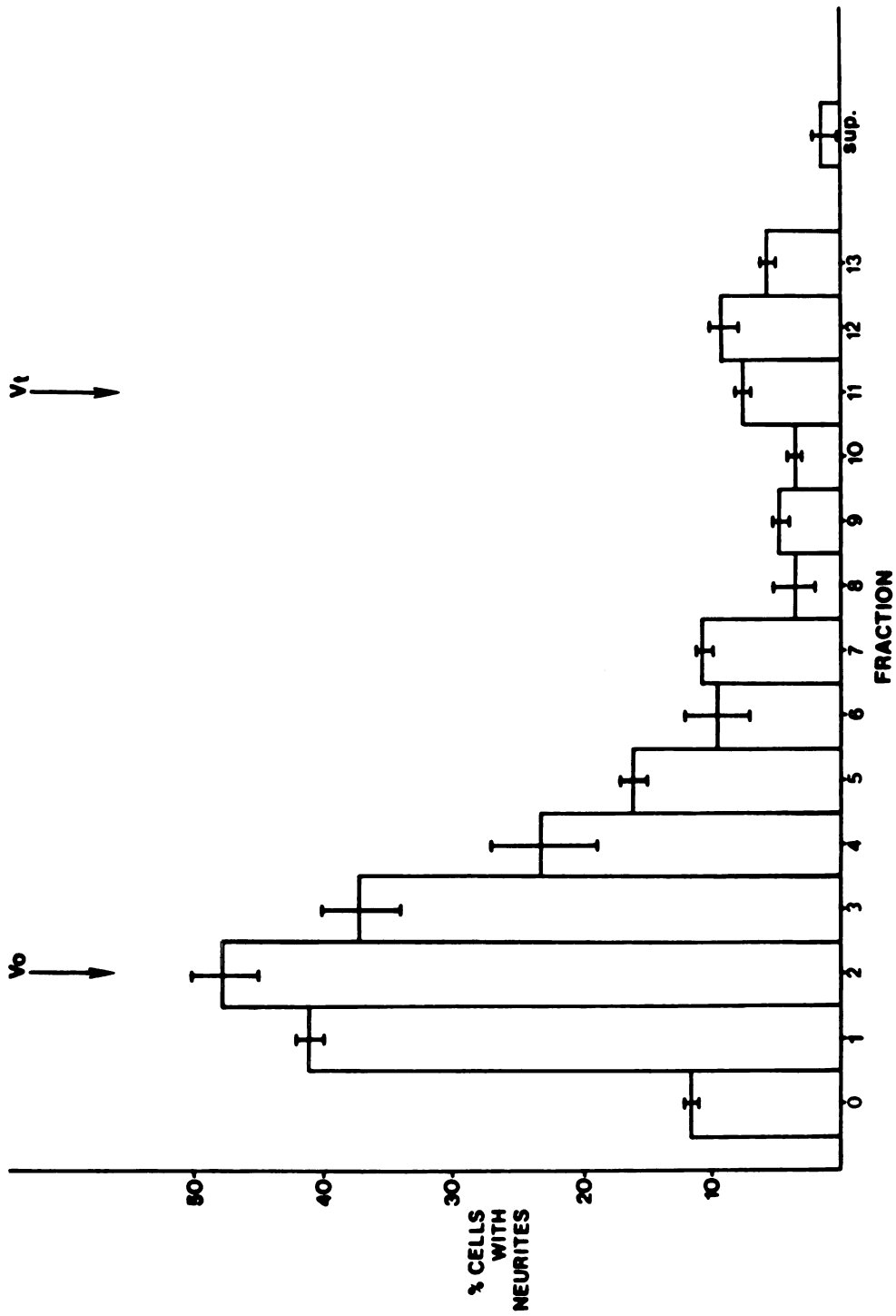

DENSITY
1.6
1.5
1.4
1.3
1.2
30
20
10
%CELLS WITH NEURITES
1
5
10
15
FRACTION

FIGURE 8.

AMMONIUM SULFATE PRECIPITATION OF MCM: FRACTIONATION OF PRECIPITATE ON SEPHAROSE CL6B

MCM was precipitated with 45% saturated ammonium sulfate as described. The pellet was resuspended in column buffer and applied to a Sepharose CL6B column (see text). The supernatant was concentrated three-fold by dialysis against polyethylene glycol (Aquacide III, Calbiochem. Behring Corp.), and dialyzed against PBS. Samples of the column fractions, as well as a sample of the concentrated supernatant, were applied to polylysine substrata and tested for neurite outgrowth-promoting activity. Motoneurons were grown for 24 hours, fixed and counted. "Sup." = concentrated ammonium sulfate supernatant, V_t = total included volume, V_o = void volume. Error bars show mean $\pm$ the deviation.


Vo
Vt
50
40
30
20
10
% CELLS WITH NEURITES
0
1
2
3
4
5
6
7
8
9
10
11
12
13
sup.
FRACTION

near the void volume of the column. (The exclusion limit of Sepharose 6B, as given by its manufacturer [Pharmacia], is approximately 4×10^6 daltons for globular proteins and approximately 10^6 daltons for polysaccharides.) This experiment is illustrated in Figure 8. The void volume fraction, containing the partially purified substratum factor, was designated "NOPA" (for "neurite outgrowth-promoting activity"). The chromatographic properties on Sepharose CL-6B of the NOPA from MCM, corresponded to those of the neurite outgrowth-promoting factor from BCE-CM (Lander et al., 1982; 1983).

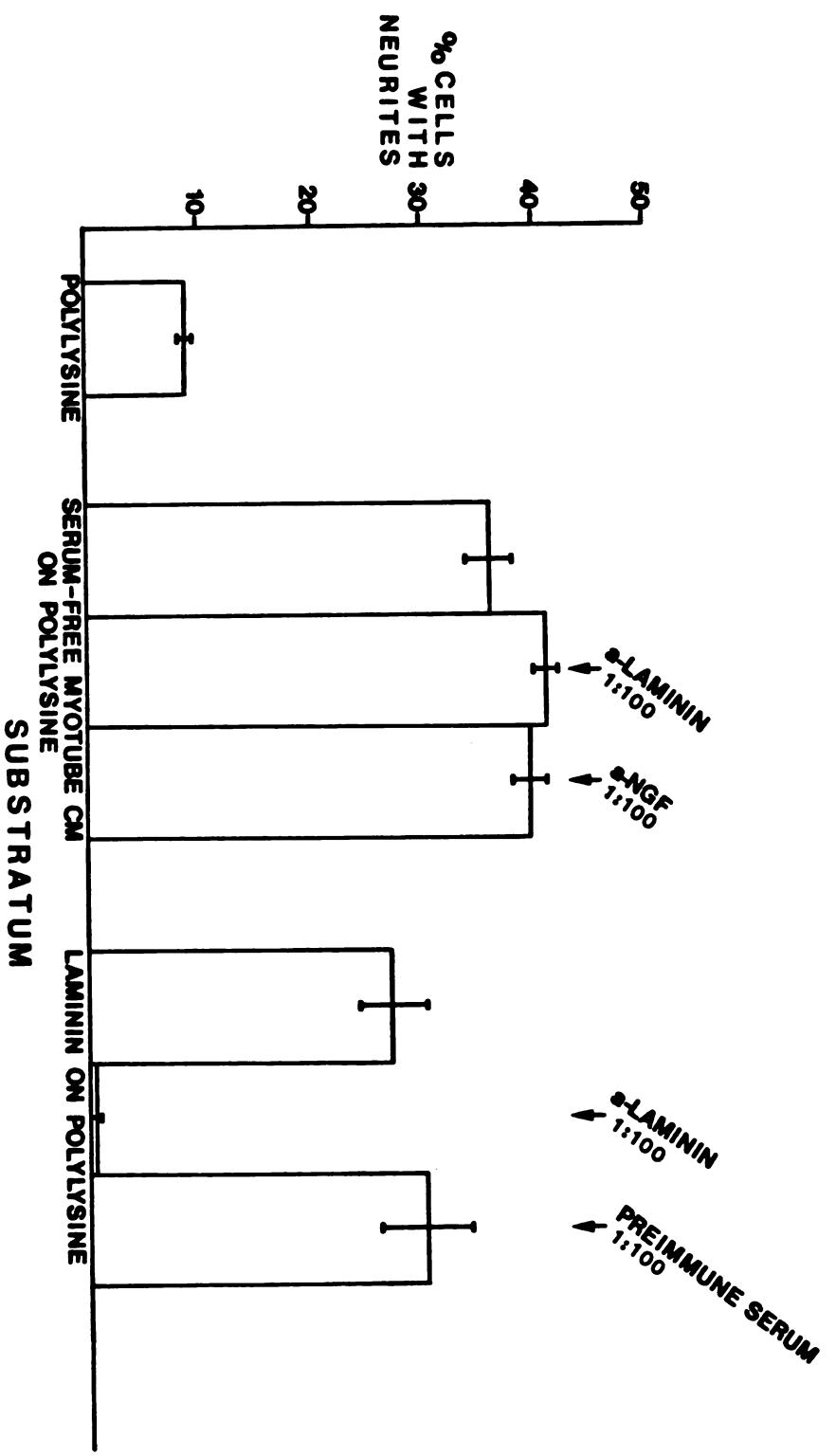
c. EFFECTS OF ANTIBODIES TO LAMININ AND NERVE GROWTH FACTOR ON MOTONEURON NEURITE OUTGROWTH

Conditioned medium was not the only substance that could bind to a substratum and promote neurite outgrowth by motoneurons. The basement membrane glycoprotein laminin was also effective when applied to a polylysine substratum at a concentration of 10 ug/ml (Figure 9). The experiments shown in Figure 9 demonstrate that laminin's neurite outgrowth-promoting effect could be blocked by anti-laminin antibodies present in the culture medium. These same antibodies did not block the neurite outgrowth-promoting effect of the MCM_{SF} factor. Similar results were obtained when cultures of rat sympathetic neurons were treated with antibodies to laminin: the antibodies blocked neurite outgrowth on laminin but not on the BCE factor (Lander et al., 1983). Preimmune serum and antiserum to Nerve Growth Factor, both included in the neuronal culture medium,

FIGURE 9.

EFFECTS OF ANTISERA ON MOTONEURON NEURITE OUTGROWTH

Sorted motoneurons were plated onto polylysine substrata that had been treated with MCM_{SF} or laminin (10 ug/ml). Motoneuron growth medium contained 3% horse serum and was supplemented as indicated with preimmune or immune rabbit sera diluted 100-fold. Motoneurons were fixed at 24 hours and the neurite outgrowth in duplicate cultures was evaluated. Error bars represent mean $\pm$ the deviation. The same results were obtained with sera diluted 50-fold (data not shown).


% CELLS WITH NEURITES
10
20
30
40
50
POLYLYSINE
SERUM-FREE MYOTUBE CM ON POLYLYSINE
LAMININ ON POLYLYSINE
SUBSTRATUM
α-LAMININ 1:100
α-NGF 1:100
α-LAMININ 1:100
PREIMMUNE SERUM 1:100

had no effect on motoneuron neurite outgrowth.

7. DISCUSSION

Myotube conditioned medium (MCM) contains a motoneuron neurite outgrowth-promoting factor which has been shown to be effective when adsorbed to the culture substratum. In the preliminary characterization detailed above, this factor was shown to be similar to the neurite outgrowth-promoting factor (BCE factor) purified by Lander and colleagues from bovine corneal endothelial cell conditioned medium, and known to be present in media conditioned by many other cell types (Lander et al., 1982; 1983; Adler et al., 1981). Its effects were evident at short times (24 hours), it was susceptible to the same enzymes, had about the same buoyant density in cesium chloride, and behaved similarly in gel filtration experiments. The factor appeared to contain protein and heparan sulfate, as does the BCE factor. Finally, in the same manner as the BCE factor, the substratum-binding, neurite outgrowth-promoting activity from MCM was mimicked by conditioned media from other cell types, and MCM could act upon neurons other than motoneurons. Thus, motoneuron neurite outgrowth in response to substratum-bound MCM did not appear to be a response to a factor produced only by target tissue, but rather a response typical of many types of neurons, to a substance secreted by many cell types.

There are, as yet, no characterizations by other laboratories of the response of purified motoneurons to the substratum-binding, neurite outgrowth-promoting activity in myotube conditioned medium.

However, other investigators have reported that neurite outgrowth from whole spinal cord explants or dissociated cells is enhanced by the presence of various conditioned media and/or tissue extracts, and have cited these effects as evidence for existence of putative "motoneuron trophic factors". Effects on neurite outgrowth by spinal cord explants derived from embryonic frog (Pollack et al., 1981), embryonic chick (Obata and Tanaka, 1980; Tanaka et al., 1982; Hsu et al., 1982; Nurcombe and Bennett, 1983; Nurcombe et al., 1984), and embryonic rat (Dribin and Barrett, 1980, 1982a,b) have been studied. Experiments demonstrating neurite outgrowth-promoting activities for dissociated cells from the spinal cord of embryonic rat (Smith and Appel, 1983) and embryonic chicken (Henderson et al., 1981, 1983, 1984; Tanaka and Obata, 1982; Longo et al., 1982) have also been reported. Molecular characterizations of these neurite outgrowth-promoting activities have been attempted in only a few cases. Where such attempts have been made, there is little consensus about the molecular nature of activities affecting neurite outgrowth by spinal cord cells. This is not surprising, given two important facts: First, only a few investigators have attempted to separate the effects of substratum-binding, neurite outgrowth-promoting factors, from the effects of molecules that might act via the culture medium to affect the growth and differentiation of spinal cord cells. Second, the interpretability of all the studies cited above suffers from the fact that motoneurons comprise only a small percentage of all spinal cord cells. In whole spinal cord cultures, even when it is possible to identify

motoneurons (e.g., Bennett et al., 1980), it is impossible to distinguish direct effects of exogenous substances on motoneurons from indirect effects mediated by other classes of spinal cord cells. In fact, it is certain that contaminating (i.e. non-motoneuron) spinal cord cells themselves affect the state of neurite outgrowth in these cultures. It has been reported that neurite outgrowth by dissociated spinal cord cells is enhanced when these cells are cultured in the presence of spinal cord extracts or co-cultured with spinal cord explants (Tanaka and Obata, 1982). In addition, this report has been substantiated by findings in the present study: If polylysine substrata were treated with serum-free medium conditioned over cultures of spinal cord dividing cells (see Section A.2.c. of this chapter), these substrata could support motoneuron neurite outgrowth as well as MCM-treated substrata (data not shown).

In spite of the confusion generated by differences in experimental strategies, some support for the results reported in Section A of this chapter can be found in the literature. Several investigators have reported neurite outgrowth by spinal cord cells in response to conditioned-medium components that bind to polycationic substrata (Longo et al., 1982; Nurcombe and Bennett, 1983; Dribin and Barrett, 1982a). Using neurite outgrowth by rat spinal cord explants as their bioassay, Dribin and Barrett (1982a) have reported a substratum-binding, neurite outgrowth-promoting activity that is present in conditioned medium from cultures of myotubes or fibroblasts. Gel filtration chromatography of the conditioned medium indicated

that this activity has a molecular weight in excess of 300,000 Da. These results are consistent with the variety of sources for, and the large size of, the substratum-binding, neurite outgrowth-promoting activity for motoneurons that was characterized in Section A of this chapter.

B. IDENTIFICATION OF LAMININ AS THE ACTIVE FACTOR

Laminin, a basement membrane glycoprotein, has also been shown to promote neurite outgrowth from many types of neurons (e.g., Manthorpe et al., 1983). Fractionation of BCE-CM, on the basis of its ability to promote outgrowth by rat sympathetic neurons, showed that laminin -- in association with laminin-binding molecules -- comprised the "neurite outgrowth-promoting factor" in this conditioned medium (Lander et al., 1985a). Because of the common features of the neurite outgrowth-promoting activities in MCM and BCE-CM (detailed above), because the large size of the MCM factor was consistent with the possibility that it was laminin, and because there was evidence that cultures of chick myotubes could secrete laminin (e.g. Bayne et al., 1984), it was decided to determine whether laminin was also responsible for the neurite outgrowth-promoting effect that MCM exerted on motoneurons.

1. MATERIALS AND METHODS

a. MATERIALS

Goat anti-rabbit immunoglobulin (Ig) was from Cappel. Gelatin was coupled to Sepharose 4B (Phamacia) at 1 mg gelatin/ml resin (Ruoslahti et al., 1982). All other reagents were obtained from Sigma (St. Louis, Missouri).

b. CULTURE OF MOTONEURONS

Motoneurons were purified from chick embryos by retrograde transport and fluorescence-activated cell sorting, as described in Chapter 2. Culture surfaces consisted of 96-well tissue culture plates (Costar) that were exposed to poly-D-lysine as described (Chapter 3, Section A.3.) and treated with samples to be tested for neurite outgrowth-promoting activity (50 ul/well) overnight at 4°C. Wells were washed extensively, then motoneurons were plated into them at a density of 3000-5000 cells per well and maintained for 24 hours in serum-free motoneuron growth medium (Chapter 3, A.3.).

c. MYOTUBE CONDITIONED MEDIUM

Chick myotube cultures were prepared according to the method of Konigsberg (1979). Plating procedures and myotube growth medium were described previously (Chapter 3, Section A.2.a.). After fusión of myotubes, cultures were periodically treated with growth medium containing 10 um cytosine-beta-D-arabinsofuranoside in order to minimize fibroblast contamination. For preparation of myotube conditioned medium (MCM), cultures were washed twice in

plain Dulbecco's Modified Eagle's Medium containing 0.45% glucose (DME), and incubated in DME supplemented with 1% horse serum (HS), 25 ug/ml ovotransferrin (Otf), 2mM glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin. 7-8 ml of medium per 100 mm-diameter tissue culture plate was conditioned for 3-5 days and then harvested. Metabolically-labelled MCM was prepared by rinsing cultures with methionine-free DME, and labelling in methionine-free DME supplemented with 1% HS, ^{35}S-methionine (40 uCi/ml, 1110 Ci/mmol; Amersham), unlabelled methionine (final 21 uM), 25 ug/ml Otf, and glutamine and antibiotics as above. Cultures were maintained for 2 days, then fed with 1 ml of methionine-containing DME. After 24 hours, the labelled MCM was harvested, filtered (0.2 um), and treated with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mM), EDTA (3mM), pepstatin (10^{-7} M), and N-ethyl maleiimide (NEM; 2 mM).

d. FRACTIONATION OF MYOTUBE CONDITIONED MEDIUM

A modification of the procedure of Lander et al. (1985a) was used to fractionate the active factor in MCM. 90 ml of MCM, metabolically labelled with ^{35}S-methionine, were mixed with 130 ml of cold MCM. The pooled conditioned medium was depleted of fibronectin by mixing it with 6.5 ml of packed gelatin-Sepharose and gently stirring the mixture for 2 hours at room temperature. The gelatin-Sepharose was then removed from the MCM by centrifugation. (Without this step, insoluble aggregates formed during subsequent procedures and neurite outgrowth-promoting activity was lost.) The fibronectin-

depleted MCM was then brought to 45% saturating ammonium sulfate, and precipitated material was removed by centrifugation and dialyzed against 0.1 M NaCl,0.05 M Tris-HCl, pH 7.4. The dialyzed material (7 ml) was mixed overnight with 2 ml of DEAE cellulose (Whatman DE52). This mixture was poured into a column, the unbound material was eluted, and the column was rinsed with 2 ml of 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4. Neither the supernatant from the ammonium sulfate precipitation, nor the unbound material from the ion exchange step, had neurite outgrowth-promoting activity. Material eluted from the DEAE with 1 M NaCl,0.05 M Tris-HCl, pH 7.4, contained the neurite outgrowthpromoting activity, and this fraction (1.3 ml) was chromatographed on a column (28 X 1.5 cm) of Sepharose CL-4B equilibrated in buffer (0.5 M NaCl, 0.05 M Tris-HCl, pH 7.4) containing 0.2 mg/ml hemoglobin and 0.1% Triton X-100 (to minimize nonspecific adsorption of material to the column), and the protease inhibitors EDTA (2 mM), PMSF (1 mM), pepstatin (10^{-7} M), and NEM (2 mM). 1 ml fractions were collected at 3.5 ml per hour; alternate fractions were tested for neurite outgrowth-promoting activity, laminin immunoreactivity, and run on SDS gels.

e. IMMUNOCHEMICAL TECHNIQUES

A rabbit antiserum to purified EHS laminin was generated, and affinity-purified antibodies were prepared by chromatographing the serum on laminin coupled to Affigel 10 (Bio-Rad; Richmond, California) at 0.8 mg/ml of gel. Immunoprecipitations were performed by mixing 0.4 ml of MCM with 10.8 ug affinity-purified anti-laminin

and carrier non-immune immunoglobulin (Ig; 24 ug). The mixture was incubated overnight at 4°C, and precipitating antiserum (goat anti-rabbit Ig) was then added to the equivalence point (determined to be 25 ul/ug carrier Ig) for a further 24 hours at 4°C. The precipitate was removed by centrifugation. Enzymed linked immunoassays (EIA's; Hudson and Hay, 1980) were performed on samples applied to polylysine-coated tissue culture wells, using affinity-purified anti-laminin at 1.5 ug/ml. Assays were developed using peroxidase-conjugated goat anti-rabbit antiserum (Cappel) and 2,2'-azino-di-(3ethylbenzthiazoline sulfonic acid) as the chromagen. Results were quantified spectrophotometrically at 415 nm.

f. ELECTROPHORESIS

The sodium dodecyl sulfate (SDS)-polyacrylamide gel system of Laemmli (1970) was used. Beta-mercaptoethanol was omitted, except where specified. Separating gels (13 x 15.5 cm) were 3-15% exponential acrylamide gradients. Stacking gels (13 x 2.5 cm) were 2.8% acrylamide. Gels were cast on GelBond PAG solid supports (Marine Colloids). Labelled material was detected by fluorography using preflashed Kodak XAR-2 film at -70°C (Bonner and Laskey, 1974).

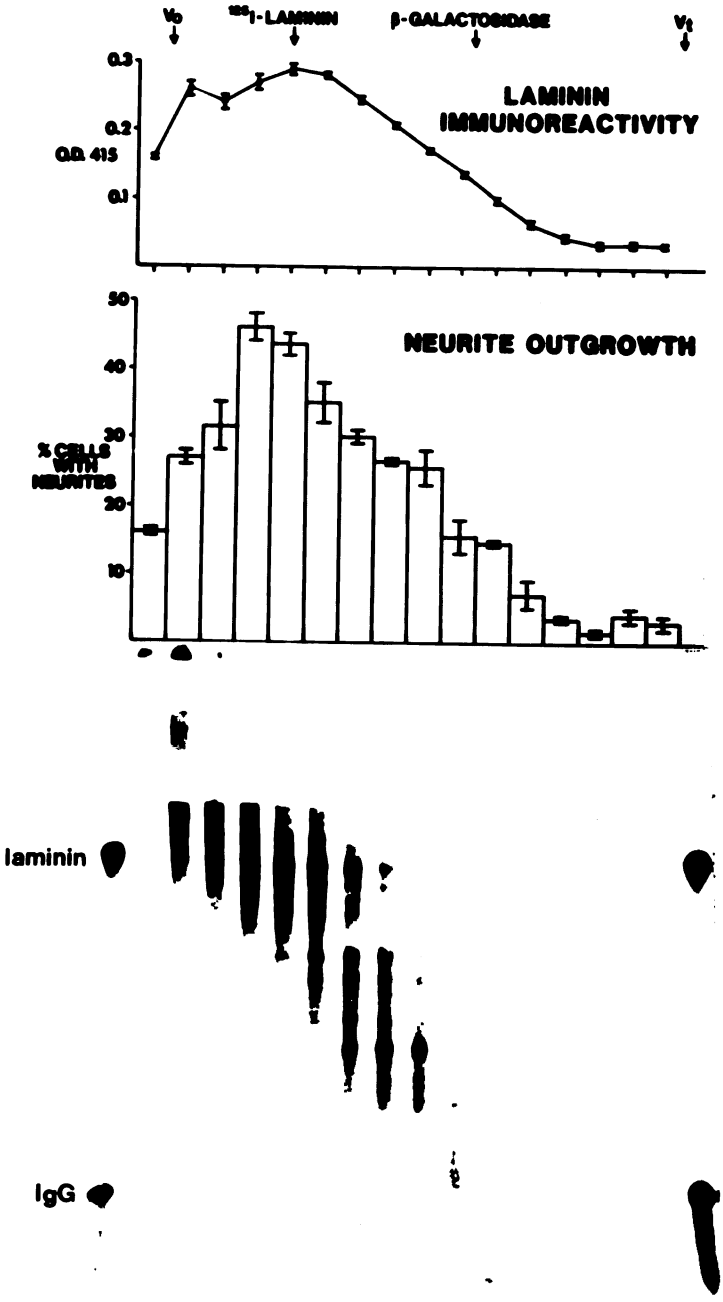
2. RESULTS

Partial purification of the "neurite outgrowth-promoting factor" in MCM demonstrated the presence of laminin in all active fractions. The results are shown in Figure 10. The peak of substratum-binding,

FIGURE 10.

FRACTIONATION OF NEURITE OUTGROWTH-PROMOTING ACTIVITY ON SEPHAROSE CL-4B

MCM, metabolically labeled with ^{35}S-methionine, was concentrated and fractionated on a column of Sepharose CL-4B as described in the text. The column was calibrated with laminin (obtained from mouse EHS sarcoma) and beta-galactosidase. High molecular weight aggregates of beta-galactosidase served to mark the void volume (V_o), and $^{35}SO_4$ was used to mark the total included volume (V_t). The two top panels show assays for laminin immunoreactivity (EIA's, described in the text) and for motoneuron neurite outgrowth-promoting activity, for each of the fractions run on the gel shown in the bottom panel. Motoneuron neurite outgrowth was quantified as the percentage of cells bearing neurites $\geq$ 2 cell diameters in length. Data are presented as the averages of duplicate cultures, $\pm$ the deviation. Standards for this nonreducing gel, shown in the two outside lanes, were ^{125}I-laminin and ^{125}I-mouse immunoglobulin G (IgG).


Vo
125I-LAMININ
β-GALACTOSIDASE
Vt
LAMININ IMMUNOREACTIVITY
O.D. 415
0.3
0.2
0.1
NEURITE OUTGROWTH
% CELLS WITH NEURITES
50
40
30
20
10
laminin
IgG
19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
FRACTION

neurite outgrowth-promoting activity corresponded closely both to the presence of a protein which comigrated with authentic laminin in the gel, and to a peak of laminin immunoreactivity. Photographs of motoneurons grown for 24 hours on polylysine substrata treated with different column fractions demonstrate the extensive neurite outgrowth seen with positive fractions (Fig. 11A) and the absence of neurite outgrowth seen when motoneurons were grown on the negative fractions (Fig. 11B). Thus, laminin appeared to be present in the fractions of MCM that possessed neurite outgrowth-promoting activity.

Earlier experiments showed that anti-laminin antibodies fail to block neurite outgrowth on substrata treated with MCM (Chapter 3, Section A.6.c.). Although this suggested that laminin might not be responsible for the neurite outgrowth-promoting activity in MCM, it seemed likely that the mouse laminin to which anti-laminin antibodies were raised might not share many functionally important determinants with chicken laminin, so that the blocking experiments would be unlikely to succeed. However, it seemed possible that chick and mouse laminins would share some antigenic determinants, so that anti-mouse laminin antibodies could be used to remove laminin from MCM. If removal of laminin from MCM correlated with the loss of neurite outgrowth-promoting activity, then the importance of laminin for this activity would be confirmed. Accordingly, MCM was subjected to immunoprecipitation with affinity-purified anti-laminin antibodies. Neurite outgrowth-promoting activity was indeed removed from MCM by this procedure (Fig. 12). In order to determine whether the immunoreactive component removed

FIGURE 11.

MOTONEURON NEURITE OUTGROWTH

ON FRACTIONATED MYOTUBE CONDITIONED MEDIUM

Motoneurons were grown on polylysine substrata treated with fractions from the column illustrated in Figure 10 (see text for culture conditions). After 24 hours in culture, the cells were fixed and washed as previously described. A. Motoneurons grown on substratum treated with fraction 27. B. Motoneurons grown on substratum treated with fraction 47.

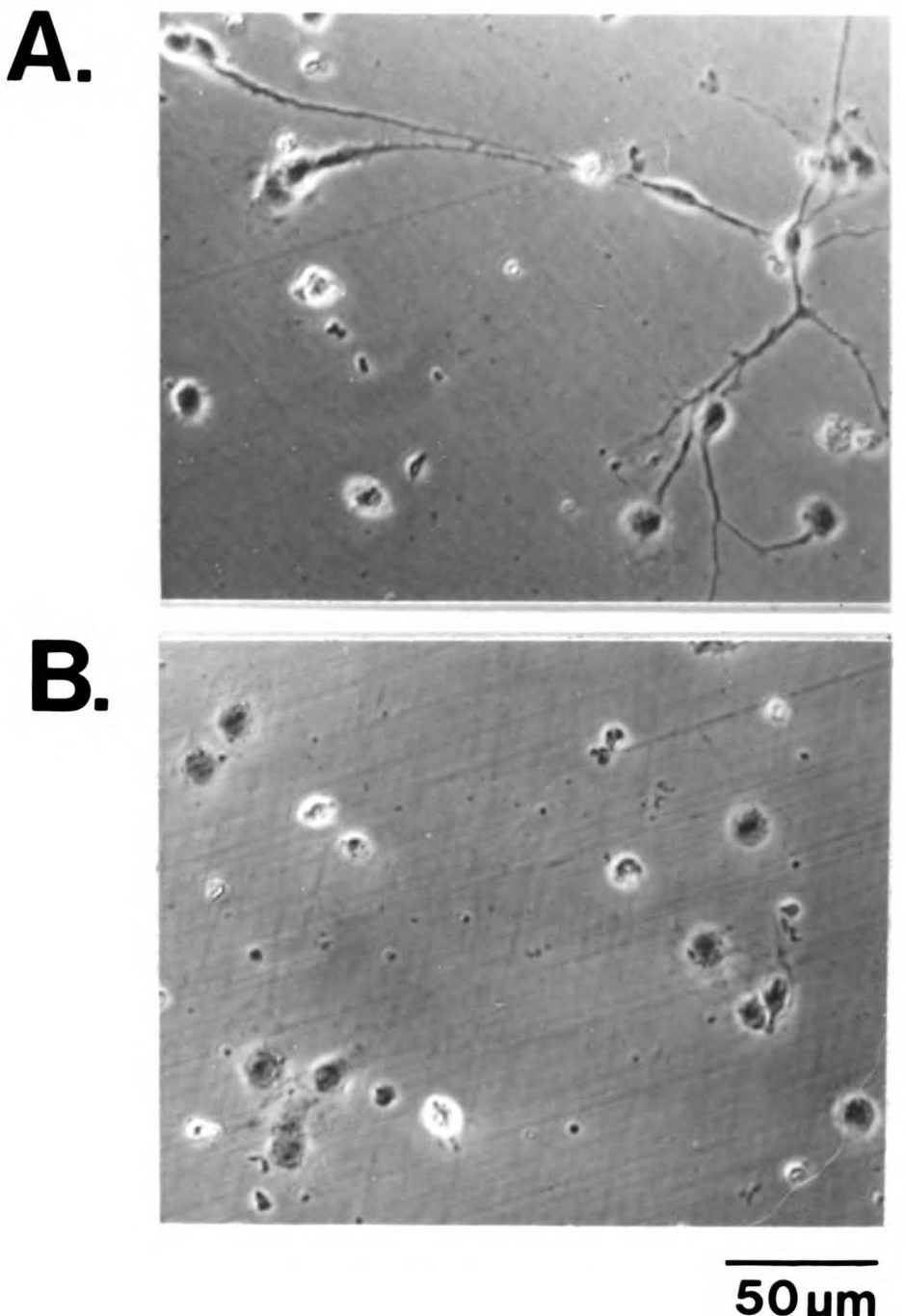
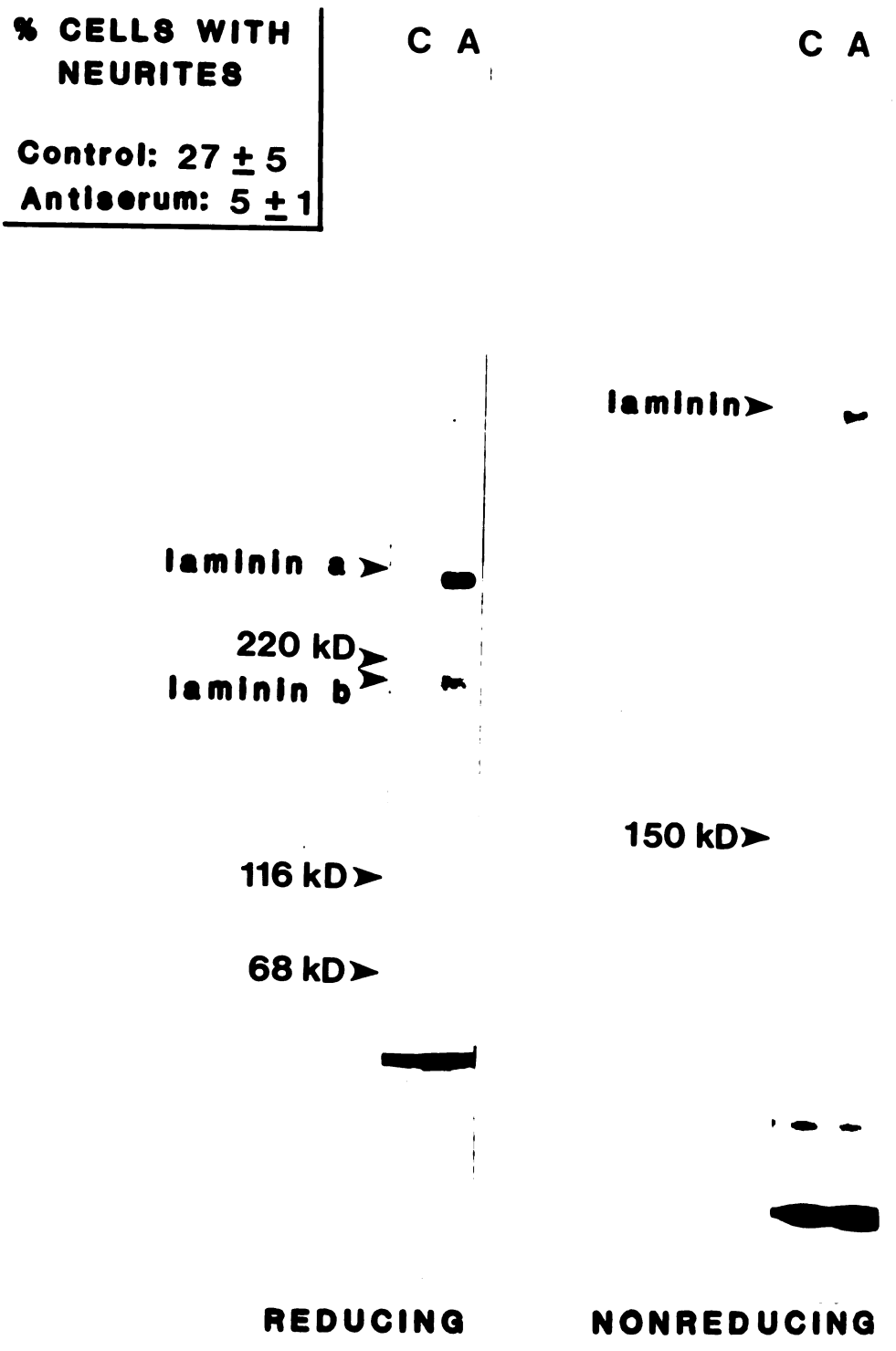

A.
B.
50µm

FIGURE 12.

IMMUNOPRECIPITATION OF NEURITE OUTGROWTH-PROMOTING ACTIVITY FROM MCM

The inset shows results of motoneuron neurite outgrowth assays of substrata treated with supernatants from immunoprecipitations of MCM. MCM was subjected to immunoprecipitation either with ("Antiserum") or without ("Control") anti-laminin antibodies. Proteins removed from MCM by this procedure were identified by SDS-polyacrylamide gel analysis of identical immunoprecipitates from ^{35}S-methionine labelled MCM. Adjacent lanes from reducing and nonreducing gels are shown. C= control immunoprecipitation (anti-laminin antibodies omitted), A= experimental immunoprecipitation (affinity-purified anti-laminin antibodies were used). The molecular weight standards for the nonreducing gel were mouse EHS laminin (850-1,000 kD) and mouse immunoglobulin G (150 kD). Standards for the reducing gel were mouse EHS laminin heavy chain ("laminin a", 400 kD), cellular fibronectin (220 kD), laminin light chain ("laminin b", 200-220 kD), B-galactosidase (116 kD), and bovine serum albumin (68 kD).


% CELLS WITH NEURITES
Control: 27 ± 5
Antiserum: 5 ± 1
C A
C A
laminin
laminin a
220 kD
laminin b
150 kD
116 kD
68 kD
REDUCING
NONREDUCING

from MCM was, in fact, laminin, identical immunoprecipitations were performed using ^{35}S-methionine labeled MCM. The precipitated material (washed once in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.4) was subjected to electrophoresis under both reducing and non-reducing conditions (Fig. 12). In non-reducing gels, the specifically-immunoprecipitated material appeared as a single protein band that comigrated with authentic laminin (purified from mouse EHS sarcoma). This band was converted, upon reduction, to 2 bands with M_r's of ca. 400 k and ca. 200 k, appropriate for the heavy and light chains of laminin.

3. DISCUSSION

Fractionation of the partially-purified, neurite outgrowth-promoting factor in MCM revealed the presence of laminin in the active fractions. Furthermore, immunoprecipitation experiments showed that removal of laminin from MCM removed its neurite outgrowth-promoting activity. These results suggest that laminin in MCM is responsible for the ability of substrata treated with this conditioned medium to promote neurite outgrowth by motoneurons.

These results are in agreement with those of Lander et al. (1985 a,b), who reported that immunoprecipitation of laminin from BCE-CM, as well as several other types of conditioned media, eliminated the ability of these conditioned media to promote neurite outgrowth by rat sympathetic neurons. Fractionation of metabolically-labelled BCE-CM demonstrated that laminin was the only molecule present in all active fractions and absent from inactive

fractions. Those authors suggested that laminin is responsible for the substratum-binding, neurite outgrowth-promoting activity of all conditioned media that have this activity. The results presented in the second half of this chapter suggest that this is indeed the case for the neurite outgrowth-promoting activity in MCM that acts upon chick spinal motoneurons, and are consistent with the previously-demonstrated ability of purified laminin to promote motoneuron neurite outgrowth (Chapter 3, Section A.6.c.).

Enzymatic treatments of MCM, performed as part of the preliminary characterization of the motoneuron neurite outgrowth-promoting factor, suggested that heparan sulfate is associated with at least some of the activity in unfractionated MCM (see Section A of this chapter). However, evidence for the presence of the core protein of a heparan sulfate proteoglycan, associated with laminin in the active fractions, was not obtained when labelled column fractions were digested with heparitinase and subjected to electrophoresis under conditions in which the protein core of the BCE-CM heparan sulfate proteoglycan was easily visualized (data not shown; cf. Lander et al., 1985a). It is possible that the protein core of such a heparan sulfate proteoglycan from MCM is too small to be detected in the gels used in the present study. Such a small core protein for a heparan sulfate proteoglycan is not inconceivable; a recent study has demonstrated the presence of a heparan sulfate proteoglycan in mouse basement membranes with a protein core of an estimated 5-12 x 10^3 Da in molecular weight (Fujiwara et al., 1984). In addition, since the heparitinase digestions of labelled

MCM were performed after the MCM had been through several fractionation procedures, it is possible that heparan sulfate proteoglycan was lost during one of these steps. To resolve this question, it would be necessary to perform all fractionation and enzymatic digestion procedures using MCM that had been metabolically labelled with ^{35}S-sulfate, in order to trace the presence of sulfated proteoglycans and glycosaminoglycans. However, the fact that immunoprecipitation of laminin removes the neurite outgrowth-promoting activity from MCM, coupled with the knowledge that the heparan sulfate proteoglycan in the purified BCE-CM factor is not required for that factor's activity (Lander et al., 1985a), suggest that such experiments would offer little additional information about the essential component of the motoneuron neurite outgrowth-promoting activity in MCM. It appears that laminin is responsible for such activity in this conditioned medium, as for most conditioned media known to possess a substratum-binding, neurite outgrowth-promoting activity (Lander et al., 1985b).

CHAPTER 4.

CONDITIONED MEDIUM EFFECTS OF MOTONEURONS: ANALYSIS OF SURVIVAL-ENHANCING ACTIVITY

CHAPTER 4.

CONDITIONED MEDIUM EFFECTS ON MOTONEURONS: ANALYSIS OF SURVIVAL-ENHANCING ACTIVITY

A. THE SURVIVAL-ENHANCING ACTIVITY IN MYOTUBE CONDITIONED MEDIUM IS DISTINCT FROM ITS SUBSTRATUM-BINDING, NEURITE OUTGROWTH-PROMOTING ACTIVITY

1. MATERIALS AND METHODS

a. CELL CULTURE

Cultures of nonneuronal cells were as described in Chapter 3, Section A.2. Conditioned medium was also prepared as described in that section of Chapter 3. Motoneuron culture conditions will be listed separately for each experiment in this section (Section A) of Chapter 4.

b. EVALUATION OF MOTONEURON SURVIVAL

After the recorded number of days in culture, motoneuron cultures were fixed in PBS containing 2.5% glutaraldehyde and 5% sucrose, and washed into 0.9% NaCl prior to counting. At least 200 cells in a minimum of 10 random fields were counted for each culture, and two cultures were counted for each experimental condition reported. Motoneuron survival is reported as the percentage of cells bearing 1 or more processes greater than 2 cell diameters in

length ("% Cells with Neurites"). Cells with obvious fibroblast morphology were not counted. All other cells, including dead cells, were counted. Since intact cells that did not have neurites were counted in the same category as dead cells (i.e., as total cells), any motoneurons without processes, which may have been alive at the time of culture fixation and evaluation, would not be counted as surviving in this assay. However, because no simple, foolproof method for evaluating the state of cells without processes was available, it was felt that counting cells with neurites would be a rapid and conservative alternative for evaluating motoneuron survival. The reported percentage of cells with neurites, therefore, reflects the number of intact motoneurons, alive and bearing processes, at the recorded time in culture. Using this method, reliable and reproducible results were obtained.

2. RESULTS

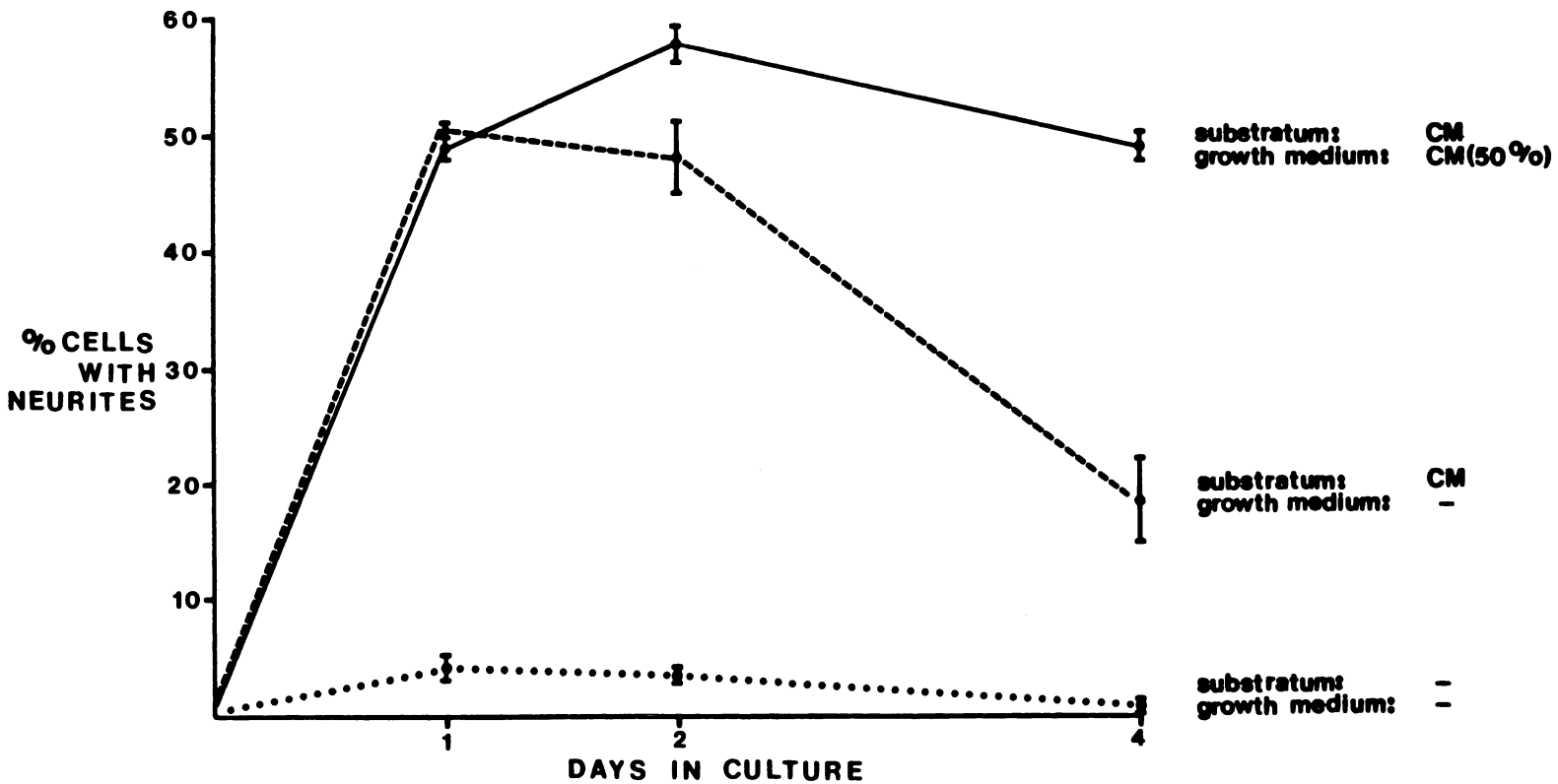
a. MYOTUBE CONDITIONED MEDIUM IN THE CULTURE MEDIUM ALLOWS MOTONEURONS TO SURVIVE

Although purified motoneurons survived and extended neurites for one to two days when grown on substrata treated with serum-free myotube conditioned medium (MCM_{SF}), they began to deteriorate unless this conditioned medium was also included in their culture medium. This is illustrated in Figure 13, which shows the numbers of healthy cells with neurites in motoneuron cultures grown for 4 days. Motoneurons grown in serum-free medium on polylysine substrata

FIGURE 13.

EFFECT OF MCM_{SF} ADDED TO MOTONEURON CULTURE MEDIUM

Motoneurons were grown under different substratum and medium conditions. Duplicate cultures were fixed at 1, 2 and 4 days. The percentage of healthy cells with neurites was determined. (.....) untreated polylysine substratum, unsupplemented serum-free growth medium; (----------) polylysine substratum treated with MCM_{SF}, unsupplemented serum-free growth medium; (_____) polylysine substratum treated with MCM_{SF}, serum-free growth medium supplemented with MCM_{SF} (to 50%). Error bars represent mean $\pm$ the deviation.


60
50
40
30
20
10
% CELLS WITH NEURITES
1
2
4
DAYS IN CULTURE
substratum: CM
growth medium: CM(50%)
substratum: CM
growth medium: –
substratum: –
growth medium: –

never developed neurites to any appreciable extent. Motoneurons grown on substrata that had been treated with MCM_{SF} extended neurites and remained healthy for about 2 days in culture, but by day 4 had deteriorated, as shown by the decrease in the number of healthy neurons with neurites in the cultures. However, if serum-free myotube conditioned medium was included in the culture medium, as well as being present on the substratum, motoneurons grew and remained healthy for at least 4 days in serum-free culture.

This apparent trophic activity of myotube conditioned medium could be explained in several ways: (1) Myotube conditioned medium could contain a particular trophic factor, which was necessary for motoneuron survival in culture; or (2) motoneurons might take up and use up the substratum-bound factor from myotube conditioned medium. If so, they might require a continuous supply of the factor. This could be provided by the conditioned medium that had been added to the culture medium. Finally, (3) the survival-promoting effect of myotube conditioned medium might not be due to the presence in it of any specific trophic factor, but might instead be due to a general medium conditioning effect. Several experiments were performed to test these hypotheses.

b. ADDITION OF NEURITE OUTGROWTH-PROMOTING FRACTION TO THE CULTURE MEDIUM DOES NOT ENHANCE MOTONEURON SURVIVAL

In order to determine whether the addition of MCM_{SF} to the culture medium was of benefit to motoneurons simply because it provided them with a continuous supply of the substratum-binding

factor, the following was done: The substratum-binding factor was partially purified from MCM by ammonium sulfate precipitation and gel filtration as described in Chapter 3 (Section A.5.d. and Figure 8). Motoneurons were cultured on polylysine substrata that had been treated with this partially-purified fraction of the substratum-binding factor. In addition, this partially purified fraction (NOPA, for "Neurite Outgrowth-Promoting Activity) was then added back to the medium of some motoneuron cultures, and replenished every 2-3 days for 6 1/2 days, to see whether it could enhance motoneuron survival. The results are shown in Figure 14. While the addition of MCM_{SF} to motoneuron growth medium promoted survival, the addition of the neurite outgrowth-promoting fraction of MCM (NOPA) had no effect. It was unlikely that this lack of effect reflected toxic or inhibitory substances that were present in the NOPA fraction, since the addition of both MCM_{SF} and the NOPA fraction to cultures was as effective as MCM_{SF} alone. Instead, it must be concluded that MCM_{SF} supplied motoneurons with something more than just the substratum-binding factor.

The extent of the difference between motoneurons grown in the presence and absence of MCM_{SF} was reflected dramatically in cell body size and neurite morphology. This is shown in Figure 15. At short-times (1-2 days) in culture, neurons grown on the neurite outgrowth-promoting fraction of MCM (NOPA) looked similar, whether or not MCM_{SF} was included in the culture medium (15D, G). However, by 4 days in culture, cells on the NOPA substratum still had neurites, but appeared to be deteriorating (15H). This was

FIGURE 14.

MOTONEURON SURVIVAL IS NOT ENHANCED WHEN THE NEURITE OUTGROWTH-PROMOTING FRACTION OF MCM IS ADDED BACK TO THE MEDIUM

The neurite outgrowth-promoting fraction of MCM (NOPA) was prepared from 25 ml of MCM by ammonium sulfate precipitation and gel filtration. It was recovered in a final volume of 5 ml, in a buffer (200 mMNaCl, 20 mM Hepes, pH 7.4) containing 0.2 mg/ml hemoglobin (see Chapter 3, Section A.5.d. and Figure 8). Motoneurons were grown under the following conditions and fixed and evaluated at the indicated times: (—■--) untreated polylysine substratum, unsupplemented serum-free growth medium; (—■—) NOPA-treated substratum, unsupplemented serum-free growth medium; (—●—) NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}; (—··—●—·) NOPA-treated substratum, serum-free growth medium supplemented with 20% NOPA fraction and sufficient distilled water to readjust the salt concentration to 145 mOsM; (····○·····) NOPA-treated substratum, serum-free growth medium supplemented with <u>both</u> MCM_{SF} (50%) and NOPA (20%) plus distilled water as above. The percentage of healthy cells with neurites is given as the mean of duplicate cultures ± the deviation.

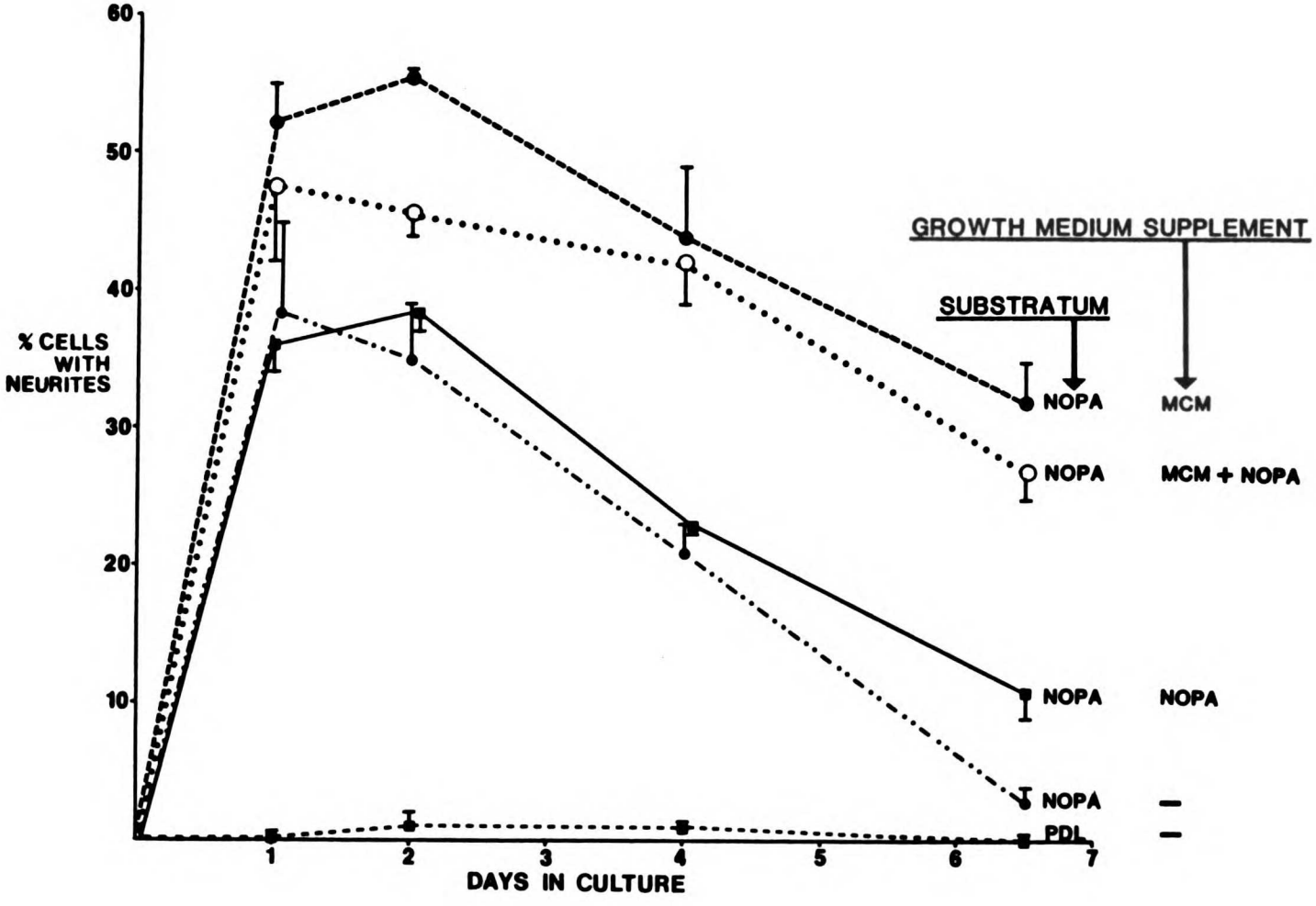

60
50
40
30
20
10
% CELLS WITH NEURITES
1
2
3
4
5
6
7
DAYS IN CULTURE
GROWTH MEDIUM SUPPLEMENT
SUBSTRATUM
NOPA
MCM
NOPA
MCM + NOPA
NOPA
NOPA
NOPA
—
PDL
—

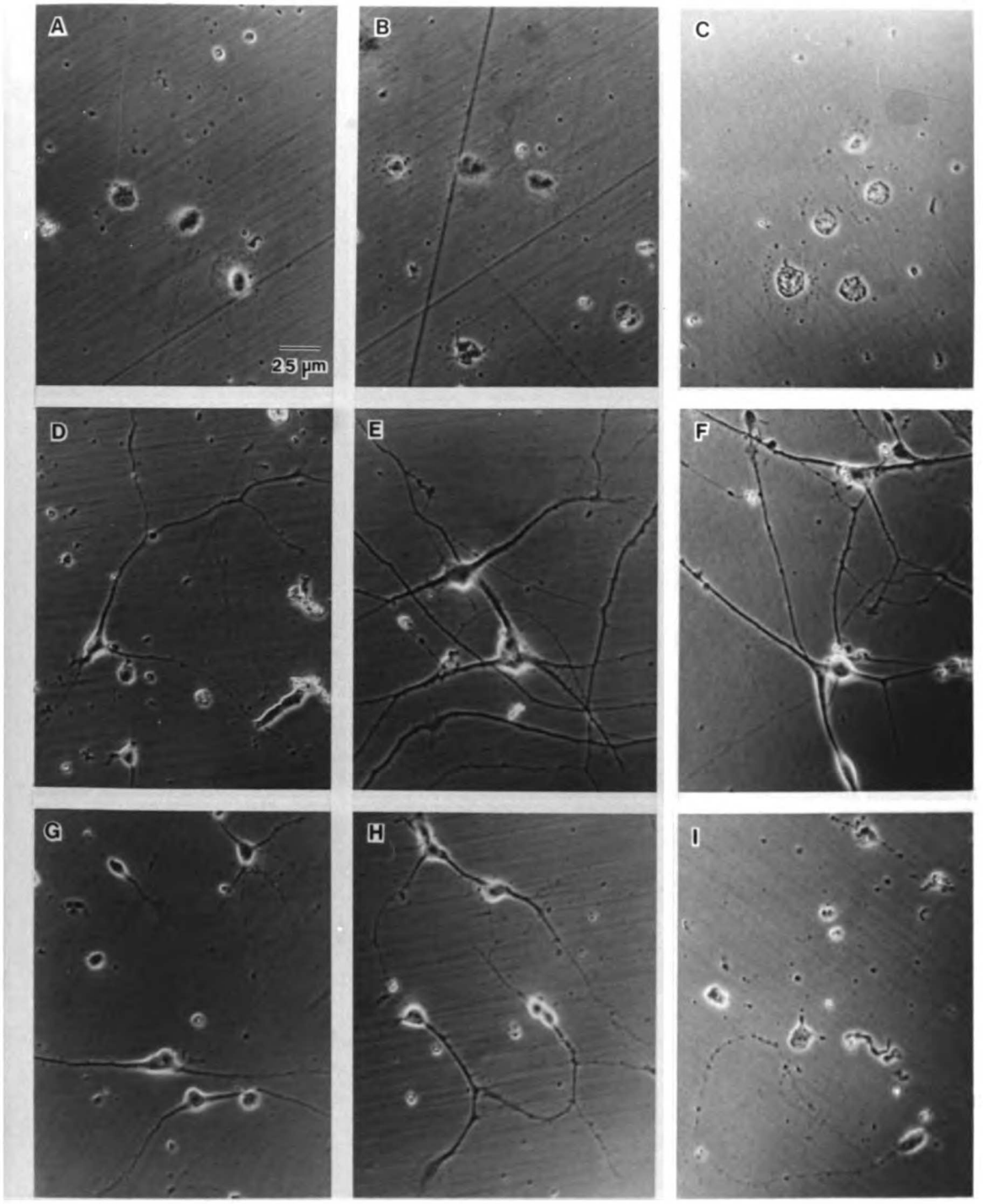
FIGURE 15.

MOTONEURONS GROWN UNDER CONDITIONS DESCRIBED IN FIGURE 14

A-C: untreated polylysine substratum, unsupplemented serum-free growth medium, cells fixed at 1 day (A), 4 days (B), and 6 1/2 days (C).

D-F: NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}; cells fixed at 1 day (D), 4 days (E), and 6 1/2 days (F).

G-I: NOPA-treated substratum, serum-free growth medium supplemented with 20% NOPA as described in Figure 13; cells fixed at 1 day (G), 4 days (H), and 6 1/2 days (I).


A
B
C
25 µm
D
E
F
G
H
I

especially apparent when these cells were compared with motoneurons grown for 4 days on NOPA in the presence of MCM_{SF} (15E). These motoneurons had larger cell bodies and larger numbers of longer, thicker neurites. By 6 1/2 days in culture these differences in cell morphology and survival were even greater (15 F,I) and were reflected in the graphs of Figure 14. Since changes in morphology indicating deterioration were noticed before this deterioration was advanced enough to show in cell counts, the graphs in Figure 14 are probably delayed with respect to the real time course of motoneuron survival under these conditions.

c. EFFECTS OF OTHER CONDITIONED MEDIA ON MOTONEURON SURVIVAL

Another possible explanation of the apparent trophic activity of myotube conditioned medium could be a general medium conditioning effect, such as the secretion by other cells of nutritive substances which the motoneurons did not themselves make in culture, or which they made in insufficient quantities for their own utilization (e.g. Ham, 1973). If the survival-enhancing effect of myotube conditioned medium was due to such a general conditioning effect, then media conditioned by other cell types might be able to substitute for MCM_{SF}. To test this possibility, motoneurons were plated onto polylysine substrata treated with the NOPA fraction of MCM and grown for 6 1/2 days in growth medium supplemented with serum-free conditioned medium from myotubes (MCM_{SF}), spinal cord dividing cells ($SCCM_{SF}$), or fibroblasts (FCM_{SF}) (Figure 16A). Survival of motoneurons over 6 1/2 days in culture was enhanced to

FIGURE 16.

EFFECT OF DIFFERENT CONDITIONED MEDIA ON MOTONEURON SURVIVAL

Motoneurons were grown under the following conditions and fixed and evaluated at the indicated times:

A. (⊙) untreated polylysine substratum, unsupplemented serum-free growth medium.

(⊙) NOPA-treated substratum, unsupplemented serum-free growth medium.

(■) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$.

(●) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF}.

(▲) NOPA-treated substratum, serum-free growth medium supplemented with 50% MCM_{SF}.

B. (△) NOPA-treated substratum, serum-free growth medium supplemented with 20% MCM_{SF}.

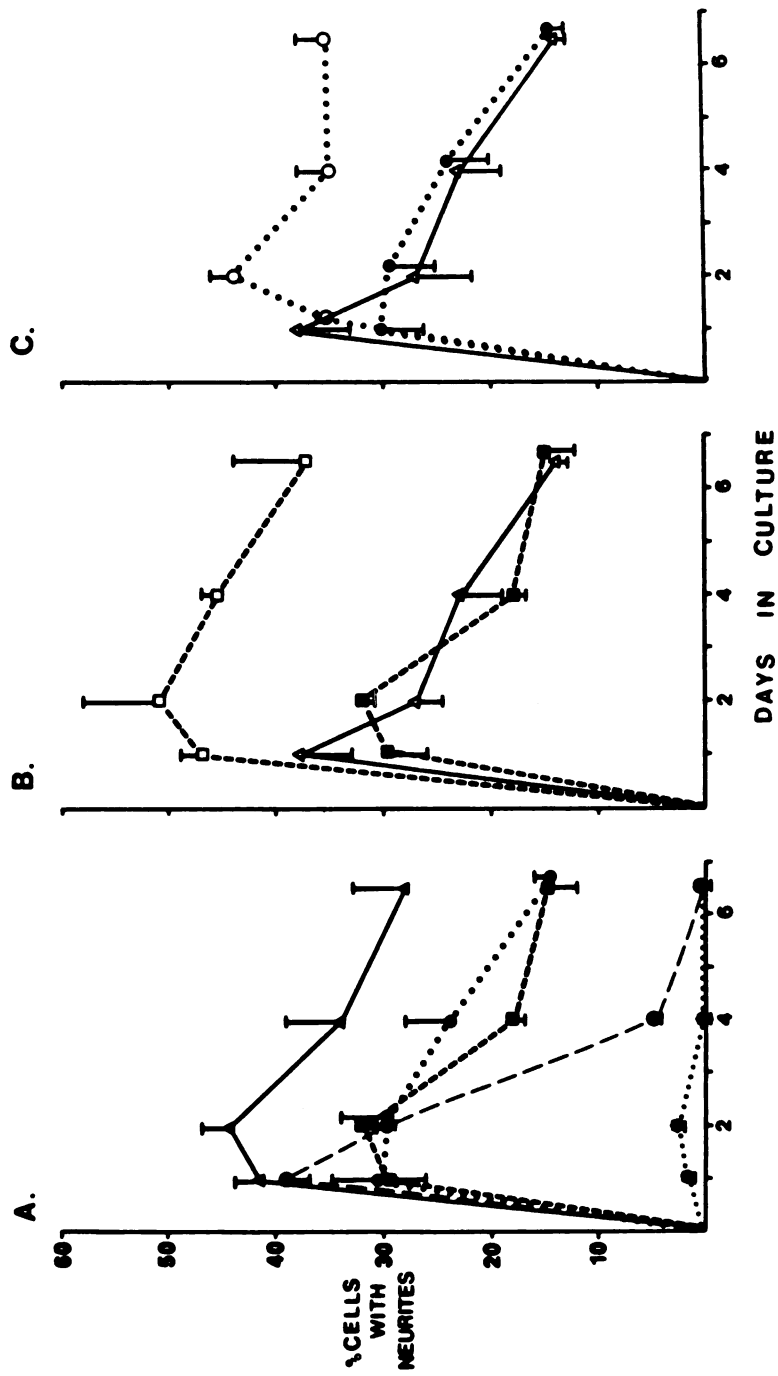
(■) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$.

(□) NOPA-treated substratum, serum-free growth medium supplemented with 50% $SCCM_{SF}$ plus 20% MCM_{SF}.

C. (△) NOPA-treated substratum, serum-free growth medium supplemented with 20% MCM_{SF}.

(●) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF}.

(○) NOPA-treated substratum, serum-free growth medium supplemented with 50% FCM_{SF} plus 20% MCM_{SF}.


A.
B.
C.
% CELLS WITH NEURITES
60
50
40
30
20
10
2
4
6
DAYS IN CULTURE

some extent by the presence of any of these conditioned media. However, motoneurons grown in MCM_{SF} showed initial neurite outgrowth greater than that of motoneurons grown in the other conditioned media. Furthermore, the percentage of healthy motoneurons at 6 1/2 days in culture was greater in cultures supplemented with MCM_{SF} than in cultures supplemented with the other conditioned media.

The results at 1 day in several experiments indicated that motoneurons grown in $SCCM_{SF}$ and FCM_{SF} had their initial outgrowth depressed relative to that of cells grown in MCM_{SF} or even in the absence of any conditioned medium supplement (16A). To see whether this apparent effect might be due to the presence of toxic or inhibitory substance(s) in the $SCCM_{SF}$ and FCM_{SF}, motoneurons were grown in these media combined with small amounts of MCM_{SF} (final medium: 50% $SCCM_{SF}$ or FCM_{SF}, 20% MCM_{SF}). Motoneurons grown in the combined conditioned media showed initial neurite outgrowth and long-term survival that equalled or exceeded that seen in medium supplemented with standard amounts (50%) of MCM_{SF} (B,C).

Since the initial depression of neurite outgrowth seen when motoneurons were grown in $SCCM_{SF}$and FCM_{SF}-supplemented media could be overcome by the addition of low levels of MCM_{SF}, it was unlikely that large amounts of toxic or inhibitory substances were present in these media.

3. DISCUSSION

In addition to its neurite outgrowth-promoting activity,

myotube conditioned medium added to the culture medium of motoneurons enhanced motoneuron survival over periods of greater than two days in culture. Reports from several other groups confirm the presence in myotube conditioned medium or muscle extracts of a survival-promoting activity for embryonic chick spinal cord cells (e.g., Nurcombe et al., 1984; Tanaka and Obata, 1982; Longo et al., 1982; Doherty et al., 1985). In some cases, embryonic chick motoneurons have been identified in mixed cultures by the presence in their somata of horseradish peroxidase, taken up via retrograde axonal transport from material injected into limb muscles in ovo. When the percentage of surviving labelled motoneurons was determined after one or two days in culture, it was found that myotube conditioned medium or muscle extracts enhanced the survival of identified motoneurons (Bennett et al., 1980; Slack and Pockett, 1982; Eagleson and Bennett, 1983). The results of these studies are consistent with the present finding that myotube conditioned medium enhances motoneuron survival. There were several possible explanations of this apparent trophic activity of myotube conditioned medium, and two were examined in different tests:

Motoneurons might take up and use up the substratum-binding factor during the course of long-term cultures, and therefore might require a continuous supply of the factor to maintain their neurites and remain healthy. To rule out this possibility, the substratum-binding factor was partially purified from MCM and added back to the medium of motoneuron cultures every 2 to 3 days for 6-1/2 days. Cultures supplemented in this way fared no better

than cultures grown in medium without supplement. In support of this, other investigators have reported that the survival-enhancing activity for spinal cord cells, found in muscle extracts or myotube conditioned medium, can be distinguished from the neurite outgrowth-promoting activity present in these extracts or conditioned media (Longo et al., 1982; Nurcombe et al., 1984; Doherty et al., 1985).

It was also possible that the survival-enhancing effect of MCM added to motoneuron culture medium was due to a general medium conditioning effect. Such an effect could be due to the secretion by other cultured cells of nutritive substances which the motoneurons did not themselves make in culture, or which they made in insufficient quantities for their own utilization. This possibility was certainly consistent with the results reported here, although it was not the only possible explanation for them. A finding that medium conditioned by myotubes was the only type of conditioned medium capable of supporting motoneuron survival would have suggested that a target-specific trophic molecule was involved. A finding that any type of conditioned medium was as effective as myotube conditioned medium would have argued in favor of a general medium-conditioning effect. In fact, the experimental data lay somewhere between these two possibilities. MCM_{SF}, $SCCM_{SF}$, and FCM_{SF} all enhanced motoneuron survival, but more motoneurons survived for extended periods in MCM_{SF} than in FCM_{SF} or $SCCM_{SF}$. The results of these tests of different conditoned media were complicated, and must be interpreted with caution. Despite their ability to enhance motoneuron survival over the course of one week, FCM_{SF} and $SCCM_{SF}$

appeared to depress initial neurite outgrowth (24 hours) compared to controls (serum-free growth medium alone). This indicated that substances that interfered with initial cell attachment, survival, or neurite outgrowth might be present in FCM_{SF} and $SCCM_{SF}$. Considering that the effects of such substances would be superimposed on any survival-enhancing effects, it was not possible, on the basis of these data, to determine accurately the relative quantities of survival-enhancing substance(s) in the three conditioned media.

Finally, it was possible that the survival-enhancing activity of myotube conditioned medium was due to the presence in it of a "motoneuron trophic factor". However, survival of all motoneurons over the course of one week in culture did not occur, even under the optimum conditions tested in these experiments (motoneuron growth medium supplemented with 50% MCM_{SF}). This suggested that motoneurons might require further trophic support than that provided by MCM_{SF}. It may have been be that myotubes in culture did not secrete an appropriate "motoneuron trophic factor". Alternatively, myotube conditioned medium might have contained too little of such a trophic factor, or the factor might have been present in too degraded a state, to be completely effective. Conclusive answers to these questions will require the identification of putative "motoneuron trophic factors" and assays for their presence in myotube conditioned medium.

B. PHYSICAL AND ENZYMATIC CHARACTERIZATION OF THE SURVIVAL-ENHANCING ACTIVITY IN MYOTUBE CONDITIONED MEDIUM

The preliminary characterization of the survival-enhancing activity in myotube conditioned medium indicated that this was distinct from its substratum-binding, neurite outgrowth-promoting activity. However, simple tests designed to determine whether this ability of MCM to enhance motoneuron survival could be attributed to a general medium conditioning effect, or the presence in MCM of a specific "motoneuron trophic factor", were not conclusive. It was decided that further characterization of the survival-enhancing activity would be useful in determining the course of future experiments directed toward answering this question.

1. CELL CULTURE AND CONDITIONED MEDIA

a. MYOTUBE CONDITIONED MEDIUM

Serum-free myotube conditioned medium (MCM_{SF}), which was used in all of the experiments described below where supplementation of serum-free growth medium is described, was prepared as described in Section A.5.a. of Chapter 3. Myotube conditioned medium made with 1% horse serum was used to prepare the neurite outgrowth-promoting fraction (NOPA) for motoneuron culture substrata (see below); the production of this conditioned medium is described in Chapter 3, Section B.3.

b. MOTONEURON CULTURE SUBSTRATA

Motoneurons were cultured in multiwell plates (96-well clusters, Costar) that were exposed to poly-D-lysine (Sigma, 1 mg/ml in 0.1 M sodium borate, pH 8.4) overnight, washed with deionized distilled water, and sterilized under ultraviolet light. The culture surfaces were then incubated overnight at 4°C with 50 ul/well of the "neurite outgrowth-promoting activity" fraction (NOPA fraction) from myotube conditioned medium (cf. Chapter 3, Section A.6.b.), prepared as follows: 25 ml of myotube conditioned medium made with 1% horse serum was mixed with the protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mM), EDTA (3 mM), and pepstatin (10^{-7} M). To deplete the MCM of fibronectin, 1.5 ml of packed gelatin-Sepharose (1 mg/ml resin capacity; see Chapter 3, Section B.1.) was then added to the MCM and the mixture was gently stirred for 2 hours at room temperature. The gelatin-Sepharose was removed by centrifugation, and the fibronectin-depleted MCM was cooled to 4°C and brought to 45% saturating ammonium sulfate. The solution was left to stir for 1 hour at 4 °C, after which the precipitated material was removed by centrifugation. The precipitate was then resuspended in 1.3 ml of buffer (0.2 M NaCl, 0.02 M Hepes, pH 7.4) containing 0.2 mg/ml hemoglobin (Sigma Type IV) and protease inhibitors as described above. This material was spun to remove insoluble material, after which ultra-pure sucrose (Schwarz-Mann) was added to 5% (w/v) to increase the density of the solution, and sufficient phenol red (approximately 10 ul of a concentrated aqueous solution) was added to color it lightly. This material was then applied to a column

(1.5 x 8.5 cm) of Sepharose CL-6B equilibrated in the buffer described above. One (1) ml fractions were collected, and the first 4 ml of the void volume (calculated immediately before each run by calibrating the column with a 1.3 ml sample of column buffer containing sucrose and blue dextran) were pooled and used, undiluted, as the NOPA fraction.

After overnight treatment with the NOPA fraction, culture surfaces were washed 3-4 times in DME (see below), and culture medium was added to wells, shortly before motoneurons were plated into them.

c. MOTONEURON CULTURE MEDIA

All motoneuron culture medium was serum-free, and consisted of Dulbecco's Modified Eagle's Medium containing 0.45% glucose (DME), supplemented with ovotransferrin (Otf; 25 ug/ml), insulin (5 ug/ml), putrescine dihydrochloride (100 uM), progesterone (20 nM), and selenium (30 nMO (modified from Bottenstein and Sato, 1979). Glutamine (2mM), penicillin (100 U/ml), and streptomycin (100 ug/ml) were added as for all cell cultures. Motoneuron cultures were fed every two days by removing half the medium, and replacing it with fresh growth medium containing the supplements listed above. When conditioned medium was added as supplement to motoneuron culture medium, the entire mixture was brought to the concentrations of serum-free growth supplements listed above.

2. MATERIALS AND METHODS

a. MATERIALS

Insoluble trypsin (Type T-1763 Trypsin-agarose, 100 units per ml of packed beads) was obtained from Sigma (St. Louis, Missouri). Ultrafiltration membrane cones (CF 25; 25,000 Dalton cutoff) were obtained from Amicon (Danvers, Massachusetts).

b. HEAT TREATMENT OF MYOTUBE CONDITIONED MEDIUM

Aliquots of serum-free myotube conditioned medium (MCM_{SF}) were placed in a boiling water bath for 15 minutes, and then placed on ice to cool. Identical aliquots of the same batch of MCM_{SF} were used as controls. Treated or untreated aliquots of myotube conditioned medium were added to fresh growth medium (DME) to 50% of the final volume, and then supplements listed above (Chapter 4, Section B.1.b.) were added. Plain DME, with supplements listed above, served as the control growth medium.

c. PROTEASE TREATMENT OF MYOTUBE CONDITIONED MEDIUM

Trypsin-agarose was washed in 10 mM sodium acetate, pH 5.1, spun for 2 minutes at 100 x g, and then resuspended in DME. One (1) ml (100 units) of packed beads was added to 5 ml of MCM_{SF}, and the mixture was brought to 37°C and incubated, shaking slowly, for 4 hours at 37°C. As a control, trypsin beads were prepared as

described above, but were boiled for 20 minutes before being added to MCM_{SF} and incubated for 4 hours at 37°C. After incubation, the trypsin beads were removed by centrifugation, and the MCM_{SF} was filtered (0.2 um) and used immediately.

d. ULTRAFILTRATION OF MYOTUBE CONDITIONED MEDIUM

Ultrafiltration membrane cones were soaked for 6 hours in deionized distilled water prior to use. To minimize nonspecific adsorption to the filters by components of serum-free myotube conditioned medium, the filters were blocked before used with 1 mg/ml hemoglobin (human hemoglobin, 2X crystallized; Sigma) in PBS as follows: 7 ml of hemoglobin solution were placed in the membrane, and spun at 800 x g for 10 minutes. Additional hemoglobin solution (approximately 5 ml) was added to replace the solution that had passed through the membrane, and the procedure was repeated. Before use, the retained hemoglobin was removed from the filter and the filter was washed in plain DME to remove nonadsorbing hemoglobin. This procedure also served to check the integrity of each membrane before use, since hemoglobin was easily detectable by its color, and it could easily be determined whether any had passed through the filter.

The "retained" fraction of MCM_{SF} was prepared as follows: 7 ml of MCM_{SF} was placed in an ultrafiltration cone and spun for 10 minutes at 800 x g. Plain DME (approximately 4-5 ml) was then added to the retained portion to bring the volume back up to 7 ml, and the membrane was spun again. This rinsing procedure was repeated

twice, to ensure that no low molecular weight components of the myotube conditioned medium remained in the retained fraction. The retained fraction was reconstituted to its original volume, with plain DME, before use.

Material that passed through the ultrafiltration membrane was obtained by filtering separately a second aliquot of the same batch of MCM_{SF} until all liquid had passed through the filter. The flow-through fraction was then used just as it came through the filter.

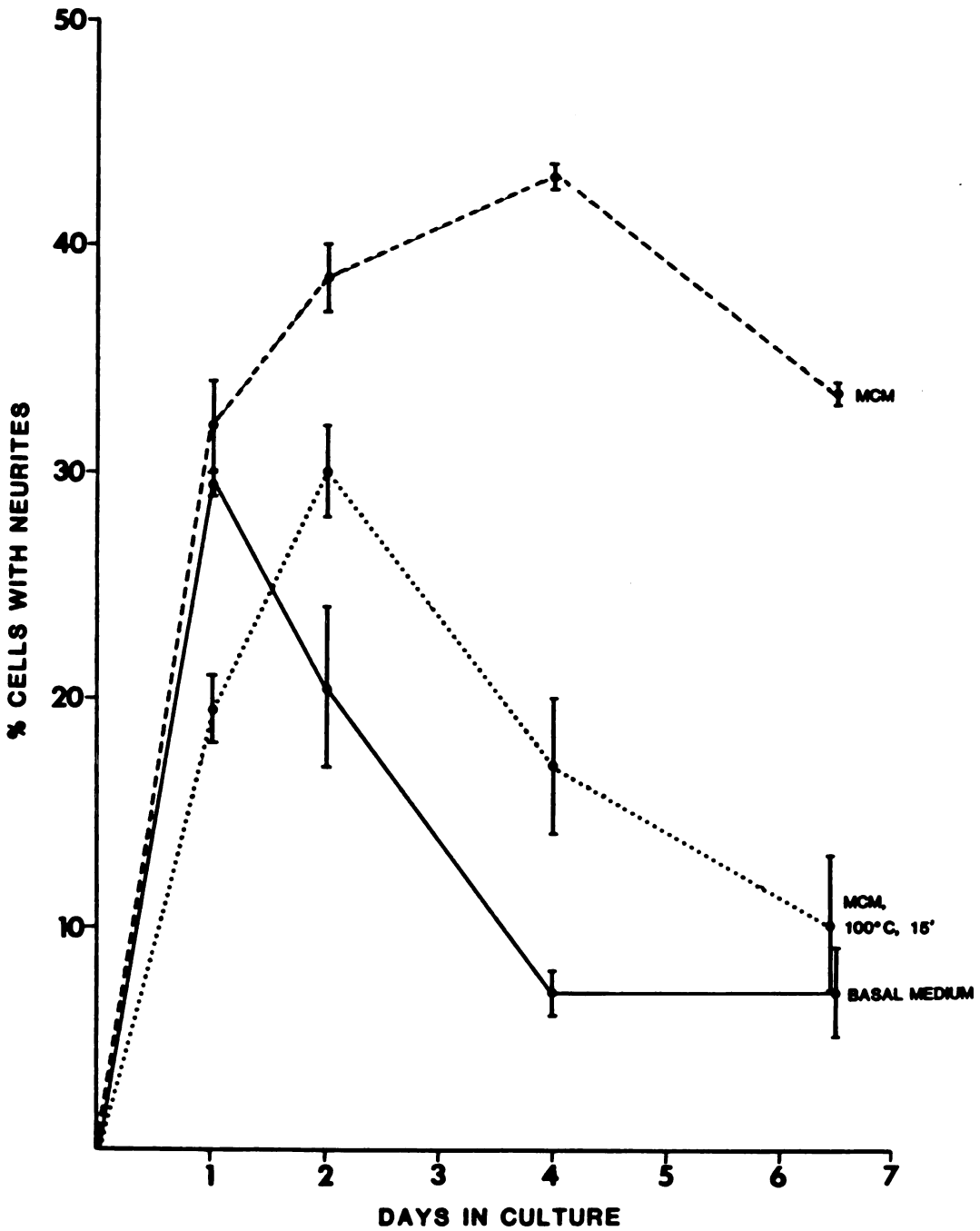
3. RESULTS

The ability of myotube conditioned medium to enhance the survival of motoneurons was destroyed by boiling, as shown in Figure 17. Motoneurons were grown for one week in serum-free medium which contained either no supplement, 50% serum-free myotube condtioned medium, or 50% serum-free myotube conditioned medium that had been subjected to 100°C for 15 minutes. Survival of motoneurons was better in cultures in which the growth medium was supplemented with untreated MCM_{SF}, than in cultures supplemented with boiled MCM_{SF} or unsupplemented cultures. Over the course of the first 4 days in culture, motoneuron survival appeared to be slightly better in growth medium supplemented with boiled MCM_{SF} than in growth medium containing no conditioned medium supplement whatsoever. However, this difference disappeared by the end of a week in culture. By this time, motoneurons grown in untreated myotube conditioned medium were faring almost as well as they had at 24 hours in culture, but very few motoneurons were alive in cultures grown in unsup-

FIGURE 17.

EFFECT OF BOILING ON THE SURVIVAL-ENHANCING ACTIVITY IN MCM_{SF}: SURVIVAL OF MOTONEURONS IN SUPPLEMENTED MEDIA

Motoneurons were grown for one week in serum-free medium, on polylysine-treated culture substrata that had been incubated with the neurite outgrowth-promoting fraction from MCM (see text). Experimental culture media were supplemented to 50% of the final volume with untreated serum-free myotube conditioned medium (MCM_{SF}) (----------), or MCM_{SF} that had been boiled for 15 minutes (.....). Serum-free growth medium with no conditioned medium added served as a control (_____). Data are given as the mean $\pm$ the deviation.


50
40
30
20
10
% CELLS WITH NEURITES
1
2
3
4
5
6
7
DAYS IN CULTURE
MCM
MCM,
100°C, 15'
BASAL MEDIUM

plemented medium or medium supplemented with boiled MCM_{SF}.

Similarly, incubation with trypsin-agarose removed the ability of myotube conditioned medium to enhance motoneuron survival. This is shown in Figure 18. That the loss of the survival-enhancing activity was not due to nonspecific adsorption of conditioned medium components to the agarose is shown by the control in which boiled trypsin-agarose was incubated with MCM_{SF}; myotube conditioned medium retained its ability to enhance motoneuron survival after this treatment. Thus, the survival-enhancing activity in myotube conditioned medium appears to consist, in large part at least, of protein.

Figure 19 shows the results of an experiment in which MCM_{SF} was subjected to ultrafiltration. Most of the survival-enhancing activity in myotube conditioned medium passed through a filter with a 25,000 Dalton cutoff. This suggests that much of the survival-enhancing activity is possessed by a component or components of MCM_{SF} with molecular weight less than 25,000 Daltons. This result should be interpreted with caution, however, since not all survival-enhancing activity could be recovered. After a week in culture, motoneurons grown in medium supplemented with the flow-through fraction fared better than motoneurons grown in the retained fraction; however, the extent of motoneuron survival in medium supplemented with the flow-through fraction was not as great as that in medium supplemented with untreated MCM_{SF}. In addition, motoneurons grown in the flow-through fraction did not appear to be as healthy as motoneurons grown in unfractionated MCM_{SF}; their neurites appeared to

FIGURE 18.

EFFECT OF TRYPSIN ON THE SURVIVAL-ENHANCING ACTIVITY IN MCM_{SF}: SURVIVAL OF MOTONEURONS IN SUPPLEMENTED MEDIA

Motoneurons were grown for one week in serum-free medium, on polylysine-treated culture substrata that had been incubated with the neurite outgrowth-promoting fraction of MCM (see text). Experimental culture media were supplemented to 50% of the final volume with untreated serum-free myotube conditioned medium (MCM_{SF}) (----------), or MCM_{SF} that had been incubated with trypsin-agarose for 4 hours at 37°C (.....). Serum-free growth medium supplemented with MCM_{SF} that had been incubated with inactivated trypsin-agarose (boiled for 20 minutes)(.____.____.), or with no conditioned medium added (_____), served as controls. Data are given as the mean ± the deviation.

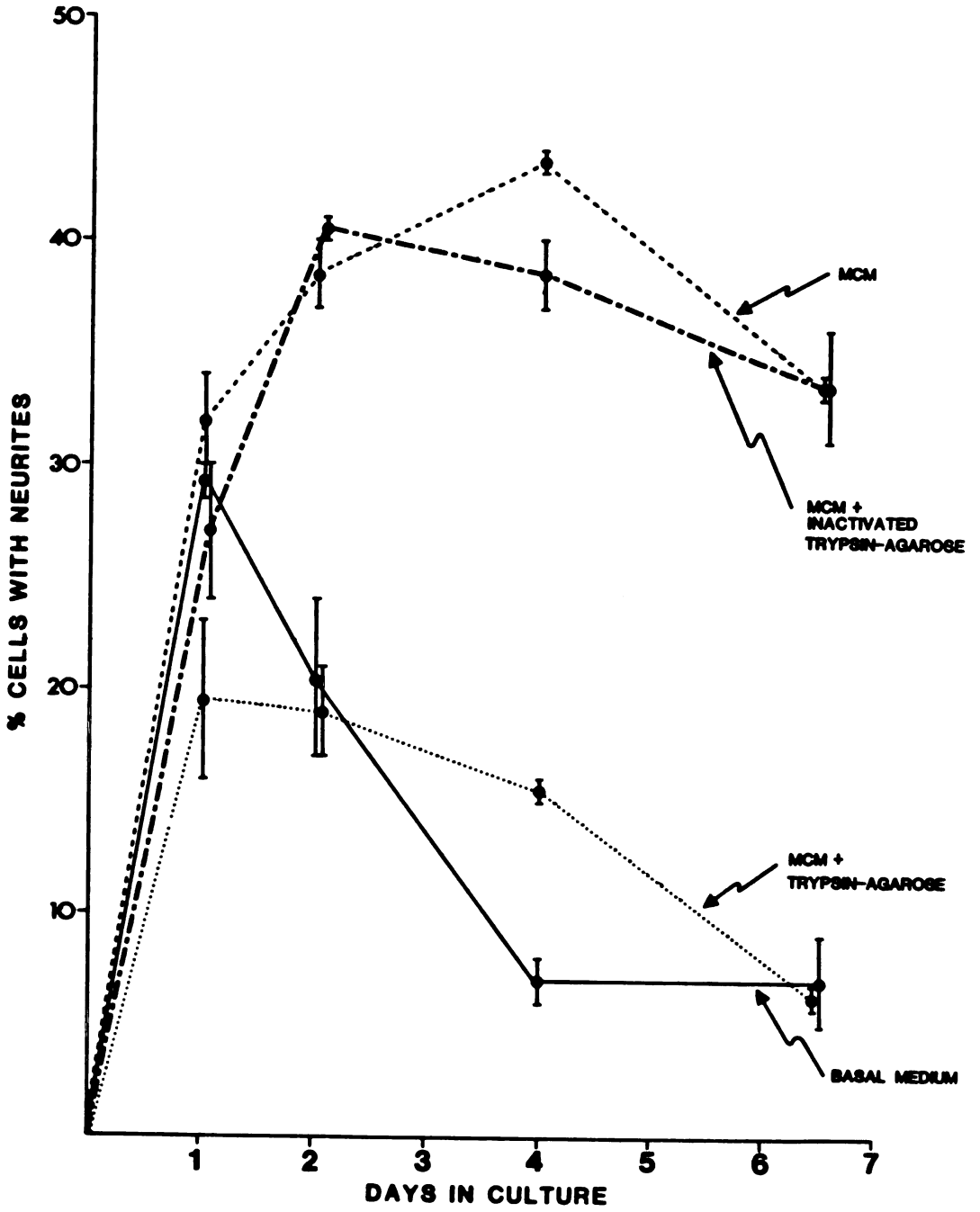
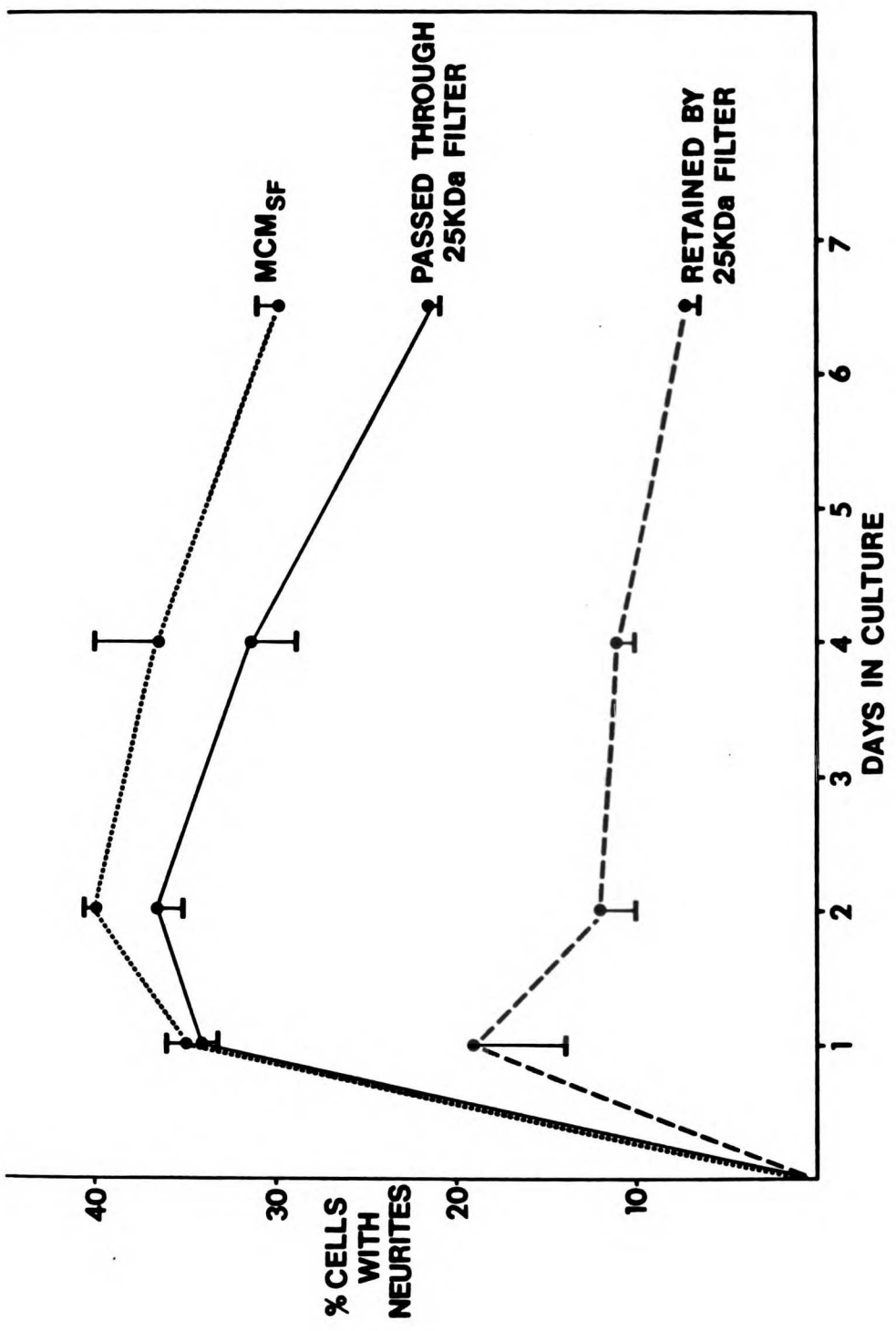

50
40
30
20
10
% CELLS WITH NEURITES
1
2
3
4
5
6
7
DAYS IN CULTURE
MCM
MCM +
INACTIVATED
TRYPSIN-AGAROSE
MCM +
TRYPSIN-AGAROSE
BASAL MEDIUM

FIGURE 19.

ULTRAFILTRATION OF MCM_{SF}:

SURVIVAL OF MOTONEURONS IN SUPPLEMENTED MEDIA

Motoneurons were grown for one week in serum-free medium, on polylysine-treated culture substrata that had been incubated with the neurite outgrowth-promoting fraction of MCM (see text). Serum-free culture media were supplemented to 50% of the final volume with untreated serum-free myotube conditioned medium (MCM_{SF})(----------), the fraction of MCM_{SF} that passed through an ultrafiltration membrane with a 25,000 Da cutoff (_____), or the fraction of MCM_{SF} that was retained by an ultrafiltration membrane with a 25,000 Da cutoff (___ ___ ___). Data are given as the mean $\pm$ the deviation.


MCM$_{SF}$
PASSED THROUGH 25KDa FILTER
RETAINED BY 25KDa FILTER
% CELLS WITH NEURITES
40
30
20
10
1
2
3
4
5
6
7
DAYS IN CULTURE

be thinner and their cell bodies smaller, than those of motoneurons grown in whole conditioned medium.

4. DISCUSSION

Ultrafiltration of myotube conditioned medium was performed in order to estimate the size fraction in which survival-enhancing activity from MCM_{SF} could be recovered. This method was chosen in order to avoid the dilution that would be introduced by a method such as gel filtration. Pilot experiments had demonstrated that the full survival-enhancing activity in a given batch of MCM_{SF} could not be recovered when this conditioned medium was diluted more than two-fold (data not shown), making poor choices of experiments in which the survival-enhancing activity might be diluted.

The destruction of myotube conditioned medium's survival-enhancing activity for motoneurons by boiling and by trypsin suggest that this activity may reside in a protein, or proteins. The results of the ultrafiltration experiment further suggest that much of the survival-enhancing activity resides in a component of MCM_{SF} that is less than 25,000 Daltons in molecular weight. As a first working hypothesis, then, it is proposed that the ability of myotube conditioned medium to enhance motoneuron survival depends upon the presence in it of a protein, or proteins. This protein or proteins may have a molecular weight of less than 25,000 Daltons. This estimate of molecular weight must be taken with caution, however, since all of the activity in myotube conditioned medium was not recovered by the ultrafiltration procedure. The possibility

of loss during this procedure of larger components of myotube conditioned medium, which might also have possessed motoneuron survival-enhancing activity, cannot be dismissed.

Tanaka and Obata (1982) have reported that myotube conditioned medium contains survival-enhancing activity for spinal cord cells taken from 6 and 7 day chick embryos. The activity from conditioned medium was trypsin-sensitive but proved difficult to resolve by gel filtration chromatography; they reported three peaks of activity, with approximate M_rs of 40,000, 70,000, and 150,000. The relevance of their work to that reported above is in any case difficult to assess, since they assayed survival only at 1 day in culture. It is probably impossible to resolve accurately any survival-enhancing effects on motoneurons at such short times, since motoneurons grown on a neurite outgrowth-promoting substratum can survive for one or two days in serum-free medium in the absence of any conditioned medium or tissue extracts (see Section A of this chapter).

Doherty and colleagues (1985) have also reported that the presence of muscle cell conditioned medium (in this case, from human biopsies) enhances the survival of dissociated spinal cord neurons in mixed spinal cord cultures from 7 day chick embryos. In this study, neuronal survival in the cultures was assessed after 5 days, using a quantitative immunoassay for neurofilament protein. The addition of myotube conditioned medium to their cultures resulted in an 80% increase in neurofilament protein, relative to untreated control cultures, after 5 days in culture. The survival-enhancing activity they reported was heat- and trypsin-sensitive, and was

retained by an ultrafiltration membrane with a nominal retention limit of 30,000 Daltons. It is apparent that some aspect of neuronal growth was affected by the addition of myotube conditoned medium to these cultures. However, motoneurons can have comprised no more than 5% of the total cells in their cultures (see Chapter 1, Footnote 3), so that the effects they report may have been on other neurons in the cultures.

The simplest explanation for the lack of agreement between the characterization of the motoneuron survival-enhancing activity reported in Section B of this chapter, and the activities reported by other investigators and detailed above, is that other investigators have not looked at cultures of motoneurons. Instead, they have observed effects on cultures of which motoneurons comprise only a small part. However, it would be imprudent to dismiss the work on whole spinal cord cultures completely. The characterization of motoneuron survival-enhancing activity in the present study cannot be regarded as definitive. Difficulties in recovering biological activity from fractionated conditioned medium are well known to be a major problem in such studies, and this one was no exception. For example, problems in recovering full activity during the ultrafiltration experiment, or after small dilutions of MCM_{SF}, have already been mentioned. The reported characterization of the motoneuron survival-enhancing activity from MCM_{SF} was undertaken in order to provide some indication of which future experiments might prove fruitful in the attempt to identify molecules responsible for sustaining the growth of embryonic motoneurons *in vitro* and *in vivo*.

CHAPTER 5.

TESTS OF A PUTATIVE "MOTONEURON TROPHIC FACTOR": EFFECTS ON MOTONEURON SURVIVAL

CHAPTER 5.

TESTS OF A PUTATIVE "MOTONEURON TROPHIC FACTOR": EFFECTS ON MOTONEURON SURVIVAL

A. INTRODUCTION

When a muscle in a mature animal is partially denervated, the undamaged axons in the motor nerve sprout and reinnervate the denervated muscle fibers. The sprouting that occurs at the neuromuscular junction (called "terminal sprouting") can also be produced by treating the muscle with botulinum toxin or by rendering the muscle inactive with other pharmacological agents (Brown and Ironton, 1977; Brown et al., 1980; Betz et al., 1980). This sprouting of terminal fibers, in response to muscle inactivity in the mature animal, has been likened to the rescue of embryonic motoneurons from naturally-occurring cell death by pharmacological paralysis of the embryo (e.g., Sanes, 1984). As with the hypothesis that naturally-occurring motoneuron death might be regulated by a soluble "motoneuron trophic factor", it has been suggested that terminal sprouting might be regulated by a muscle-derived "sprouting factor" (cf. Brown et al., 1981).

Gurney (1984) has reported the production of several rabbit antisera that suppress botulinum toxin-induced sprouting of motor neuron terminals in the mouse gluteus muscle. The antisera were produced against a 40,000-60,000 Dalton fraction of conditioned medium from cultured denervated rat hemidiaphragm. Analysis of the immunoreactive material in conditioned medium by Western blots

showed that the antisera shared a common antigen, a protein of approximately 56,000 Daltons molecular weight.

The hypothesis that this 56,000 Da protein might be of significance in Amyotrophic Lateral Sclerosis (ALS), a disease that affects both upper and lower motoneurons, was indirectly evaluated in the following manner: Gurney tested sera from 19 ALS patients in his toxin-induced sprouting system, and found that 8 out of 19 of these sera suppressed terminal sprouting. Sera from 6 healthy individuals had no effect. Of the 8 sera that were effective in inducing terminal sprouting, 3 were analyzed on Western blots of the conditioned medium from denervated rat hemidiaphragm. All three reacted with a protein of approximately 56,000 Da, whereas the 6 sera from healthy individuals did not react with this protein (Gurney, 1984). These data were interpreted as evidence for active suppression of terminal sprouting in some cases of ALS, with the additional suggestion that ALS may have an autoimmune component against the human equivalent of the 56,000 Dalton antigen from rat.

Gurney and Apatoff (1984) subsequently reported, in abstract form, the fractionation of conditioned medium from cultured denervated rat hemidiaphragm and partial purification of the 56,000 Da antigen. The antigen was reported to co-purify with a survival activity (assayed at 24 hours only) for dissociated spinal cord cells from 4 day chick embryos. A monoclonal antibody, which reacted with the 56,000 Da antigen in Western blots, blocked the survival activity of the conditioned medium, and suppressed terminal sprouting in the

toxin-induced sprouting system, was reported to have been produced. This monoclonal antibody was used to affinity purify, from the rat muscle conditioned medium, the survival activity for chick spinal cord cells.

Although the monoclonal antibody described above has not been distributed to other investigators (M.E. Gurney, personal communication), the purified 56,000 Da protein has been made available for testing. The results of tests of the ability of this protein to enhance motoneuron survival are described in this chapter.

B. MATERIALS AND METHODS

1. DESCRIPTION OF THE TESTED PROTEIN FRACTION

Aliquots of two samples of the 56,000 Da protein were received frozen on dry ice and were immediately stored at -70°C until used. The material had been purified from denervated rat hemidiaphragm conditioned medium, and will be referred to as "RHDCM" or "the RHDCM factor". The final fraction was eluted from a Q300 HPLC column in 5mM $NaHPO_4$ (pH 7.5), and appeared pure by gel electrophoresis. Two different samples were received for testing: One aliquot of "Q300 8-24-84 50ug", containing 50 ug of the protein in a 3.2 ug/ml solution; and several aliquots of "Q300 8-24-84 BSA", containing the 56,000 Da protein at a concentration of 0.32 mg/ml in a 1 mg/ml solution of BSA. Because there were several aliquots of the "Q300 8-24-84 BSA" preparation available, these were thawed only once and the entire aliquot was used for a given

experiment. It was necessary to thaw and refreeze the "Q300 8-24-84 50 ug" aliquot once in order to test its effects on sorted motoneurons on two different occasions.

2. MOTONEURON CULTURE SUBSTRATA

Motoneurons were grown in polylysine-treated 96-well plates that had been incubated overnight with the neurite outgrowth-promoting fraction of myotube conditioned medium, as described in Chapter 4 (Section B.1.b.).

3. MOTONEURON CULTURE MEDIA

All motoneuron culture medium was serum-free, and consisted of Dulbecco's Modified Eagle's medium with 0.45% glucose, supplemented with ovotransferrin, insulin, putrescine, progesterone, selenium, glutamine, penicillin, and streptomycin, as described in Chapter 4 (Section B.1.c.). Motoneuron cultures were fed every two days by removing half the medium, and replacing it with fresh growth medium containing the supplements listed above, plus the RHDCM factor at the concentrations indicated. All media were prepared immediately before motoneurons were plated, sterilized through cellulose acetate filters (0.2 um) to minimize nonspecific loss of material, and aliquots were then frozen in a dry ice-ethanol bath and stored at -70°C until they were used to feed the motoneuron cultures. When bovine serum albumin (BSA, Sigma Fraction V) was added to the medium as carrier protein, it was added to a final concentration of

100 ug/ml. When serum-free myotube conditioned medium (MCM_{SF}) was added as a supplement to culture media, it was added to a final concentration of 50%, and then the serum-free growth supplements listed above were added to their customary concentrations.

4. EVALUATION OF MOTONEURON SURVIVAL

Motoneuron survival is reported as the percentage of cells bearing 1 or more processes greater than 2 cell diameters in length, as described in Chapter 4 (Section A.1.b.). For the experiments described in this chapter, at least 400 cells in a minimum of 20 random fields were counted for each culture, and two cultures were counted for each experimental condition reported.

C. RESULTS

Comparisons of the percentage of motoneurons surviving in serum-free cultures, in the presence or absence of the RHDCM factor, are given in Tables 2-5. In none of these tests of either sample of the factor, was survival ever greater in cultures whose media were supplemented with the factor, than in unsupplemented cultures. Concentrations of the factor that were tested ranged from approximately one-tenth the concentration at which survival-enhancing activity for chick spinal cord cells was reported to be one-half maximal (M.E. Gurney, personal communication), to levels 100 times greater than this reported half-maximal level. Testing of greater concentrations was not possible because available quantities of the

TABLE 2.

TEST OF RHDCM FACTOR "Q300 8-24-84 BSA" (11-7-84):

SURVIVAL OF SORTED MOTONEURONS

Conditions: No BSA as carrier protein. All media are serum-free and all substrata are NOPA fraction of MCM (see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
320 ng/ml	1	24.5 ± 1.5	23.5 ± 2.5
	2	17.5 ± 2.5	17.5 ± 0.5
	4	9.5 ± 1.5	11.5 ± 1.5
	6.5	4.5 ± 0.5	5 ± 2
32 ng/ml	1	24.5 ± 1.5	23.5 ± 2.5
	2	19.5 ± 0.5	17.5 ± 0.5
	4	11 ± 1	11.5 ± 1.5
	6.5	6 ± 2	5 ± 2
3.2 ng/ml	1	22.5 ± 0.5	23.5 ± 2.5
	2	20.5 ± 0.5	17.5 ± 0.5
	4	15.5 ± 1.5	11.5 ± 1.5
	6.5	5.5 ± 0.5	5 ± 2

[1] Reported one-half maximal activity for survival of cells in 4 day spinal cord cultures is at 4.5 ng/ml.

[2] Data are given as the mean ± the deviation.

TABLE 3.

TEST OF RHDCM FACTOR "Q300 8-24-84 BSA" (11-13-84):

SURVIVAL OF SORTED MOTONEURONS

Conditions: 100 ug/ml BSA as carrier protein in all media. All media are serum-free and all substrata are NOPA fraction of MCM (see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
320 ng/ml	1	34 ± 1	43 ± 1
	2	32.5 ± 1.5	38.5 ± 0.5
	4	27.5 ± 2.5	28 ± 5
	6.5	34.5 ± 1.5	29 ± 4
32 ng/ml	1	38.5 ± 2.5	43 ± 1
	2	35 ± 4	38.5 ± 0.5
	4	34 ± 2	28 ± 5
	6.5	28 ± 1	29 ± 4
3.2 ng/ml	1	39 ± 2	43 ± 1
	2	36.5 ± 1.5	38.5 ± 0.5
	4	27.5 ± 0.5	28 ± 5
	6.5	33 ± 2	29 ± 4
0.32 ng/ml	1	31 ± 2	43 ± 1
	2	44.5 ± 2.5	38.5 ± 0.5
	4	33.5 ± 0.5	28 ± 5
	6.5	29.5 ± 0.5	29 ± 4

[1] Reported one-half maximal activity for survival of cells in 4 day spinal cord cultures is at 4.5 ng/ml.

[2] Data are given as the mean ± the deviation.

TABLE 4.

TEST OF RHDCM FACTOR "Q300 8-24-84 50 ug" (12-5-84):

SURVIVAL OF SORTED MOTONEURONS

Conditions: 100 ug/ml BSA as carrier protein in all media. All media are serum-free and all substrata are NOPA fraction of MCM (see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
320 ng/ml	1	18.5 ± 0.5	24.5 ± 0.5
	2	17 ± 2	23.5 ± 3.5
	4	17.5 ± 0.5	30 ± 0.5
	6.5	5 ± 2	22.5 ± 0.5
32 ng/ml	1	18 ± 3	24.5 ± 0.5
	2	20 ± 1	23.5 ± 3.5
	4	25.5 ± 3.5	30 ± 0.5
	6.5	15.5 ± 0.5	22.5 ± 0.5
3.2 ng/ml	1	20.5 ± 1.5	24.5 ± 0.5
	2	21 ± 1	23.5 ± 3.5
	4	20 ± 3	30 ± 0.5
	6.5	17 ± 2	22.5 ± 0.5
0.32 ng/ml	1	17.5 ± 1.5	24.5 ± 0.5
	2	21.5 ± 0.5	23.5 ± 3.5
	4	23.5 ± 0.5	30 ± 0.5
	6.5	11.5 ± 2.5	22.5 ± 0.5

[1] Reported one-half maximal activity for survival of cells in 4 day spinal cord cultures is at 2 ng/ml.

[2] Data are given as the mean ± the deviation.

TABLE 5.

TEST OF RHDCM FACTOR "Q300 8-24-84 50 ug" (1-9-85):

SURVIVAL OF SORTED MOTONEURONS

Conditions: No BSA as carrier protein. All media are serum-free and all substrata are NOPA fraction of MCM (see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
320 ng/ml	1	23 ± 1	32.5 ± 1.5
	2	22.5 ± 2.5	22.5 ± 2.5
	4	12.5 ± 2.5	6.5 ± 0.5
	6.5	<1	<1
32 ng/ml	1	23 ± 1	32.5 ± 1.5
	2	26 ± 1	22.5 ± 2.5
	4	10.5 ± 2.5	6.5 ± 0.5
	6.5	<1	<1
3.2 ng/ml	1	26 ± 1	32.5 ± 1.5
	2	17.5 ± 2.5	22.5 ± 2.5
	4	9.5 ± 1.5	6.5 ± 0.5
	6.5	<1	<1
0.32 ng/ml	1	24.5 ± 0.5	32.5 ± 1.5
	2	22.5 ± 2.5	22.5 ± 2.5
	4	8.5 ± 1.5	6.5 ± 0.5
	6.5	<1	<1

[1] Reported one-half maximal activity for survival of cells in 4 day spinal cord cultures is at 2 ng/ml.

[2] Data are given as the mean ± the deviation.

factor were limiting.

Two graphs are provided to illustrate the survival of purified motoneurons cultured for one week in serum-free medium, in the presence or absence of the highest concentration of the RHDCM factor that was tested (320 ng/ml). When no BSA was added to the medium (Figure 20), motoneuron survival dropped off quickly from the second day in unsupplemented cultures or in cultures supplemented with the RHDCM factor. In cultures maintained in medium supplemented with myotube conditioned medium, motoneuron survival remained at high levels throughout the course of a week in culture.

When BSA was added to the medium as carrier protein (Figure 21), an unexpected result was obtained: Levels of motoneuron survival in all cultures stayed at high levels for one week, regardless of the supplement added. In these BSA-containing media, motoneuron survival at 4 days still appeared to be better in cultures maintained in media supplemented with MCM_{SF}, than in unsupplemented media or media containing up to 320 ng/ml of the RHDCM factor. However, this difference was not detectable at one week in culture.

D. DISCUSSION

Motoneuron survival was not enhanced by the presence of up to 320 ng/ml of the RHDCM factor in serum-free culture media. One possible explanation for this result, which cannot be discounted, is that the RHDCM factor was rendered inactive by storage and/or culture conditions. This protein has been reported to be extremely unstable. For example, two different samples of the protein,

FIGURE 20.

TEST OF RHDCM FACTOR "Q300 8-24-82 BSA" (11-13-84): SURVIVAL OF MOTONEURONS IN SUPPLEMENTED AND UNSUPPLEMENTED SERUM-FREE MEDIA

Motoneurons were grown for one week in serum-free medium, on polylysine-treated culture substrata that had been incubated with the neurite outgrowth-promoting fraction of MCM (see Chapter 4, Section B.1.b.). Experimental culture media were supplemented to 50% of the final volume with serum-free myotube conditioned medium (MCM_{SF}) (----------), or various concentrations of the RHDCM factor. Shown is motoneuron survival in a concentration of the factor that was approximately 100 times the reported concentration required for one-half maximal survival of cells in 4 day chick embryo spinal cord cultures (320 ng/ml; see Table 3) (___.___.___). Serum-free growth medium, with no conditioned medium added, served as a control (_____). Data are given as the mean $\pm$ the deviation.

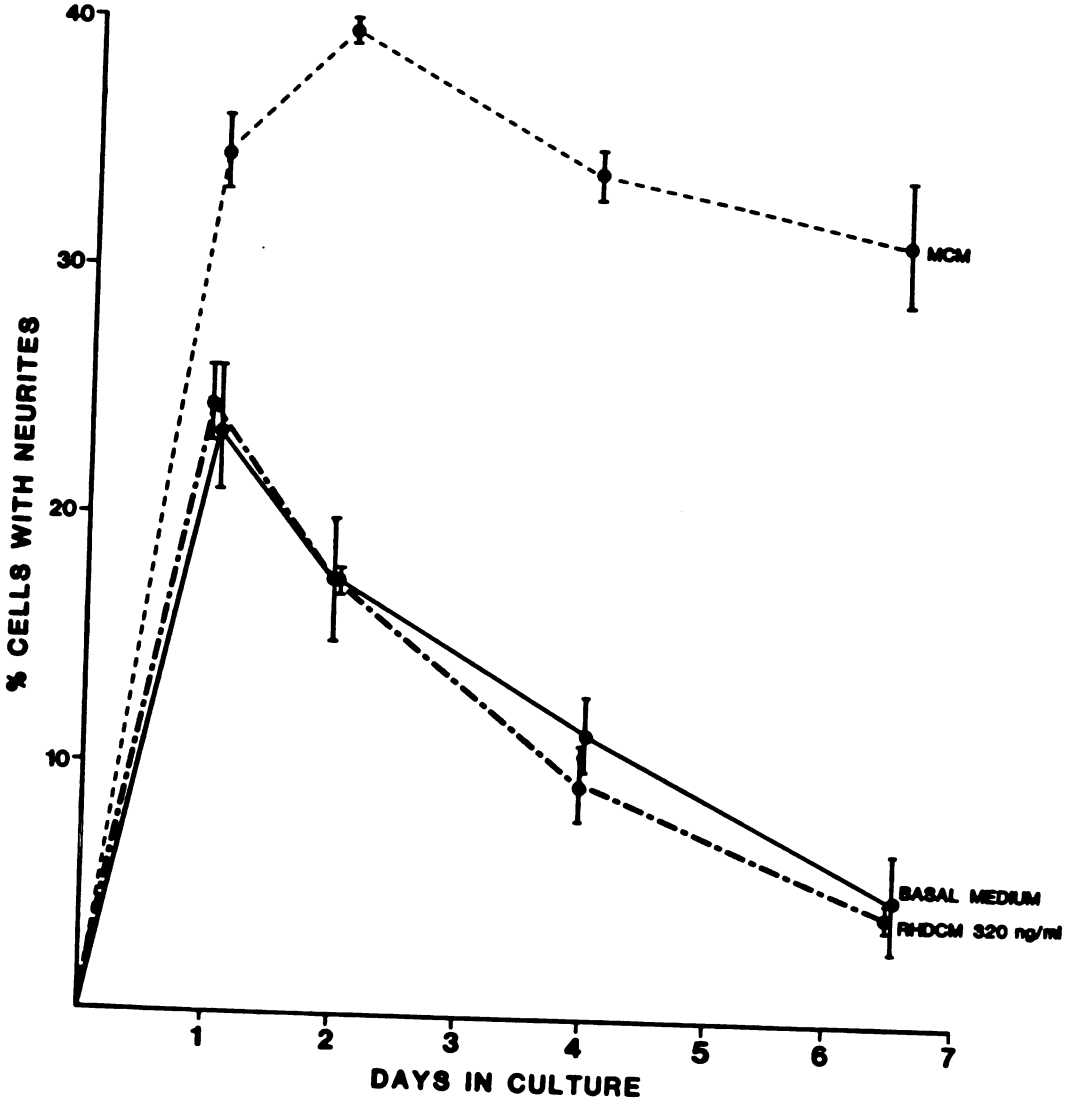
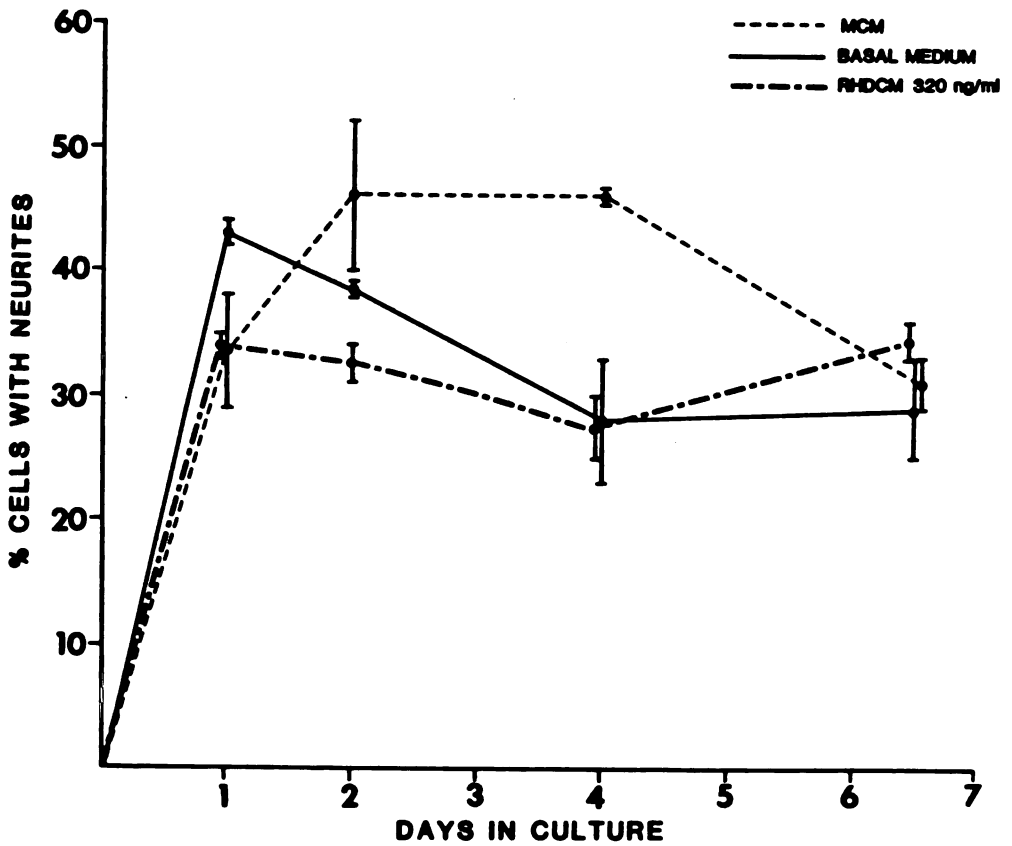

40
30
20
10
% CELLS WITH NEURITES
MCM
BASAL MEDIUM
RHDCM 320 ng/ml
1
2
3
4
5
6
7
DAYS IN CULTURE

FIGURE 21.

TEST OF RHDCM FACTOR "Q300 8-24-84 BSA" (11-7-84):
SURVIVAL OF MOTONEURONS IN SERUM-FREE MEDIA CONTAINING
BOVINE SERUM ALBUMIN

Motoneurons were grown for one week in serum-free medium containing 100 ug/ml of bovine serum albumin (BSA; Sigma Fraction V), on polylysine-treated culture substrata that had been incubated with the neurite outgrowth-promoting fraction of MCM (see Chapter 4, Section B.1.b.). Experimental culture media were supplemented to 50% of the final volumne with serum-free myotube conditioned medium (MCM_{SF}) (----------), or various concentrations of the RHDCM factor. Shown is motoneuron survival in a concentration of the factor that was approximately 100 times the reported concentration required for one-half maximal survival of cells in 4 day chick embryo spinal cord cultures (320 ng/ml; see Table 2) (___.___.___). Serum-free growth medium, containing 100 ug/ml BSA but with no conditioned medium added, served as a control (_____). Data are given as the mean $\pm$ the deviation.


MCM
BASAL MEDIUM
RHDCM 320 ng/ml
% CELLS WITH NEURITES
60
50
40
30
20
10
1
2
3
4
5
6
7
DAYS IN CULTURE

tested on the day of preparation for their ability to enhance cell survival in spinal cord cultures, were reported to have half-maximal levels of activity of about 0.7 ng.ml. After overnight storage at 4°C, this activity fell to 1.25 ng/ml, and activity was lost completely after storage for 6 days at 4°C (M.E. Gurney, personal communication). Even when stored for 5 days at -20°C, in a solution of 50% glycerol, the protein was reported to lose its activity (M.E. Gurney, personal communication). In an attempt to prevent the loss of activity known to occur at temperatures above -20°C, motoneuron culture media were prepared immediately before their first use, then rapidly frozen in a dry ice-ethanol bath and stored at -70°C until they were thawed for feeding the cultures. However, even with this precaution, activity may still have been lost by storage of the media at -70°C; Gurney has reported (personal communication) that storage of the "Q300 8-24-84 BSA" preparation for 18 days at -70°C resulted in total loss of its activity. Because of technical difficulties with cell sorting equipment, it was not possible to perform the first test of the RHDCM factor until about 6 weeks after the material was received. It is therefore quite possible that the factor's activity was lost in storage.

In any case, the reported instability of this protein makes the possibility of detecting motoneuron survival-enhancing activity, using the culture conditions employed in the present study (i.e. growth for one week at 37°C), highly unlikely. If overnight storage of the protein at 4°C reduces its reported activity by one-half, then it might be expected to retain activity for no

more than a few hours at 37°C. It might be necessary to replace the culture medium with a much higher frequency (e.g., two or three times per day), or to test much higher concentrations of the factor than were available, in order to detect any activity. A possible alternative might be to alter motoneuron culture conditions, making them so stringent that motoneuron survival for even one day would be impossible unless the cells were "rescued" by the presence of a powerful growth-supporting substance. Such a stringent bioassay would have involved growing the motoneurons on some substratum other than the neurite outgrowth-promoting fraction of MCM, since motoneurons grown on such a substratum can survive for one or two days in serum-free medium in the absence of any supplements (see Chapter 4, Section A.2.a. and Figure 13). The development of a stringent bioassay using a different culture substratum was not attempted in the present study, because it was reported that the assays of the protein's survival-enhancing activity were routinely performed using spinal cord cells cultured on a neurite outgrowth-promoting substratum (M.E. Gurney, personal communication).

A second possible explanation for the results of the present study, is that the RHDCM factor does not have any effect on motoneuron survival. Since the survival-enhancing activity of the factor was originally assessed on cultures of dissociated spinal cord cells from 4-5 day chick embryos, it is conceivable that the effect Gurney has reported was exerted on some cell type other than motoneurons. In fact, since motoneuron survival on a neurite outgrowth-promoting substratum is at high levels after 24 hours

in culture, whether or not the growth medium is supplemented with conditioned medium (see Chapter 4), it is likely that the reported effects of the RHDCM factor were exerted on a different class of spinal cord cells. Given the difficulties, encountered in the present study, of accurately testing the available preparation of the RHDCM factor, the resolution of this question must await further testing of freshly-prepared material.

Two experiments in the present study presented an unexpected finding: 100 ug/ml of bovine serum albumin (BSA; Sigma Fraction V), added to serum-free culture medium, appeared to maintain motoneuron survival at the levels observed in 1 day cultures, regardless of other supplements added to the medium (compare Figures 20 and 21). BSA was added to the culture medium at the suggestion of M.E. Gurney (personal communication); it was intended to act as a carrier protein when low concentrations of the RHDCM factor were tested in serum-free media. BSA also appeared to stimulate the growth of contaminating nonneuronal cells in the cultures (data not shown); sorted motoneuron cultures are on the average about 80% pure (see Chapter 2), but when these cultures are maintained in the defined media used in most experiments in the present study, there is little or no proliferation of cells that have obvious fibroblast-like morphology (presumably contaminating meningeal fibroblasts and astrocytes). The high levels of survival in cultures grown in BSA-supplemented media suggest that some contaminant(s) of the BSA used, or the BSA itself, can act to enhance the survival of cells in these cultures. Whether the apparent effect on motoneurons was

a direct one, or whether it was mediated indirectly through the increased numbers of nonneuronal cells, is not clear. What the contaminant(s) of BSA that exert this effect might be, is not known.

Interestingly, Kaufman and Barrett (1983) have reported that fractionation of fetal calf serum at acid pH (3.6) yields a broad peak of material with the ability to support the long-term (3 weeks) survival of dissociated spinal cord cells from embryonic rats. In gel filtration experiments the survival activity eluted with apparent molecular weight in the range of 50,000 to 80,000 Daltons. The major peak of activity migrated near the BSA standard in the reported column profile. Given the source and size range of the activity reported by Kaufman and Barrett, it is reasonable to suggest that material possessing this activity could be present in commercial preparations of BSA, such as that used in the present experiments. It is also intriguing that the major peak of activity from fetal calf serum, reported by Kaufman and Barrett, was close to the RHDCM factor in apparent molecular weight: 55,000 as compared to 56,000 Daltons. The major peak of activity reported by Kaufman and Barrett was analyzed for size only by gel filtration, and it was contained in a broader peak of material possessing growth-promoting properties. However, it would be interesting to determine whether fetal calf serum contains a 55,000 Da protein that is recognized by the monoclonal antibody reported by Gurney and Apatoff (1984).

CHAPTER 6.

TESTS OF THE "CILIARY NEURONOTROPHIC FACTOR": EFFECTS ON MOTONEURON SURVIVAL

CHAPTER 6.

TESTS OF THE "CILIARY NEURONOTROPHIC FACTOR": EFFECTS ON MOTONEURON SURVIVAL

A. INTRODUCTION

Neurons in the embryonic chick ciliary ganglion, a structure of the parasympathetic nervous system, undergo a period of naturally-occurring cell death analogous to that of embryonic chick spinal motoneurons. Approximately one-half of the neurons originally present in the ganglion die between days 8 and 14 of embryogenesis, and this period of cell death is nearly synchronous with the establishment of peripheral connections by ganglionic neurons (Landmesser and Pilar, 1974b). As in the case of naturally-occurring motoneuron death, several observations argue that the naturally-occurring death of ciliary ganglionic neurons is regulated by interactions of these neurons with their intraocular target tissues. Ganglionic neurons that have been deprived of their peripheral target, by removal of the eye primordium prior to the onset of the period of naturally-occurring cell death, differentiate in normal numbers and send functional axons into the postganglionic nerve prior to the onset of the period of naturally-occurring neuronal cell death. However, naturally-occurring ciliary ganglion neuron death is greatly exacerbated by eye removal; over 90% of the neurons subsequently die in such cases (Landmesser and Pilar, 1974a). Implantation of an additional eye primordium reduces the extent of naturally-occurring ciliary ganglion neuron death (Narayanan and

Narayanan, 1978).

Early studies of cultured ciliary ganglion neurons demonstrated that their survival *in vitro* was dependent on the presence of muscle cells or muscle cell conditioned medium (Helfand et al., 1976; Nishi and Berg, 1977; Nishi and Berg, 1979). Since muscle cells of the eye are the normal target tissues of ciliary ganglion neurons, this finding was consistent with the notion that a target tissue-derived trophic factor might mediate ciliary ganglion neuron survival *in vivo*, during embryogenesis, and *in vitro*, in dissociated cell culture. Several groups have assayed the effects of tissue extracts and conditioned medium on the survival of cultured ciliary ganglion neurons, in an attempt to isolate candidates for a "ciliary ganglion neuron trophic factor". Extracts from embryonic chick eye (Adler et al., 1979; Nishi and Berg, 1981) and bovine cardiac muscle (Bonyhady et al., 1980), both parasympathetic target tissues, have been found to contain activities that enhance the survival of cultured ciliary ganglion neurons. Results from these groups suggest that a protein with an apparent molecular weight of about 20,000 Daltons is responsible for this activity (Bonyhady et al., 1980; Nishi and Berg, 1981; Barbin et al., 1984). Barbin and his colleagues have succeeded in purifying what they call the "Ciliary Neuronotrophic Factor" (CNTF) from extracts of choroid, iris-ciliary body, and pigment epithelium dissected from chick embryo eyes. The purificatiion scheme involves successive steps of homogenization, ion exchange chromatrography, separation on a sucrose density gradient, separation on a polyacrylamide gel gel containing SDS, and elution

of the active factor from the SDS gel (Barbin et al., 1984). Samples of the purified CNTF fraction were received from M. Manthorpe, and this CNTF was tested for its ability to enhance the survival of motoneurons purified by cell sorting.

B. MATERIALS AND METHODS

1. DESCRIPTION OF THE TESTED PROTEIN FRACTION

Two aliquots of lyophilized CNTF, each containing 0.8 ug of CNTF protein (reported to be 6400 Trophic Units[4]; M. Manthorpe, personal communication) plus 150 ug carrier protein (bovine serum albumin), were received for testing. The aliquots were stored at -70°C until use. The final CNTF fraction had been eluted from a preparative SDS-polyacrylamide gel by homogenization of a gel slice, which contained the CNTF protein, in phosphate-buffered saline containing bovine serum albumin. Gel pieces were removed by centrifugation and the material was then incubated with Extracti-Gel D (Pierce Chemical Co., Rockford, Illinois) to remove residual SDS. This material was then lyophilized and shipped (M. Manthorpe, personal communication). To make motoneuron culture medium, an aliquot was diluted into 1 ml of basal medium (see below). Half of this diluted material was frozen in a dry ice-ethanol bath and stored at -70°C for later use; the other half was diluted for use in culture

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[4] One Trophic Unit is defined by Barbin et al. (1984) as that amount of CNTF, per milliliter of culture medium, which supports one-half maximal survival of ciliary ganglion neurons at 24 hours in culture.

media. Thus, some CNTF had undergone an additional round of freezing and thawing before being used to feed motoneuron cultures.

2. NEURONAL CULTURE SUBSTRATA

In an initial experiment, motoneurons and control cultures of ciliary ganglion neurons were grown in polylysine-treated 96-well plates that had been incubated overnight with the neurite outgrowth-promoting fraction of myotube conditioned medium, as described in Chapter 4 (Section B.1.b.). For convenience, all subsequent test cultures were grown in polylysine-treated 96-well plates that had been incubated overnight with 50 ul per well of a 10 ug/ml solution of purified EHS laminin (see Chapter 3, Section A.1.) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline.

3. NEURONAL CULTURE MEDIA

In an initial experiment, motoneuron cultures and control ciliary ganglion neuron cultures were grown in medium consisting of Dulbecco's Modified Eagle's Medium with 0.45% glucose (DME), supplemented with 1% horse serum, 25 ug/ml ovotransferrin, 10 ug/ml insulin, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 ug/ml streptomycin. In subsequent experiments, the concentration of horse serum was reduced to 0.5%, and instead of the supplements listed above, to the DME plus 0.5% horse serum were added the customary serum-free growth medium supplements (see Chapter 4, Section B.1.c.): ovotransferrin, insulin, putrescine,

progesterone, selenium, glutamine, penicillin, and streptomycin. Motoneuron cultures were fed every two days by removing half the medium, and replacing it with fresh growth medium containing the listed supplements, plus the reported concentration of CNTF. All media were prepared the day before motoneurons or control ciliary ganglion neurons were plated, sterilized through cellulose acetate filters (0.2 um) to minimize nonspecific loss of material, and aliquots were then frozen in a dry ice-ethanol bath and stored at -70°C until they were used to feed the motoneuron cultures.

4. EVALUATION OF NEURONAL SURVIVAL

Motoneuron survival is reported as the percentage of cells bearing 1 or more processes greater than 2 cell diameters in length, as described in Chapter 4 (Section A.1.b.). In control cultures of ciliary ganglion neurons, survival was determined as the percentage of spherical, phase-bright cells bearing 1 or more processes greater than 2 cell diameters in length. Cultures of ciliary ganglion neurons, evaluated for neuronal survival after 24 hours, were grown under the same culture conditions as sorted motoneurons in all experiments described below. The level of survival of ciliary ganglion neurons in the various tested media provided a control for the efficacy of the sample of CNTF being tested, by allowing an estimate to be made of the CNTF concentration for which ciliary neuron survival was one-half maximal on the day of the test.

C. RESULTS

More motoneurons survived when grown for one week in CNTF-supplemented media, than in medium without CNTF. Table 6 and Table 7 list the percentages of surviving motoneurons in cultures maintained in supplemented and unsupplemented media for 1, 2, 4, and 6.5 days. For every concentration of CNTF tested in these two experiments, more motoneurons survived in the presence of CNTF than in its absence. Motoneurons survival over the course of one week in culture, in basal medium supplemented with CNTF, serum-free myotube conditioned medium (MCM_{SF}), or containing no supplement, is illustrated in Figure 22. In some cases, however, the difference between motoneuron survival in CNTF-supplemented cultures versus unsupplemented cultures was quite small, and probably not significant. Furthermore, the enhancement of motoneuron survival sometimes appeared to be greater in lower concentrations of CNTF (compare responses at 40 ng/ml CNTF versus 4 ng/ml CNTF in Table 6). Since the differences between motoneuron survival in supplemented versus unsupplemented media appeared to be greatest after 4 days in culture, whether the supplement was CNTF or MCM_{SF}, it was decided to standardize tests of CNTF to a 4-day survival assay. Limiting assessment of motoneuron survival to a single time point allowed a larger number of CNTF concentrations to be tested in a given experiment. This was essential for obtaining accurate dose-response data, since the number of motoneurons that could be obtained from a given sort preparation was limited (see Chapter 2, Section E.).

Survival of motoneurons, grown for 4 days in 9 different concentrations of CNTF, is detailed in Table 8. for every

TABLE 6.

TEST OF THE "CILIARY NEURONOTROPHIC FACTOR" (2-27-85):

SURVIVAL OF SORTED MOTONEURONS

Conditions: Basal growth medium was DME with 1% horse serum with ovotransferrin, insulin, glutamine, pyruvate, penicillin, and streptomycin. Substratum was neurite outgrowth-promoting fraction of MCM (see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
40 ng/ml	1	14.5 ± 2.5	10.5 ± 0.5
	2	17.5 ± 0.5	11 ± 2
	4	10.5 ± -.5	4.5 ± 1.5
	6.5	3 ± 2	<1
8 ng/ml	1	16.5 ± 2.5	10.5 ± 0.5
	2	20.5 ± 0.5	11 ± 2
	4	10.5 ± 0.5	4.5 ± 1.5
	6.5	3.5 ± 3.5	<1
4 ng/ml	1	18.5 ± 1.5	10.5 ± 0.5
	2	17.5 ± 0.5	11 ± 2
	4	14 ± 1	4.5 ± 1.5
	6.5	6.5 ± 0.5	<1
2 ng/ml	1	14.5 ± 0.5	10.5 ± 0.5
	2	22.5 ± 0.5	11 ± 2
	4	15.5 ± 0.5	4.5 ± 1.5
	6.5	3.5 ± 0.5	<1

[1] For this experiment, data from control ciliary ganglion cultures is not available. However, this preparation of CNTF was the same as that used in the experiment shown in Table 7.

[2] Data are given as the mean response ± the deviation.

TABLE 7.

TEST OF THE "CILIARY NEURONOTROPHIC FACTOR" (3-6-85):

SURVIVAL OF SORTED MOTONEURONS

Conditions: Basal growth medium was customary serum-free growth medium for motoneurons, plus 0.5% horse serum (see text). Substratum was laminin (10 ug/ml; see text).

CONCENTRATION OF FACTOR[1]	DAYS IN CULTURE	% CELLS WITH NEURITES (C.W.N.)[2]	% C.W.N. IN MEDIUM WITHOUT FACTOR[2]
40 ng/ml	1	28 ± 2	24.5 ± 3.5
	2	33 ± 4	21.5 ± 0.5
	4	28.5 ± 0.5	16.5 ± 1.5
	6.5	19.5 ± 1.5	8.5 ± 0.5
8 ng/ml	1	28.5 ± 0.5	24.5 ± 3.5
	2	33.5 ± 0.5	21.5 ± 0.5
	4	21 ± 1	16.5 ± 1.5
	6.5	18 ± 1	8.5 ± 0.5
4 ng/ml	1	29.5 ± 3.5	24.5 ± 3.5
	2	29 ± 2	21.5 ± 0.5
	4	20.5 ± 0.5	16.5 ± 1.5
	6.5	13 ± 1	8.5 ± 0.5
2 ng/ml	1	22.5 ± 3.5	24.5 ± 3.5
	2	26.5 ± 0.5	21.5 ± 0.5
	4	19 ± 1	16.5 ± 1.5
	6.5	17 ± 2	8.5 ± 0.5

[1] In this experiment, one-half maximal response of control ciliary ganglion cultures, grown in identical media, was at 4 ng/ml.

[2] Data are given as the mean response ± the deviation.

FIGURE 22.

TEST OF THE "CILIARY NEURONOTROPHIC FACTOR":

SURVIVAL OF MOTONEURONS IN SUPPLEMENTED AND UNSUPPLEMENTED MEDIA

Motoneurons were grown for one week in DME containing 0.5% horse serum and the customary serum-free growth medium supplements, on lamin substrata (see text). Experimental culture media were supplemented to 50% of the final volume with serum-free myotube conditioned medium (MCM_{SF}) (---------), 4 ng/ml CNTF (.....), or 40 ng/ml CNTF (___.___.___). Basal medium, with no conditioned medium added, served as a control (_____). Data are given as the mean $\pm$ the deviation.

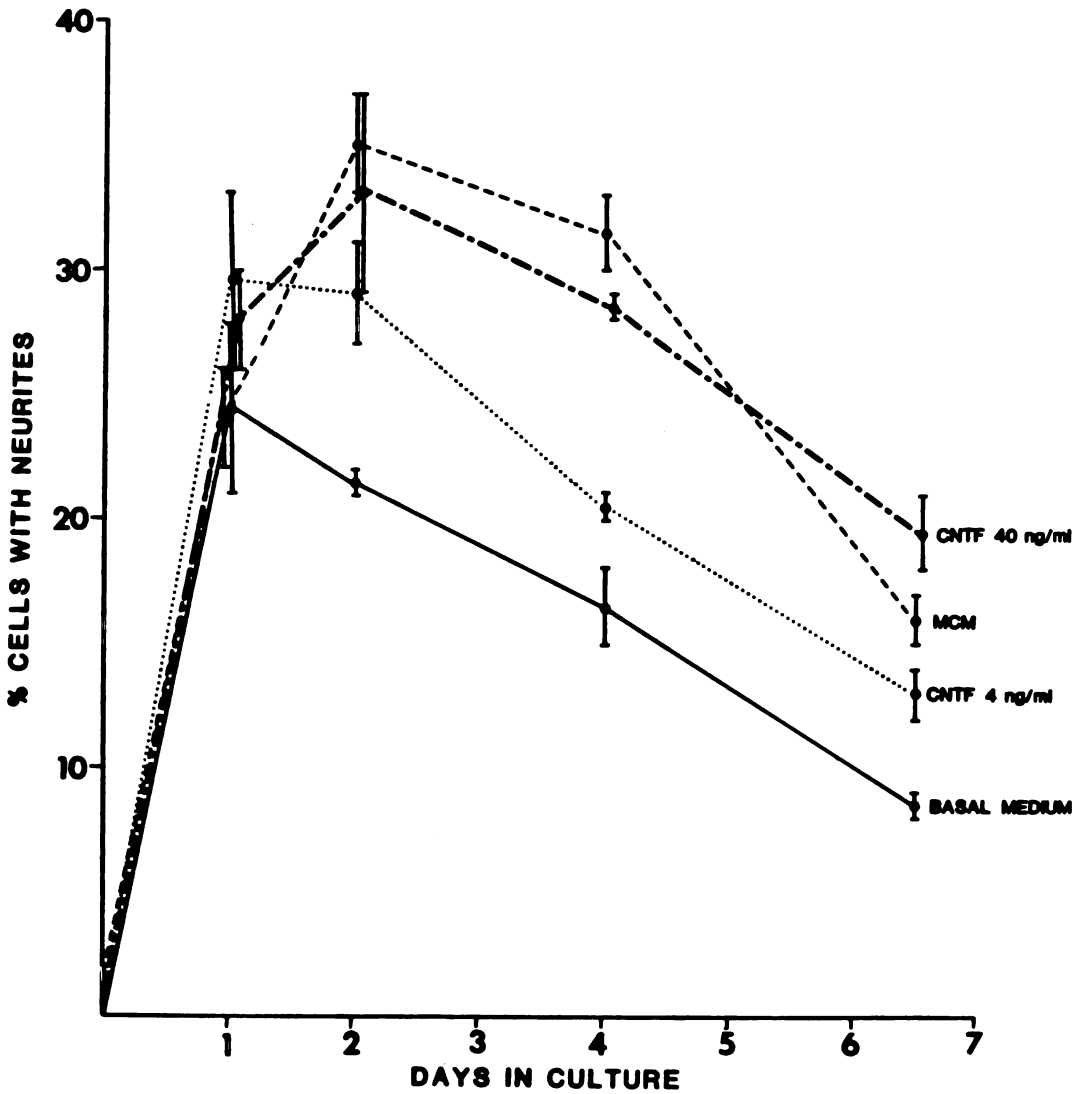
40
30
20
10
% CELLS WITH NEURITES
1
2
3
4
5
6
7
DAYS IN CULTURE
CNTF 40 ng/ml
MCM
CNTF 4 ng/ml
BASAL MEDIUM

TABLE 8.

TEST OF THE "CILIARY NEURONOTROPHIC FACTOR" (3-20-85):

SURVIVAL OF SORTED MOTONEURONS

Conditions: Basal growth medium was customary serum-free growth medium for motoneurons plus 0.5% horse serum (see text). Substratum was laminin (10 ug/ml; see text).

CONCENTRATION OF FACTOR[1]	% CELLS WITH NEURITES[2]	% OF RESPONSE IN MCM_{SF}[3]	% OF RESPONSE IN UNSUPPLEMENTED MEDIUM[4]
80 ng/ml	10.5 ± 0.5	46	210
16 ng/ml	9 ± 1	39	180
8 ng/ml	10 ± 1	43	200
4 ng/ml	15.5 ± 1.5	67	310
1.6 ng/ml	15.5 ± 0.5	67	310
0.8 ng/ml	16.5 ± 3.5	72	330
0.4 ng/ml	14 ± 1	61	280
0.16 ng/ml	7.5 ± 0.5	33	150
0.08 ng/ml	9.5 ± 0.5	41	190

1 In this experiment, one-half maximal response for control ciliary ganglion neuron cultures grown in identical media was at 0.8 ng/ml. 0.16 ng/ml was the reported concentration of this CNTF preparation at which response of ciliary neurons was one-half maximal (M. Manthorpe, personal communication).

2 Data are given as the mean response ± the deviation.

3 Percentage of cells with neurites, in cultures grown in media supplemented with 50% MCM_{SF}, was 23 ± 4.

4 Percentage of cells with neurites, in cultures grown in basal growth medium, was 5 ± 1.

concentration of CNTF tested, motoneuron survival was greater than that in unsupplemented medium, but less than that in medium supplemented with serum-free myotube conditioned medium (MCM_{SF}). As in a previous experiment, CNTF-enhanced motoneuron survival was reduced in the higher concentrations of CNTF. The enhancement of motoneuron survival was greatest between 0.4 and 4 ng/ml of CNTF, and decreased at CNTF concentrations above and below this range.

D. DISCUSSION

Since ciliary ganglion neurons are cholinergic neurons that synapse upon muscle, factors enhancing their survival might also be expected to enhance the survival of spinal motoneurons. In the present study, the purified "Ciliary Neuronotrophic Factor" (CNTF) enhanced motoneuron survival over the course of one week in culture, although this effect was not generally as great as that of myotube conditioned medium added to the motoneuron culture medium. In fact, higher concentrations of CNTF gave a paradoxical result: Although motoneuron survival was still better in medium supplemented with concentrations of CNTF above 4 ng/ml than in basal medium, survival in these higher concentrations was no better than survival in very low concentrations (e.g. 0.1 ng/ml; see Table 8).

A possible explanation for this paradoxical result is that the final CNTF fraction tested in these experiments contained low levels of a toxic substance, so that higher concentrations of this fraciton had a deleterious effect on motoneuron survival. This explanation is quite plausible. The final fraction of CNTF tested in these

experiments had been eluted from a preparative polyacrylamide gel containing the detergent sodium dodecyl sulfate (SDS). Any remaining SDS in the CNTF preparation could be expected to be toxic to motoneurons, especially when they were grown in media containing very little protein (e.g., 1% or 0.5% horse serum). For several reasons, a toxic effect might not be noticed in the ciliary ganglion neuron cultures performed by Manthorpe and colleagues to titrate CNTF preparations that were shipped for testing on motoneurons. First, their assays were routinely performed in the presence of 20% horse serum (Barbin et al., 1984). Residual SDS in the CNTF fraction would probably remain bound to serum proteins under these conditions, and not affect ciliary ganglion neurons in the cultures. Second, motoneuron cultures were fed several times during the course of a 4 to 7 day assay, so levels of unbound detergent in the culture media would increase over time in motoneuron cultures. In contrast, CNTF effects on ciliary ganglion neurons were assessed after 1 day in culture, so effects of toxic contaminants of the CNTF preparation would not be exacerbated by repeated feedings. Third, motoneuron cultures might be more sensitive to toxic contaminants of the CNTF preparation than ciliary ganglion neuron cultures. Motoneuron cultures may be somewhat enfeebled as a result of the extensive dissociation and sorting procedures the motoneurons must undergo; this might account for their greater susceptibility to toxic substances. In 24-hour cultures of ciliary ganglion neurons performed in the present experiments, ciliary neuron survival increased with increasing CNTF concentrations in the expected

manner (data not shown). Whether this reflects the fact that ciliary ganglion cell cultures are indeed less sensitive to the effects of toxic contaminants than are cultures of sorted motoneurons, or whether this apparent lack of toxicity is due to the fact that these cultures were kept for only one day, remains to be determined. The resolution of this question awaits future tests of motoneuron survival-enhancing activity in material from purification stages before and after the fractionation step on SDS-polyacrylamide gels. Dose-response tests of material without CNTF activity, extracted from gels in a procedure identical to that with which CNTF is extracted, will determine whether the Extracti-Gel D procedure is indeed ineffective in removing toxic contaminants from material obtained by gel homogenization.

The consistent effect of the ciliary neuronotrophic factor in enhancing motoneuron survival, while not large is nonetheless intriguing. Does CNTF affect only a subpopulation of motoneurons, and this is why the effect seen is small? Alternatively, is CNTF alone insufficient to fully support motoneuron survival, but in combination with other trophic substances, may enhance motoneuron survival in vitro to as great or greater an extent than myotube conditioned medium? Or is the effect of CNTF never as great as that of myotube conditioned medium because toxic substances in the CNTF preparation never let it achieve better than a certain effect? The initial results, presented in this chapter, are encouraging and should stimulate future studies. A critical test of a role for CNTF in spinal motoneuron development will be an examination of the

effects on these neurons of its inactivation (by antibodies) or supplementation (by systemic administration) during embryogenesis.

CHAPTER 7.

SUMMARY AND CONCLUSIONS

CHAPTER 7.

SUMMARY AND CONCLUSIONS

A. SUMMARY

The material presented in this dissertation concerns the development of a method for purifying embryonic spinal motoneuros and the examination of factors afecting the survival and differentiation of these neurons in culture. Chapter 2 contains a description of the motoneuron purification procedure. This procedure involves preparation of a lectin-fluorochrome conjugate, dissection and organ culture of chick embryos, injection of embryonic limb muscles with the fluorescent conjugate, dissociation and fractionation of spinal cord cells, and use of a fluorescence-activated cell sorter to separate fluorescently-labelled spinal motoneurons from unlabelled spinal cord cells. Included in this chapter are descriptions of the way in which specific labelling of motoneurons by the injection procedure was verified. The sorting parameters used in the present study, and the estimated purity of motoneuron cultures obtained by using these sorting parameters, are also discussed.

Conditioned medium effects on the survival and differentiation of cultured motoneurons were examined first. Medium conditioned over cultures of embryonic chick myotubes was found to bind to polylysine tissue culture substrata and promote extensive neurite outgrowth by motoneurons cultured on these treated substrata. This substratum-binding, neurite outgrowth-promoting activity of myotube conditioned medium (MCM) was analyzed in Chapter 3. A preliminary

characterization of the activity in MCM indicated that it was similar in several ways to another neurite outgrowth-promoting factor, found in bovine corneal endothelial cell conditioned medium (BCE-CM): First, both factors bound to polylysine substrata and promoted neurite outgrowth by either embryonic chick motoneurons or neonatal rat sympathetic neurons. Second, the neurite outgrowth-promoting activity in MCM was similar to the BCE-CM factor in its susceptibilities to enzymes; for both factors, protein and heparan sulfate appeared to be important for neurite outgrowth-promoting activity. Similarities in buoyant density and chromatographic properties between the two factors are also discussed.

Subsequent studies, presented in the second half of Chapter 3, showed that laminin in MCM was responsible for its ability to promote motoneuron neurite outgrowth. Although the presence in motoneuron cultures of anti-laminin antibodies did not block the ability of MCM to promote motoneuron neurite outgrowth, these antibodies could be used to immunoprecipitate all neurite outgrowth-promoting activity from MCM. Immunoprecipitations from MCM, which had been metabolically labelled with ^{35}S-methionine, indicated that the specifically-immunoprecipitated material was indeed laminin: Nonreducing SDS-polyacrylamide gels of immunoprecipitated material showed a single protein band that comigrated with mouse laminin; upon reduction this was converted to two bands that migrated close to the heavy and light chains of mouse laminin. Furthermore, when MCM was fractionated by ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, the peak of neurite outgrowth-

promoting activity was associated with a peak of laminin immunoreactivity. This peak of neurite outgrowth-promoting activity also contained a protein that comigrated with mouse laminin in nonreducing SDS-polyacrylamide gels.

In addition to its neurite outgrowth-promoting activity, MCM in the culture medium of motoneurons enhanced their survival over periods greater than two days. This survival-enhancing activity was analyzed in Chapter 4. The survival-enhancing activity of MCM was found to be distinct from its neurite outgrowth-promoting activity, since supplementation of motoneuron culture media with the neurite outgrowth-promoting fraction of MCM did not enhance motoneuron survival, whereas unfractionated MCM did. Medium conditioned over cultures of spinal cord dividing cells and fibroblasts also possessed some survival-enhancing activity for motoneurons. The motoneuron survival-enhancing activity from MCM was characterized by heat treatment and protease treatment; destruction of the activity by boiling and by trypsin suggested that this activity may reside in a protein, or proteins. Results of experiments in which MCM was subjected to ultrafiltration further suggest that much of the survival-enhancing activity resides in a component or components of MCM that are less than 25,000 Daltons in molecular weight.

Tests of a putative "motoneuron trophic factor" are described in Chapter 5. This 56,000 Dalton protein had originally been identified as a protein recognized by several rabbit antisera that blocked motor nerve sprouting at the neuromuscular junction;

antibodies directed against this protein were also found in the sera of several patients with Amyotrophic Lateral Sclerosis (Gurney, 1984). The protein was subsequently purified and reported to enhance the survival of cells in cultures of dissociated spinal cord cells from 4-5 day chick embryos (Gurney and Apatoff, 1984). In the present study, this purified protein was found to have no effect on the survival of motoneurons over the course of one week in culture. Several possible explanations for this result are discussed.

The "Ciliary Neuronotrophic Factor" (CNTF), a 20,000 Dalton protein purified by Barbin and colleagues (1984), was also tested for its ability to enhance motoneuron survival. CNTF has been shown by several groups to support the survival of cultured ciliary ganglion neurons. The experiments testing its effects on motoneurons are described in Chapter 6. CNTF was shown to enhance motoneuron survival at all concentrations tested. However, the effect was relatively small (usually less than that of MCM), and paradoxical effects were obtained at higher concentrations of the tested protein fraction. Possible explanations for these results, and experiments designed to test these explanations, are discussed.

B. PURIFICATION OF MOTONEURONS BY RETROGRADE TRANSPORT AND FLUORESCENCE-ACTIVATED CELL SORTING: LIMITATIONS OF THE PRESENT METHOD AND DIRECTIONS FOR FUTURE STUDIES

The method used for purifying embryonic chick spinal motoneurons in the present study was successful, insofar as it yielded

for study viable populations of motoneurons that were reasonably pure. However, there are several limitations of the method, and these warrant discussion.

The method is not simple. As used, it involves several complicated dissection and culture procedures performed over the course of two days, some very costly equipment, and technical support staff. The first complicated procedure is the dissection and injection of the embryos. This must be done carefully to avoid leakage of the fluorescent conjugate to areas other than the limb muscle masses, and it must be done quickly to avoid compromising the viability of the embryos. In the present experiments, the embryos were removed from the egg altogether to make the limbs more accessible and the injections therefore faster. Nonetheless, it was never possible, even after a great deal of experience with the technique, to complete the dissection and injection procedures in less than 15 minutes per embryo. Thus, only a limited number of embryos could be injected for a given preparation of motoneurons (this number was also limited by available time on the cell sorter): about 14 embryos could be injected for each motoneuron preparation. Since the number of sorted motoneurons obtained was between 10,000 and 25,000 per embryo, the yield for a given preparation was never more than about 300,000 motoneurons. This limited the type and number of experiments that could be performed.

Since the embryos were removed from the eggs to allow the injections to be made, they had to be maintained in organ culture

to allow their motoneurons to be labelled by retrograde axonal transport of the injected fluorescent conjugate. The organ culture procedure itself was quite simple, but it required time (about one day) and an incubator specialized for the task. An advantage of this technique was that embryos were already partially dissected and pinned out in dishes when the time came to remove their spinal cords the next day. This allowed the final dissections to be performed quickly (about 7 minutes per embryo), which was useful in speeding up the dissociation and sorting procedures.

The final dissociation and sorting procedure was complicated and required two people for the present study; in fact, it is probably not possible for the same investigator to prepare and sort the spinal cord cells in these experiments. Obtaining viable motoneurons from the procedure requires that dissections be performed very quickly and spinal cord dissociates be sorted in small batches, to reduce as much as possible the time that elapses between dissecting spinal cords and collecting and plating the sorted motoneurons. Thus, in order to obtain a workable number of motoneurons in less than 5 hours of sorting time, one person must be dissecting spinal cords and preparing dissociated cells while another oversees the sorting of a previously-prepared cell suspension. In addition, the preparation of the cell sorter -- involving sterilization of the cell suspension pathways and focusing of the lasers -- must be accomplished before any analysis and sorting can begin.

The major limitations of the present method are therefore its complexity, the large amount of time required, and the low yield.

This last is the problem of greatest scientific interest, and potential methods for its solution are the main focus of the remainder of this discussion.

Fluorescence-activated cell sorting could be used to obtain greater numbers of motoneurons from spinal cord dissociates, if an antibody that recognized a motoneuron-specific, cell-surface antigen were available. Such an antibody could be bound to the surfaces of motoneuron somata in suspensions of dissociated spinal cord cells. After labelling the motoneurons with a fluorescent second antibody, these dissociates could be sorted using the fluorescence-activated cell sorter. This labelling procedure would be superior to labelling by retrograde transport: Most motoneurons could be labelled, each motoneuron would be labelled to a greater extent, and the level of fluorescent signal detectable by the cell sorter would be much higher for label present on the cell surface rather than within the cell soma. Yield from this procedure would be higher because of greater sorting efficiency (due to increased signal), and because a larger number of labelled cells could easily be prepared by such a simple method.

A hybridoma cell line, which secretes an antibody that might be of use in a motoneuron purification procedure, has recently been made. Tanaka and Obata (1984) have reported the production of a monoclonal antibody that recognizes spinal motoneurons in chick embryos between Hamburger and Hamilton stages 17 and 31 (about days 3 to 7; Hamburger and Hamilton, 1951) of incubation. The antibody, termed "SC 1", binds to a trypsin-sensitive antigen on the surfaces

of embryonic motoneurons and ventral epithelial cells in the spinal cord, as well as to dorsal root ganglion cells and cells in the sympathetic ganglia. Because the spinal cord can be dissected free of dorsal root and autonomic ganglia, only the spinal cord ventral epithelial cells would be contaminants of a preparation purified using this antibody. It is difficult to estimate quantitatively what degree of purity could be obtained in cultures isolated by cell sorting using this antibody marker. However, available photomicrographs (Tanaka and Obata, 1984) indicate that motoneurons probably outnumber the ventral epithelial cells by about 2 to 1 at the later stages shown (Hamburger and Hamilton stages 27-30), so it seems safe to estimate that cultures could be obtained which contained at least 50% motoneurons. The trypsin sensitivity of the antigen is a potential drawback to its use, but this problem could probably be circumvented by using other proteolytic enzymes in the dissociation procedure. Alternatively, the monoclonal antibody could be used to affinity purify the antigen; polyclonal antisera raised against the purified antigen might recognize trypsin-resistant sites on the molecule, so that these sera would be compatible with a trypsin dissociation of spinal cord cells in the motoneuron purification procedure.

There are alternative approaches to purifying motoneurons using an antibody to a cell-surface molecule, that do not require the use of a fluorescence-activated cell sorter. Chromatographic procedures for separating cells, based on the presence of the cell surface membranes of specifically-reactive molecules, are available.

Many of these have been developed for use in immunological studies of antigen-specific lymphocytes. These methods usually involve passing the cells over solid supports to which specific cell recognition molecules have been coupled. Such methods can be used to deplete cell populations of certain cell types, or to purify specific cell types from among heterogeneous populations of cells (cf. Hudson and Hay, 1980). Cells have been enriched to purities of over 90%, starting from mixed cell populations containing less than 2% antigen-positive cells, by these types of procedures (e.g., Meier et al., 1982). A problem with many of the procedures is poor yield; the study of Meier et al. (1982) did indeed achieve high levels of purity (>90%), but the yield was less than 7% . However, the development of techniques such as magnetic chromatography is beginning to eliminate many of the problems associated with eluting selected cells from chromatographic supports; cell elution has been responsible for most of the yield problems in these studies (e.g., Molday and Molday, 1984). Thus, given the right antibody marker, there are many approaches available for purifying motoneurons from spinal cord dissociates. The development of a reliable motoneuron purification procedure, using such an approach, would greatly facilitate future studies of motoneuron development.

C. IN VITRO STUDIES OF SPINAL MOTONEURON DEVELOPMENT AND THE SEARCH FOR "MOTONEURON TROPHIC FACTORS"

It cannot yet be stated that the study of purified motoneurons grown in culture has led to the identification of a "motoneuron

trophic factor". However, a factor affecting the outgrowth of neuronal processes by cultured motoneurons has been identified in the studies described in this dissertation. Furthermore, these studies have demonstrated that motoneurons cannot survive in vitro for periods greater than 1 or 2 days without the addition of conditioned medium to their culture medium. The substance or substances in conditioned medium that are responsible for this effect on motoneuron survival have yet to be identified, and tests of their ability to affect motoneuron survival during embryogenesis lie in the future.

1. LAMININ AND ITS ROLE IN MOTONEURON PROCESS OUTGROWTH

Results discussed in Chapter 4 suggest that laminin in myotube conditioned medium is responsible for the ability of substrata treated with this conditioned medium to promote neurite outgrowth by cultured motoneurons. The role of laminin in motoneuron process outgrowth in vivo is more difficult to assess. Little is known about the distribution of laminin in the embryo. One study has demonstrated its presence in mouse embryos as early as the 8-cell stage of development, and has proposed a role for laminin in the polarization of blastomeres and their subsequent differentiation (Wu et al., 1983). Only one group has attempted to identify laminin in regions of early axon outgrowth in the chick embryo. At the stages during which motoneuron axons are first growing out of the ventral root, they found laminin, but not fibronectin, in the regions of the ventral root pathway: the external limiting membrane of the neural tube, the cellular matrix between the neural tube

and somites, and the surfaces of somites. These authors suggested that the growth cones of motoneuron axons could be in contact with a laminin substratum throughout their entire migration from spinal cord to peripheral muscle masses (Rogers et al., 1984). Whether laminin actually provides adhesive guidance cues for motoneuron axons along early axonal pathways, however, remains to be determined.

One approach to testing laminin's role in motoneuron axon guidance might be to attempt to block or disrupt motoneuron axon outgrowth in vivo using anti-laminin antibodies. Such an approach has been used with success to disrupt other developmental processes involving axon outgrowth or cell migration: Antibodies to the "Neuronal Cell Adhesion Molecule" can disrupt the normal projection of retinal ganglion cell axons to the tectum in chicken embryos (Thanos et al., 1984), and antibodies to fibronectin have been shown to disrupt gastrulation in the amphibian embryo (Boucaut et al., 1984a). However, attempts to disrupt motoneuron axon outgrowth with anti-laminin antibodies may be difficult: The results presented in this and other studies indicate that the substratum-binding, neurite outgrowth-promoting activity of various conditioned media -- which is known to depend upon the presence of laminin in those conditioned media (Lander et al., 1985b) -- cannot be blocked by antibodies to purified mouse tumor laminin (Lander et al., 1983, 1985b; Manthorpe et al., 1983; Edgar et al., 1984). It may be necessary to make antibodies to the particular laminins found in these "neurite outgrowth-promoting factors", or to the laminin-binding molecules they also contain (cf. Lander et al., 1985a),

in order to obtain a reagent that can successfully block laminin function *in vivo*.

Laminin is present at very early stages of development, and it is virtually ubiquitous in epithelial basement membranes in the mature animal. Because of this, it would probably not be feasible to completely eliminate laminin in a developing animal (e.g., by creating a "laminin-less" mutant) and look at the consequences for motoneuron axon guidance. However, it may be possible to saturate neuronal laminin receptors in embryos by injecting a cell-binding fragment of laminin into them; the effects of these fragments on neuronal process outgrowth could then be observed. Such an approach has been used to disrupt cellular interactions with fibronectin in developing animals: Injection of a decapeptide that contains the cell-recognition sequence of fibronectin has been shown to inhibit amphibian gastrulation and avian neural crest cell migration *in vivo* (Boucaut et al., 1984b). A peptide containing the collagen-binding sequence of fibronectin, injected under the same conditions, had no effect on gastrulation or crest cell migration. These experiments suggest that interactions of these migrating cells with a specific sequence within the fibronectin molecule are important in these two developmental processes. The elegance of the approach lies in the blockade of specific molecular interactions by the injection of peptides containing particular functional domains of that molecule. This approach avoids the problems associated with complete inactivation of a large, multifunctional molecule such as fibronectin, where specific effects can be obscured by the disruption

of processes unassociated with the phenomenon under investigation. It may be the best approach for probing the developmental functions of laminin, which is an enormous protein with at least three distinct molecular and cellular interactions: binding to cells, binding to heparan sulfate proteoglycans, and binding to Type IV collagen (cf. Timpl et al., 1982, 1983). It should be noted that Edgar and his colleagues (1984) have recently tested different proteolytic fragments of laminin for their ability to promote neurite outgrowth by chick sympathetic neurons. The results of this study are complicated and suggest that more than one region of the laminin molecule may possess neurite outgrowth-promoting activity. Tests of the roles of specific laminin sequences in axon outgrowth *in vivo* may therefore prove more difficult to perform than were the tests of fibronectin sequences.

2. PROBLEMS ASSOCIATED WITH *IN VITRO* STUDIES OF "MOTONEURON TROPHIC FACTORS"

The experiments discussed in Chapter 4 make it apparent that myotube conditioned medium (MCM) contains at least two activities which affect motoneuron development *in vitro*: a neurite outgrowth-promoting activity (in which laminin has proved to be the active factor), and a survival-enhancing activity. A preliminary characterization of the survival-enhancing activity was undertaken and is described in Chapter 4, but purification of this activity was not attempted. One of the most important reasons for not pursuing this purification was the finding, in preliminary

experiments, that full survival-enhancing activity could not be obtained from a given batch of MCM when it was diluted five-fold (i.e., supplementation of motoneuron growth medium to 20% MCM was not as effective at enhancing motoneuron survival as was supplementation to 50% MCM). This finding that the motoneuron survival-enhancing activity is not present in high concentration in MCM, coupled with the difficulties and time involved in generating primary cultures of chick myotubes, make myotube conditioned medium an unattractive source from which to purify the activity.

Conditioned medium has usually proved to be a poor source from which to purify putative neuronal trophic factors, and investigators have sought other sources which are available in greater quantity and are easier to fractionate. For example, the trophic factor from sensory neurons purified by Barde and his colleagues (1982) was originally described as a survival-enhancing activity present in C6 glioma cell conditioned medium (Barde et al., 1978). Purification of the activity from the conditioned medium source appears not to have been attempted, and with good reason when the purification data from pig brain are examined: 2 micrograms of trophic factor protein were obtained from 3 kilograms of brain (Barde et al., 1982). The purified protein represented about one-ten millionth of the starting protein in the pig brain preparation. The concentration of the factor is probably higher in glioma cell conditioned medium (GCM) than in whole pig brain. However, even assuming that the concentration of the factor

in GCM is 1 ng/ml, tens of liters of conditioned medium would be required for the purification of 1 ug of trophic factor protein by conventional methods.[5] The time and effort required to produce such large amounts of conditioned medium suggests that a more attractive source from which to attempt purifications would be tissue extracts.

Extracts from embryonic limb muscles would seem an obvious source of a muscle-derived "motoneuron trophic factor". During the course of the studies reported in this dissertation, a single, unsuccessful attempt was made to identify motoneuron survival-enhancing activity in soluble extracts of limb muscles taken from 10 day chick embryos (data not shown). The effort was abandoned because of time constraints, lack of effect obtained, and because dissections of large quantities of embryonic muscle tissue proved to be as laborious as generating primary cultures of myotubes. It may be that whole embryo extracts would prove to be a easier source of putative motoneuron survival-enhancing activities.

Other investigators have studied the effects of muscle extracts, from tissue obtained from newborn chickens or adult rats, on the survival and neurite outgrowth of cultured spinal cord cells and

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[5] The purified factor is half-maximally effective at about 0.5 ng/ml (Barde et al., 1982). Since GCM is maximally effective at a 50% final concentration in sensory neuron growth medium (Barde et al., 1978), the factor can be assumed to be present in GCM at a concentration of at least 1 ng/ml. Assuming a 10 ug/ml protein concentration in GCM, the factor would have to be purified by a factor of at least 10^4. Assuming a yield of less than 10%, as might be expected for a 10,000-fold purification, at least ten liters of conditioned medium would be required to purify 1 ug of the trophic factor protein.

explants (Tanaka et al., 1982; Henderson et al., 1983, 1984; Nurcombe et al., 1984). Some effect of muscle extracts in promoting neurite outgrowth and survival have been reported in these studies. There is thus some hope that such extracts may prove to be a source of factors affecting the survival and development of motoneurons grown in culture. If reports from two laboratories prove correct, denervated muscle may be a tissue source enriched for such putative trophic factors (Henderson et al., 1983; Nurcombe et al., 1984). Unfortunately, however, the active factors reported in these studies have not been characterized, making it impossible to compare these studies with one another or to studies of conditioned medium effects on spinal cord cultures. Much more work is needed before it will be possible to tell whether the activities present in muscle extracts are similar to the survival-enhancing activity from MCM characterized in the present study. It will also be important for future assays of trophic activities in these extracts to be performed using cultures of purified motoneurons, to avoid confusion from contaminating activities that might affect other classes of spinal cord cells. Ultimately, the role of any putative "motoneuron trophic factor" must be verified *in vivo*.

3. ARE CNTF AND THE RHDCM FACTOR "MOTONEURON TROPHIC FACTORS"?

It is unfortunate that the tests of two putative neuronal trophic factors, reported in Chapters 5 and 6, cannot be considered conclusive: It is not yet known whether CNTF and the RHDCM factor act as "motoneuron trophic factors" *in vitro*. The characterization

of the survival-enhancing activity from myotube conditioned medium, presented in Chapter 4, did not suggest that the RHDCM factor (also derived from muscle conditioned medium) would be a likely candidate for the source of this activity: the apparent size of the survival-enhancing activity from MCM (< 25,000 Da) was considerably smaller than that of the RHDCM factor (56,000 Da). The failure of this factor to enhance survival of cultured motoneurons did not contradict this notion. It may well be that the RHDCM factor is important in the process of motor nerve terminal sprouting -- the ability of antibodies to this protein to suppress such sprouting certainly suggests this (Gurney, 1984; Gurney and Apatoff, 1984) -- but has nothing to do with motoneuron survival during embryogenesis. As was discussed in Chapter 5, further tests of this factor, in vivo and in vitro, are necessary in order to decide this issue.

At present, the "Ciliary Neuronotrophic Factor" (CNTF) can be considered a potential "motoneuron trophic factor", in vitro at least. This result is not entirely surprising given the fact that this protein was purified from muscle tissue on the basis of its ability to support the survival of ciliary ganglion neurons, which are both cholinergic and motor. The paradoxical effects on motoneuron survival of higher concentrations of CNTF may well prove to be artifacts of the factor's preparation, as was discussed in Chapter 5; or it may be that CNTF alone cannot fully support motoneuron survival in vitro. The proof of CNTF as a "motoneuron trophic factor" (and, indeed, as a "ciliary ganglion neuron trophic factor"), awaits the proper studies in vivo.

BIBLIOGRAPHY

Adler, R., K.B. Landa, M. Manthorpe, and S. Varon. (1979) Cholinergic neuronotrophic factors: Intraocular distribution of trophic activity for ciliary neurons. Science 204, 1434-1436.

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

Adler, R. and S. Varon (1982) Neuronal survival in intact ciliary ganglia *in vivo* and *in vitro*: Ciliary neuronotrophic factor as a target surrogate. Dev. Biol. 92, 470-475.

Akers, R.M., D.F. Mosher, and J.E. Lilien (1981) Promotion of retinal neurite outgrowth by substratum-bound fibronectin. Dev. Biol. 86, 179-188.

Angeletti, P.U. and R. Levi-Montalcini (1970) Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. Proc. Natl. Acad. Sci. U.S.A. 65, 114-121.

Barald, K.F. and D.K. Berg (1979) Autoradiographic labeling of spinal cord neurons with high affinity choline uptake in cell culture. Dev. Biol. 72, 1-14.

Barbin, G., M. Manthorpe and S. Varon (1984) Purification of the chick eye ciliary neuronotrophic factor. J. Neurochem. 43, 1468-1478.

Barde, Y.-A., D. Edgar and H. Thoenen (1980) Sensory neurons in culture: Changing requirements for survival factors during embryonic development. Proc. Natl. Acad. Sci. U.S.A. 77, 1199-1203.

Barde, Y.-A., D. Edgar and H. Thoenen (1982) Purification of a new

neurotrophic factor from mammalian brain. The EMBO J. 1, 549-553.

Barde, Y.-A., D. Edgar and H. Thoenen (1983) New neurotrophic factors. Ann. Rev. Physiol. 45, 601-612.

Barde, Y.-A., R.M. Lindsay, D. Monard and H. Thoenen (1978) New factor released by cultured glioma cells supporting survival and growth of sensory neurones. Nature 274, 818.

Baron-Van Evercooren, A., H.K. Kleinman, S. Ohno, P. Marangos, J.P. Schwartz and M.E. Dubois-Dalcq (1982) Nerve growth factor, laminin and fibronectin promote nerve growth in human fetal sensory ganglia cultures. J. Neurosci. Res. 8, 179-193.

Bayne, E.K., M.J. Anderson, and D.M. Fambrough (1984) Extracellular matrix organization in developing muscle: Correlation with acetylcholine receptor aggregates. J. Cell Biol. 99, 1486-1501.

Bennett, M.R., Lai, K., and V. Nurcombe (1980) Identification of embryonic motoneurons in vitro: Their survival is dependent on skeletal muscle. Brain Res. 190, 537-542.

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

Berg, D.K. and G.D. Fischbach (1978) Enrichment of spinal cord cell cultures with motoneurons. J. Cell Biol. 77, 83-98.

Betz, W.J., J.H. Caldwell, and R.R. Ribchester (1980) Sprouting of active nerve terminals in partially inactive muscles of the rat. J. Physiol. 303, 281-297

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

Bonyhady, R.E., I.A. Hendry and C.E. Hill (1982) Reversible dissociation of a bovine cardiac factor that supports survival of avial ciliary ganglionic neurones. J. Neurosci. Res. 7, 11-21.

Bonyhady, R.E., I.A. Hendry, C.E. Hill and I.S. McLennan (1980) Characterization of a cardiac muscle factor required for the survival of cultured parasympathetic neurones. Neurosci. Letts. 18, 197-201.

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

Bottenstein, J.E., S.D. Skaper, S. Varon and G.H. Sato. (1980) Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented medium. Exp. Cell Res. 125, 183-190.

Boucaut, J.-C., T. Darribere, H. Boulekbache and J.P. Thiery (1984a) Prevention of gastrulation but not neurulation by antibodies to fibronectin in amphibian embryos. Nature 307, 364-367.

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

Brown, M.C., R.L. Holland and W.G. Hopkins (1981) Motor nerve sprouting. Ann. Rev. Neurosci. 4, 17-42.

Brown, M.C., R.L. Holland and R. Ironton (1980) Nodal and terminal

sprouting from motor nerves in fast and slow muscles of the mouse. J. Physiol. 306, 493-510.

Brown, M.C. and R. Ironton (1977) Motor neurone sprouting induced by prolonged tetrodotoxin block of nerve action potentials. Nature 265, 459-461.

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

Chu-Wang, I.-W., and R.W. Oppenheim (1978a) Cell death of motoneurons in the chick embryo spinal cord. I. A light and electron microscopic study of naturally occurring and induced cell loss during development. J. Comp. Neurol. 177, 33-58.

Chu-Wang, I.-W., and R.W. Oppenheim (1978b) Cell death of motoneurons in the chick embryo spinal cord. II. A quantitative and qualitative analysis of degeneration in the ventral root, including evidence for axon outgrowth and limb innervation prior to cell death. J. Comp. Neurol. 177: 59-86.

Chun, L.L.Y. and P.H. Patterson (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.

Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. Proc. Natl. Acad. Sci. U.S.A. 40, 1014-1018.

Cohen, S., R. Levi-Montalcini and V. Hamburger (1954) A nerve growth-stimulating factor isolated from sarcomas 37 and 180. Proc. Natl.

Acad. Sci. U.S.A. 40, 1014-1018.

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

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

Coughlin, M.D. and J.A. Kessler (1982) Antiserum to a new neuronal growth factor: Effects on neurite outgrowth. J. Neurosci. Res. 8, 298-312.

Cowan, W.M. and P.G.H. Clarke (1976) The development of the isthmo-optic nucleus. Brain Behav. Evol. 13, 345-375.

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

Doherty, P., J.G. Dickson, T.P. Flannigan and F.S. Walsh (1985) Human skeletal muscle cells synthesize a novel neuronotrophic factor specifically reactive with spinal neurons. In preparation.

Dribin, L.B., and J. N. Barrett (1980) Conditioned medium enhances neuritic outgrowth from rat spinal cord explants. Dev. Biol. 74, 184- 195.

Dribin, L.B., and J. N. Barrett (1982a) Two components of conditioned medium increase neuritic outgrowth from rat spinal cord explants. J. Neurosi. Res. 8, 271-280.

Dribin, L.B. and J.N. Barrett (1982b) Characterization of neuritic outgrowth-promoting activity of conditioned medium on spinal cord

explants. Dev. Br. Res. 4, 435-441.

Dumas, M., M.E. Schwab and H. Thoenen (1979) Retrograde axonal transport of specific macromolecules as a tool for characterizing nerve terminal membranes. J. Neurobiol. 10, 179-197.

Eagleson, K.L. and M.R. Bennett (1983) Survival of purified motor neurones in vitro: Effects of skeletal muscle-conditioned medium. Neurosci. Letts. 38, 187-192.

Ebendal, T., L. Olson, A. Seiger and K.-O. Hedlund (1980) Nerve growth factors in the rat iris. Nature 286, 25-28.

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

Fujiwara, S., H. Wiedemann, R. Timpl, A. Lustig and J. Engel (1984) Structure and interactions of heparan sulfate proteoglycans from a mouse tumor basement membrane. Eur. J. Biochem. 143, 145-157.

Gorin, P.D. and E.M. Johnson (1979) Experimental autoimmune model of nerve growth factor deprivation: Effects on developing peripheral sympathetic and sensory neurons. Proc. Natl. Acad. Sci. U.S.A. 76, 5382-5386.

Greene, L.A. (1977) Quantitative in vitro studies on the nerve growth factor (NGF) requirement of neurons. II. Sensory neurons. Dev. Biol. 58, 106-113.

Gurney, M.E. (1984) Suppression of sprouting at the neuromuscular junction by immune sera. Nature 307, 546-548.

Gurney, M.E. and B. Apatoff (1984) Activity of a muscle-derived growth factor for spinal neurons in vitro and in vivo. Soc. Neurosci. Abstr. 10, 1051.

Ham, R.C. (1973) Dilution plating and nutritional consideration. A. Animal cells. In Tissue Culture: Methods and Applications (P.F. Kruse, Jr., and M.K. Petterson, Jr., eds.) © 1973 Academic Press, New York, N.Y.

Hamburger, V. (1958) Regression versus peripheral control of differentiation in motor hypoplasia. Am. J. Anat. 102, 365-410.

Hamburger, V. (1975) Cell death in the development of the lateral motor column of the chick embryo. J. Comp. Neurol. 160, 535-546.

Hamburger, V., J.K. Bruno-Bechtold, and J. Yip (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J. Neurosci. 1, 60-71.

Hamburger, V., and H. L. Hamilton (1951) A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Hamburger, V. and R. Levi-Montalcini (1949) Proliferation, differentiation and degeneration in the spinal ganglia of the chick embryo under normal and experimental conditions. J. Exp. Zool. 111, 457-502.

Harrison, P.J., H. Hultborn, E. Jankowska, R. Katz, B. Storai, and D. Zytnicki (1984) Labelling of interneurons by retrograde transsynaptic transport of horseradish peroxidase from motoneurons in rats and cats. Neuroscience Letters 45, 15-19.

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

Helfand, S.L., G.A. Smith and N.K. Wessells (1976) Survival and development in culture of dissociated paracympathetic neurons from ciliary ganglia. Dev. Biol. 50, 541-547.

Henderson, C.E., M. Huchet and J.-P. Changeux (1981) Neurite outgrowth from embryonic chicken spinal neurons is promoted by media conditioned by muscle cells. Proc. Natl. Acad. Sci. USA 78, 2625-2629.

Henderson, C.E., M. Huchet and J.-P. Changeux (1983) Denervation increases a neurite-promoting activity in extracts of skeletal muscle. Nature 302, 609-611.

Henderson, C.E., M. Huchet and J.-P. Changeux (1984) Neurite-promoting activities for embryonic spinal neurons and their developmental changes in the chick. Dev. Biol. 104, 336-347.

Hendry, I.A. (1975) The response of adrenergic neurones to axotomy and nerve growth factor. Brain Res. 94, 87-97.

Hendry, I.A. and J. Campbell (1976) Morphometric analysis of rat superior cervical ganglion after axotomy and nerve growth factor treatment. J. Neurocytol. 5, 351-360.

Hendry, I.A., K. Stockel, H. Thoenen and L.L. Iversen (1974) The retrograde axonal transport of nerve growth factor. Brain Res. 68, 103-121.

Hollyday, M. and V. Hamburger (1976) Reduction of the naturally occurring motor neuron loss by enlargement of the periphery.

J. Comp. Neurol. 170, 311-320.

Hollyday, M. and V. Hamburger (1977) An autoradiographic study of the formation of the lateral motor column in the chick embryo. Brain Res. 132, 197-208.

Hsu, L., D. Natyzak and G.L. Trupin (1982) Neuronotrophic effects of skeletal muscle fractions on spinal cord differentiation. J. Embryol. Exp. Morph. 71, 83-95.

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

Kaufman, L.M. and J.N. Barrett (1983) Serum factor supporting long-term survival of rat central neurons in culture. Science 220, 1394-1396.

Kessler, J.A., P. Cochard and I.B. Black (1979) Nerve growth factor alters the fate of embryonic neuroblasts. Nature 280, 141-142.

Kimura, I., Hasegawa, T., and E. Ozawa (1982) Indispensability of iron-bound chick transferrin for chick myogenesis in vitro. Dev. Growth Differen., 24: 369-379.

Konieczny, S.F., J. McKay and J.R. Coleman (1982) Isolation and characterization of terminally differentiated chicken and rat skeletal muscle myoblasts. Dev. Biol. 91, 11-26.

Konigsberg, I.R. (1979) Skeletal myoblasts in culture. Meth. Enzymol. 58, 511-526.

Korsching, S. and H. Thoenen (1983) Nerve growth factor in sympathetic ganglia and corresponding target organs of the rat: Correlation with density of sympathetic innervation. Proc. Natl. Acad. Sci. U.S.A. 80, 3513-3516.

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

Landa, K.B., R. Adler, M. Manthorpe and S. Varon (1980) Cholinergic neuronotrophic factors. III. Developmental increase of trophic activity for chick embryo ciliary ganglion neurons in their intraocular target tissues. Dev. Biol. 74, 401-408.

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

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

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

Landmesser, L. (1978) The development of motor projection patterns in the chick hind limb. J. Physiol. 284, 391-414

Landmesser, L. and G. Pilar (1974a) Synpase formation during embryogenesis on ganglion cells lacking a periphery. J. Physiol. 241, 715-736.

Landmesser, L. and G. Pilar (1974b) Synaptic transmission and cell death during normal ganglionic development. J. Physiol. 241, 737-749.

Laurie, G.W., C.P. LeBlond and G.R. Martin (1982) Localization of type IV collagen, laminin, heparan sulfate proteoglycan, and fibronectin to the basal lamina of basement membranes. J. Cell Biol. 95, 340-344.

Levi-Montalcini, R. (1952) Effects of mouse tumor transplants on nervous system. Ann. N.Y. Acad. Sci. 55, 330-343.

Levi-Montalcini, R., L. Aloe, E. Mugnaini, F. Oeschi and H. Thoenen. (1975) Nerve growth factor induces volume increase and enhances tyrosine hydroxylase synthesis in chemically axotomized sympathetic ganglia. Proc. Natl. Acad. Sci. U.S.A. 72, 595-599.

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

Levi-Montalcini, R. and P.U. Angeletti (1968) Nerve growth factor. Physiol. Rev. 48, 534-569.

Levi-Montalcini, R. and B. Booker (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.

Levi-Montalcini, R. and V. Hamburger (1951) Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool. 116, 321-362.

Levi-Montalcini, R. and V. Hamburger (1953) A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. J. Exp. Zool. 123, 233-288.

Levi-Montalcini, R., H. Meyer and V. Hamburger (1954) In vitro experiments on the effects of mouse sarcomas 180 and 37 on the spinal and sympathetic ganglia of the chick embryo. Cancer Res. 14, 49-57.

Linker, A., and P. Hovingh (1972) Heparinase and heparatinase from Flavobacteria. Meth. Enzymol., 28, 902-910.

Longo, F.M., M. Manthorpe and S. Varon (1982) Spinal cord neuronotrophic factors (SCNTFs): I. Bioassay of schwannoma and other conditioned media. Dev. Brain Res. 3, 277-294.

Manthorpe, M., R. Adler and S. Varon (1981) Cholinergic neuronotrophic factors. VI. Age-dependent requirements by chick embryo ciliary ganglionic neurons. Dev. Biol. 85, 156-163.

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

Marshall, L.M., J.R. Sanes and U.J. MCMahan (1977) Reinnervation of original synaptic sites on muscle fiber basement membrane after disruption of the muscle cells. Proc. Natl Acad. Sci. U.S.A.

74, 3073-3077.

Masuko, S., H. Kuromi, and Y. Shimada (1979) Isolation and culture of motoneurons from embryonic chicken spinal cords. Proc. Natl. Acad. Sci. U.S.A. 76: 3537-3541.

McPheeters, M. (1982) Autoradiographic and fluorescent labelling of neuron classes in chick embryo spinal cord and sensory ganglia: Isolation and culture of motoneurons. Ph.D. Thesis, Univ. of Utah, Salt Lake City.

McPheeters, M., and L.M. Okun (1980) Identification and isolation in vitro of presumptive motoneurons marked by retrograde transport of a new fluorescent tracer. Soc. Neurosci. Abstr. 6, 733.

Meier, D.H., C. Lagenaur and M. Schacner (1982) Immunoselection of oligodendrocytes by magnetic beads. I. Determination of antibody coupling parameters and cell binding conditions. J. Neurosci. Res. 7, 119-134.

Molday, R.S. and L.L. Molday (1984) Separation of cells labeled with immunospecific iron dextran microspheres using high gradient magnetic chromatography. FEBS Letts. 170, 232-238.

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

Muller, H.W., S. Beckh and W. Seifert (1984) Neurotrophic factor for central neurons. Proc. Natl. Acad. Sci. U.S.A. 81, 1248-1252.

Narayanan, C.H. and Y. Narayanan (1978) Neuronal adjustments in developing nuclear centers of the chick embryo following transplantation of an additional optic primordium. J. Embryol.

Exp. Morphol. 44, 53-70.

Nishi, R. and D.K. Berg (1977) Dissociated ciliary ganglion neurons in vitro: Survival and synapse formation. Proc. Natl. Acad. Sci. U.S.A. 74, 5171-5175.

Nishi, R. and D.K. Berg (1979) Survival and development of ciliary ganglion neurones grown alone in cell culture. Nature 277, 232-234.

Nishi, R., and D. Berg (1981) Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. J. Neurosci. 1, 505-513.

Nurcombe, V. and M.R. Bennett (1982) Evidence for neuron-survival and neurite-promoting factors from skeletal muscle: Their effects on embryonic spinal cord. Neurosci. Letts. 34, 89-93.

Nurcombe, V. and M.R. Bennett (1983) The growth of neurites from explants of brachial spinal cord exposed to different components of wing bud mesenchyme. J. Comp. Neurol. 219, 133-142.

Nurcombe, V., M.A. Hill, K.L. Eagleson, and M.R. Bennett (1984) Motor neuron survival and neuritic extension from spinal cord explants induced by factors released from denervated muscle. Brain Res. 291, 19-28.

Obata, K., and H. Tanaka (1980) Conditioned medium promotes neurite growth from both central and peripheral neurons. Neurosci. Letts. 16, 27-33.

Okado, N. and R.W. Oppenheim (1984) Cell death of motoneurons in the chick embryo spinal cord. IX. The loss of motoneurons following removal of afferent inputs. J. Neurosci. 4, 1639-1652.

Okun, L.M. (1981) Identification and isolation *in vitro* of neurons marked *in situ* by retrograde transport. In *New Approaches in Developmental Neurobiology*, Society for Neuroscience, Bethesda, Maryland, pp. 109-121.

Oppenheim, R.W. (1981a) Neuronal death and some related regressive phenomena during neurogenesis: A selective historical review and progress report. In *Studies in Developmental Neurobiology*: Essays in Honor of Viktor Hamburger (W.M. Cowan, ed.) New York, Oxford University Press, pp. 74-133.

Oppenheim, R.W. (1981b) Cell death of motoneurons in the chick embryo spinal cord. V. Evidence on the role of cell death and neuromuscular function in the formation of specific peripheral connections. J. Neurosci. 1, 141-151.

Pittman, R., and R.W. Oppenheim (1979) Cell death of motoneurons in the chick embryo spinal cord. IV. Evidence that a functional neuromuscular interaction is involved in the regulation of naturally occurring cell death and the stabilization of synapses. J. Comp. Neurol. 187, 425-446.

Pollack, E.D., W.L. Muhlach and V. Liebig (1981) Neurotropic influence of mesenchymal limb target tissue on spinal cord neurite growth *in vitro*. J. Comp. Neurol. 200, 393-405.

Purves, D. and J.W. Lichtman (1980) Elimination of synapses in the developing nervous system. Science 210, 153-157.

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

Ruoslahti, E., E.G. Hayman, M. Peirschbacher and E. Engvall (1982) Fibronectin: Purification, immunochemical properties, and biological activities. Meth. Enzymol. 82, 803-831.

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

Sanes, J.R. (1984) More nerve growth factors? Nature 307, 500.

Sanes, J.R., L.M. Marshall and U.J. McMahan (1978) Reinnervation of original synaptic sites on muscle fiber basement membrane after disruption of the muscle cells. Proc. Natl. Acad. Sci. U.S.A. 74, 3073-3077.

Schnaar, R.L. and A.E. Schaffner (1981) Separation of cell types from embryonic chicken and rat spinal cord: Characteristization of motoneuron-enriched fractions. J. Neurosci. 1, 204-217.

Shelton, D.L. and L.F. Reichardt (1984) Expression of the B-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. Proc. Natl. Acad. Sci. U.S.A. 81, 7951-7955.

Slack, J.R. and S. Pockett (1982) Motor neurotrophic factor in denervated adult skeletal muscle. Brain Res. 247, 138-140.

Smith, R.G. and S.H. Appel (1983) Extracts of skeletal muscle increase neurite outgrowth and cholinergic activity of fetal rat spinal

motor neurons. Science 219, 1079-1081.

Summerbell, D. and R.V. Stirling (1981) The innervation of dorso-ventrally reversed chick wings: Evidence that motor axons do not actively seek out their appropriate targets. J. Embryol. Exp. Morphol. 61, 233-247.

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

Tanaka, H. and K. Obata (1982) Survival and neurite growth of chick embryo spinal cord cells in serum-free culture. Dev. Brain Res. 4, 313-321.

Tanaka, H. and K. Obata (1984) Developmental changes in unique cell surface antigens of chick embryo spinal motoneurons and ganglion cells. Dev. Biol. 106, 26-37.

Tanaka, H., M. Sakai and K. Obata (1982) Effects of serum, tissue extract, conditioned medium, and culture substrata on neurite appearance from spinal cord explants of chick embryo. Dev. Brain Res. 4, 303-312.

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

Thoenen, H. and Y.-A. Barde (1980) Physiology of nerve growth factor. Physiol. Rev. 60, 1284-1335.

Timpl, R., J. Enge., and G.R. Martin (1983) Laminin -- A multifunctional protein of basement membranes. T.I.B.S. 8,

207-209.

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

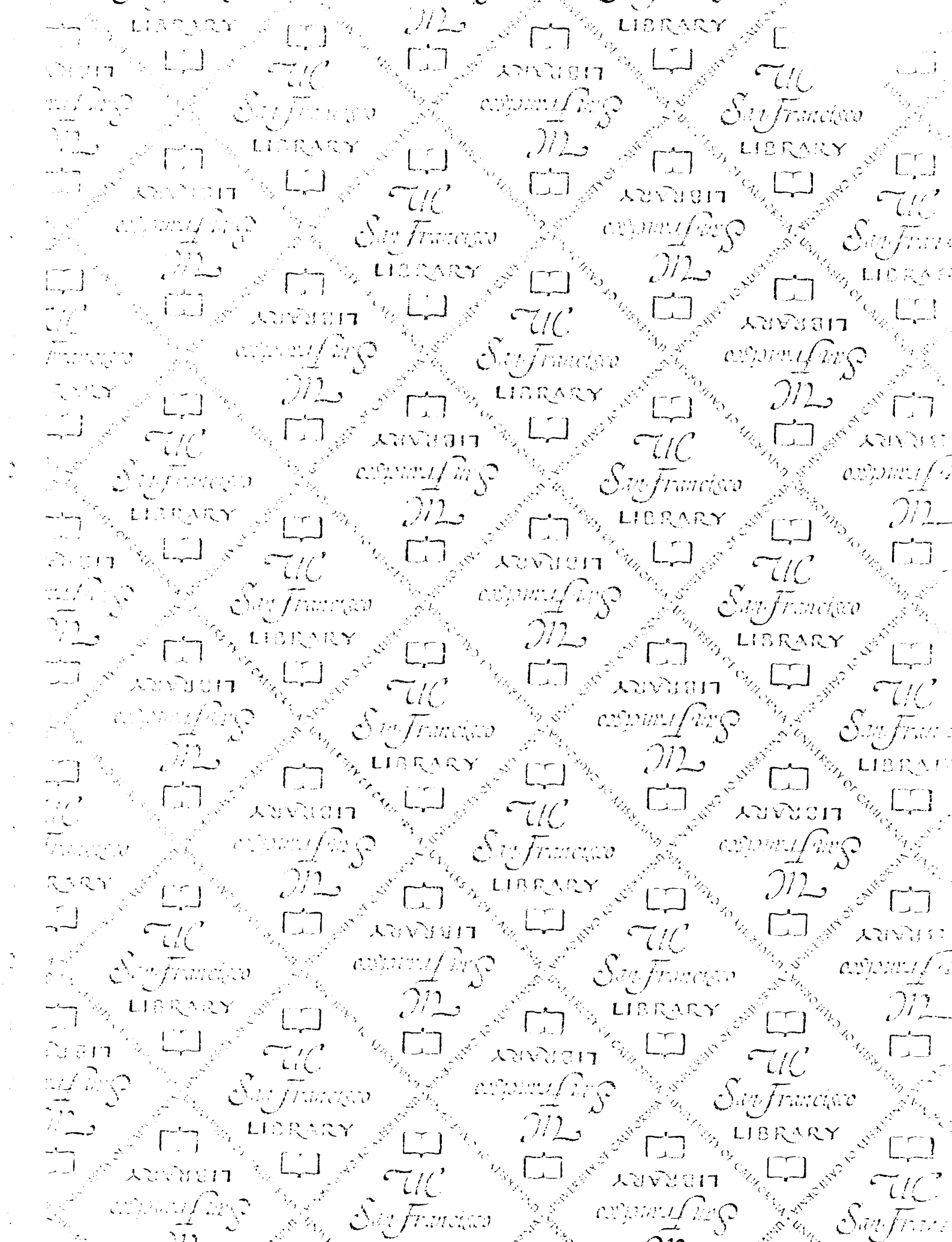
Timpl, R., H. Rohde, P.G. Robey, S.I. Rennard, J.-M. Foidart and G.R. Martin (1979) Laminin -- A glycoprotein from basement membranes. J. Biol. Chem. 254, 9933-9937.

Varon, S., Manthorpe., M. & Adler, R. (1979) Cholinergic neuronotrophic factors: I. Survival, neurite outgrowth and choline acetyltransferase activity in monolayer cultures from chick embryo ciliary ganglia. Brain Res. 173, 29-45.

Varon, S., S.D. Skaper, G. Barbin, I. Selak and M. Manthorpe (1984) Low molecular weight agents support survival of cultured neurons from the central nervous system. J. Neurosci. 4, 654-658.

Wu, T.-C., Y.-J. Wau, A.E. Chung and I. Damjanov (1983) Immunohistochemical localization of entactin and laminin in mouse embryos and fetuses. Dev. Biol. 100, 496-505.

Yanker, B.A. and E.M. Shooter (1982) The biology and mechanism of action of nerve growth factor. Ann. Rev. Biochem. 51, 845-868.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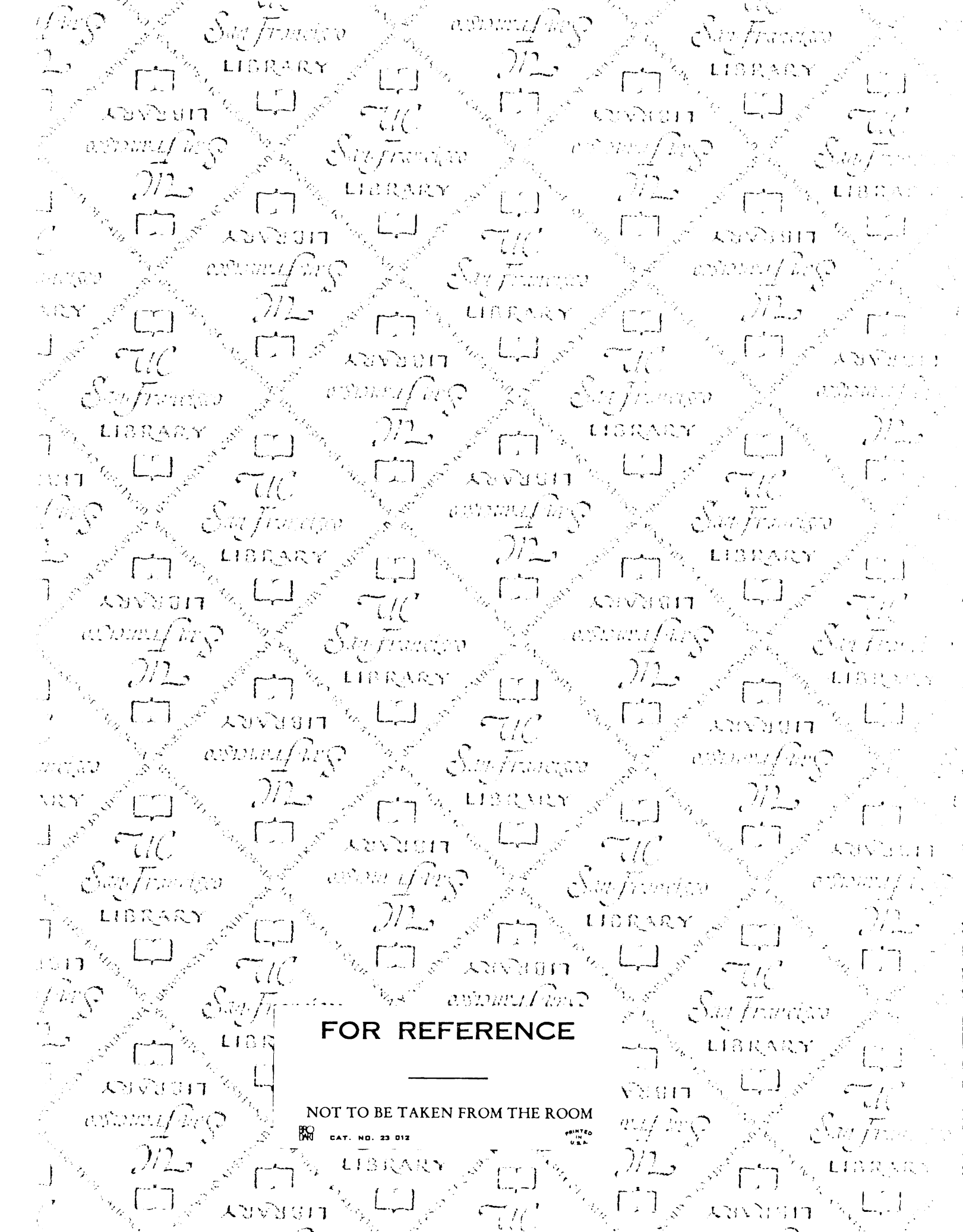
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