17. F. H. Reynolds, Jr., G. J. Todaro, C. Fryling, J. R. Stephenson, *Nature (London)* **292**, 259 (1981).
18. M. S. Collett and R. L. Erikson, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2021 (1978).
19. J. R. Stephenson and G. J. Todaro, in *Viral Oncology*, G. Klein, Ed. (Raven, New York, 1982).
20. H. Marquardt and J. Todaro, *J. Biol. Chem.* **257**, 5220 (1982).
21. H. Marquardt *et al.*, in preparation.
22. A. B. Schreiber, Y. Yarden, J. Schlessinger, *Biochem. Biophys. Res. Commun.* **101**, 517 (1981).
23. G. N. Gill and C. S. Lazar, *Nature (London)* **293**, 305 (1981).
24. D. R. Twardzik, J. Ranchalis, G. J. Todaro, *Cancer Res.* **42**, 590 (1982).
25. A. B. Roberts *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3494 (1980).
26. U. K. Laemmli, *Nature (London)* **227**, 680 (1970).
27. B. R. Oakley, D. R. Kirsch, N. R. Morris, *Anal. Biochem.* **105**, 361 (1980).
28. We thank J. E. Ranchalis for technical assistance and J. DeLarco for the antiserum to EGF.

19 October 1981; revised 22 February 1982

Nerve Fiber Growth on Defined Hydrogel Substrates

Abstract. *Cultured neurons become attached to hydrogel substrates prepared from 2-hydroxyethylmethacrylate but grow few nerve fibers unless fibronectin, collagen, or nerve growth factor is incorporated into the hydrogel. Antibodies to fibronectin inhibit nerve fiber growth on hydrogels containing fibronectin, which suggests that growing neurons interact directly with proteins trapped in the hydrogel. The adhesive requirements for attachment of neurons appear distinct and possibly less specific than those for fiber growth. Defined hydrogel substrates offer a controlled method for analyzing complex substrates that support nerve fiber growth and neuronal differentiation.*

The extent to which cultured neurons extend nerve fibers is known to depend on the culture substrate, but this dependence is poorly understood. Interactions of the cell surface with the substrate, especially at growth cones (that is, at the attached motile tips of growing nerve fibers), appear to determine the rate and direction of growth (*1*). Substrates that support fiber extension are thought to be suitably "adhesive" (*2*), but the factors responsible for this adhesiveness have not been defined in molecular terms. Attachment of neuronal somata to various substrates is commonly used as a measure of substrate adhesiveness (*3*), but it is not clear whether the molecular interactions involved in the attachment of somata are identical, or even related, to those that mediate nerve fiber extension.

The uncertainty results in part from difficulty in preparing defined substrates that support nerve fiber outgrowth. Dishes precoated with purified proteins and related macromolecules such as fibronectin, collagen, and polyamino acids support excellent nerve fiber outgrowth (*1, 2, 4*). However, analysis of the molecular interactions required for nerve fiber outgrowth is complicated by variability in the binding of macromolecules to the dishes (*5*) and in the subsequent dissociation of the macromolecules or their removal by cells (*6*), and by the difficulty that neurons from several sources extend nerve fibers when seeded on tissue culture dishes that have not been precoated. Moreover, the upper surfaces of nonneuronal cells (*7*) as well as materials deposited on the dish by such cells (*8*) have been shown to promote luxuriant fiber outgrowth. Thus, in primary neuronal cultures that are invariably contaminated by large numbers of nonneuronal cells (glia and fibroblasts), the substrate, whether it has been deliberately precoated or not, may be substantially modified by undefined products of nonneuronal cells.

We report here the results of an effort to elucidate the molecular properties of substrates necessary for nerve fiber outgrowth. Hydrogels prepared from 2-hydroxyethylmethacrylate (HEMA) permit neuron attachment, but support little nerve fiber growth. When the gels are modified by the inclusion of appropriate macromolecules within the gel matrix, neurons grow extensive nerve fibers. Small molecules such as mannose (molecular weight 180) can be easily incorporated into HEMA gels, whereas satisfactory, stable coating of culture dishes by incubation with solutions of small molecules is seldom feasible. As a result it is possible to study, in a controlled fashion,

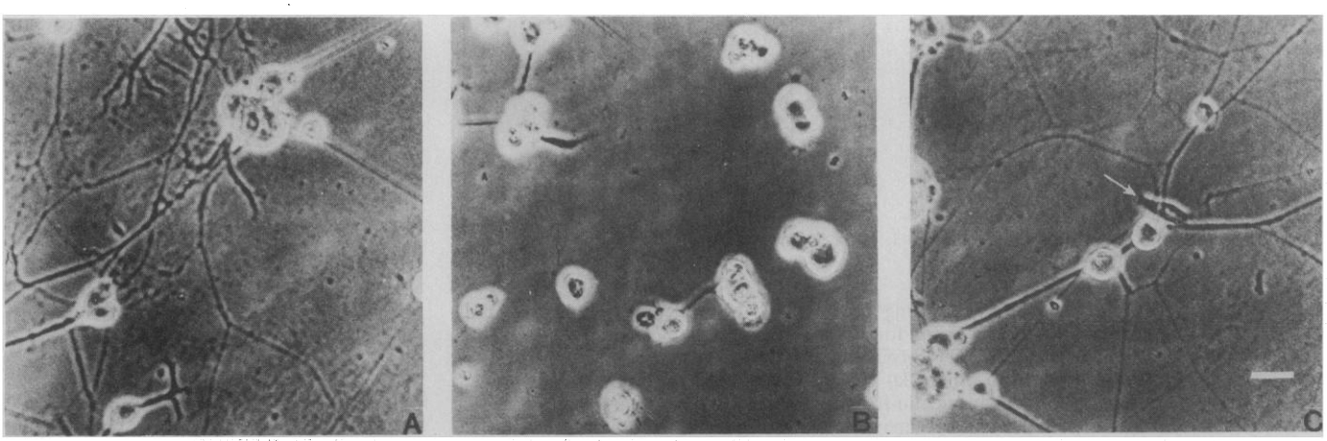

Fig. 1. Growth of neurons on HEMA gels containing collagen. Cells from chick embryo dorsal root ganglia were prepared by trypsin treatment and trituration of dorsal root ganglia from chick embryos (*14*). A suspension of cells in culture medium containing fibronectin-free horse serum was seeded onto circles of HEMA-collagen gels (1 mg/ml in tris-buffered saline, *p*H 7.4) or onto circles of HEMA with tris-buffered saline. There was no significant difference in the number of cells attached to the two types of gels. (A) On HEMA-collagen gels after 36 hours in culture 40 percent of the neurons had nerve fibers that averaged 208 μm in length per cell (N = 41). (B) After 36 hours 16 percent of the neurons on simple HEMA gels had grown nerve fibers that averaged 49 μm in length (N = 60). Total axon length per 100 neurons, determined by multiplying the fraction of growing neurons by the total nerve fiber length per cell, was 784 μm per 100 for neurons on simple HEMA gels and 8320 μm per 100 neurons on HEMA-collagen gels. After 36 hours the cells on simple HEMA gels were harvested from the gel by mild trypsin treatment (0.0125 percent trypsin in divalent cation-free Hanks balanced salt solution, *p*H 7.2, for 10 minutes at 23°C), then centrifuged and resuspended in culture medium (as above). These harvested cells were reseeded onto HEMA-collagen gels (1 mg/ml) and within 24 hours 30 percent of the neurons had extended fibers (C) that averaged 290 μm in length (N = 31; total axon length, 8600 μm per 100 neurons). A spread fibroblast (arrow) is visible in this photomicrograph.

0036-8075/82/0521-0897$01.00/0

the effects of a wide variety of defined HEMA substrates on nerve fiber growth in culture.

We found that fibronectin, β nerve growth factor (βNGF), and several types of collagen are effective at supporting nerve fiber growth when incorporated into the HEMA matrix. A variety of other macromolecules were much less effective, including: wheat germ agglutinin, a plant lectin known to bind to neurons (*9*), cytochrome *c*, and two cationic polyamino acids. Nerve fiber outgrowth is as extensive in cultures depleted of nonneuronal cells as in cultures containing large numbers of nonneuronal cells, but on HEMA-fibronectin gels nerve fiber growth is blocked by antiserum to fibronectin. Taken together these results suggest a direct and selective interaction of the nerve fiber surface with defined molecules embedded in the gels.

Synthetic polymers of HEMA belong to a class called hydrogels that contain large amounts of water (*10*). HEMA gels are well tolerated as implanted prostheses; for this reason, and because of their optical clarity and resilience, they are commonly used as soft contact lenses. The culture substrates we used were prepared according to the methods of Civerchia-Perez *et al.* (*11*). The collagens incorporated into the gels were types I, III, and IV; other molecules included fibronectin, βNGF, cytochrome *c*, and wheat germ agglutinin (*12*). HEMA gels have a pore size of approximately 0.4 nm (*10*). Polymerization of HEMA in the presence of a variety of radioactively labeled molecules ([^{125}I]βNGF, [^{125}I]α-bungarotoxin, [^{3}H]concanavalin A, [^{35}S]methionine, [^{3}H]mannose) showed that even molecules of low molecular weight become almost permanently trapped in the gels (*13*).

Suspensions of cells from dorsal root ganglia of chicken embryos were seeded directly onto HEMA-gel substrates (*14*). There was no significant difference in the number of cells that attached to simple HEMA-gel substrates or to substrates containing proteins. There was little nerve fiber growth, and nonneuronal cells did not flatten on simple HEMA gels; however, on HEMA gels containing fibronectin or collagen, neurons extended long fibers, and nonneuronal cells spread upon the substrate (Fig. 1). When spherical cells that had been cultured on simple HEMA gels for 24 hours were resuspended by trypsinization and then reseeded onto HEMA gels containing fibronectin, they rapidly extended nerve fibers (Fig. 1).

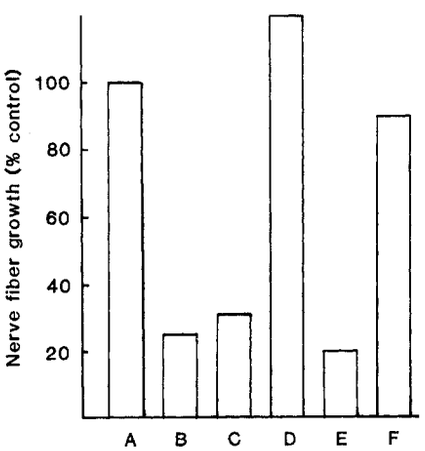

Fig. 2. Nerve fiber growth on HEMA-fibronectin gels: effects of enrichment of cultures for neurons and of antiserum to fibronectin. Cells from chick embryo dorsal root ganglia were seeded onto HEMA-fibronectin gels or simple HEMA gels (Fig. 1). After 24 hours the cultures were fixed with 3 percent paraformaldehyde in 100 m*M* phosphate buffer, *p*H 7.4, and the lengths of nerve fibers were determined by observation through a light microscope fitted with an eyepiece micrometer. In this and all other experiments nerve fibers from neurons not in contact with other cells were measured so that the neurons were clearly growing on the gel substrate and not on other cells. The extent of growth was 581 μm per 100 neurons in controls (A) and 116 μm per 100 neurons in cultures on the simple HEMA gels (E). Data are expressed as a percentage of growth on control HEMA-fibronectin gels. (A) HEMA-fibronectin gels (1 mg/ml). (B) HEMA-fibronectin gels (1 mg/ml) that were pretreated with antiserum to fibronectin for 3 hours at 37°C and washed three times with Hanks balanced salt solution. (C) HEMA-fibronectin gels upon which cells were cultured in medium containing antiserum to fibronectin. (D) The same as (C) except the medium contained complete horse serum rather than fibronectin-free horse serum as in (A), (B), and (C). (E) Simple HEMA gels. (F) In a separate experiment dorsal root ganglia cells were preplated on uncoated petri dishes to which the nonneuronal cells attached firmly but the neurons much less firmly. After the cells were cultured overnight the neurons were selectively dislodged by simply directing a stream of medium over the culture. A set of cultures on HEMA-fibronectin gels equivalent to those on petri dishes was also prepared and fixed after 24 hours of growth. The ratio of neurons to nonneurons was 0.80 in cultures before preplating and increased to 9.2 after preplating. The preplated cells enriched for neurons were reseeded on HEMA-fibronectin gels. Control cultures consisted of preplated cells cultured on HEMA-fibronectin gels from which neurons and fibroblasts were nonselectively harvested, and which had a neuron to fibroblast ratio of 1.5. The extent of nerve fiber growth after 24 hours by the enriched cultures on HEMA-fibronectin gels was not significantly different from control cultures that had six times more nonneurons (F) and was greater than the extent of growth by the originally cultured (not preplated) neurons that had ten times more nonneurons.

The best nerve fiber growth occurred on HEMA-gel substrates containing fibronectin, collagen, or βNGF. The extent of nerve fiber growth on these substrates depended on the concentration of protein added to the gel (range tested: 0.05 to 1 mg/ml). Types I, III, and IV collagen did not differ significantly in their ability to support fiber growth (data not shown). Cytochrome *c* (0.2 to 1 mg/ml), a soluble protein with an isoelectric point similar to βNGF, and wheat germ agglutinin (0.1 to 1 mg/ml), which binds avidly to neurons (*9*), both supported little fiber growth (data not shown). Poly-L-lysine (1 mg/ml) and poly-L-ornithine (1 mg/ml), both widely used to render culture substrates more adhesive for cells, were also ineffective, as were the polyanionic glycosaminoglycans, chondroitin sulfate (1 mg/ml), hyaluronic acid (0.25 mg/ml), and heparin (1 mg/ml).

Only about 50 percent of the cells in our standard cultures from dorsal root ganglia are neurons. However, by preplating cells overnight on plastic petri dishes, to which nonneuronal cells attach firmly but neurons attach poorly, it is possible to selectively remove the neurons. Using this approach we have obtained a tenfold enrichment of neurons in our cultures (Fig. 2). Elimination of most of the nonneuronal cells had no significant effect on the extent of nerve fiber growth on HEMA-fibronectin gels (Fig. 2F). There is thus no evidence that products of nonneuronal cells are required either to render the substrate adhesive for neuronal growth cones or to enhance the fiber growth response of neurons.

Additional evidence that the growing nerve fibers interact directly with specific proteins embedded in the HEMA gels was provided by experiments with antibodies against fibronectin. Prior incubation of HEMA-fibronectin gels with antiserum to fibronectin markedly inhibited fiber extension by neurons (Fig. 2B), but did not cause neurons to detach from the gel surface. Nerve fiber growth on HEMA gels with fibronectin was inhibited to an even greater extent by addition of antiserum to a medium containing fibronectin-free serum (Fig. 2C). The antiserums had no inhibitory effect when added to medium supplemented with whole serum containing fibronectin (Fig. 2D). The failure of fibronectin-antifibronectin complexes to support fiber outgrowth suggests a direct interaction of the nerve fiber surface with molecules embedded in the substrate.

It is not known whether the various collagens or βNGF also interact directly with the surface of the growth cone.

Collagens of all types are known to bind the fibronectin present in culture media supplemented with whole serum (*15*). The collagen in HEMA gels may therefore be an effective substrate for fiber growth because of its ability to collect fibronectin. If this were so, we would expect other molecules that bind fibronectin to be effective when incorporated into HEMA gels. Yet heparin, which does bind fibronectin (*16*) did not promote fiber growth. Furthermore, βNGF, which we have found to be active in supporting fiber growth and has been shown by others to affect the extent and direction of nerve fiber outgrowth (*17*), is not known to bind fibronectin. It seems likely that βNGF interacts directly with its own receptors on the cell surfaces of neurons and their processes. Wheat germ agglutinin, which also binds to the surfaces of these neurons (*9*) more extensively than does βNGF, might be expected to be more adhesive but it supports little nerve fiber growth. At least two sets of distinct molecular interactions between the growth cone and the culture substrate appear to be effective in permitting nerve fiber growth.

From these studies we have distinguished two types of adhesive interactions between cultured neurons and their substrates: one that permits attachment of neurons and a second, more specific, adhesive interaction required for nerve fiber growth. Beyond being useful for experiments with well-characterized macromolecules, hydrogel substrates should be valuable in analyzing complex substrates including microexudates and cell surfaces to identify those components that support and direct nerve fiber growth in vivo. In addition, it should be possible to prepare stable gradients of macromolecules that may stimulate chemotaxis of growth cones (*17*). Finally, the well-known biocompatibility of HEMA gels used as tissue implants suggests that HEMA gels might be used for the manufacture of prostheses that would promote nerve fiber regeneration. Severed nerves realigned by HEMA-gel cuffs containing macromolecules appropriate to support regeneration might improve the chances of functional reinnervation.

S. T. CARBONETTO
M. M. GRUVER
Department of Pharmacology, State University of New York–Upstate Medical Center, Syracuse 13066
D. C. TURNER
Department of Biochemistry, State University of New York–Upstate Medical Center

References and Notes

1. P. C. Letourneau, *Dev. Biol.* **44**, 77 (1975).
2. ———, *ibid.*, p. 92.
3. D. I. Gottlieb and L. Glaser, *Annu. Rev. Neurosci.* **3**, 303 (1980).
4. R. M. Akers, D. F. Mosher, J. E. Lilien, *Dev. Biol.* **86**, 179 (1981).
5. E. A. Turley and S. Roth, *Cell* **17**, 109 (1979).
6. B. Geiger and Z. Avnur, *ibid.* **25**, 121 (1981).
7. M. A. Ludueña, *Dev. Biol.* **33**, 268 (1973); R. Adler and S. Varon, *ibid.* **86**, 69 (1981).
8. F. Collins, *ibid.* **79**, 247 (1980).
9. S. Carbonetto and Y. Argon, *ibid.* **80**, 364 (1980).
10. S. D. Bruck, *J. Biomed. Mater. Res.* **7**, 387 (1973).
11. L. Civerchia-Perez *et. al.*, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2064 (1980). To a mixture of 0.5 ml of 2-hydroxyethylmethacrylate (Aldrich) and 0.5 ml of ethylene glycol (Aldrich) was added 0.5 ml of tris-buffered saline (TBS; 50 m*M* tris, *p*H 7.4) or 0.5 ml of TBS containing 0.05 to 1.0 mg of a protein to be incorporated into the gel. Polymerization was initiated by adding 25 μl of an aqueous solution of ammonium persulfate (60 mg/ml; Biorad) and 25 μl of an aqueous solution of sodium metabisulfite (12 mg/ml; Sigma). The mixture was pipetted between two microscope slides separated at each end by cover slips and it was allowed to polymerize for 1 to 2 hours at 40°C. After polymerization the gels were removed and washed for 12 hours or longer in at least three changes of TBS (approximately 100 ml per gel per wash). Individual HEMA-gel substrates were prepared by cutting circles about 17 mm in diameter from the slabs and placing each circle in a 35-mm-diameter petri dish. The gels were sterilized by ultraviolet irradiation for 30 minutes and subsequently equilibrated with Eagle's minimum essential medium (EMEM, Gibco) for 1 hour in the CO_2 incubator. A modified procedure for preparing HEMA gels with 0.75 ml of ethylene glycol and only 0.25 ml of the same protein solutions in TBS also yielded hydrogels that supported extensive fiber outgrowth.
12. The collagens, pure calf skin type I, human type III, and bovine renal glomerular basement membrane type IV, were a gift from J. W. Freytag, E. I. du Pont de Nemours. All collagens were dissolved in 50 m*M* acetic acid and then dialyzed exhaustively against TBS before use. Fibronectin eluted in 4*M* urea from a gelatin affinity column [M. Chiquet, E. C. Puri, D. C. Turner, *J. Biol. Chem.* **254**, 5475 (1979)] was dialyzed exhaustively against TBS before use. Sources of other reagents were: cytochrome *c*, heparin, chondroitin sulfate, hyaluronic acid, and wheat germ agglutinin from Sigma; polylysine and polyornithine from Miles-Yeda; βNGF was the generous gift of R. W. Stach, State University of New York, Upstate Medical Center. Native collagen was denatured by boiling the solubilized collagen for 10 minutes.
13. After polymerization of HEMA gels approximately 20 to 40 percent of the radioactivity was loosely associated with the gel in each case and was removed with the first wash (1 hour at 37°C). The rest of the radioactivity remained associated with the gel with little or no diffusion from the gel over the next 24 to 48 hours at 37°C.
14. Dissociated cells from chick embryo dorsal root ganglia were prepared as described [S. Carbonetto and R. W. Stach, *Dev. Brain Res.* **3**, 463 (1982)]. Cells were seeded at a density of approximately 0.5×10^6 cells per milliliter in EMEM supplemented with horse serum (10 percent, Gibco), βNGF (10 ng/ml), arabinosylcytosine (280 ng/ml, Sigma), and gentamicin (50 μg/ml, Schering). Cells seeded onto HEMA gels containing βNGF were cultured in medium from which βNGF was omitted. Unless otherwise noted, fibronectin-free horse serum was used in the medium. Approximately 75 μl of suspension was pipetted onto each hydrogel. Cells were permitted to attach at 37°C in a CO_2 incubator. After 3 to 4 hours an additional 1.5 ml of medium was added to each dish and the cultures were returned to the incubator for 1 to 2 days.
15. E. Engvall and E. Ruoslahti, *Int. J. Cancer* **20**, 1 (1977).
16. B. A. Bray, I. Mandl, G. M. Turino, *Science* **214**, 793 (1981).
17. P. C. Letourneau, *Dev. Biol.* **66**, 183 (1978); R. W. Gundersen and J. N. Barrett, *J. Cell Biol.* **87**, 546 (1980).
18. We thank R. W. Stach and J. DeGeorge for helpful suggestions. Work in our laboratories is supported by grants to S.T.C. from NSF (BNS 80-21860) and the Amyotrophic Lateral Sclerosis Society of America and by grants to D.C.T. from the Muscular Dystrophy Association and from NIH (2S07RR0540220).

11 January 1982; revised 4 March 1982

Anticonvulsant Action of Excitatory Amino Acid Antagonists

Abstract. *Compounds that antagonize neuronal excitation induced by dicarboxylic amino acids were tested in two animal models of epilepsy, namely sound-induced seizures in DBA/2 mice and threshold pentylenetetrazol seizures in Swiss mice. Sound-induced seizures could be prevented by intracerebroventricular injection of compounds that block excitation due to* N*-methyl-*D*-aspartic acid. The most potent such compound, 2-amino-7-phosphonoheptanoic acid, was anticonvulsant in both test systems when given either intraperitoneally or intracerebroventricularly. Specific antagonists of excitation that is caused by amino acids provide a new class of anticonvulsant agents.*

In focal epilepsy, in reflex epilepsy, and in primary generalized epilepsy with tonic or clonic motor signs, the development of clinically evident convulsive activity depends on the recruitment of normal neurons into paroxysmal patterns of firing (*1*). Since this process depends on excitatory neurotransmission it can be prevented by antagonists of excitatory neurotransmitters. The dicarboxylic amino acids in the brain are universally excitatory when applied by microiontophoresis to the mammalian central nervous system (*2*). The most abundant of these amino acids, glutamic and aspartic acids, appear to act as excitatory neurotransmitters in many brain areas, including the neocortex, hippocampus, cerebellum, and sensory afferent pathways (*3*). Studies with analogs of glutamic and aspartic acids, including various cyclic compounds, have led to the description of three classes of receptors for dicarboxylic amino acids: receptors that are most potently activated by *N*-methyl-D-aspartic acid (NMDA); those that are activated preferentially by quisqualic acid; and those that are activated by kainic acid (*2*, *4*). Comparison of the effects of various antagonists either in the spinal cord or the rat cortex shows that activation by *N*-methyl-D-aspartic acid is preferentially blocked by 2-amino-5-phosphonopentanoic acid and 2-

0036-8075/82/0521-0899$01.00/0