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.

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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).

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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-

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Table 1. Anticonvulsant activity of excitatory amino acid antagonists in DBA/2 mice. Groups of DBA/2 mice (N = 6 to 10), 21 to 28 days old, were injected intracerebroventricularly under light ether anesthesia with 10 μl of drug solution or phosphate buffer alone (*p*H 7.3). Auditory stimulation (electric bell generating 109 dB at mouse level) was applied 45 minutes later, for 60 seconds or until tonic extension was observed, and the incidence and timing of the phases of the seizure response were recorded. These included an initial wild running phase (WR) followed by myocolonus, tonic flexion, and extension and, frequently, respiratory arrest. Seizure response was scored as previously documented (*15*) and comparisons between groups of control and drug-treated animals were made by using Fisher's exact probability test. Successive doses, with a geometric factor of 3, were tested until an adequate log dose-response curve (three to six points) could be constructed for each antagonist for each phase of the seizure response, and ED_{50} values were graphically determined. A rank order of anticonvulsant potency was allocated to each antagonist for comparison with iontophoretic data (*5*).

Antagonist	Minimum dose to suppress WR* (μmole)	ED_{50} (μmole)			Relative anti-convulsant potency	Relative NMDA antagonist potency†
		WR	Clonus	Tonus		
D-α-Aminoadipic acid	(0.25)‡				O	
Glutamic acid diethylester	(3.3)‡				O	
γ-D-Glutamylglycine	0.1	0.058	0.046	0.054	X	
(±)-2-Amino-4-phosphonobutyric acid	(0.5)‡				O	(X)
(±)-2-Amino-5-phosphonopentanoic acid	0.1	0.046	0.022	0.025	X	XX
(±)-2-Amino-6-phosphonohexanoic acid	0.25	0.17	0.14	0.15	(X)	(X)
(±)-2-Amino-7-phosphonoheptanoic acid	0.01	0.004	0.0018	0.0008	XXX	XXX
(+)-2-Amino-7-phosphonoheptanoic acid	0.033	0.0135	0.0018	0.0018	XX	XX
(−)-2-Amino-7-phosphonoheptanoic acid	0.0033	0.0022	0.0008	0.0008	XXXX	XXXX

*$P < .01$. †See Perkins and co-workers (*5*). ‡Inactive.

amino-7-phosphonoheptanoic acid, whereas activation by quisqualic acid is preferentially blocked by L-glutamic acid diethylester (*4*, *5*). The dipeptide γ-D-glutamyl glycine antagonizes excitation due to kainic acid or to NMDA but not that due to quisqualic acid (*2*, *4*).

We have investigated the role of excitatory amino acids in the development of seizure responses by administering selective antagonists (*6*) of these excitants to DBA/2 mice, an inbred strain in which, within a critical age range, a fixed sequence of seizure responses can be induced by a loud sound (*7*) and to mice, not genetically seizure prone, injected with a minimal convulsant dose of pentylenetetrazol (*8*).

To avoid differential effects of the blood-brain barrier on the antagonist and to allow comparison with data derived from iontophoretic experiments, we used intracerebroventricular as well as intraperitoneal injections.

Table 1 shows that the "quisqualic acid receptor" antagonist glutamic acid diethylester does not provide protection against audiogenic seizures. D-α-Aminoadipate, a selective but relatively weak NMDA receptor antagonist (*4*), is also inactive in this test system. However, the phosphono derivatives of aliphatic amino acids that antagonize NMDA-induced excitation block all stages of the audiogenic seizure response. The phosphonoheptanoic acid derivative is the most potent of the series. The relative anticonvulsant potencies of these compounds match their relative potencies as antagonists of excitation caused by iontophoretic administration of NMDA to the rat cortex (*5*). The greater activity of the D-(−) isomer of 2-amino-7-phosphonoheptanoic acid compared with the (+) isomer also corresponds to the relative activities of the two isomers after iontophoretic application. This correlation suggests that excitatory neurotransmission mediated by the NMDA receptor plays an important role in the initiation or spread of epileptic neuronal hyperactivity. The fact that γ-D-glutamylglycine is active against all phases of the audiogenic seizure response is consistent with its antagonism of NMDA-induced excitation (although an action on kainic acid receptor–mediated excitation cannot be excluded). Evaluation of the possible role of actions on kainic acid receptors requires more specific kainic acid receptor antagonists than are available at present.

Excitatory amino acids are involved in sensory afferent transmission, including that of the auditory system–VIIIth cranial nerve relay in the cochlear nucleus (*9*). The audiogenic seizure model is critically dependent on a functionally intact auditory system. Blockade of transmission within this pathway could give a misleading appearance of anticonvulsant activity. The data in Table 1 suggest that

Table 2. Effect of (±)-2-amino-7-phosphonoheptanoic acid on audiogenically induced and pentylenetetrazol-induced seizures in mice. Studies of audiogenic seizures were performed as outlined in the legend to Table 1. For intraperitoneal administration the antagonist was injected with 0.1 ml of saline 45 minutes before testing. Antagonism of pentylenetetrazol (PTZ; threshold) seizures was studied in random-bred Swiss mice (Tuck T/O strain; 28 days old; 20 to 23 g). Mice in groups (N = 10) received pentylenetetrazol (85 mg/kg; 0.85 percent solution in 0.9 percent sodium chloride) subcutaneously in a loose fold of skin on the back of the neck 45 minutes after intracerebroventricular or intraperitoneal administration of drug or vehicle. During the 30-minute observation period sustained rhythmic clonic jerking with tonic spasms occurred in 90 to 100 percent of control mice. The incidence and timing of clonic episodes was recorded, with absence of sustained clonic jerking (no episode of 5 seconds duration or longer) being defined as protection (*8*). The ED_{50} values and 95 percent fiducial limits were estimated by using the method of moving averages (*16*) with data from four successive dose levels.

Antagonist	Route of administration	Minimum dose to suppress WR*	ED_{50}			ED_{50} PTZ (95 percent fiducial limits)
			WR	Clonus	Tonus	
(±)-2-Amino-7-phosphono-heptanoic acid	Intracerebroventricular	0.01 μmole	0.004	0.0018	0.0008	0.64 (0.19–2.13) μmole
	Intraperitoneal	0.33 mmole/kg	0.18	0.04	0.04	1.18 (0.97–1.43) mmole/kg

*$P < .01$.

this might be a contributory factor but cannot be the principal mechanism (*10*).

The test for threshold pentylenetetrazol seizures in Swiss mice indicates a direct anticonvulsant action of (±)-2-amino-7-phosphonoheptanoic acid (Table 2). This seizure model is not critically dependent on sensory afferent transmission. Because pentylenetetrazol has a diffuse action in the nervous system it is not an optimal test system for an agent preventing the progressive recruitment of normal neurons by excitatory neurotransmission (*11*).

An anticonvulsant action of (±)-2-amino-7-phosphonoheptanoic acid was found in both test systems after systemic administration (Table 2). The greater efficacy of the compound against audiogenic seizures when it was given intracerebroventricularly suggests that access to critical sites of action is facilitated by this method of administration (*12*).

These findings indicate that selective antagonists of amino acid–induced excitation provide an anticonvulsant action comparable both in terms of efficacy (*13*) and acute toxicity (*14*) to that of some drugs in clinical use. Testing of such antagonists in man for their efficacy against reflex epilepsy and focal or generalized seizures must await further study of their selectivity of action and short- and long-term toxicity.

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References and Notes

1. A. R. Wyler and A. A. Ward, in *Epilepsy: A Window to Brain Mechanisms*, J. S. Lockard and A. A. Ward, Eds. (Raven, New York, 1980), pp. 51–68; A. J. Gabor, R. P. Scobey, C. G. Wehrli, *J. Neurophysiol.* **42**, 1609 (1979).
2. D. R. Curtis and G. A. R. Johnston, *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **69**, 98 (1974); J. C. Watkins and R. H. Evans, *Annu. Rev. Pharmacol. Toxicol.* **21**, 165 (1981); A. C. Foster, G. E. Fogg, E. E. Mena, C. W. Cotman, *Brain Res.* **229**, 246 (1981).
3. R. W. Baughman and C. D. Gilbert, *Nature (London)* **287**, 848 (1980); F. Fonnum, J. Storm-Mathisen, I. Divac, *Neuroscience* **6**, 863 (1981); T. W. Stone, *Br. J. Pharmacol.* **66**, 291 (1979); C. W. Cotman, A. Foster, T. Lanthorn, in *Glutamate as a Neurotransmitter*, G. Di Chiara and G. L. Gessa, Eds. (Raven, New York, 1981), pp. 1–27.
4. J. Davies, R. H. Evans, A. A. Francis, A. W. Jones, J. C. Watkins, in *Neurotransmitters and Their Receptors*, U. Z. Littauer, Y. Dudai, I. Silman, V. I. Teichberg, Z. Vogel, Eds. (Wiley, Chichester, 1980), pp. 333–347; H. McLennan, in *Glutamate as a Neurotransmitter*, G. Di Chiara and G. L. Gessa, Eds. (Raven, New York, 1981), pp. 253–262.
5. M. N. Perkins, T. W. Stone, J. F. Collins, K. Curry, *Neurosci. Lett.* **23**, 333 (1981); T. W. Stone, M. N. Perkins, J. F. Collins, K. Curry, *Neuroscience* **6**, 2249 (1981).
6. Sources of compounds: γ-D-glutamylglycine (Cambridge Research Biochemical, Huntingdon, United Kingdom). The (±)-2-amino-ω-phosphonocarboxylic acids were prepared by modification of the method of J. R. Chambers and A. F. Isbell [*J. Org. Chem.* **29**, 832 (1964)]. Satisfactory microanalyses have been obtained for C, H, N, and P, and the purity of the compounds confirmed by ^{13}C and ^{31}P nuclear magnetic resonance spectroscopy. Resolution of the racemic compounds was achieved by means of a modification of the method described by R. H. Evans, A. A. Francis, K. Hunt, D. J. Oakes, and J. C. Watkins [*Br. J. Pharmacol.* **67**, 591 (1979)]. The (−) isomer was found to have $[\alpha]_{546}^{25} = -8.8°$ (H_2O) and the (+) isomer $[\alpha]_{546}^{25} = +5.7°$ (H_2O). The absolute configurations were assigned by using circular dichroism spectra. The optical purity of the (−) and (+) forms has not been determined.
7. R. L. Collins, in *Experimental Models of Epilepsy*, D. P. Purpura, J. K. Penry, D. Tower, D. M. Woodbury, R. Walker, Eds. (Raven, New York, 1972), pp. 347–372; G. M. Anlezark, R. W. Horton, B. S. Meldrum, *Biochem. Pharmacol.* **27**, 2821 (1978); T. N. Seyfried, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 2399 (1979).
8. E. A. Swinyard, in *Anticonvulsant Drugs*, J. Mercier, Ed. (Pergamon, Oxford, 1972), pp. 47–65.
9. R. J. Wenthold and R. L. Gulley, *Brain Res.* **138**, 11 (1977); R. J. Wenthold, *ibid.* **162**, 338 (1979); M. R. Martin, *Neuropharmacology* **19**, 519 (1980).
10. Low doses of 2-amino-7-phosphonoheptanoic acid preferentially suppress the later phases of the seizure response (that depend on diffuse spread of excitation) while leaving the early wild-running phase intact. An action that was exclusively on auditory transmission would suppress all phases of the response together (as seen after administration of γ-D-glutamyl glycine). D-α-Aminoadipic acid potently inhibits excitation in the cochlear nucleus induced either by synaptic stimulation or by exogenous amino acids (*10*), but it is inactive against audiogenic seizures. This apparent contradiction may indicate that the maximum dose of D-α-aminoadipic acid that we are able to administer in solution through the ventricles is insufficient to act on the cochlear nucleus.
11. The pentylenetetrazol (threshold) seizure test discriminates among anticonvulsant drugs in favor of benzodiazepines, barbiturates, and branched chain fatty acids [see R. L. Krall, J. K. Penry, B. G. White, H. J. Kupferberg, E. A. Swinyard, *Epilepsia* **19**, 409 (1978)]. These drugs are believed to act by enhancing inhibition due to γ-aminobutyric acid; see W. E. Haefely, *Agents Actions* **7**, 353 (1977); and B. Meldrum, in *The Molecular Basis of Neuropathology*, A. N. Davison and R. H. S. Thompson, Eds. (Arnold, London, 1981), pp. 265–301.
12. In a recent study of focal cortical epilepsy in the rat, in which cortical superfusion of "glutamate antagonists" was used [J. Coutinho-Netto, A. S. Abdul-Ghani, J. F. Collins, H. F. Bradford, *Epilepsia* **22**, 289 (1981)], a decrease in limb jerks was seen with four compounds. This phenomenon was not shown to correlate with specific postsynaptic antagonist actions of the compounds used; indeed, the two most potent compounds were probably acting by other mechanisms: D,L-pyroglutamic acid by inhibition of GABA-transaminase activity, and (+)-2-amino-4-phosphonobutyric acid by depression of synaptic activation not involving direct action on the glutamic acid or aspartic acid receptor [R. H. Evans, A. W. Jones, J. C. Watkins, *Br. J. Pharmacol.* **74**, 907P (1981)].
13. The median effective dose (ED_{50}) for sodium valproate against pentylenetetrazol (threshold) seizures is 0.9 mmole/kg (*13*), and against the wild-running phase of sound-induced seizures in DBA/2 mice, 2.0 mmole/kg [G. Anlezark, R. W. Horton, B. S. Meldrum, M. C. B. Sawaya, *Biochem. Pharmacol.* **25**, 413 (1976)].
14. The signs of acute toxicity were sedation, reduced or impaired locomotor activity, and ataxia. After administration of γ-D-glutamyl glycine (0.1 μmole intracerebroventricularly), toxic signs were severe for 20 to 30 minutes but mild at the time of testing. No toxic signs were produced by (±)-2-amino-7-phosphonoheptanoic acid after intracerebroventricular administration of doses protecting against audiogenic seizures. Marked sedation followed the highest intracerebroventricular doses of (±)-2-amino-7-phosphonoheptanoic acid. Reduced motor activity was still evident at the time of testing with pentylenetetrazol after anticonvulsant doses had been given.
15. R. W. Horton, J. F. Collins, G. M. Anlezark, B. S. Meldrum, *Eur. J. Pharmacol.* **59**, 75 (1979).
16. C. S. Weil, *Biometrics* **8**, 249 (1952).
17. We thank K. Curry for technical assistance, and the Medical Research Council, the Wellcome Trust, and the Science Research Council for financial support.

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