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REQUEST FOR A STANDARD PATENT AND NOTICE OF ENTITLEMENT

The Applicant identified below requests the grant of a patent to the nominated person identified below for an invention described in the accompanying standard complete patent specification.

Applicant and Nominated Person:

THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES of National Institutes of Health, Office of Technology Transfer, Patent Branch, Bethesda, Maryland 20892, United States of America

Invention Title:

ACTIVITY-DEPENDENT NEUROTROPHIC FACTOR

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Applicant states the following:

The nominated person has entitlement from the actual inventors by assignment.

The nominated person has entitlement from the basic applicants listed in the Declaration under Article 8 of the PCT.

The basic applications were the first made in a Convention country in respect of the invention the subject of the application.

22 December, 1995

THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY
THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES
by PHILLIPS ORMONDE & FITZPATRICK
Patent Attorneys
By:

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(56) Prior Art Documents
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(57) Claim

1. A purified non-neuronal activity-dependent neurotrophic factor (ADNF) protein that increases the survival of neuron cells, said protein having a molecular weight of 16,000 to 18,000 daltons.

20. A purified non-neuronal ADNF protein produced by a process comprising the steps of:

i) culturing cells producing neurotrophic factors;

ii) collecting culture medium from said cells;

iii) fractionating components of said medium by ion exchange chromatography;

iv) assaying the fractions resulting from step (iii) for the ability to increase in vitro neuronal survival;

v) subjecting fractions from step (iv) found to possess said activity to gel permeation chromatography;

vi) assaying the fractions resulting from step (v) for the ability to increase in vitro neuronal survival; and

vii) collecting active fractions from step (vi).

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(54) Title: ACTIVITY-DEPENDENT NEUROTROPHIC FACTOR

(57) Abstract

The present invention relates to a purified non-neuronal activity-dependent neurotrophic factor (ADNF) protein that increases the survival of spinal cord neuron cells, cerebral cortical cells and hippocampal neuron cells which has a molecular weight of 16,000 to 18,000 Daltons as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and a basic pI of about 8.1. The protein of the present invention can be used in the treatment of neurological deficiencies and for the prevention of AIDS related neuronal cell death.

ACTIVITY-DEPENDENT NEUROTROPHIC FACTOR

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to activity-dependent neurotrophic factors (ADNF). In particular, the present invention relates to purified non-neuronal ADNF and uses thereof for the treatment of neurological deficiencies and for the prevention of AIDS related neuronal cell death, or dystrophy.

2. Background Information

Neuronal maturation and survival are dependent on protein growth factors during development. The present inventors have sought to isolate a novel growth factor that increases the survival of activity-dependent spinal cord neurons. These neurons are activity-dependent in the sense that their survival is also influenced by electrical activity (Brenneman et al., (1985) J. Pharmacol. Exp. Therap. 233, 402-408; Brenneman et al., (1983) Dev. Brain Res. 9, 13-27;and Brenneman et al., (1984) Dev. Brain Res. 15, 211-217). Previous studies carried out by the present inventors have indicated that a part of the molecular basis of this activity-dependence is the action of vasoactive intestinal peptide (VIP), a neuropeptide which is released during electrical activity (Brenneman D.E. and Eiden L.E. (1986) Proc. Natl. Acad Sci. U.S.A 83, 1159-1162; and Brenneman et al., (1985) Peptides 6 (suppl. 2) 35-39). Previous work also indicated that VIP increased the survival of activity-dependent spinal cord neurons by releasing a protein growth

factor from non-neuronal spinal cord cells (Brenneman et al., (1987) J. Cell Biology, 104, 1603-1610). The working hypothesis supported by the present invention is that VIP interacts with its receptors on glial cells (Gozes et al., (1989) Soc. Neurosci. Abs. 15, 216) to induce the secretion of neuronal survival factor(s) (Brenneman et al., (1990) J. Neurosci. Res. 25, 386-394; and Gozes, I. and Brenneman, D.E. (1989) Molecular Neurobiology, 3, 201-236).

Previously, growth factors such as NGF (Levi-Montalcini, R. and Angeletti, P.V. (1968) Physiol. Rev. 48, 534-569), CNTF (Lin et al., (1989) Science 246, 1023-1025), FGF (Walicke et al., (1986) Proc. Natl. Acad. Sci. USA 83, 3012-3016) and BDNF (Leibrock et al., (1989) Nature 341, 149-152) have been isolated and shown to provide neurotrophic support for specific populations of neurons. The growth factor of the present invention (ADNF) differs from all other known growth factors on the basis of: 1) specificity of the target population of neurons; 2) structural characteristics of the protein, such as, total amino acid composition, and chromatographic properties; and 3) relationship to vasoactive intestinal peptide and electrical activity.

SUMMARY OF THE INVENTION

It would be desirable ~~It is an object of the present invention~~ to provide a purified ADNF protein. The purified protein of the present invention increases the survival of activity-dependent spinal cord neurons and cerebral cortical neurons and prevents neuronal cell death resulting from HIV infection.



Various other advantages of the present invention will become apparent from the following description of the invention and the drawings.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises", are not intended to exclude other additives or components or integers.

In one embodiment, the present invention relates to a purified non-neuronal activity-dependent neurotrophic factor (ADNF) protein that increases the survival of neuron cells which has a molecular weight of 16,000 to 18,000 Daltons or peptides thereof comprising a portion of the protein. The present invention further relates to antibodies, monoclonal and polyclonal, specific for the ADNF protein or fragment thereof.

In another embodiment, the present invention relates to a pharmaceutical composition comprising the purified ADNF protein of the present invention or peptide thereof or synthetic analogue thereof and a pharmaceutically acceptable carrier.

In a further embodiment, the present invention relates to methods of treating neurological deficiencies and methods of preventing neuronal cell death in a patient comprising administration of a pharmaceutical composition according to the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 demonstrates the survival-promoting activity in unfractionated conditioned medium from astroglial cultures: effect on spinal cord neurons. Spinal cord test cultures were treated for five days with varying amounts of conditioned medium. All test cultures were co-treated with 1 μM tetrodotoxin to block electrical

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activity and inhibit the release of vasoactive intestinal peptide. Treatment was started on day 9 in vitro. Each value is the mean ± SEM of 3 determinations.

Fig. 2 shows the identification of the neurotrophic fraction eluted by increasing salt concentrations from DEAE-ion exchange column (closed circles). Each value is the mean of 3 determinations ± SEM. A 5-day test period is begun nine days after plating of the embryonic SC/DRG tissue. Column fractions (1:10,000) are added together with 1 μM tetrodotoxin. Significant increases in neuronal cell counts are observed in the 2M NaCl eluate. Open circles represent O.D. 280 absorbance of each fraction.

Fig. 3 shows size separation of the DEAE (2M NaCl eluate) activity-dependent neurotrophic fraction by gel permeation chromatography (closed circles). A 5-day test period is started nine days after plating of the embryonic SC/DRG tissue. Column fractions (1:10,000) are added together with 1 μM tetrodotoxin. Significant increases in neuronal cell counts are observed in column fractions 22 and 31. Open circles represent O.D. 280 absorbance of each fraction.

Fig. 4 represents molecular weight estimation from the gel permeation (sizing) chromatography. Samples from one FPLC size separation column are subjected to electrophoreseis through 2% Tris Tricine polyacrylamide gel (Analytical Biochem. 166: 368, 1987). A series of four molecular standards of known weight were used to calibrate the column. In the range from 67,000 (bovine serum albumin) to

13,700 (Ribonuclease A), the column separation as a function of elution volume was linear. Based on these separations and the observed elution volume of 15.5ml for ADNF, the molecular weight is estimated to be 16,000 Daltons. The ~~vertical~~ line shown in Figure 4 indicates the average elution volume for ADNF under these conditions. The other markers employed were ovalbumin (43,000) and Chymotrypsin (25,000).

Fig. 5 shows purification of the low molecular weight neurotrophic activity by hydrophobic interaction chromatography (Closed circles). The same experiment as in Fig. 1 and 2 is performed. Results shown indicate that fraction 20 contains the neurotrophic activity. **Open circles represent O.D. 280 absorbance of each fraction.**

Fig. 6 is a summary of the purification scheme of ADNF, showing the starting material, its total biological activity and the specific activity of the neuronal survival factor. The increase in specific activity is shown for each purification step. Starting from the original secreted proteins, a 1650-fold purification was achieved to apparent homogeneity.

Fig. 7 demonstrates isoelectric focusing of the reverse phase FPLC purified neurotrophic activity. Samples from the reverse phase FPLC are subjected to isoelectric focusing (pH range 3.5-9.5), and silver stained. While fraction 31 from the size separation column (Lane 1) shows three basic protein bands, fraction 20 of the reverse phase (Lane 2) column displays only one protein exhibiting a pI of about 8.1.

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Fig. 8 represents Tris tricine polyacrylamide gel electrophoresis (Anal. Biochem. 166, 368, (1987)) of intact conditioned medium (lane 1) and purified ADNF (lane 2) indicating an apparent molecular weight 18,500 Daltons for purified ADNF.

Fig. 9 demonstrates the ability of ADNF to prevent neuronal cell death associated with human immunodeficiency virus (HIV) external envelope glycoprotein. Murine hippocampal cultures were treated for 5 days with 1 pM gp120 (strain: RF II). Cells were counted by image analysis and confirmed by visual examination.

Fig. 10 demonstrates that ADNF prevents tetrodotoxin-induced death in cerebral cortical cultures in a dose responsive manner. Purified ADNF was added to cultures 9 days after plating the neurons. The duration of the experiments was 5 days, with only one treatment of ADNF given at the beginning of the test period. To block electrical activity, 1μM tetrodotoxin was given. Each value is the mean of three determinations ± SEM.

Fig. 11 demonstrates that ADNF can increase the survival of developing cerebral cortical neurons in cell culture. The same assay was performed as was described in Fig. 10, except that no tetrodotoxin was added.

Fig 12 demonstrates the effect of antiserum to ADNF on survival of cerebral cortical neurons. Neutralizing antiserum was obtained by serial injections of purified ADNF into mice. As

shown, the anti-ADNF produced neuronal cell death in cerebral cortical cultures.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to a purified glial-derived, VIP-released growth factor protein. Using a combination of ion exchange chromatography, molecular sieving technologies employing FPLC, reverse phase chromatography, polyacrylamide gel electrophoresis, isoelectric focusing and microsequencing, the present inventors have identified and isolated a VIP-releasable monomer protein from non-neuronal cells that has activity-dependent neurotrophic activity for the survival of nerve cells, for example, spinal cord neurons, hippocampal neurons and cerebral cortical cultures. Furthermore, this protein plays a role in the prevention of neuronal cell death produced by the external envelope glycoprotein of the human immunodeficiency virus (HIV). This protein has been designated activity dependent neurotrophic factor (ADNF). The term "purified" as used herein refers to an electrophoretically pure protein as based on both isoelectric focusing gels and Tris tricine polyacrylamide gels.

The present inventors have isolated ADNF from non-neuronal rat cells (that is, rat glial cells), however, one skilled in the art can also isolate ADNF or homologous factors thereof from other species, including humans, without undue experimentation based on the present disclosure. For example, ADNF could be isolated from human tissue by preparing human astroglial cultures from either fetal cerebral cortex or from adult brain

at autopsy. Cultures would be treated with VIP as described hereinbelow. Biochemical isolation could then proceed as described for the rat tissue. In an alternative example, a human astrocyte cDNA library could be screened with either an oligonucleotide probe derived from the rat amino acid sequence of ADNF or rat cDNA for ADNF. Conservation between the rat gene and the human would allow hybridization to occur, thus permitting the isolation of cDNA for human ADNF and subsequent sequencing. With the cDNA known sequence, the library could be re-screened to obtain full length clones. The encoded protein could then be obtained from the full length cDNA using recombinant baculovirus or vaccinia virus expression systems (Sambrook et al., (1989)).

The purified ADNF has been structurally characterized as having a molecular weight of about 16,000 to 18,000 Daltons as determined by gel permeation chromatography (see Figure 4) Tris tricine polyacrylamide gel electrophoresis (see Figure 8), a basic pI of about 8.1 (see Figure 7) and the amino acid composition given in Table I. ADNF is inactivated by trypsin and is heat-sensitive. As one skilled in the art will appreciate, the amino acid composition of purified ADNF may vary slightly due to allelic and species variations. The present invention also relates to peptide fragments of ADNF comprising at least 5 or 6 amino acids. Peptides that increase the survival of neuron cells may be used as an anti-HIV therapeutic agent.

The present invention further relates to DNA sequences encoding the ADNF protein of the present invention or fragments thereof which are at least 15 or 18 nucleotides in length. It is contemplated that probes could be designed based

on the purified protein and utilized to obtain the cloned cDNA. Using recombinant methods, the DNA clone can be used to produce recombinant ADNF. Accordingly, the present invention also relates to DNA sequences encoding ADNF, to recombinant constructs including the DNA sequences and to host cells transformed therewith. Further, the invention relates to methods of using the transformed host cells to produce ADNF.

The present invention also relates to antibodies specific for the purified ADNF protein or fragments thereof. Using standard methodology, one skilled in the art can easily generate antibodies (monoclonal and/or polyclonal) to ADNF. For example, polyclonal antibodies can be elicited injecting test animals, such as rabbits, with the ADNF protein of the present invention and adjuvant. The animals are then bled and screened for the production of antibodies specific for the ADNF protein. Monoclonal antibodies specific for ADNF can be produced using known hybridization and screening techniques.

The purified ADNF of the present invention has been shown to protect neuronal cells against death. ADNF has the ability to increase the growth and survival of developing spinal cord neurons and cerebral cortical neurons. ADNF further protects neuronal cell viability by preventing neuronal cell death produced by the external envelope protein of the HIV virus.

Due to its ability to increase growth and survival of neurons, ADNF may have extensive use in the treatment of neurological deficiencies which result, for example, from neuronal development, aging, neurodegenerative diseases or spinal cord injury. Since the source of the protein is the cerebral cortex and the neuronal

test system consists of cerebral cortical neurons as well as spinal cord neurons, it is likely that the ADNF protein has a broad spectrum of target neurons which respond to or require it for survival and/or growth. Preliminary studies suggest that blockade of vasoactive intestinal peptide (VIP) activity in vivo can result in morphological damage of nerve cells, impairment of acquisition of reflexes and loss of learning and memory mechanisms (Gozes et al., (1990) Soc. Neurosci. Abs. in press.; and Panililio et al., (1990) Soc. Neurosci. Àbs. in press). Treatment with ADNF promoting neuronal cell growth and survival may elevate the damages resulting from VIP blockage and other neurological deficiencies.

Neurological deficiencies can be treated by administering to a patient the ADNF protein of the present invention or a biologically active fragment thereof, in an amount sufficient to promote survival or growth of neuronal cells.

The protein can be administered in the form of a pharmaceutical composition comprising the purified protein or a derivative of ADNF, such as, a D-Ala derivative, and a pharmaceutically acceptable carrier. The protein is present in the composition in an amount sufficient to promote survival or growth of neuronal cells when the composition is administered to a patient.

The protein can be administered into the spinal fluid. Alternatively, the protein can be administered intravenously or intranasally in a lipophilic form. For example, the protein can be administered in a lipophic emulsion carrier. The protein may also be modified to increases its lipophilicity.

In addition, studies conducted with developing hippocampal neurons grown in cell

culture have indicated that ADNF can prevent neuronal cell death associated with the external envelope protein of the HIV virus, the causative agent of AIDS. Thus, the protein of the present invention can be used as a therapeutic agent in the treatment of AIDS.

Neuronal cell death in patients infected with an HIV virus may be prevented by administering to that patient the purified protein of the present invention or a biologically active fragment thereof, in an amount sufficient to prevent cell death and a pharmaceutically acceptable carrier. The amount of ADNF to be administered will depend on the route of administration utilized and on the patient's condition but can be easily determined by the patient's physician.

The following non-limiting examples are provided to aid in the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedure set forth, without departing from the true spirit of the invention.

EXAMPLES

Techniques of Isolation and Detection:

To isolate activity dependent factors, two different cell cultures were used. The source of the neurotrophic factors was rat cortical astrocytes prepared according to previously described methods (McCarthy, K.D. and Partlow, L.M. (1976) Brain Res. 114, 391-414; and Evans et al., (1984) J. Neurochem. 43, 131-138). Three and a half week old cultures (confluent 75cm^2

flasks) were washed three times with phosphate buffered saline (PBS) and conditioned medium was collected (10 ml/flask) during a 3 hour incubation with 0.1 nM VIP. The medium was then centrifuged (3000 x g for 10 min) and dialyzed (10 kDalton cutoff) against 50 mM sodium phosphate buffer, pH 7.0.

The second type of cell culture, mouse spinal cord cultures, were used to test for biological activity of the conditioned medium. Dissociated mouse spinal cord cultures (obtained from 12-day-old embryos) were plated (0.5 million cells/35 mm dish) in medium consisting of 10% fetal calf serum and 10% heat inactivated horse serum in minimum essential medium (MEM). After 24 hours, the medium was changed to 5% horse serum in MEM supplemented with defined medium components (Romijn et al., (1982) Dev. Brain Res. 2, 583-589). After nine days in vitro, the cultures were given a complete change of medium and treated with 1 μM tetrodotoxin and varying concentrations of fractionated conditioned medium for five days. Neuronal cell counts were conducted after immunocytochemical identification with antisera against neuron specific enolase (Levi-Montalcini, R. and Angeletti, P.V. (1968) Physiol. Rev. 48, 534-569). Counts were made in 30 fields from predetermined coordinate locations without knowledge of the treatment group.

The concentration-dependence effects of unfractionated, dialyzed conditioned medium on neuronal survival is shown in figure 1.

The survival factor (ADNF) is inactivated by trypsin and is also heat-sensitive.

The dialyzed material was then loaded onto a DEAE-Sephacel (Pharmacia AB Biotechnology, Uppsala, Sweden) column (0.75 cm diameter and 3 cm

length) pre-equilibrated with 50 mM sodium pyro-phosphate buffer, pH 7.0. About 300 ml of dialyzed material (1-2 mg of protein) was loaded onto the column, and washed sequentially with 40 ml of 50 mM sodium pyro-phosphate buffer (pH 7.0) and then the same buffer supplemented with increasing concentrations of NaCl: 0.1M, 0.26M, 0.5M, 1.0M, 2M and 3M. Fractions were extensively dialyzed against deionized water (at 4° C) and tested at two dilutions: 1:10,000 and 1:100,000. Maximal biological activity was discovered at the 2M NaCl fraction (Fig. 2). This fraction was thereafter used for further purification.

Following the ion exchange chromatography, the 2M NaCl fraction was further purified using gel permeation chromatography. For size separation, a Superose™12 (pre-packed HR 10/30) column in fast performance liquid chromatography (FPLC system, Pharmacia) was used. The DEAE-Sephacel eluate at 2M NaCl was dialyzed extensively against deionized water, lyophilized and resuspended in 0.5 ml of 50 mM sodium phosphate (pH 7.3) containing 0.15M NaCl. 0.25 ml aliquots (corresponds to 150 ml of original material) were loaded onto the column. Fractions (0.5 ml) were collected from the column. Two peaks of activity were discovered, one at fraction 22 and another at fraction 31 (fractions were tested at 1:10,000 dilutions). The estimated molecular weights of fraction 22 was 150,000 Daltons and of fraction 31 was 16,000 Daltons (see Fig. 3 and Fig. 4).

Identification of Novel Activity-Dependent Neurotrophic Factor (ADNF):

The activity-dependent factors, isolated and characterized as described above, were subjected to analysis of size and structure.

Fractions 22 and 31 obtained as described above were dialyzed extensively against deionized water, lyophilized, filtered and subjected to Tris tricine polyacrylamide gel electrophoresis (Anal. Biochem. 166: 368, 1987). Purified ADNF resulted in one band, migrating at about 18,500 Daltons (see Fig. 8).

To purify further the protein associated with biological activity, fraction 31 of the sizing-FPLC column, hydrophobic interaction-FPLC (Alkyl Superose™HR5/5, Pharmacia) was used. The sample (fraction 31, 0.5 ml) was dialyzed extensively against deionized water and lyophilized. It was thereafter resuspended in water and diluted with a buffer to a final concentration of 0.1 M sodium phosphate buffer, pH 7.0 containing 1.43 M $(NH_4)_2SO_4$. Prior to loading, the column was washed with 0.1 M phosphate buffer (pH 7.0) and then equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 2.0 M $(NH_4)_2SO_4$. Elution was performed with a linear gradient of salt removal (2.0-0.0 M) over 25 min. Samples were tested for biological function, using the spinal cord cell culture system, as described above. Results shown in Fig. 5 demonstrate that the neuronal survival activity was concentrated in fraction 20.

Isoelectric focusing was performed using LKB ampholine gel plates (pH range 3.5-9.5, Pharmacia) and electrophoresis was carried out for 3 hours in a flat bed apparatus (multiphor, Pharmacia), voltage set: 1500 Volts, amperage set: 50 mA. Gels were stained with silver stain (BioRad). Results showed three protein bands in fraction 31 (sizing column-FPLC) and one protein band in fraction 20 of the reverse phase (hydrophobic interaction) column. All protein bands are of basic nature (see Fig. 7), the

approximate pI of the protein band in fraction 20 was 8.1

In addition, the purified growth factor was analyzed for its total amino acid composition. In 0.84 μg of purified material, the amino acid composition given below in Table I was observed.

TABLE I

Amino Acid	Residues/mol
Asx	39
Thr	6
Ser	18
Glx	28
Pro	8
Gly	41
Ala	12
Val	10
Met	2
~~Iso~~ Ile	8
Leu	14
Tyr	4
Phe	6
His	3
Trp	ND
Lys	8
Arg	8
Cys	ND

Partial sequence analysis of ANDF was obtained by V8-protease digests as previously described (Proc. Natl. Acad. Sci. USA 82: 6507-6511, (1985)) and the peptides were separated on a HPLC by reverse phase chromatography. The following fragments were obtained:

Fragment I -X-X-X-Gln-Pro-X-Thr-Lys-Asn-Gly

Fragment II -Leu-X-X-X-Ser-Ala-Leu-Leu-Arg-Ser-~~Iso~~ Ile-Pro-Ala-Leu

Fragment III -Pro-Ala-Leu-Asp-Ser-Leu-Lys-Pro-Ala-Asn-Glu-

Identification of Activity of ADNF:

Developing hippocampal neurons were grown in cell culture as previously described (Forsythe and Westbrook, (1988) J. Physiol. London 396, 515-533). The cultured cells were treated with 1pM ADNF and subjected to 1pM gp120 (strain: RF II). Cells were counted by image analysis and confirmed by visual examination. The results are shown in Figure 9.

To test ADNF for anti-gp120 activity previously employed methods were used (Brenneman et al., (1988) Drug Dev. Res. 15, 361-369). Briefly, one week old hippocampal cultures (Forsythe and Westbrook (1988) J. Physiol. Lond. 396, 515-533), were incubated for 5 days with 1 pM gp120 (strain: RF II). Cultures were then fixed in glutaraldehyde, counted by image analysis and observations confirmed by visual examination. As the test agent 1 pM of ADNF was used. The ADNF was added in a vehicle consisting of phosphate buffered saline containing 0.01% bovine serum albumin.

To further test the ability of purified ADNF to increase the survival of developing neurons, another cell type was chosen, cerebral cortical neurons. Cerebral cortical cultures were prepared by a slight modification of the techniques described by Forsythe and Westbrook, 1988; the cerebral cortex was utilized instead of hippocampus and new born rats were used instead of E16 mice. For the assay, purified ADNF was added to cultures 9 days after plating. One treatment of

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ADNF was given at the beginning of the five-day test period. Survival of cells which have been treated with or without 1uM tetrodoxin are shown in Fig. 10 and 11 respectively.

Effect of antiserum to ADNF on neuronal survival of cerebral cortical neurons:

Neutralizing antiserum was obtained by serial injections of purified ADNF into mice. As shown in Fig. 12, the anti-ADNF produced neuronal cell death in cerebral cortical cultures. The antibody-induced cell death could be prevented by co-administration of ADNF. Control anti-serum produced no neuronal cell death at the same dilutions employed with the ADNF.

All publications mentioned hereinabove are hereby incorporated by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A purified non-neuronal activity-dependent neurotrophic factor (ADNF) protein that increases the survival of neuron cells, said protein having a molecular weight of 16,000 to 18,000 daltons.

2. A peptide that increases the survival of neuron cells, said peptide comprising a portion of the protein according to claim 1.

3. A peptide according to claim 2, said peptide comprising the amino acid sequence Leu-X-X-X-Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala-Leu.

4. A peptide according to claim 2 or 3, wherein the neuron cells are spinal cord neuron cells, hippocampal neuron cells or cerebral cortical neuron cells.

5. An antibody specific for a peptide according to any one of claims 2 to 4.

6. An antibody according to claim 5, wherein said antibody is a monoclonal antibody.

7. A purified protein according to claim 1, wherein the neuron cells are spinal cord neuron cells, hippocampal neuron cells or cerebral cortical neuron cells.

8. A purified protein according to claim 1 or 7, said protein having the amino acid composition given in Table I.

9. A purified protein according to claim 1, 7 or 8, wherein said protein is purified from rodent non-neuronal cells.

10. A purified protein according to claim 1, 7 or 8 wherein said protein is purified from human non-neuronal cells.

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11. A purified protein according to claim 9 or 10, wherein said non-neuronal cells are glial cells.

12. An antibody specific for the purified protein according to any one of claims 1 and 7-11.

13. An antibody according to claim 12, wherein said antibody, is a monoclonal antibody.

14. A pharmaceutical composition comprising a purified protein according to any one of claims 1 and 7-11 or a synthetic analogue thereof and a pharmaceutically acceptable carrier.

15. A pharmaceutical composition comprising a peptide according to any one of claims 2-4 or a synthetic analogue thereof and a pharmaceutically acceptable carrier.

16. A method of treating neurological deficiencies comprising administering to a patient with said deficiency a pharmaceutical composition according to claim 14 or 15 in an amount effective to increase the survival of neuronal cells.

17. A method according to claim 16 wherein said neurological deficiencies are the result of development, aging, neurodegenerative diseases or spinal cord injury.

18. A method of preventing neuronal cell death in a patient comprising administering to said patient a pharmaceutical composition according to claim 14 or 15 in an amount effective to prevent said cell death.

19. A method according to claim 18, wherein said patient is infected with human immunodeficiency virus.

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20. A purified non-neuronal ADNF protein produced by a process comprising the steps of:

i) culturing cells producing neurotrophic factors;

ii) collecting culture medium from said cells;

iii) fractionating components of said medium by ion exchange chromatography;

iv) assaying the fractions resulting from step (iii) for the ability to increase in vitro neuronal survival;

v) subjecting fractions from step (iv) found to possess said activity to gel permeation chromatography;

vi) assaying the fractions resulting from step (v) for the ability to increase in vitro neuronal survival; and

vii) collecting active fractions from step (vi).

21. A purified non-neuronal activity dependent neurotrophic factor (ANDF) protein or a peptide comprising a portion of said protein substantially as hereinbefore described with reference to any one of the examples.

DATED: 22 December, 1995

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Attorneys for:
THE GOVERNMENT OF THE UNITED STATES OF AMERICA
AS REPRESENTED BY THE SECRETARY,
DEPARTMENT OF HEALTH AND HUMAN SERVICES

AUSTRALIAN PATENT OFFICE

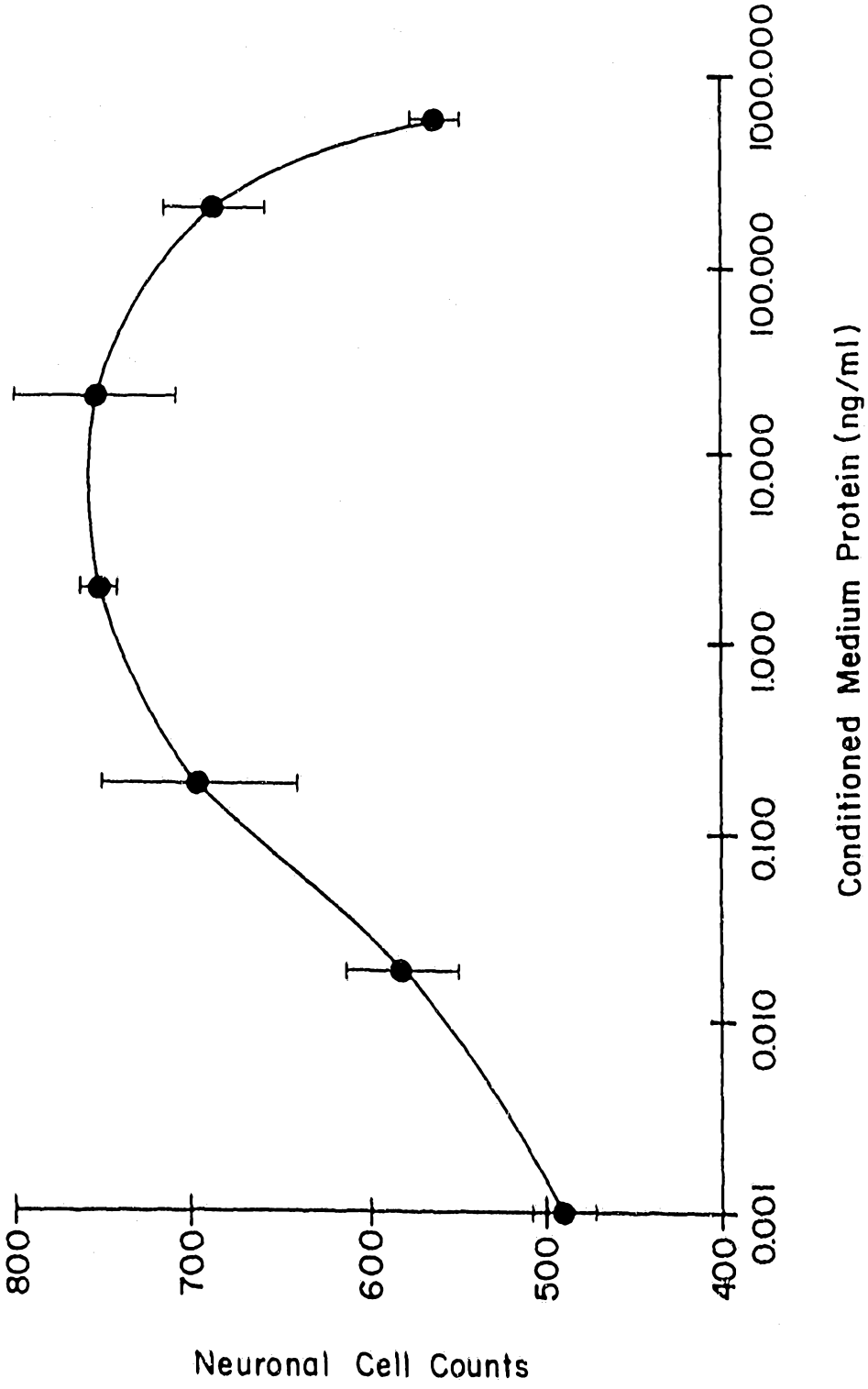

800
700
600
500
400
Neuronal Cell Counts
0.001
0.010
0.100
1.000
10.000
100.000
1000.000
Conditioned Medium Protein (ng/ml)

FIG. 1

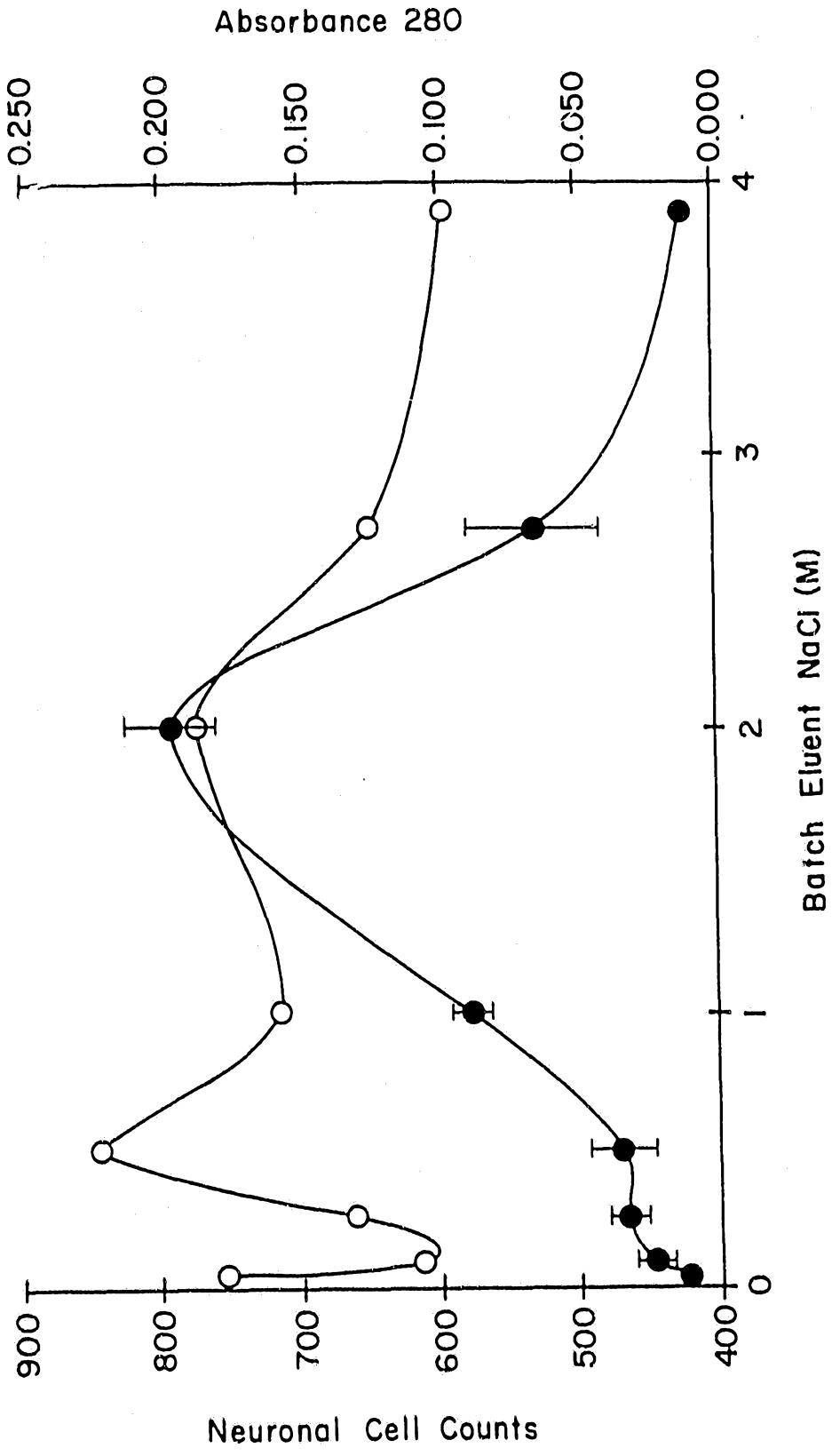

Absorbance 280
0.250
0.200
0.150
0.100
0.050
0.000
900
800
700
600
500
400
0
1
2
3
4
Batch Eluent NaCl (M)
Neuronal Cell Counts

FIG. 2

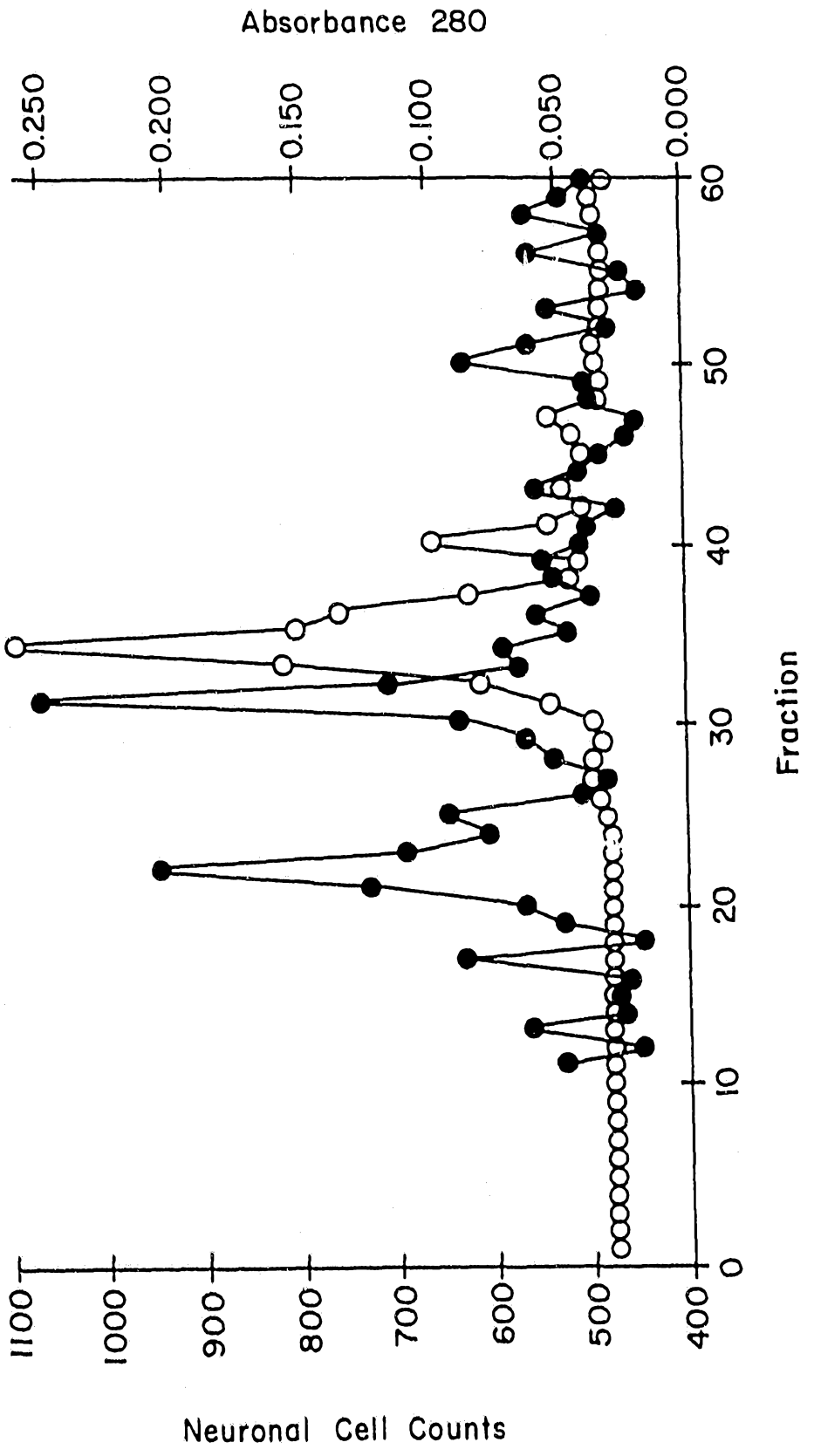

Absorbance 280
0.250
0.200
0.150
0.100
0.050
0.000
60
50
40
30
20
10
0
Fraction
1100
1000
900
800
700
600
500
400
Neuronal Cell Counts

FIG. 3

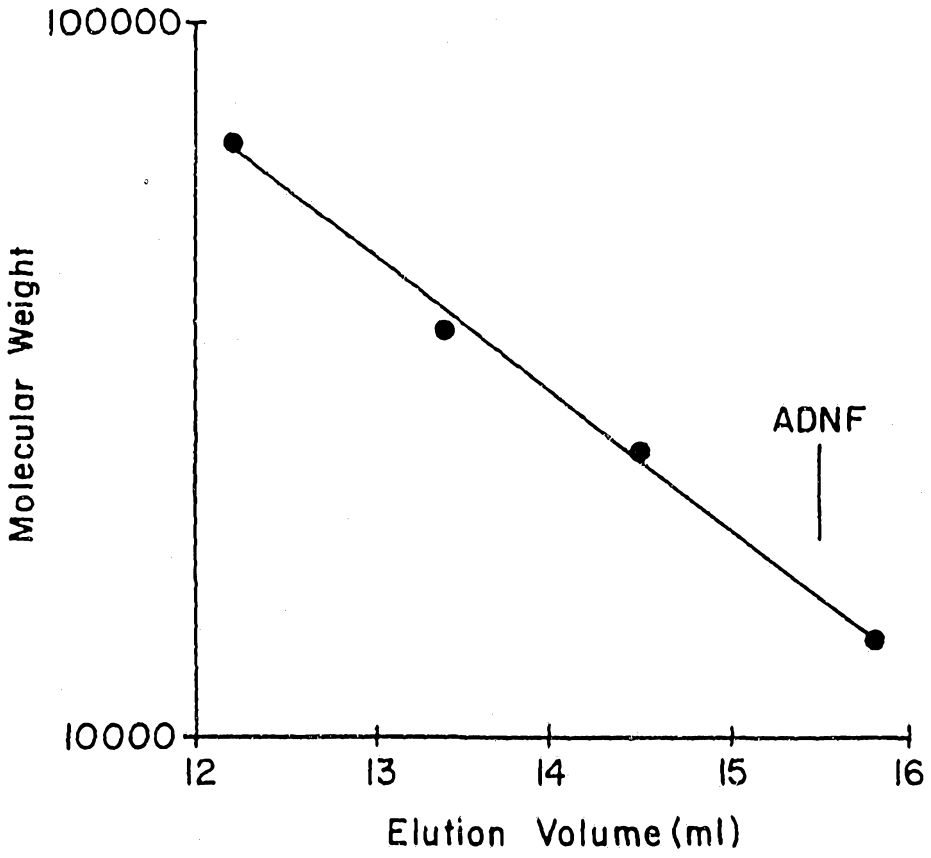

100000
10000
Molecular Weight
ADNF
12
13
14
15
16
Elution Volume (ml)

FIG. 4

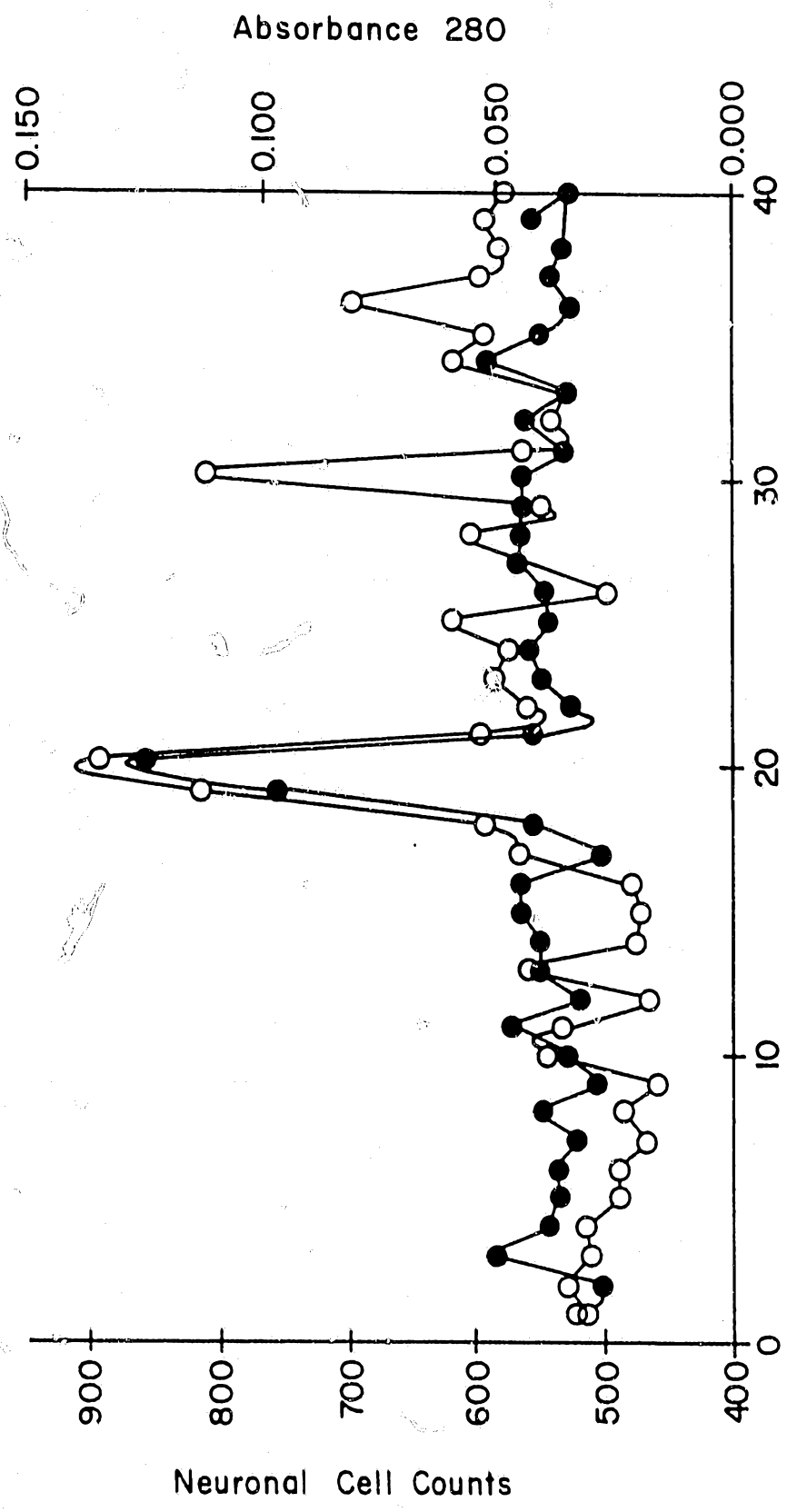

Absorbance 280
0.150
0.100
0.050
0.000
40
30
20
10
0
Fraction
900
800
700
600
500
400
Neuronal Cell Counts

FIG. 5

SUMMARY

STEP	PROTEIN	UNITS	SPECIFIC ACTIVITY
1. TOTAL	5400ug	43 million	8 U/ng
2. DEAE	467 ug	41.5 million	89 U/ng
3. Sizing	5 ug	5.5 million	1100 U/ng
4. Reverse Phase	0.28 ug	3.7 million	13,200 U/ng

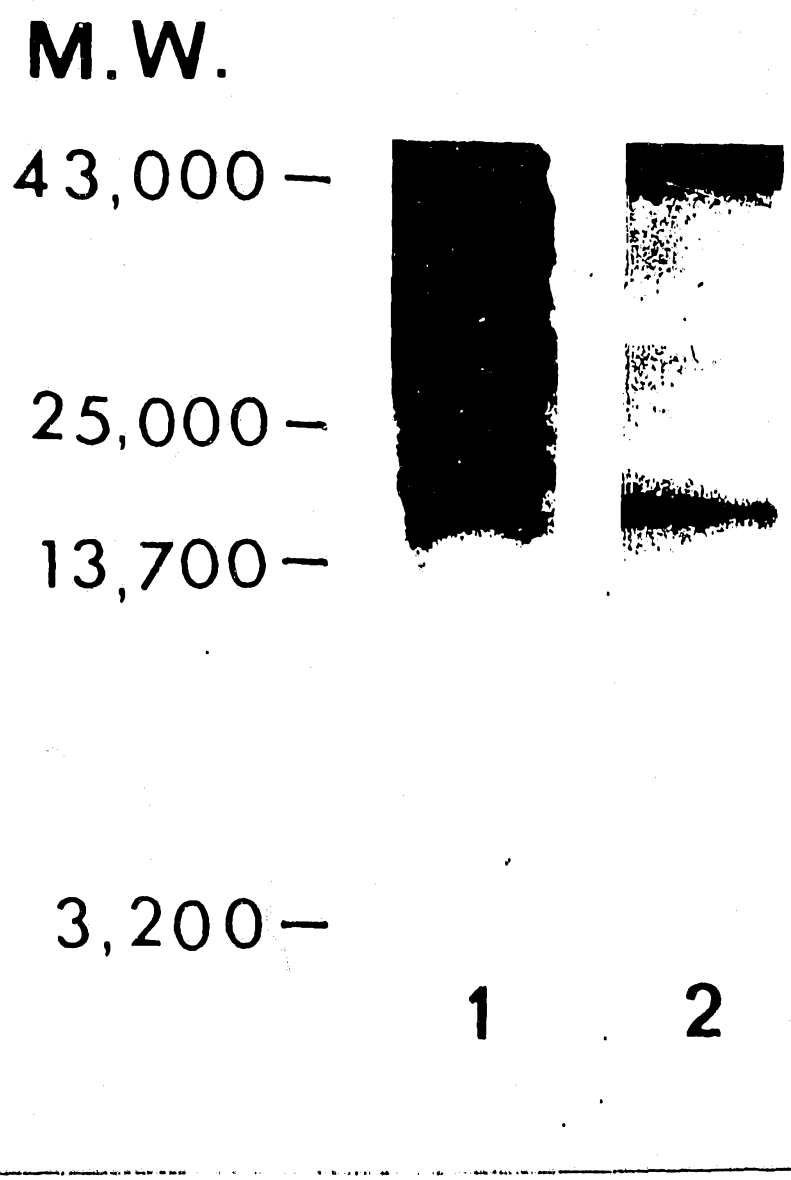
Yield: 0.005%
Purification: 1650 x

FIG. 6

FIG. 7



FIG. 8



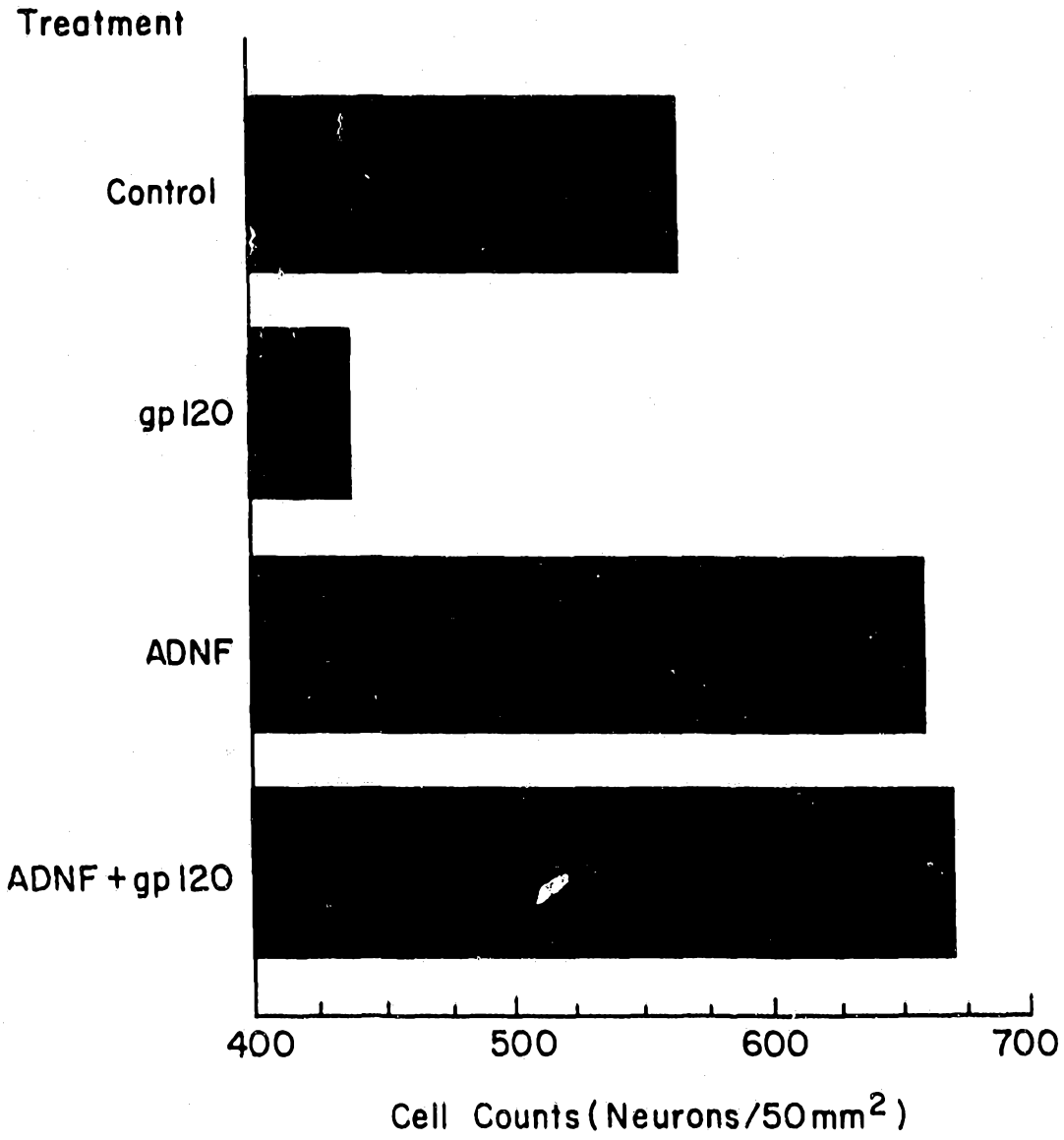

Treatment
Control
gp120
ADNF
ADNF + gp120
400
500
600
700
Cell Counts (Neurons/50mm²)

FIG. 9

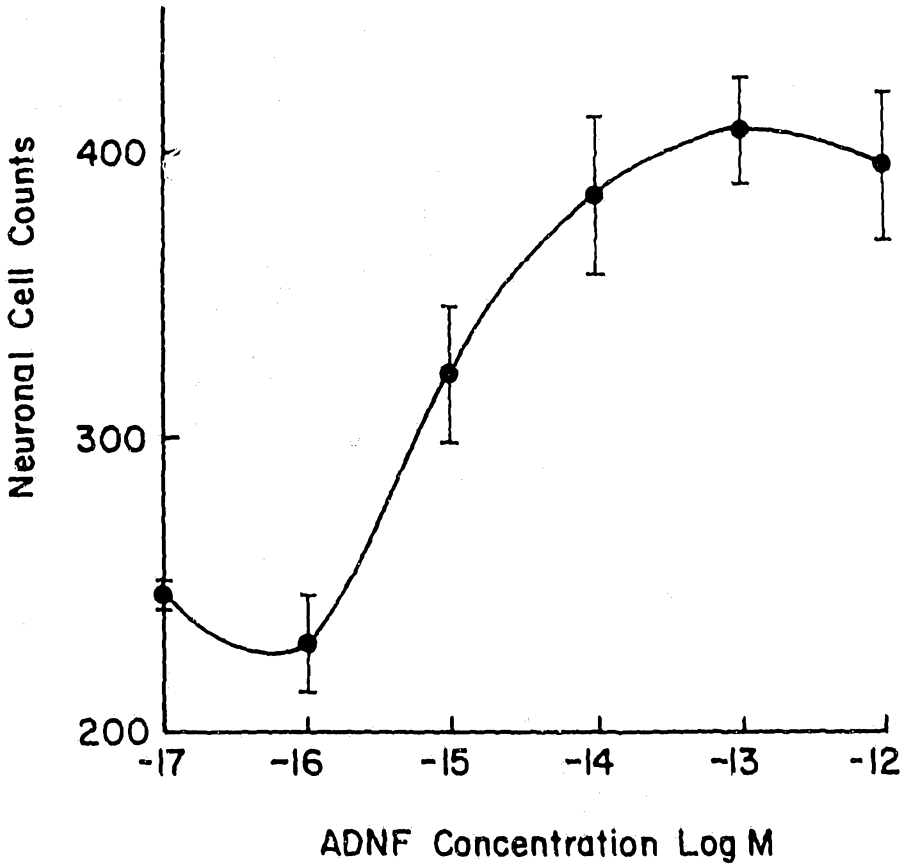

Neuronal Cell Counts
400
300
200
-17
-16
-15
-14
-13
-12
ADNF Concentration Log M

FIG. 10

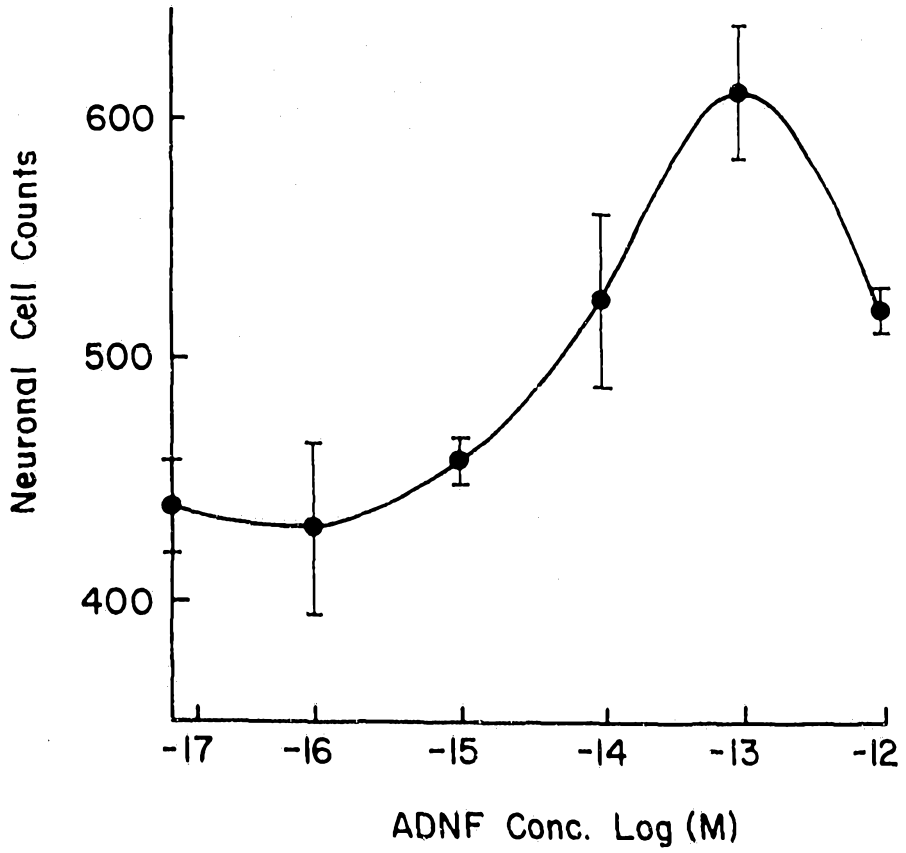

Neuronal Cell Counts
600
500
400
-17
-16
-15
-14
-13
-12
ADNF Conc. Log (M)

FIG. 11

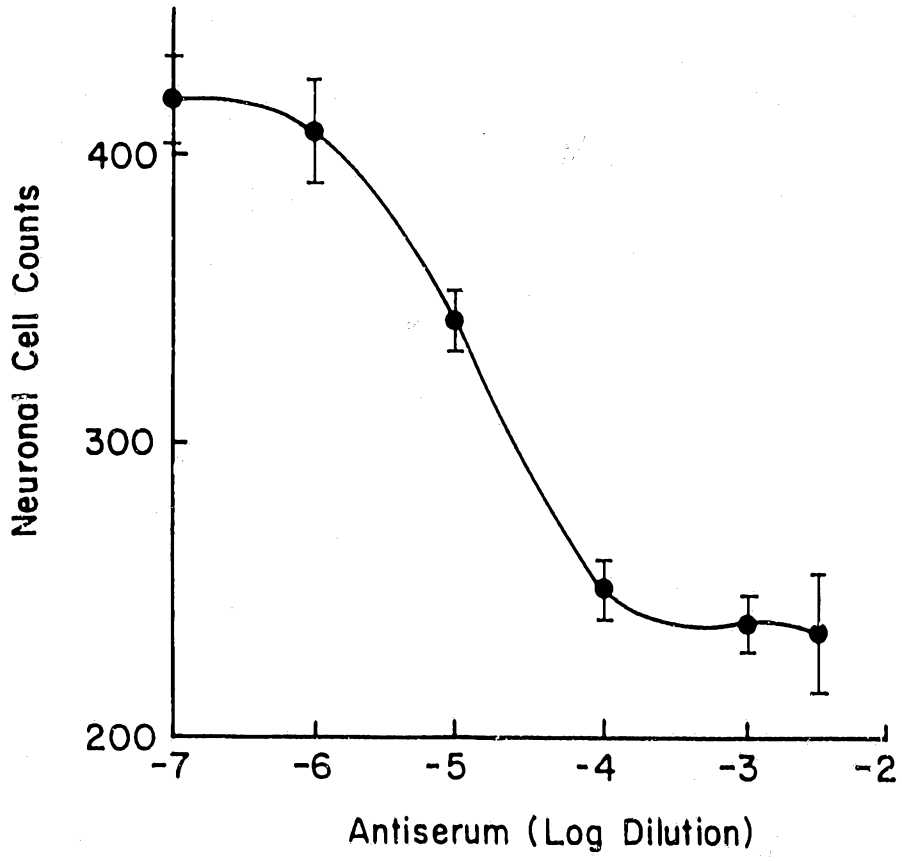

Neuronal Cell Counts
400
300
200
-7
-6
-5
-4
-3
-2
Antiserum (Log Dilution)

FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/03109

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :A61K 37/00; C07K 5/00
US CL :514/12, 21; 530/300, 387
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21; 530/300, 387

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US, A, 4,923,696, Appel et al., 08 May 1990, see entire document.	1-7,18 8-17
X Y	Proceedings of the National Academy of Science, USA, Volume 82, issued February 1985, KLAGSBRUN ET AL, "Heparin Affinity of anionic and cationic capillary endothelial cell growth factors: Analysis of hypothalamus-derived growth factors and fibroblast growth factors", pages 805-809, see entire document.	1,3,6,7 1-18

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 20 JULY 1992	Date of mailing of the international search report 29 JUL 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer BENNETT CELSA Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*