Purification and Metabolic Effects of a Nerve Growth-promoting Protein from Snake Venom

STANLEY COHEN

From the Washington University Department of Zoology, St. Louis, Missouri

A factor which specifically stimulates the growth of sensory and sympathetic nerve cells has been under investigation in this laboratory for a number of years. The growth stimulation is demonstrable both in tissue culture, on isolated nerve ganglia, and in the living chick embryo.

The factor was originally discovered in certain mouse sarcomas (2-5) and the biological activity was found to reside in a protein fraction obtained from these tumors (6). A much more potent source of the nerve growth factor has recently been found in the salivary glands and salivary secretions of a variety of species. These include snake venom (7), the venom of the Gila monster, the saliva and salivary glands of the mouse, and the salivary glands of the rat and hamster (1). The venom and mouse salivary gland factors duplicated the growth promoting effects of the tumor both in tissue culture and in vivo, when they were injected into the chick embryo (8, 9).

This paper is concerned with two main problems: (a) the chemical nature of the venom growth factor and (b) its mode of action and metabolic effect on the nerve cell.

EXPERIMENTAL

Materials and Methods

Materials—Dried moccasin and cobra venoms (Agkistrodon piscivorus and Naja naja) were obtained from the Ross Allen Reptile Institute. The other venoms used were kindly furnished by Dr. E. A. Zeller. Dr. Karl Slotta provided a sample of crystallized crototoxin. The author is also indebted to Dr. P. Berg for a sample of inorganic pyrophosphatase and to Dr. M. Friedkin for the Ca [bis(p-nitrophenyl)phosphate].

The carboxymethyl cellulose (CM-cellulose) cation exchanger was prepared according to the procedure of Peterson and Sober (10) and contained 0.6 m.eq. per gm. The anion cellulose absorbent (DEAE-cellulose, Solka-Floc) was obtained from the Brown Company. The crystalline enzymes were products of the Worthington Biochemical Corporation. The radioactive glucose, lysine, and ademine were obtained from the Volk Radiochemical Company. The radioactive measurements were made with a Nuclear-Chicago model D47 gas flow counter.

Tissue Culture Assay For Nerve Growth-promoting Activity—Hanging-drop tissue culture preparations were made consisting of 1/4 plasma (rooster), 1/4 Parker's synthetic medium 1066 (Connaught Medical Research Laboratories) containing thrombin (0.5 mg. per ml.), and 1/4 isotonic sodium chloride with the material to be tested in a final total volume of 0.075 ml. Each culture contained three or four sensory ganglia isolated from an 8- to 9-day chick embryo. As previously reported (11) the effects were observed after 18 hours of incubation at 37°C, and the amount of fiber outgrowth was recorded in a semiquantitative scale as 1+ to 4+.

The assay was sensitive to 2-fold changes in concentration of the active material; smaller changes were not detected by gross observation of the ganglia.

Enzymatic Determinations—Phospholipase A activity was measured by determining the rate at which lyssolecithin was liberated from purified egg lecithin. The incubation mixture was similar to that of Hayaishi and Kornberg (12) except that calcium chloride (0.01 M) and sodium chloride (0.1 M) were present. The lyssolecithin was determined as the iodine complex (12). A unit of enzyme was defined as the amount hydrolyzing 0.1 pmole of substrate in 1 hour.

Phosphodiesterase activity was determined by the procedure of Sinsheimer and Koerner (13) with Ca [bis(p-nitrophenyl)phosphate] as substrate. A unit of enzyme was that which liberated 0.1 pmole p-nitrophenol in one hour.

5-Nucleotidase was assayed by measuring the inorganic phosphate liberated from adenosine 3-phosphate (13). A unit of enzyme is that activity which liberated 10 pmoles of inorganic phosphorus in 1 hour.

L-Amino acid oxidase was assayed with L-leucine as substrate with the conditions of incubation reported by Kearney and Singer (14). The reaction was followed by measuring the ammonia liberated by nesslerization. A unit of activity is that which liberated 1 pmole of ammonia N in 30 minutes.

The ribonuclease assay was modified from that described by McDonald (15). The incubation was carried out at pH 7.7 for 30 minutes at 37°C. The undigested RNA was precipitated with MacFadyen's reagent, and after centrifugation the increase in optical density at 260 mg of the supernatant fluid was determined in a l-cm. cell. A unit of activity was defined as the activity which under these conditions produces an optical density increase of 1.0 in 30 minutes.

DNase activity was determined in a manner identical to that used for RNase except that the undigested DNA was precipitated with an equal volume of 7 per cent perchloric acid.

DPN nucleotidase activity was determined by measuring the rate of DPN destruction with alcohol dehydrogenase according to the procedure of Kornberg and Priger (16). One unit is defined as the amount causing the cleavage of 1 pmole of substrate per hour.

ATPase was determined by measuring the inorganic pyrophosphate liberated after incubation of the venom with ATP according to the procedure of Zeller (17). The pyrophosphate was measured as inorganic phosphate after incubation with inorganic...
pyrophosphatase. One unit was that amount which liberated 1 μmole of inorganic pyrophosphate per hour.

Hyaluronidase was assayed turbidimetrically (18). A unit was that activity which decreased the turbidity producing capacity of 0.2 mg. of hyaluronic acid to that of 0.1 mg. in 30 minutes.

The proteolytic activity was determined at pH 7.8 with denatured hemoglobin as substrate according to the procedure of Anson (19) for the estimation of trypsin. One unit of activity was defined as that which liberated 1 μmole of tyrosine in 30 minutes. The tyrosine was measured with the Folin phenol reagent.

One unit of activity was that amount which decreased the turbidity producing capacity of 1 Mmole of tyrosine in 30 minutes. The tyrosine was measured with the Folin phenol reagent (20). The enzyme assays were run in order to determine the relative enzymatic composition of the purified growth factor and the crude venom. For each assay a standard curve was prepared with the use of the crude venom. All incubations were at 37°. The protein content was measured with the Folin phenol reagent (20) with bovine albumin as a standard.

Determinations of Radioactivity in Protein, RNA, DNA, and Carbon Dioxide Fractions From Tissue Cultures—To obtain the total protein fraction the ganglia and plasma clot were dissolved by the addition of 0.2 ml. of 0.1 M NaOH and the protein was precipitated with 1 ml. of 10 per cent trichloroacetic acid. The precipitated protein was centrifuged, washed repeatedly with 2 per cent trichloroacetic acid, and was finally dissolved in 0.1 ml. of 0.25 M Na₂CO₃. The entire solution was plated and counted. The culturing and washing procedure was controlled in separate experiments by preparing cultures with the use of ganglia which had been killed by boiling.

The RNA and DNA fractions were obtained by treating the cultures with 1 ml. of cold 10 per cent trichloroacetic acid. The precipitated plasma clot together with the embedded ganglia were transferred to centrifuge tubes and the RNA and DNA fractions were isolated by the Schmidt-Thannhauser-Schneider procedure, as described by Volkin and Cohn (21), with the cation exchanger to remove the alkali. The final DNA fractions were boiled to decompose the trichloroacetic acid and were plated and counted. Again the entire procedure was controlled by preparing cultures containing heat-killed ganglia.

The total amount of carbon dioxide liberated by the ganglia was collected and counted as follows. At the end of the incubation period, 0.2 ml. of 0.1 M NaOH was added to the cultures through a small, paraffin-sealed pinhole in the covering glass plate. The mixture was then transferred to a Conway dish, and the carbon dioxide liberated by sulfurous acid was collected on a glass dish containing 0.1 ml. of a 0.1 M Ba(OH)₂-0.05 M BaCl₂ mixture. The plates were then dried and counted.

RESULTS

Distribution of Nerve Growth Factor in Venoms of Various Species—Samples of dried venom from species of the three families of poisonous snakes were dissolved in isotonic sodium chloride and assayed for their growth-stimulating properties in tissue culture. A series of 2-fold dilutions of each venom was examined and the results (Table I) were expressed as the number of μg. of venom per ml. of tissue culture medium required to show a 3+ response. All of the venoms examined showed similar nerve growth-promoting properties. The crude venoms of the Elapidae and Viperidae were from 2 to 4 times as potent as those of the Crotalidae.

Freshly prepared serum from Agkistrodon piscivorus showed no growth-promoting activity. (Dilutions up to 1:1,000,000 were assayed).

Purification of Growth Factor—The venom of the moccasin (Agkistrodon piscivorus) was used as the starting material for the purification of the growth factor. The procedure has been modified from that outlined previously (7). One gm. of venom and 18 gm. of urea were dissolved in 37 ml. of water containing 3.5 ml. of 0.1 M NaOH. The solution was allowed to stand for 90 minutes at 0°. All subsequent procedures were carried out at a temperature of 0-3°. The urea step was included since its omission resulted in a considerable loss of biological activity during fractionation. Saturated ammonium sulfate (at 0°, adjusted to pH 7.6 with ammonium hydroxide) was added until a final concentration of 46.5 per cent saturation was obtained. The mixture was allowed to stand for 30 minutes and was then centrifuged at 10,000 × g for 5 minutes. The precipitate was discarded. To the supernatant fluid ammonium sulfate was added to a final concentration of 68 per cent saturation. The mixture was again allowed to stand for 15 minutes and was centrifuged. The yellowish supernatant fluid was discarded. The precipitate containing the growth factor was dissolved in 25 ml. of water and dialyzed overnight against distilled water. The material was centrifuged and the slight precipitate discarded. The supernatant fluid was again fractionated with the above saturated ammonium sulfate solution (in the absence of urea); the active material was precipitated between 45.5 and 50 per cent saturation. After centrifugation the precipitate was dissolved in 20 ml. of water and the solution was again dialyzed overnight against distilled water. This fraction contained 155 mg. of protein and possessed over 50 per cent of the original biological activity. It should be remembered that the tissue culture assay was sensitive only to 2-fold changes in the concentration of the growth factor.

The growth factor was then further purified by adsorption and elution from the anion and cation exchangers, DEAE- and CM-cellulose. A column 2.0 cm. in diameter containing 3 gm. of the DEAE-cellulose was prepared and equilibrated with 0.2 M potassium phosphate buffer at pH 6.0. The column was then washed with distilled water. The dialyzed ammonium sulfate fraction (containing approximately 5 mg. of protein per ml.) was
passed through the resin at a flow rate of 0.1 to 0.2 ml per minute followed by a wash of 1.5 column volumes of distilled water. Under these conditions the growth factor was not adsorbed by the resin. The eluate contained 67 mg of protein and all of the biological activity. The protein remaining on the column could be quantitatively eluted with 1.0 m sodium chloride and was biologically inactive. The column was regenerated by washing with a mixture of 0.5 m NaCl and 0.5 m NaOH, followed by distilled water.

A column 2.0 cm in diameter containing 3 gm of CM-cellulose was then prepared and washed with a mixture of NaCl and NaOH, each at a concentration of 0.5 m, followed by distilled water. The eluate from the DEAE-cellulose column was then applied at a flow rate of 0.1 to 0.2 ml per minute. The column was washed with 20 ml of water. The combined eluate contained 3 mg of protein and was inactive. The column was then washed with 0.2 m NaCl and 5 ml portions were collected and analyzed for protein and biological activity. The growth factor appeared in Tubes 3 to 7. The combined fractions (25 ml) contained 32 mg of protein and over 50 per cent of the biological activity applied. The active solution was then dialyzed against distilled water in preparation for adsorption on a third column.

A column containing 3 gm of DEAE-cellulose was prepared as previously described except that it was equilibrated with distilled water after washing with the NaCl-NaOH mixture. The dialyzed eluate from the CM-cellulose was then applied. Under these conditions the biological activity remains adsorbed on the column. The resin was washed with 20 ml of 0.001 m NaCl and the eluate discarded. NaCl, 0.05 m, was then passed through the column, and 5 ml fractions were collected. The biological activity appeared in Tubes 3 and 4. The combined fractions (10 ml) contained over 50 per cent of the activity applied to the column and 5.2 mg of protein.

Thus, approximately 25 per cent of the nerve growth-promoting activity present in 1 gm of the crude venom was recovered in 5.2 mg of protein, a purification of about 40-fold. This preparation was used in the enzymatic and metabolic studies reported in this paper.

Properties of Growth Factor—We have previously reported (7) that the growth factor was nondialyzable, heat-labile (5 minutes at 90° in isotonic sodium chloride, pH 7.4), destroyed by acid (0.1 N HCl for 1 hour at 26°), stable to alkali (0.1 N NaOH for 1 hour at 26°), and stable to 6 N urea (1 hour at 0°).

The behavior of the material was examined in a Spinco analytical ultracentrifuge. The results are shown in Fig. 1. Only a single component was detectable, with an s20 of 2.2 S; the molecular weight was estimated to be on the order of 20,000. The centrifugation was continued until the boundary had completely sedimented. The supernatant fluid was removed and the residue redissolved in an isotonic sodium chloride. Tissue culture assays of these fractions showed that the supernatant fluid did not contain the growth factor; all of the activity was present in the sedimented fraction.

The ultraviolet absorption spectrum of the material is shown in Fig. 2. The 280/260 absorption ratio was found to be 1.3. A solution containing 1.0 mg per ml in distilled water showed an optical density of 1.03 at 280 mp in a cell with a path length of 1 cm.

Upon acid hydrolysis (6 N HCl for 10 hours in an autoclave) and two-dimensional paper chromatography of 150 μg aliquots, the amino acid pattern was qualitatively identical to a similar chromatogram prepared with crystalline bovine albumin. No reducing sugars could be detected on the chromatograms, although the original material showed the presence of 1.6 per cent hexose as determined by the orcinol procedure, with galactose as a standard at 540 mp (22).

Two additional lines of evidence support the view that the biological activity is associated with a protein: (a) the destruction of the biological activity upon incubation with proteolytic enzymes, and (b) the loss of biological activity upon incubation with antisera to snake venom.

The growth-promoting activity of the purified factor is completely destroyed by incubation with each of the crystalline proteolytic enzymes, trypsin, chymotrypsin, and papain. In these experiments 80 μg of the purified factor were incubated with 20 μg of each of the proteases in 0.2 ml of 0.1 m phosphate buffer, pH 7.0, for 4 hours at 28°. The papain digestion mixture also contained 60 μg of cysteine. Controls were run in which the enzyme was omitted. After the incubation was completed, aliquots were assayed. Under these conditions there was no diminution of the activity in the control tubes whereas only traces of activity remained after proteolytic digestion. Under similar conditions incubation with 100 μg of RNase or DNase did not affect the biological activity.

The possibility that the proteolytic enzymes were acting by...
Nerve Growth-promoting Protein from Snake Venom

Vol. 234, No. 5

1132

The nervous response of the ganglia was excluded, since uninhibited mixtures of the growth factor and the proteases were as active as the control. The failure of the proteases to act on the factor in the tissue cultures is understandable in view of the dilution of the enzyme mixture in the cultures (1:2000) and the presence of factors in the medium. The possibility that the enzymes were liberating an inhibitor of some sort during the incubation was excluded, at least in the case of trypsin, by adding soybean trypsin inhibitor and fresh growth factor after the incubation had been completed. No inhibition of the added growth factor was detected.

The biological activity of the crude venom was abolished by antiserum to snake venom (7). These results were confirmed with the purified growth factor. Of the protein, 40 μg. were preincubated for 1 hour at 26° with varying amounts of commercial antiserum (Wyeth Laboratories, Inc.) in 10 ml. of isotonic sodium chloride, and aliquots were then assayed. The biological activity was completely inhibited by 5 mg. of the antiserum preparation. In control experiments the further addition to the mixture of an equivalent amount of the growth factor restored over 50 per cent of the original activity, indicating that the antiserum was not inhibiting the response of the ganglia in some nonspecific fashion. Normal horse serum has no effect.

Comparison of Enzymatic Activities of Crude Venom and Purified Growth Factor—The possibility that the growth-promoting properties of venom were due to the activity of one of the enzymes known to be present in snake venom was then investigated. The enzymatic composition of animal toxins has been reviewed by Zeller (28).

The results of the enzymatic assays on crude venom (Aqalectrodon piscivorus) and the 40-fold purified growth factor are shown in Table II. It was expected that if the biological activity was due to a particular enzyme, its specific activity (units per mg.) should also be approximately 40 times higher in the purified fraction than in the crude venom. The results show that the specific activities of all of the enzymes examined were lower in the purified fraction. Indeed, only two of the enzymes, protease and RNase, showed any appreciable activity. The possibility that these enzymatic activities might still be associated with the growth factor was further reduced by the fact that whereas the growth factor is stable in 0.1 m NaOH (28°, 1 hour) the protease and RNase activities are completely destroyed by this treatment.

Crystalline crotoxin (phospholipase A) showed no growth-promoting activity. The fact that both the white and yellow venoms of Vipera aspis showed similar biological activity confirms the elimination of l-amino acid oxidase since the former venom is free of this enzyme. Cholinesterase, while present in the Viperaeae, is not found in the Viperidae or Crotalidae. Similarly, catalase, while found in some venoms is not present in Vipera russelli (24). The addition of beef liver catalase to the cultures had no effect. The protease activity of A. piscivorus has been reported to be 30 times greater than C. adamanteus (25) whereas both have identical growth-promoting properties. These authors also report considerable variations in clotting ability and bradykinin formation among the Crotalidae in contrast to their very similar growth-promoting activities.

Effect of Protein Hormones on Nerve Growth—In view of the failure to demonstrate any association of enzymatic activity with the growth factor, and the presence of a similar agent in mouse sarcomas and salivary glands, it was of interest to see whether any of the protein hormones would stimulate nerve growth. Purified preparations of insulin and glucagon were kindly furnished by the Lilly Research Laboratories. Preparations of melanophore-stimulating hormone, growth hormone, follicle-stimulating hormone, corticotropin, gonadotropin, and thyroid-stimulating hormone were furnished by the Armour Laboratories. Tissue cultures were prepared containing these hormones in concentrations from 0.0001 to 100 μg. per ml. No nerve growth-promoting effect was observed in any instance.

Site of Action of Growth Factor—The outgrowth of nerve fibers in tissue culture under the influence of the venom factor might be due to (a) a direct effect on the ganglion itself or (b) an indirect effect as a result of some interaction of the growth factor with the medium. Conceivably, the latter might involve the removal of an inhibitor or the liberation of some stimulating substance. The following experiments were performed to decide between these alternatives.

Plasma, 3 ml., and synthetic medium (containing thrombin), 3 ml., were incubated separately with 2 μg. of the growth factor in 1.5 ml. of isotonic sodium chloride for 8 hours at 37°. (This period of time was sufficient to allow for the initiation of nerve growth in control cultures.) Equal volumes of the two solutions were mixed to assay for biological activity. In other aliquots, the antiserum to the venom was added before mixing for the assay. The presence of the antiserum completely abolished the nerve-stimulating effect of the mixture. These results indicate that the venom does not remove an inhibitor from the medium and that if the venom produces some other stimulating substance, it must have a very transient existence.

The possibility that the venom was inducing the cells of the ganglion to produce (and secrete into the medium) a growth stimulating agent was examined by incubating 40 ganglia for 8 hours at 37° in 0.1 ml. of the synthetic medium containing the venom protein. The medium was then assayed for biological activity in the presence and absence of the antiserum. Since the addition of the antiserum completely abolished the activity, this hypothesis was ruled out.

Nutritional Requirements for Nerve Fiber Outgrowth—We have reported (6) that the outgrowth of nerve fibers in tissue culture resulting from the presence of the tumor factor, although com-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Crude venom</th>
<th>Nerve growth factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg.</td>
<td>units/mg.</td>
</tr>
<tr>
<td>Phospholipase A</td>
<td>6.2</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Phosphodiesterase</td>
<td>11</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>5-Nucleotidase</td>
<td>19</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
<td>14</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>4.2</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Diprophosphorydine nucleotidease</td>
<td>13.8</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Adenosine triphosphatase</td>
<td>19.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>15.6</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Protease</td>
<td>16.7</td>
<td>12.6</td>
</tr>
</tbody>
</table>
pletely inhibited by iodoacetate (10^{-4} M) was not prevented by fluoride (10^{-3} M), cyanide (10^{-5} M), or dinitrophenol (10^{-4} M). These results have been confirmed with the growth factor obtained from venom.

The possibility was therefore considered that the outgrowth of fibers was due simply to a redistribution of existing cytoplasm with no associated metabolic effects. Consequently, we tried to see (a) whether there were any nutritional requirements for nerve outgrowth and (b) whether the growth factor had any effect on either the oxidation of glucose or the synthesis of protein and nucleic acids in the cells of the ganglion or both.

To examine the carbohydrate requirements for nerve growth, cultures were prepared with plasma which had been dialyzed for 24 hours against isotonic sodium chloride containing 200 units of penicillin and 20 μg. of streptomycin per ml. Eagle's synthetic medium (containing thrombin) was prepared (26) with the substitution of sodium chloride or a variety of possible "energy sources" for glucose. The carbohydrates and other added metabolites were present in the cultures at a final concentration of 10^{-3} M. The cultures were prepared as described, with 0.025 ml. of plasma, 0.025 ml. of synthetic medium, and 0.025 ml. of the growth factor (0.015 μg.) in isotonic sodium chloride.

The presence of glucose or mannose is required for the outgrowth of nerve fibers. In the absence of any added energy source the outgrowth of fibers was initiated but then ceased abruptly. The glucose could not be replaced by r-fructose, r- or r-arabinose, r-ribose, r-galactose, gluconic acid, gluconic acid, malic acid, α-ketoglutaric acid, succinic acid, or fumaric acid. Lactate and pyruvate could partially replace the glucose requirement. Typical results are illustrated in Figs. 3 to 6. It is not known to what extent the failure of some of the substrates to support growth was due simply to permeability.

To examine the amino acid requirements for nerve growth, a basal culture medium was prepared containing the dialyzed plasma and Earle's salt solution (with glucose and thrombin). However, even under these conditions, in the absence of any added amino acids, the growth factor elicited the usual halo of outgrowth of nerve fibers after 18 hours of culture. We were thus presented with two alternatives; (a) amino acids were not required for the outgrowth of the nerve fibers or (b) amino acids were required, but could be obtained from the protein of the medium, or degenerating cells of the explant, or from internal reserves.

The following experiment, involving the use of the amino acid analogue p-fluorophenylalanine, indicated that at least one amino acid, phenylalanine, was required for growth. Under the above basal conditions the addition of 0.2 mM l-p-fluorophenylalanine almost completely inhibited nerve growth. This inhibition was specifically reversed by the addition of l-phenylalanine (0.5 mM). The addition of a mixture of amino acids (arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, and glutamine) each at a concentration of 0.5 mM did not reverse the inhibition. These results are shown in Figs. 7 to 10.

Although other amino acid analogues have not been tested, it seems probable that there is a more general requirement for amino acids during nerve outgrowth.

**Effect of Venom Protein on Synthesis of Protein and Nucleic Acids and Oxidation of Glucose**—Isotopically labeled substrates were used to obtain, more directly, information concerning the synthetic and metabolic events occurring in the nerve cells under the influence of the growth factor.

For these experiments the cultures were prepared in large depression slides in pairs, each culture containing five identical contralateral sensory ganglia isolated from the lumbar sacral plexus of a 9-day chick embryo. The total protein content of the five ganglia averaged 30 μg., and the protein content of the two sets of ganglia agreed to within 10 per cent.

The effect of the growth factor on the incorporation of C^{14}L-lysine into the protein of the ganglion was then examined. Each set of five ganglia was treated in an identical manner except for the omission of the growth factor in the control cultures. Each culture contained 0.05 ml. of the dialyzed plasma, 0.03 ml. of the growth factor (0.025 μg. in isotonic sodium chloride), 0.02 ml of 1-C^{14}DL-lysine (5.8 μg. in isotonic sodium chloride), and 0.05 ml. of modified Eagle's synthetic medium. (The Eagle's medium was prepared with the omission of lysine and the incorporation of glucose at a level of 3 mg. per ml. and thrombin at a concentration of 0.5 mg. per ml.) The sealed cultures were allowed to incubate for 20 hours at 37° and the total protein fraction was separated and counted.

The effects of the growth factor on the incorporation of 8-C^{14}L-adenine into the RNA and DNA and the incorporation of 6-C^{14}L-glucose to C^{14}O_{2} were also examined.

The results of these experiments are shown in Table III. Between 0.35 and 0.45 μg. of lysine was incorporated into the protein fraction of the five ganglia in the control cultures. Under the influence of the venom the incorporation was increased 58 to 72 per cent in three experiments.

In view of the fact that sodium fluoride does not inhibit the outgrowth of the nerve fibers, but does inhibit the secondary migration of cells from the explant, the effect of 0.01 M NaF on the incorporation of lysine into the protein fraction was also examined. It can be seen from Table III that although the control level of lysine incorporation was reduced, the growth factor increased the incorporation by 84 to 94 per cent.

Between 0.033 and 0.038 μg. of adenine was incorporated into the RNA of the control cultures. In the presence of the growth factor the incorporation was increased 40 to 69 per cent. Only traces of the adenine could be detected in the DNA fractions of both the control and venom-treated ganglia, of the order of 100 times lower than into the RNA. Whether or not there is any effect of the venom on the synthesis of DNA cannot be seen from these data.

Similarly, the factor increased the oxidation of carbon 1 of glucose by 41 to 54 per cent. Less of the C-6 of glucose was oxidized by the ganglia and the increase due to the presence of the venom ranged from 12 to 26 per cent.

**DISCUSSION**

The experiments herein reported were designed to determine the chemical nature of the growth-stimulating factor present in venom, its site of action, and its effect on some of the metabolic processes occurring in the nerve cell.

It is clear that the factor isolated from the venom is a protein. It is heat-labile, nondialyzable, acid-labile, and may be fractionated by standard procedures of protein separation. The biological activity is destroyed by incubation with a variety of proteolytic enzymes. The factor is antigenic as evidenced by the inhibition of the growth-promoting activity by antisnake venom serum.
Figs. 3-6. Microphotographs of living sensory ganglia (from 9-day chick embryos) after 18 hours of incubation

Fig. 3. A control culture containing glucose but no growth factor.

Fig. 4. The culture contained glucose (or mannose) together with the growth factor.

Fig. 5. The culture contained the growth factor without added glucose. Cultures prepared with a variety of possible energy sources (see text) had a similar appearance.

Fig. 6. The culture contained the growth factor with added lactate (or pyruvate).
The biological activity is associated with a particle with a sedimentation constant of 2.2 S. Paper chromatography of an acid hydrolysate of the growth factor revealed a typical protein amino acid pattern. The ultraviolet absorption spectrum of the material is consistent with this conclusion.

Although the mechanism by which this protein stimulates the outgrowth of nerve fibers, both in tissue culture and in the chick embryo, is not known, it appears to act directly on the nerve cells. Our tissue culture studies have shown that it neither removes an inhibitor from the medium nor reacts with the medium or the

Figs. 7-10. Microphotographs of living sensory ganglia (from 9-day chick embryos) after 18 hours of incubation. All of the cultures contained the growth-promoting protein.

Fig. 7. The basal (amino acid-free medium) was employed (see text)

Fig. 8. The basal medium was supplemented with D.p-fluorophenylalanine

Fig. 9. The basal medium contained fluorophenylalanine and L-phenylalanine

Fig. 10. The basal medium contained fluorophenylalanine and a mixture of 11 other amino acids
Evidence that the growth factor actually stimulates oxidative and synthetic processes in the nerve ganglion was obtained by studying the fate of labeled substrates added to the tissue culture medium. The incorporation of lysine into protein, adenine into RNA, and the oxidation of glucose to carbon dioxide were all increased in the presence of the venom protein. No data are available to distinguish between net synthesis and turnover. The interpretation is further complicated by the facts that the ganglia are not pure cultures of nerve cells, and that a variable amount of necrosis occurred in the central parts of the ganglia during the incubation period.

The results obtained by the use of metabolic inhibitors have revealed some unexpected aspects of the metabolism of glucose by the embryonic sensory ganglia. The presence of cyanide does not prevent either the outgrowth of nerve fiber or the increase in the quantity of C14-glucose oxidized to C14-carbon dioxide (preliminary unpublished experiments). This suggests the operation of a noncytochrome oxidase-linked oxidative process. The findings that the outgrowth of fibers is not inhibited by fluoride and that more of carbon 1 of glucose appears in the carbon dioxide than of carbon 6, indicates the operation, at least to some extent, of a direct oxidative pathway of glucose breakdown.

Our recent findings that the salivary glands of rodents possess a similar factor, and that it stimulates nerve growth in ganglia isolated both from the chick embryo as well as from the mouse (7), raises the question of the physiological role (if any) played by these growth factors in the normal economy of the organism.

### SUMMARY

1. A protein with nerve growth-stimulating properties has been purified from snake venom. Only one component may be detected in the ultracentrifuge with a sedimentation constant of 2.2 S.

2. The factor has been found in all of the venom tested, representing species from the three families of poisonous snakes.

3. The evidence indicates that the protein acts on the nerve cell directly and that the outgrowth of nerve fibers is not due to any interaction of the growth factor with the tissue culture medium. The biological activity is not associated with any of the enzymes known to be present in venom.

4. The presence of an energy source (glucose or mannose) is required for the continuation of nerve growth. The outgrowth of fibers is inhibited by p-fluorophenylalanine and the inhibition is specifically reversed by phenylalanine. Experiments with isotopically labeled substrates have shown that concomitant with the outgrowth of nerve fibers, there is an increased incorporation of lysine into protein, adenine into ribonucleic acid, and an increased oxidation of glucose to carbon dioxide.

### REFERENCES


Purification and Metabolic Effects of a Nerve Growth-promoting Protein from Snake Venom
Stanley Cohen