Purification and Properties of a Nerve Growth-promoting Factor Isolated from Mouse Sarcoma 180*

STANLEY COHEN AND RITA LEVI-MONTALCINI

(Department of Zoology, Washington University, St. Louis, Mo.)

It was reported by Levi-Montalcini and Hamburger (7) that mouse Sarcomas 180 and 37 contain a diffusible agent which stimulates the growth of sympathetic and spinal ganglia of chick embryos. The original experiments (1, 6) consisted of the intra-embryonic and allantoic transplantation of small pieces of living tumor to 3-day embryos. Subsequently, it was observed that a marked radial outgrowth of nerve fibers from sensory and sympathetic ganglia occurred when the ganglia were explanted near fragments of living tumor in hanging-drop tissue cultures (8). The relationship of the in vivo and in vitro effects has been discussed (8).

In the following experiments, the in vitro bioassay was used in an attempt to isolate and characterize the growth-promoting factor. In a preliminary report (3) we stated that the effect of the actively growing tumor can be duplicated by a heat-labile, nondialyzable "nucleoprotein fraction" isolated from the microsomes of these tumors. In the following, we present further observations on the nature and mode of action of the active material.

MATERIALS AND METHODS

Tumor.—Sarcoma 180 was maintained by weekly passage in mice. Previously (8, 8), it was noted that the growth-promoting effects of these tumors were considerably enhanced by passage through the chick embryo and that the tumor material was obtained from intra-embryonic transplants which were placed in the body wall of 3-day chick embryos. However, this procedure yielded relatively small amounts of tumor (1–2 gm. wet weight of tumor/20 dozen eggs/week). We have now found that active extracts can be prepared from sarcoma grown on the allantoic membrane and have increased the yield approximately tenfold. In the experiments to be reported, Sarcoma 180 was transplanted to the allantoic membrane of 8-day chick embryos, and the tumors were harvested 9–10 days later. The tissue was then frozen and stored at −10° C. until a sufficient amount had been collected for fractionation. In this condition the active material is stable for at least several weeks.

Assay.—Hanging-drop tissue culture preparations were made containing one-third of plasma (rooster), one-third of Parker's synthetic medium (190 or 1066) containing thrombin (0.5 mg/ml), and one-third of the material to be tested, in a total volume of 0.075 ml. Each culture contained three to four sensory ganglia isolated from a 7-day chick embryo. As previously reported (3), the effects were observed after 18 hours of incubation at 37°C, and the amount of fiber outgrowth was recorded on a semiquantitative scale as 1+ to 4+ (see ref. [3], Figs. 1–4). The assay was sensitive to twofold changes in concentration of the active material; smaller changes were not detected by gross observation of the ganglia.

RESULTS

Preparation of the "nucleoprotein fraction."—The fractionation of homogenates of freshly harvested tumor by the differential centrifugation method of Schneider (10) resulted in the separation of a microsome fraction containing practically all the activity of the homogenate (3). When this procedure was applied to the frozen tumor, although most of the activity was associated with the microsomes, approximately one-fourth remained in the supernatant fluid after centrifugation at 100,000 × g for 1 hour. The following modified procedure was then adopted: 10 gm. (wet weight) of frozen tumor was homogenized in 30 ml. of 0.25 M sucrose in a Waring Blender for 2 minutes. Sodium hydroxide (0.1 M) was added to maintain...
tain the final pH at 7.3–7.4. The homogenate was centrifuged at 8,500 × g for 10 minutes. The supernatant was removed, and the residue was washed twice with 10 ml. of the sucrose solution. After each washing the mixture was centrifuged, and the supernatant fluids were combined. The final residue was discarded. The opalescent reddish solution was then dialyzed overnight against distilled water. These procedures were carried out in the cold (0–3°C.). The further fractionation of the material was carried out essentially as described previously (8). It involved the precipitation of the active material as an insoluble streptomycin complex, removal of the streptomycin by dialysis, and, finally, treatment with chloroform. The active material was recovered. It should be emphasized again that the quantities of enzyme and the pH of incubation were as follows: crystalline trypsin, 44 μg., pH 7.6; crystalline alpha-chymotrypsin, 44 μg., pH 7.6; crystalline papain, 44 μg., pH 7.0 (in the presence of 0.002 M cysteine); crude pancreatic protease, 44 μg., pH 7.6; lipase, 44 μg., pH 7.0; hyaluronidase, 10 μg., pH 7.0; lysozyme, 44 μg., pH 7.0; alpha- and beta-amylase, 44 μg., pH 6.6; beta-glucosidase, 44 μg., pH 6.0; beta-gluconirudase, 44 μg., pH 6.0; crystalline ribonuclease, 44 μg., pH 7.6 (in the presence of 0.15 M sodium chloride); crystalline deoxyribonuclease, 44 μg., pH 7.6 (in the presence of 0.15 M sodium chloride and 0.002 M magnesium chloride); and alkaline phosphatase, 44 μg., pH 9.3. Aliquots (25 μl.) of the digestion mixtures were assayed as described. Since the total volume of the culture was 75 μl., the enzymes were present during the growth of the ganglia at a concentration of one-third of that indicated above.

Under the conditions employed, none of the above enzymes destroyed the growth-promoting activity of the “nucleoprotein fraction.” During the digestion with ribonuclease the solution became turbid. After completion of the incubation, the pH was adjusted to 6.0, and the mixture was centrifuged. The supernatant fluid contained between 80 and 90 per cent of the material which absorbed light at 260 μμ in a cell with a path length of 1 cm.

It has been stated previously (8) that the growth factor is nondialyzable and completely destroyed by heating for 5 minutes at 80°C. Ashing of the material similarly destroyed the activity. The growth factor is destroyed in a 0.1 N acid solution but is stable in a 0.1 N alkaline solution. In these experiments 0.1 ml. of 1 N NaOH or HCl was added to 0.9 ml. of the nucleoprotein solution (in distilled water), and the mixture was allowed to stand 1 hour at 26°C. The solutions were then exactly neutralized (using phenol red as an internal indicator), and sufficient sodium chloride was added to render the solution isotonic. No growth-promoting effect was detected in the acid-treated fraction; the alkali-treated fraction was as active as the untreated control. No loss in activity could be detected after exposure of the active agent to 6 N urea for 1 hour at 0°C. Preliminary experiments indicate that free SH groups are not required for activity; exposure of the material to 0.1 M iodoacetic acid, pH 7.6, for 1 hour at 26°C. had no inactivating effect. (The iodoacetic was removed by dialysis.)

In an attempt to gain further insight into the nature of the active material, the effect of digestion of the agent with a variety of enzymes1 was examined. The enzymes were added to 3 mg. of the “nucleoprotein fraction” dissolved in 1 ml. of distilled water. If necessary, the pH was adjusted by the addition of 0.1 N NaOH or HCl. The mixture was incubated for 1 hour at 26°C. Sodium chloride was then added to make each solution isotonic. The quantities of enzyme and the pH of incubation were as follows: crystalline trypsin, 44 μg., pH 7.6; crystalline alpha-chymotrypsin, 44 μg., pH 7.6; crystalline papain, 44 μg., pH 7.0 (in the presence of 0.002 M cysteine); crude pancreatic protease, 44 μg., pH 7.6; lipase, 44 μg., pH 7.0; hyaluronidase, 10 μg., pH 7.0; lysozyme, 44 μg., pH 7.0; alpha- and beta-amylase, 44 μg., pH 6.6; beta-glucosidase, 44 μg., pH 6.0; beta-glucuronidase, 44 μg., pH 6.0; crystalline ribonuclease, 44 μg., pH 7.6 (in the presence of 0.15 M sodium chloride); crystalline deoxyribonuclease, 44 μg., pH 7.6 (in the presence of 0.15 M sodium chloride and 0.002 M magnesium chloride); and alkaline phosphatase, 44 μg., pH 9.3. Aliquots (25 μl.) of the digestion mixtures were assayed as described. Since the total volume of the culture was 75 μl., the enzymes were present during the growth of the ganglia at a concentration of one-third of that indicated above.

Under the conditions employed, none of the above enzymes destroyed the growth-promoting activity of the “nucleoprotein fraction.” During the digestion with ribonuclease the solution became turbid. After completion of the incubation, the pH was adjusted to 6.0, and the mixture was centrifuged. The supernatant fluid contained between 80 and 90 per cent of the material which absorbed light at 260 μμ. It showed no growth-promoting effect. The precipitate was partially dissolved in isotonic saline by adjusting the pH to 9.5. This turbid mixture possessed approximately three-fourths of the activity of the original solution. The reduction in activity was tentatively ascribed to the decreased solubility of the preparation. After trypsin digestion, the active factor is still heat-labile and nondialyzable. The quantities of proteolytic enzymes employed were usually sufficient to liquefy the plasma clot after 24–28 hours of incubation, thereby destroying the halo of nerve fibers.

Preparation of the “protein fraction.”—The re-

1 The enzymes were obtained from the Worthington Biochemical Co., Freehold, New Jersey, and from the Nutritional Biochemicals Co., Cleveland, Ohio.
sults obtained with ribonuclease, and preliminary experiments in which attempts were made to remove nucleic acid from the preparation by ammonium sulfate precipitation in the presence of strong urea, indicated that upon removal of the nucleic acid the active agent was only very slightly soluble under conditions necessary for its assay in tissue culture. It was then found that after preliminary digestion with trypsin most, if not all, of the nucleic acid could be removed from the “nucleoprotein fraction,” leaving the residual material active and soluble. The following procedure was adopted: To 20 ml. of the “nucleoprotein fraction” (containing approximately 3–4 mg/ml dry weight) was added 0.90 mg. of crystalline trypsin, and the mixture was incubated for 1 hour at 26°C, pH 7.6. Approximately 15 per cent of the protein was rendered nonprecipitable by 5 per cent trichloroacetic acid. Soybean trypsin inhibitor (0.90 mg.) was then added to the solution. All subsequent steps were carried out at temperatures from 0–3°C. The active material was then precipitated with saturated (at 0°C.) ammonium sulfate (pH 7.6) until the mixture was half-saturated. The supernatant fluid was discarded. The residue was washed once with 5 ml. of half-saturated ammonium sulfate and recentrifuged. The final residue was dissolved in 10 ml. distilled water and dialyzed against distilled water. Urea was then added to the solution to a final concentration of 6 N. The clear mixture was allowed to stand for 1 hour at 0°C. The active material was again precipitated with ammonium sulfate, pH 7.6. The fraction precipitating between 34 and 66 per cent saturation was collected, dissolved in 9 ml. of distilled water, and dialyzed for 24 hours to remove the urea and ammonium sulfate. After the final solution was adjusted to pH 8.0, traces of insoluble matter were removed by centrifugation. The resulting solution possessed 15 per cent of the protein, 1.5 per cent of the material absorbing light at 260 mμ, and approximately 50 per cent of the total activity of the starting “nucleoprotein fraction.” Our purest preparation contained between 25 and 50 per cent of the activity of the crude homogenate in 0.5 per cent of the dry weight of the tumor; a 50- to 100-fold purification was thus achieved.

Properties of the “protein fraction.”—In five of these “protein fraction” preparations the 280/260 absorption ratios varied from 1.20 to 1.28. A solution containing 0.90 mg/ml at pH 7.6 showed an optical density of 0.50 at 280 mμ in a cell with a path length of 1 cm. In Figures 1 and 2 are shown the growth-stimulating effects of the “nucleoprotein fraction” and of the “protein fraction” derived from it when added to the cultures at concentrations of 1.2 and .25 mg/ml, respectively. The appearance of a control culture is similar to the culture shown in Figure 5.

The growth-promoting activity of this “protein fraction” was not destroyed by incubation with ribonuclease or deoxyribonuclease (Figs. 3, 4), nor was any increase in turbidity noted. (The conditions of incubation were identical to those described above.) The active material was still stable for 1 hour in 0.1 N NaOH at 26°C. It was not affected by ultraviolet irradiation (0.5 ml. of the solution was placed in a watch glass and exposed for 15 minutes to a radiation of approximately 14 ergs/sq mm/sec, from a G. E. 30-watt sterilamp).

These data suggest that nucleic acids are not involved in the growth-promoting activity of the tumor fraction, although the possibility cannot be excluded that trace amounts of nuclease-resistant nucleic acid were present.

Attempts were made to exclude this possibility with respect to RNA by incubating the material (1 mg/ml) in 0.1 N NaOH for 24 hours at 30°C. However, a control mixture incubated in the absence of alkali retained only approximately 50 per cent of its activity. The alkali-treated sample retained 25 per cent of the activity. After dialysis against isotonic saline no change in the 280/260 absorption ratio was noted. These data indicate that the preparation contained no detectable quantities of RNA and that certainly the residual 25 per cent of activity was not associated with RNA. In attempts to eliminate the possible presence of nuclease-resistant DNA, the material was incubated with a mixture of deoxyribonuclease and phosphodiesterase from snake venom (Crotalus adamanteus). This treatment markedly enhanced the growth-promoting effect. Upon further examination it was found that snake venom alone has extremely potent nerve growth-promoting properties. These data are presented in a separate report (2).

The “protein fraction” contained 14 per cent nitrogen. Examination of the material for protein-bound hexose and hexoseamine (5) showed the presence of 8.7 per cent hexose (as glucose) and 0.8 per cent hexoseamine (as glucosamine). A portion of the protein fraction was hydrolyzed with 6 N HCl in an autoclave for 15 hours. After removal of the acid, two-dimensional paper chromatography was carried out with the solvent pairs propanol-ammonia-water (6:3:1) and the upper phase of a tertiary-amyl alcohol-water-formic acid (3:3:1) mixture. The amino acid pattern was developed with a spray of 0.1 per cent ninhydrin in n-butanol, the paper being dried at 65°C. The
chromatogram was then compared with one prepared from bovine albumin (fraction V, Armour). The two chromatograms were qualitatively identical except for the appearance of a very faint yellow spot in the tumor fraction. Since the purity of the preparation has not been established, these data have limited significance.

Treatment of the "protein fraction" with heat or acid (procedures described above) completely destroyed the activity. The growth factor was inactivated by organic solvents such as ethyl alcohol, methyl alcohol, and acetone. In these experiments the organic solvents were added to the protein fraction to a final concentration of 66 per cent. The solutions were allowed to stand for 3 hours at 30° C. The solvents were removed by distillation in a vacuum desiccator and dialysis against isotonic saline. Control experiments were run in which either the organic solvents were omitted or added at 0° C. and removed immediately. These controls showed slight, if any, inactivation of the material.

The effect of more extensive hydrolysis with proteolytic enzymes was then examined. One-mI, samples containing 1 mg. of the "protein fraction" (as protein), 0.01 M phosphate buffer (pH 7.1), 5 μg, terramycin, and 40 μg, of enzyme were incubated for 20 hours at 30° C. Crystalline chymotrypsin, crystalline papain, and commercial ficin were used. The mixtures with the latter two enzymes also contained cysteine (0.002 M). At the end of the digestion period, aliquots were examined both for biological activity in tissue culture and for the extent of proteolytic digestion. The latter was measured by precipitating the undigested protein content (9). In control experiments it was found that under these conditions 2 mg, of casein was completely digested to a nonacid precipitable form by each of the enzyme preparations employed. The results are shown in Table 1. It can be seen that the protein is only partially digested and that incubation with chymotrypsin or papain had little, if any, effect on the activity. It should be remembered that only twofold changes in activity could be detected by the assay. Ficin, which caused the most extensive proteolysis, destroyed between 50 and 75 per cent of the activity. The enzymes in the mixtures were still active after 20 hours of incubation, as shown by the digestion of added casein. A longer incubation period (4 days) caused an almost complete inactivation of the control mixture. The growth-promoting activity in the 20-hour digests containing chymotrypsin and papain was still nondialyzable.

**Observations on the nature of the nerve growth-promoting effect.**—The following series of experiments was performed to establish whether the continuous presence of the active material is necessary for the outgrowth of fibers. The possibility existed that we were dealing with a virus infection. In this instance, the nerve cells would be expected to proceed with fiber outgrowth following a short, single exposure to the growth factor.

The "nucleoprotein fraction" was used in these experiments, which were performed with both sensory and sympathetic ganglia isolated from 7- and 9-day chick embryos, respectively.

**TABLE 1**

**Effect of Digestion of the "Protein Fraction" with Proteolytic Enzymes**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Per cent of protein digested</th>
<th>Biological activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unincubated control</td>
<td>0</td>
<td>5-4+</td>
</tr>
<tr>
<td>Control incubated</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>without enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papain digestion</td>
<td>35</td>
<td>3+</td>
</tr>
<tr>
<td>Chymotrypsin digestion</td>
<td>49</td>
<td>9-</td>
</tr>
<tr>
<td>Ficin digestion</td>
<td>58</td>
<td>1-2+</td>
</tr>
</tbody>
</table>

In this instance, the nerve cells would be expected to proceed with fiber outgrowth following a short, single exposure to the growth factor. The ganglia were then cultured in the control medium. No outgrowth of nerve fibers occurred, and no difference could be observed between these ganglia and those which had been cultured with no prior treatments.

b) The ganglia were explanted in the standard medium containing the tumor factor and allowed to incubate at 37° C. for 6 hours, at which time the halo of nerve fibers had just begun to sprout. The ganglia then were excised and again cultured, both in the control medium lacking the growth factor and in a medium containing the factor. No outgrowth of fibers occurred in the control medium; nerve fibers were again produced in the medium possessing the agent (Figs. 5, 6).

c) The experiments in series (b) were repeated with ganglia which had been allowed to incubate for 50 hours to allow a marked outgrowth of fibers. The results were identical to those described in series (b). (It should be noted that the original fibers were destroyed when the ganglia were transferred. However, in series [b], most of the fibers had not yet appeared when the ganglion was transferred.)

It is clear that the continuous presence of the
growth factor in the medium is necessary for the outgrowth of the fibers. No evidence was obtained for the possibility of a virus-like infection.

The effect of metabolic inhibitors.—In preliminary experiments the effect of a variety of metabolic inhibitors on the outgrowth of nerve fibers in the presence of the “nucleoprotein fraction” was examined. The addition of sodium cyanide \(10^{-3}\text{M}\) and of dinitrophenol \(10^{-3}\text{M}\) did not inhibit the outgrowth. This indicates that oxidative phosphorylation is not necessary for fiber formation, although the possibility exists that cyanide- and dinitrophenol-insensitive oxidative processes occur. It was expected that inhibitors of glycolysis would be effective. In the presence of iodoacetate \(10^{-3}\text{M}\) no outgrowth occurred. However, the typical halo of fibers was not affected by the presence of 0.01 M sodium fluoride.

The above results were obtained after 20 hours of incubation. In normal cultures, in the presence of the growth factor, after 48 hours of incubation the nerve fibers are overgrown by cells migrating out from the explant (Figs. 7, 8). In the presence of fluoride or cyanide this secondary growth is completely inhibited, whereas the nerve fibers continue to grow. A 48-hour culture grown in the presence of the growth factor and sodium fluoride \(10^{-3}\text{M}\) is shown in Figure 9.

These results do not mean that glycolysis is not a prerequisite for fiber formation. In exploratory experiments a culture medium was prepared consisting of bovine fibrinogen, thrombin, “nucleoprotein fraction,” and Eagle’s synthetic mixture. Almost no fiber outgrowth occurred unless glucose was included.

Distribution of the growth-promoting factor.—Previous reports from this laboratory (8, 8) have indicated that prior passage of the tumors through the chick embryo were required to obtain the nerve growth-promoting effects. Later experiments have shown that Sarcoma 180 taken directly from the mouse does possess the activity, but in very variable amounts. In only one of eight attempts did the activity of extracts of these tumors approach that of extracts prepared from tumors which had been passed through the chick.

In other experiments, 10 per cent saline homogenates of the liver and brain of the adult mouse and of 11-day-old chick embryos invariably failed to elicit the response. Acetone powders of pig heart and pigeon liver in concentrations of 5 mg/ml were inactive. A yeast autolysate (prepared by the addition of 3 parts of 0.1 M sodium bicarbonate to 1 part of yeast and incubation for 20 hours at room temperature) at a concentration of 5 mg/ml was also inactive. Many of these “inactive” preparations failed to elicit the outgrowth of nerve fibers but did enhance the growth of fibroblasts. Of seven 10 per cent homogenates of the thigh muscle of adult mice, six were inactive. One preparation from muscle showed a definite nerve growth-promoting effect (approximately one-third as active as a comparable tumor extract). Previously reported experiments have already suggested that the sarcoma agent might be present in some mouse tissues in low concentrations (8).

The presence of a similar factor in snake venom will be discussed in a separate publication (3).

DISCUSSION

Most of the data thus far obtained suggest that the nerve growth-promoting factor is a protein or is bound to a protein. Our most purified fraction (a) is heat labile, (b) is acid labile, (c) can be activated by organic solvents, (d) is nondialyzable, (e) is precipitable by half-saturated ammonium sulfate, and (f) shows an absorption peak at 278 m\(\mu\) and upon hydrolysis an amino acid composition qualitatively similar to bovine albumin. The stability of the factor in 6 N urea and 0.1 N sodium hydroxide and its resistance to proteolytic digestion are not necessarily inconsistent with this hypothesis, since examples are known in which such treatment does not destroy the biological activity of a protein. For instance, growth hormone is stable in 0.1 N alkali and may be digested up to approximately 30 per cent with trypsin with no loss in biological activity (11). An unequivocal answer must await the isolation of the factor as a single component.

The failure of cyanide and dinitrophenol to inhibit the fiber outgrowth is surprising in view of the generally accepted view that nerve tissue is dependent on oxidative metabolism. However, it is known that certain early embryonic tissues can survive such treatment. The subject is reviewed by Willmer (13). The inhibition of cell migration by cyanide has been observed previously (4).

The differential effect of fluoride on nerve fiber and spindle-cell outgrowth has a parallel in the experiments of Spratt (12) on the chick embryo in vitro. Although glucose is necessary for the development of the nervous system, certain concentrations of fluoride \(5 \times 10^{-3}\text{M}\) will cause degeneration of the heart and some other mesodermal structures, whereas the central nervous system continues its development. Concentrations of cy-

---

Footnotes:

1. The Eagle synthetic medium was obtained from Microbiological Associates, Bethesda, Md.

2. The pig heart and pigeon liver preparations were obtained from the Worthington Biochemical Co., Freehold, N.J.
anide as high as $5 \times 10^{-3} \text{M}$ had no visible effect on development. Higher concentrations of fluoride and cyanide resulted in the degeneration of nervous tissues.

The mechanism by which the tumor factor induces the outgrowth of nerve fibers is not known. Experiments reported above show that the factor must be present continuously in the cultures to produce its effect.

**SUMMARY**

A heat-labile, nondialyzable factor with nerve growth-promoting properties has been partially purified from Sarcoma 180. The data indicate that the active material is a protein or is bound to a protein and that nucleic acids are not involved in its effectiveness. The factor must be continuously present in the cultures for the outgrowth of the nerve fibers to proceed. The outgrowth of nerve fibers was inhibited by iodoacetate, but not by cyanide, dinitrophenol, or fluoride.

**REFERENCES**

7. ———. A Diffusible Agent of Mouse Sarcoma, Producing Hyperplasia of Sympathetic Ganglia and Hyperneurotization of Viscera in the Chick Embryo. Ibid., 129:283–98, 1957.
FIGS. 7–9 are microphotographs of living sensory ganglia (from 7-day chick embryos) after 48 hours of incubation in hanging-drop tissue culture preparations.

Fig. 7.—Control culture in the standard medium.

Fig. 8.—The medium contains the "nucleoprotein fraction" at a concentration of 1.0 mg/ml.

Fig. 9.—The medium is identical with that described in Figure 8, but it contains, in addition, sodium fluoride at a concentration of 0.01 M.
Purification and Properties of a Nerve Growth-promoting Factor Isolated from Mouse Sarcoma 180

Stanley Cohen and Rita Levi-Montalcini

Cancer Res 1957;17:15-20.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/17/1/15