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*A NERVE GROWTH-STIMULATING FACTOR ISOLATED FROM SNAKE VENOM**

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Introduction.—In the past our efforts have been directed toward the purification of an agent contained in mouse sarcomas 180 and 37 which has remarkable growth-promoting effects on sympathetic and spinal ganglia of the chick embryo.[1, 2, 3] In the course of attempts to characterize this material, use was made of crude snake venom as a source of phosphodiesterase. Treatment of the sarcoma factor with snake venom enhanced its activity, and subsequent tests showed that snake venom alone contains a very potent growth-promoting agent. In fact, it can be shown that crude venom is approximately 3,000–6,000 times as active as crude tumor homogenates (on a dry-weight basis) in promoting nerve fiber outgrowth in spinal ganglia in vitro. In the following, we are presenting the results of a partial purification and characterization of the snake-venom factor.

Materials and Methods.—Dried moccasin snake venom (*Agkistrodon piscivorus*) was obtained from the Ross Allen Reptile Institute. For the assay of the nerve growth-promoting activity, hanging-drop tissue cultures were made containing plasma (rooster), synthetic medium 1066 (with thrombin), and the material to be tested. The details of the procedure have been presented elsewhere.[3] Each culture contained three or four sensory ganglia isolated from 7-day chick embryos. The cultures were observed after 18 hours of incubation at 37°, and the growth of the fibers was semiquantitatively recorded from 1+ to 4+ (see Figs. 1–4 of our previous publication[2]). The assay was sensitive to twofold changes in concentration of the active material; smaller changes were not detectable.

Experimental Results.—The effect of crude venom when it is added to the cultures at a concentration of 7 μg/ml is shown in Figures 1 and 2. The active component of the venom was nondialyzable. Solutions of the venom (30 μg/ml in distilled water) were then treated with alkali (0.1 *N* sodium hydroxide for 1 hour at 26°), acid (0.1 *N* hydrochloric acid for 1 hour at 26°), 6 *N* urea (1 hour at 0°), and heat (5 minutes at 90°) in a manner identical to that described elsewhere[3] for the tumor factor. The biological activity was stable to urea and alkali and was completely destroyed by the heat and acid treatments. This behavior is similar to that shown by the "protein fraction" isolated from sarcoma 180.

In exploratory experiments it was found that the effect of the venom was abolished by the anti-snake-venom serum.[4] In these experiments a mixture of venom (20 μg/ml) and antiserum (40 μg/ml) was allowed to stand for 1 hour at 26°. Aliquots

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were then tested for biological activity. The inhibitory effect of the antiserum suggests that the active factor is associated with one of the protein components of the venom.

When the solution of the crude venom was allowed to stand overnight at 20°, approximately three-fourths of the activity was destroyed. However, if the crude venom was treated with 6 *N* urea, its stability was increased, and only slight loss of activity (less than 25 per cent) was noted after standing for 18 hours at 20°. This may be due to the destruction by the urea of an inactivating enzyme in the venom. Advantage of this was taken during the following purification procedure.

One gram of venom (*A. piscivorus*) and 36 gm. of urea were dissolved in 80 ml. of water containing 3 ml. of 0.1 *N* NaOH. The solution was allowed to stand for 90 minutes at 0°. All subsequent procedures were carried out at temperatures from 0° to 3°. Saturated ammonium sulfate (at 0°, pH 7.6) was added to the solution until a final concentration of 48 per cent saturation was obtained. The mixture was allowed to stand for 15 minutes and was then centrifuged for five minutes at 16,000 × *g*. The precipitate was discarded. To the supernatant fluid ammonium sulphate was added to a final concentration of 71 per cent saturation. The mixture was again allowed to stand for 15 minutes and then was centrifuged. The yellowish supernatant fluid was discarded. The precipitate, containing the active factor, was dissolved in 40 ml. of water and dialyzed against distilled water overnight. The material was then centrifuged for 10 minutes at 16,000 × *g*, and the slight precipitate was discarded. The supernatant fluid was again fractionated with the above saturated ammonium sulfate solution (in the absence of urea); the active material precipitated between 46 and 59 per cent saturation. After centrifugation the precipitate was dissolved in 20 ml. of water and dialyzed overnight against distilled water. This material may be stored in the frozen condition for at least several weeks with no detectable loss in activity. Ninety-five milligrams (dry weight) and approximately 40 per cent of the original total activity was recovered; a fourfold purification was thus achieved.

The biological activity of this material is more stable to the denaturing effects of the organic solvents than was the factor purified from sarcoma 180. To solutions containing 30 μg/ml were added ethanol, methanol, and acetone, to a final concentration of 66 per cent. The mixtures were allowed to stand for 3 hours at 30°. The organic solvents were removed by vacuum distillation at 3° and dialysis. Over 50 per cent of the original activity was retained. Similar treatment almost completely inactivated the tumor factor.[2]

The 280/260 mμ absorption ratio in distilled water, pH 7.0, was 1.75. The addition of streptomycin (0.01 *M*) caused no visible precipitation. These data indicate that only traces, if any, of nucleic acids were present. One milligram of this material was equivalent to 1.3 mg. of protein as determined by the procedure of Lowry *et al.*,[5] using bovine albumin as a standard; subsequent fractionations were based on protein content.

The venom factor was then further purified by adsorption and elution from the anionic and cationic cellulose resins DEAE-SF (1.1 meq/gm) and CM-cellulose (0.5 meq/gm).[6] Ten milliliters of the ammonium sulfate fraction (containing 3.3 mg. of protein per milliliter) were passed through a column 1.4 cm. in diameter containing 500 mg. of the DEAE-SF at a flow rate of 0.1–0.2 ml/minute. The column was then washed with 10 ml. of distilled water. Only traces of protein and

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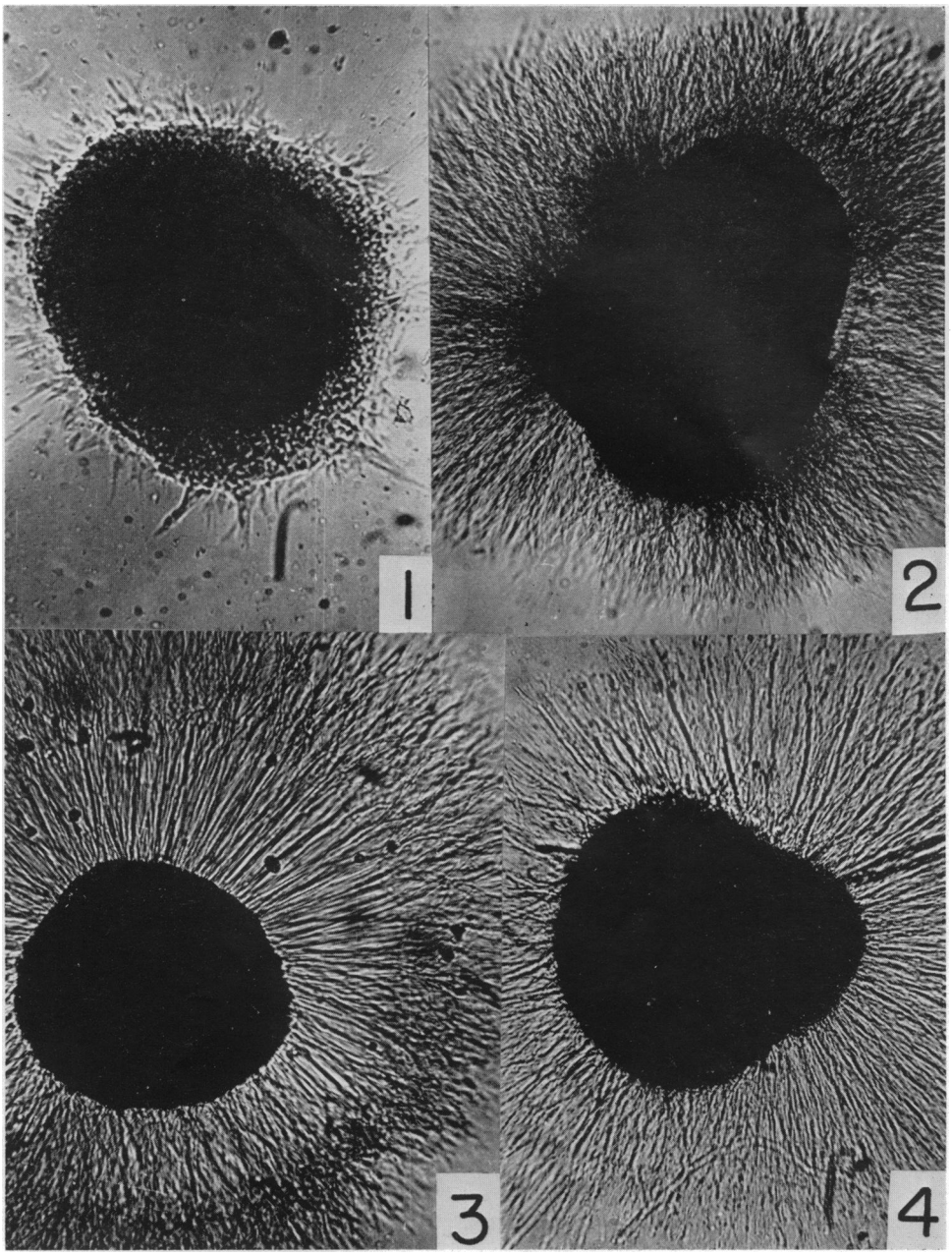

FIGS. 1–4.—Microphotographs of living sensory ganglia (from 7-day chick embryos) after 18 hours of incubation at 37°. Fig. 1, the standard control medium was used. Fig. 2, the medium contained 7 μg/ml of crude snake venom. Fig. 3, the medium contained 0.3 μg/ml of the CM fraction obtained from the venom. Fig. 4. The medium contained 250 μg/ml of the "protein fraction" purified from sarcoma 180.

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no activity were present in this combined fraction. The active material was then eluted with 20 ml. of 0.01 *M* sodium chloride in 0.01 *M* potassium phosphate buffer, pH 7.4. This fraction (DEAE fraction) contained 15 mg. of protein and almost all (over 75 per cent) of the biological activity. The 18 mg. of protein which remained on the column could be quantitatively eluted with 20 ml. of 0.5 *M* sodium chloride in the phosphate buffer. This fraction was biologically inactive.

The active fraction was then dialyzed with stirring for 5 hours against distilled water, and 10 ml. containing a total of 7 mg. of protein were applied to a column 1.4 cm. in diameter containing 500 mg. of CM-cellulose at a flow rate of 0.1–0.2 ml/minute. The column was then washed with 10 ml. of water. This combined fraction contained 4.1 mg. of protein and was inactive. The activity was then eluted with 10 ml. of 0.1 *M* sodium chloride in 0.01 *M* potassium phosphate buffer, pH 7.4. This fraction (CM fraction) contained 1.9 mg. of protein and again almost all the activity (over 75 per cent). The remaining 1 mg. of protein could be eluted with 0.5 *M* sodium chloride in phosphate buffer and was inactive.

To summarize: approximately 25 per cent of the activity present in 1 gm. of the crude venom was recovered in 11.7 mg. of a "protein" fraction, a purification of approximately twenty fold.

The effect of the CM fraction on sensory ganglia in tissue cultures at a protein concentration of 0.3 μg/ml is shown in Figure 3. For comparison, the effect of the "protein fraction" obtained from sarcoma 180 at a concentration of 250 μg/ml is shown in Figure 4. The effect of the CM fraction in promoting the outgrowth of nerve fibers in the spinal ganglia appears to be identical with that of the tumor factor. In addition, as will be shown in another publication,[7] the injection of the DEAE fraction into the yolk of the chick embryo duplicates the nerve growth-promoting properties of the sarcomas in the embryo.

The venom from the two species examined, the moccasin (*A. piscivorus*) and the rattlesnake (*Crotalus adamanteus*), were equally effective in promoting fiber outgrowth in tissue culture.

The question arises concerning the identity of the factors purified from sarcoma 180 and venom. It would seem highly improbable that two chemically identical proteins would be found in such unrelated materials. The possibility that both factors have a common enzymatic activity is under investigation.

Summary.—A heat-labile, nondialyzable factor with very potent nerve growth-stimulating properties has been partially purified from snake venom. The biological effects of the venom factor, tested in tissue culture, are very similar to the effects produced by a factor obtained from sarcoma 180. However, our most purified venom preparations have a specific activity (on a protein basis) approximately 1,000 times as high as our purest tumor fraction. The data suggest that in both instances the active material is a protein or is bound to a protein.

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[1] R. Levi-Montalcini, H. Meyer, and V. Hamburger, *Cancer Research*, **14**, 49–57, 1954.

[2] S. Cohen, R. Levi-Montalcini, and V. Hamburger, these PROCEEDINGS, **40**, 1014–1018, 1954.

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[3] S. Cohen and R. Levi-Montalcini, *Cancer Research* (in press).

[4] Antivenin (Crotalidae) polyvalent was obtained from Wyeth Laboratories, Inc.

[5] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.*, **193**, 265–275, 1951.

[6] We wish to thank Drs. A. Kornberg and H. Pahl for the diethylaminoethyl (DEAE) and carboxymethyl (CM) cellulose ion exchangers.

[7] R. Levi-Montalcini and S. Cohen, these PROCEEDINGS, **42**, 695–699, 1956.

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*BACTERIAL PROTOPLASTS INDUCED BY PENICILLIN**

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Bacterial protoplasts are believed to be cellular units that have been deprived of their rigid cell wall.[1] Accordingly, they are distinguished by their spherical shape (in bacilliform species) and their sensitivity to cytolysis in hypotonic media. Several authors[1–3] have suggested that protoplasts might be useful material for the study of biosynthesis of viruses and enzymes in a system simpler or more accessible to external modification than the intact cell. So far, only gram-positive bacteria, which are susceptible to lysozyme, have been used for such studies. A method has now been found for the efficient production of protoplasts from enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*, which are already familiar physiological and genetic subjects. The technique consists essentially of the exposure of growing cells to a medium containing penicillin, sucrose, and Mg^{++}.

Preparation of Protoplasts.—Escherichia coli strain K-12 and a variety of its mutant substrains are used in most of the experiments. The use of penicillin and sucrose was suggested by the possible analogy between penicillin and lysozyme as lytic agents and by the finding that hypertonic sucrose would interrupt bacteriolysis of *Bacillus megaterium* by lysozyme.[1] In addition, spherical bodies had been casually noted in other applications of penicillin,[4] and many authors have emphasized its use in the production of *L*-forms.[5] The following procedure was adopted after empirical trials and can doubtless be further improved.

The bacteria were grown overnight in tubes with 10 ml. of broth (Difco penassay medium) at 37° C., on a rotator. Samples of 3 ml. of the grown culture (about 2×10^9 cells/ml) were added directly to 10 ml. of broth supplemented with penicillin, 1,000 u/ml, sucrose 20 per cent, and magnesium sulfate 0.2 per cent. In 2–3 hours the cells were quantitatively converted into spheres. During this interval the optical density (measured at 650 mμ in a Coleman 14 spectrophotometer) increased about 50 per cent, but the total count (spheres or rods, estimated in a Petroff-Hausser chamber) remained constant. The spheres promptly lysed when the suspension was diluted in distilled water, and they are therefore regarded as "protoplasts."

The indicated supplements are in substantial excess, and nearly-optimal yields of protoplasts can be obtained with 5 per cent sucrose, 100 u. penicillin, and 0.1 per cent $MgSO_4$. The high magnesium requirement may depend partly on binding with the sodium citrate used in the compounding of the penicillin preparation

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