The Preparation of Nerve Growth Factors from Snake Venoms

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The growth of fibres from embryonic sensory and sympathetic ganglia of chick and from sympathetic ganglia of neonatal mouse is specifically stimulated both *in vivo* and *in vitro* by so-called NGF† present in snake venoms, mouse salivary gland and certain mouse sarcomas (Levi-Montalcini, 1964, 1965).

There is general agreement that these factors are protein in nature. Cohen (1959) isolated a protein from the venom of *Angkistrodon piscivorus* that showed a single peak on sedimentation analysis (S_{20} 2.2s) and that evoked the characteristic 'halo' effect in hanging-drop cultures of chick sensory ganglia at concentrations of approx. 10^{-7} g./ml. and above. Similarly a protein, of unspecified activity, thought to have a molecular weight of approx. 24000, has been isolated from the venom of *Crotalus adamanteus* (Angelletti, Calissano, Chen & Levi-Montalcini, 1967). In contrast, NGF from mouse salivary glands, active at 10^{-8} g./ml., is reported to have a molecular weight of approx. 140000 and to dissociate readily into several sub-units, one of which (molecular weight approx. 30000) retains 30% of the activity (Varon, Nomura & Shooter, 1967 a,b).

In order to obtain highly purified material for biological and biochemical investigations we have purified NGF from three snake venoms. We have used the conventional hanging-drop culture assay system but with collagen (rat tail) as a supporting medium instead of the usual plasma clot. We have found this to be more convenient and, though the fibres now bundle strikingly, the response to a given concentration of NGF is qualitatively similar to that on plasma clot. All quoted activities refer to a +4 response on the accepted arbitrary scale (Cohen, Levi-Montalcini & Hamburger, 1954). The solutions used for the chromatographic procedures (which were all carried out at 3°) contained 10% (w/w) of crude venom or active fraction; 0.5 M-NaCl solutions were used for gel filtration. Protein solutions were ultrafiltered and freeze-dried after each step.

Venom from *Vipera russelli* from different

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† Abbreviation: NGF, nerve growth factor(s).

suppliers or in different batches from the same supplier shows considerable variation in composition. The following refers to experiments with venom obtained from the Haffkine Institute (Bombay, India). (a) A solution of crude venom (2g.) was passed down a column (5 cm. $\times$ 100 cm.) of Sephadex G-150 (flow rate 60 ml./hr.). (b) The active fraction (eluted at 855–1134 ml., 0.62g., active at 5×10^{-8} g./ml.) was passed down a column (5 cm. $\times$ 100 cm.) of Sephadex G-75 (flow rate 60 ml./hr.). (c) The active fraction (eluted at 720–870 ml., 0.13g., active at 10^{-8} g./ml.) from (b) was chromatographed on Cellex T (BioRad Laboratories, Richmond, Calif., U.S.A.) in 0.025 M-carbonate-bicarbonate buffer, pH 10.8. A fraction (4mg.) showing activity at 10^{-14} g./ml. was obtained as the first peak.

We have examined some of the properties of three samples of NGF prepared in the above fashion: NGF-A was obtained as a sharply delineated fraction from the final step, NGF-B was the centre cut of a rather diffuse final fraction from another experiment and NGF-C was the combined side cuts from this fraction. All were non-diffusible on dialysis, had isoelectric points in the range pH 9.5–11.0 and showed activity at 10^{-14} g./ml. The Edman procedure showed the presence of *N*-terminal histidine. All three samples sedimented as a single peak in the ultracentrifuge (0.1 M-carbonate-bicarbonate buffer, pH 10.3; S_{20} values were 2.92, 2.83 and 2.88s respectively). On diffusion analysis the areas under the synthetic-boundary curves were constant with time (D_{20} values were 6.33, 6.86 and 6.72×10^{-7} cm.²/sec. respectively). The three proteins were therefore homogeneous to sedimentation and diffusion analysis. The values of the molecular weight calculated from the above data and assuming a partial specific volume of 0.745 are 42000, 38000 and 39000 respectively. The difference between these values are not considered to be significant. NGF-C was also examined at a lower concentration (4 instead of 13 mg./ml.) and at a different pH (7.5). The values of the molecular weight found were 37000 and 39500 respectively, and again are not considered to show significant differences. In view of the homogeneity of these samples in the

ultracentrifuge and of their ability to evoke response in the assay system at very low concentration we consider that we have isolated the active molecule and that we are not dealing with a carrier protein that contains only a small amount of NGF, a possibility that is difficult to exclude with less active preparations. Nevertheless, some difference can be detected between NGF-A and the other two samples. Disc electrophoresis of NGF-B or NGF-C on polymethylacrylamide gel at pH 5 with a buffer system that gives very high resolution, consisting of potassium cacodylate and 6-aminoheptanoic acid (A. R. Berry & H. ff. S. Davies, unpublished work), showed the presence of a single, rather diffuse, band. On the other hand NGF-A gives two, slightly more slowly moving, diffuse bands which are so close that we have not succeeded in separating them. These differences may reflect the presence of distinct but closely related types of NGF in the crude venom or may be the result of conformational changes.

The highly active material rapidly becomes less potent on storage in solution: an equivalent response to that given at a concentration of 10^{-14} g./ml. requires 10^{-10} g./ml. after storage in phosphate-buffered saline at pH 7.4 at a concentration of 10 mg./ml. for 6 days at 2°, but this less active material can be stored at pH 10.8, even in very dilute solution, for up to 10 weeks without further appreciable change. Freeze-dried NGF is more stable, but activity falls to the 10^{-10} g./ml. level over about 12 weeks at 2°.

Passage of NGF-B through a calibrated Sephadex column at pH 10.8 (0.1 M-carbonate-bicarbonate buffer) gave two active peaks, one, the major component, corresponding to molecular weight of approx. 24 000 and the other to a molecular weight of approx. 38 000. This result, which we treat with caution since the molecule must have an unusual shape (from the values of *S* and *D*), may mean that the protein has sub-unit structure.

The venom from *Vipera ammodytes ammodytes* (obtained as a gift from the Institute of Epidemiology, Sofia, Bulgaria) contains NGF whose activity is masked by the presence of a toxin (13%, w/w). The NGF may be obtained by the following procedure. (a) A solution of crude venom (1 g.) was passed down a column (3.2 cm. $\times$ 100 cm.) of Sephadex G-75. (b) The active fraction (second peak, 0.25 g., active at 10^{-7} g./ml.) was chromatographed on DEAE-Sephadex A-50 in 0.01 M-tris buffer, pH 8.9. (c) The active fraction, which was the first peak, from (b) was chromatographed on Cellex T in 0.01 M-guanidine hydrochloride buffer, pH 11.8. The final sample (6 mg.) obtained on application of a medium salt gradient was active at 10^{-10} g./ml. and showed behaviour on gel

electrophoresis closely resembling that of NGF-A from the venom of *Vipera russelli*.

A convenient method for the preparation of moderately active material from *Angkistrodon piscivorus* (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) is as follows. (a) A solution of crude venom (1 g.) was passed down a column (3.2 cm. $\times$ 100 cm.) of Sephadex G-75. The activity was eluted in the second and third peaks. This step was then repeated on the active fraction; the protein was eluted as two peaks, and gave material (0.125 g.) active at 10^{-6} g./ml. located between them. (b) This was chromatographed on CM-Sephadex C-25 with acetate buffer, pH 5.6, and a salt gradient (to 1.0 M-NaCl). The active fraction (20 mg., active at 10^{-7} g./ml.), gave a sharp and a fainter, more diffuse, band on starch-gel electrophoresis (pH 8.0). In the ultracentrifuge it sedimented as a single peak (*S*_{20,w} 2.2 s). The preparation is clearly very similar to that described by Cohen (1959), but whether it is an NGF of intrinsically low activity or is a protein contaminated with a small amount of highly active NGF is, as yet, impossible to decide.

Winick & Greenberg (1965) report that only sensory ganglia from 7–13-day chick embryos will respond to NGF. Using our preparations we have regularly observed partial response at 5 days and full response from 7 to 12 days. A response can also be observed even with 16½-day embryos, but the effect is less consistent, possibly because of the presence of more extraganglionic tissue. We have also found that neither embryo extract nor serum is necessary for the growth of fibres if small amounts of NGF are present in the culture medium.

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