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STUDIES ON THE NERVE GROWTH FACTOR(NGF) FROM SNAKE VENOM MOLECULAR HETEROGENEITY

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SUMMARY

The nerve growth factor from the venoms of *Crotalus adamanteus* and *Bothrops jararaca* was analyzed by chromatography and gel filtration. The data obtained suggest that purified nerve growth factor aggregates and dissociates to form active molecules of various molecular weights. Evidence was found for the existence of an active nerve growth factor, possibly a fragment or a subunit, having a molecular weight of less than 5,000.

INTRODUCTION

Venoms from the three families of poisonous snakes have been shown to contain a specific protein factor endowed with a unique nerve-growth promoting activity[1–3]. Small amounts of crude venom (0.4–0.2 μg protein) added to the culture medium of explanted chicken embryonic sensory ganglia elicit a vigorous outgrowth of nerve fibers[2]. The venom NGF was identified with a protein molecule of approximately 20,000 molecular weight. Gel filtration studies[4], however, have shown that the NGF may be present in various venoms at different levels of complexity. In the present study, venoms of two species of *Crotalidae* were analyzed comparatively.

MATERIAL AND METHODS

Venom preparations

The Butantan Institute of Brazil kindly supplied the venom of *Bothrops jararaca*. *Crotalus adamanteus* venom was purchased from Sigma Chemical Co. (St. Louis). Various amounts of these dried venoms were weighed and dissolved in the desired buffer, maintaining the temperature from 0–4°. After centrifugation for 20 min at 10,000 r.p.m. in a Sorvall centrifuge at 0–4°, the active supernatants were used for chromatography.

Assays

Nerve-growth promoting activity was assayed in the fractions by the tissue-culture method devised by LEVI-MONTALCINI[5,6] using sensory ganglia from 8-day chick embryos. Series of five-fold dilutions were tested for each fraction. The dilution of the purified fractions was performed in physiological saline containing 2 mg/ml bovine serum albumin. The presence of inert proteins at the higher dilutions stabilized the NGF response. One Biological Unit was defined as the amount of each fraction necessary to produce a 3+ response *in vitro* (nerve fiber outgrowth).

Estero-protease activity was assayed by the hydrolysis of α-N-benzoyl-L-arginine ethyl ester (BAEE) obtained from Sigma Chemical Co.[7]. Incubations were carried out in Tris–HCl buffer, 50 m*M*, pH 8.0.

Proteins were measured by the method of LOWRY *et al.*[8] using bovine serum albumin as standard. Protein concentration in chromatographic and gel filtration experiments was approximated by measuring the optical density at 280 mμ using a Zeiss spectrophotometer.

Chromatography and gel filtration

Sephadex G-100 and G-75 medium grade, and Sephadex G-25 fine grade, were obtained from Pharmacia (Uppsala). Diethylaminoethyl cellulose (DEAE-cellulose) anion exchanger and carboxymethyl cellulose (CM-cellulose) cation exchanger were both of the microgranular, preswollen type of Whatman (DE 52, CM 52). Blue Dextran (Pharmacia) and purified cytochrome c, chymotrypsin, bovine serum albumin, and tyrosine from Sigma Chemical Co. were used to calibrate the gel filtration columns. Chromatography and gel filtration were carried out at 4°.

Electrophoresis and ultracentrifugation

Electrophoresis on polyacrylamide gel was carried out by the method of DAVIES[9] and ORNSTEIN[10] with the modifications of REISFELD AND LEWIS[11] used for electrophoresis at pH 4.3. Gels were stained with Coomassie Brilliant Blue R250 at a 1% concentration in 12.5% trichloracetic acid. Ultracentrifugation was carried out in a Spinco model E ultracentrifuge at 59,000 r.p.m.

RESULTS

NGF from Crotalus adamanteus venom

The procedure adopted to analyze the NGF from this venom is summarized in Fig. 1. The supernatant from 1 g of crude venom dissolved in 6 ml of 50 m*M* Tris–HCl buffer, pH 7.3, was directly applied to a large Sephadex G-100 column equilibrated with the solvent buffer. The most active fractions, eluted in a broad region from 60,000 to 20,000 molecular weight, were collected and applied to a DEAE column after dialysis against the starting buffer, 5 m*M* Tris–HCl, pH 7.3. After thorough washing with the starting buffer, a gradient of NaCl (from 0 to 1 *M*) was applied to the column. All activity was recovered in the first unabsorbed peak and therefore together with proteins having zero or positive charge under these conditions. The active DEAE fractions were pooled and concentrated by lyophilization, and then applied to a Sephadex G-75 column at pH 7.3. The NGF activity separated in this way from a shoulder of esterase activity and other components was equilibrated at pH 5.0 with

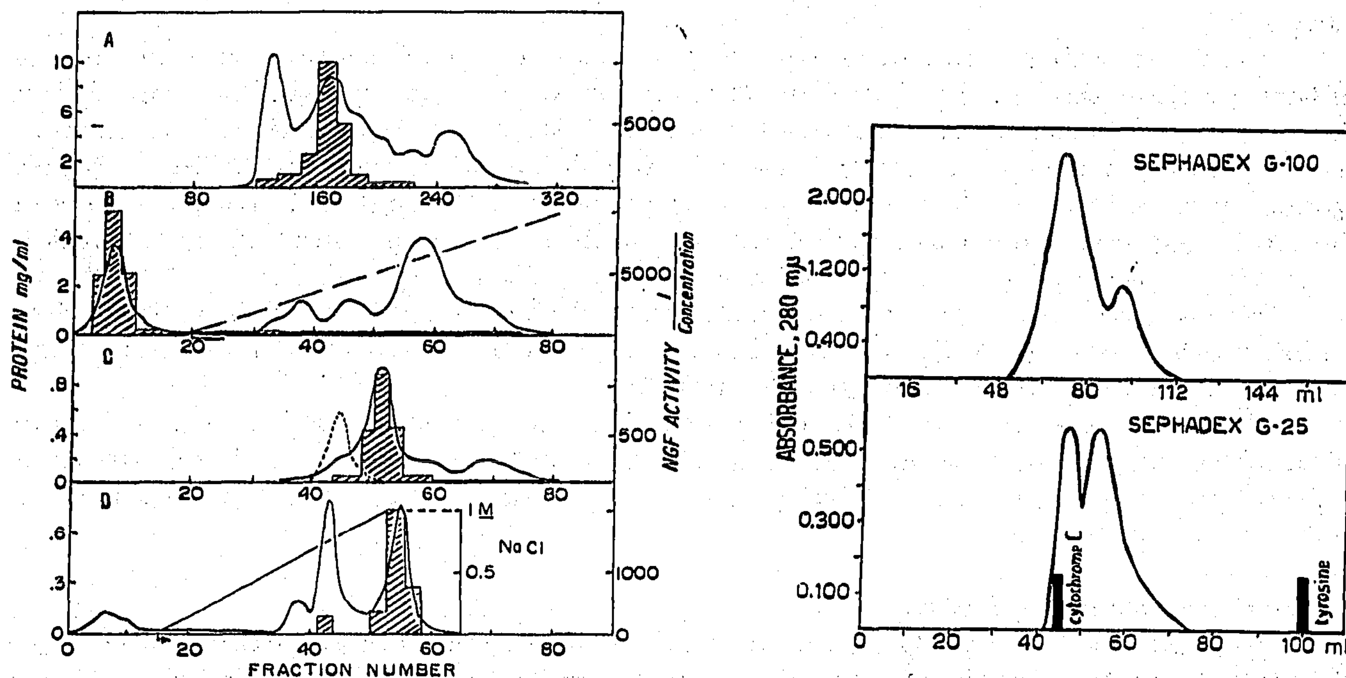

Fig. 1. Gel filtration and chromatography of NGF from *Crotalus adamanteus.* (A) Sephadex G-100 column, 115 × 3.5 cm, 50 m*M* Tris–HCl buffer, pH 7.3. (B) DEAE-cellulose column, pH 7.3. Dashed line indicates gradient of NaCl from 0 to 1 *N*. (C) Sephadex G-75 column, 100 × 1.5 cm, 50 m*M* Tris–HCl, pH 7.3. The dotted line indicates localization of esterase activity measured with BAEE as substrate. (D) CM-cellulose column, pH 5.0. Solid lines indicate protein concentration. Hatched areas localize NGF activity.

Fig. 2. Gel filtration on Sephadex G-100 and G-25 of purified *Crotalus adamanteus* NGF at pH 5.0. Bars indicate the elution volumes of cytochrome *c* and tyrosine.

acetate–acetic acid buffer, 50 m*M*. Chromatography on a carboxymethyl cellulose column was then performed using a gradient salt elution of 0 to 1 *M* NaCl. As shown in Fig. 1, most of the proteins were bound to the exchanger and were fractionated into a number of components. NGF activity was localized primarily in the third post-gradient peak, with a minor component of activity in the second.

This most active fraction was concentrated by lyophilization and applied to a Sephadex G-100 column equilibrated with acetate–acetic acid buffer, 50 m*M*, pH 5.0. As shown in Fig. 2, two main peaks appeared containing approximately equal amounts of NGF activity. The first, broad peak (A) was contained within elution volumes corresponding to a molecular weight of 30,000 to 20,000; whereas, the second component (B) was eluted in the range of 12,000 molecular weight. When this second peak of NGF activity was concentrated by lyophilization and reapplied on Sephadex G-25 column at pH 5.0, again two distinct components were obtained of approximately equal specific activity. The first was eluted in the exclusion volume and the second (C) was retarded. Ultracentrifugal analysis of components A and B gave $S_{20,w}$ values of 2.53*s* and 1.56*s* respectively (Fig. 3). As shown in Fig. 3, the sedimentation profile emerges as a single symmetrical peak, however, after 96 min the pattern of B begins to broaden and flatten assymetrically, that of A broadens, but remains symmetrical.

Another similar preparation when fractionated on Sephadex G-100 at pH 7.3, 50 m*M* Tris–HCl buffer, gave rise to three protein peaks having approximately the

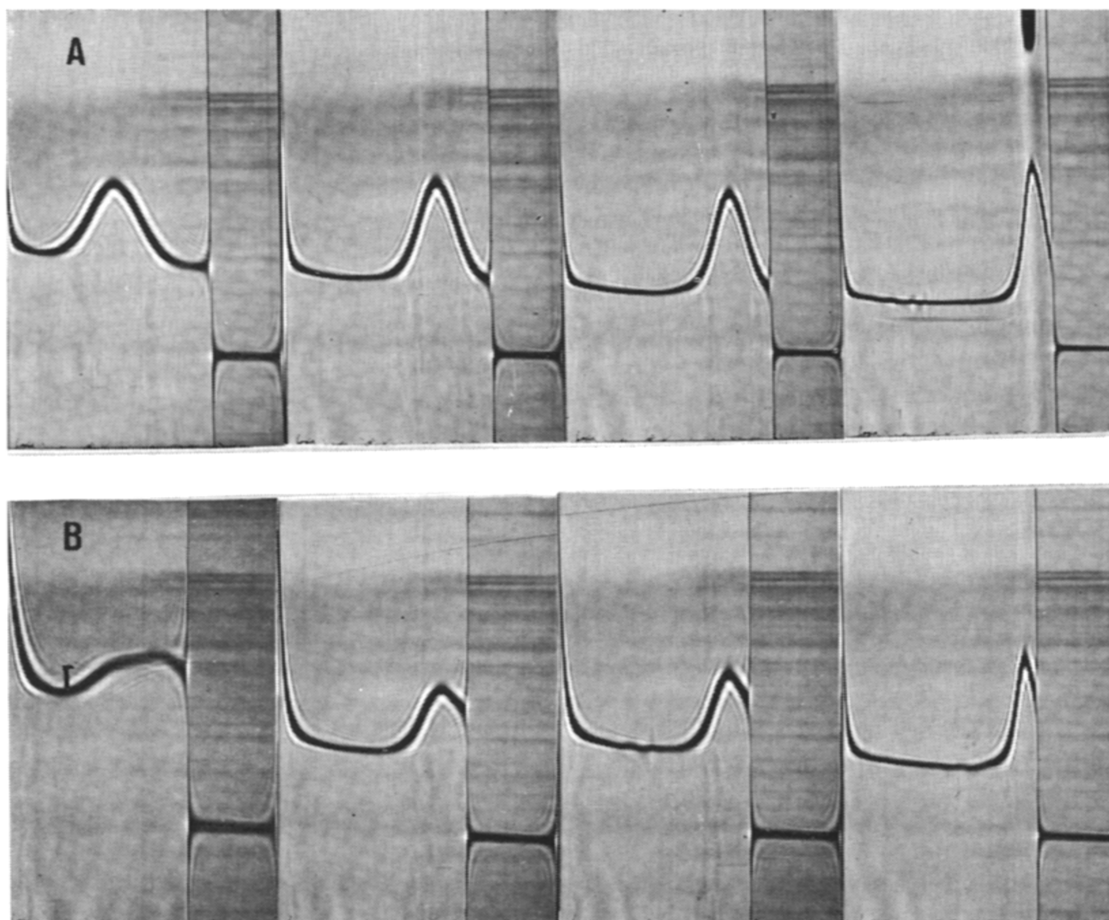

Fig. 3. Ultracentrifugation of NGF medium molecular weight components A and B (see text) from *Crotalus adamanteus* venom. Runs were performed at a rotor speed of 59,000 r.p.m. Samples were dissolved in 100 mM acetate–acetic acid buffer pH 5.0. Photographs were taken every 16 min. The photographs from left to right are at times of 112, 64, 48, and 16 min, respectively.

same NGF specificity. As seen in Fig. 4, the first peak was excluded from the G-100 and is thus of molecular weight greater than 100,000. The second peak appeared at the same point as the A component of the other preparations, and had a similar $S_{20,w}$ of 2.56. The third broad peak D appeared later in the chromatogram as compared to B of the earlier preparations, and when rechromatographed on Sephadex G-25 appeared as a single, slightly retained peak.

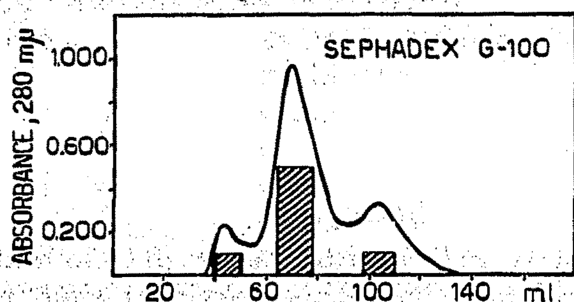

Fig. 4. Gel filtration of a purified *Crotalus adamanteus* NGF on Sephadex G-100 at pH 7.3. Hatched areas indicate NGF activity.

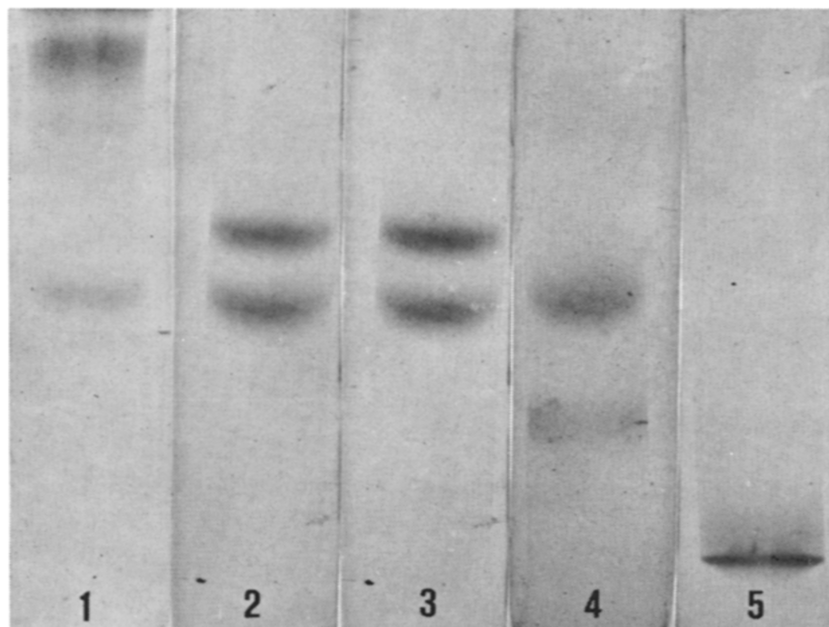

Fig. 5. Electrophoresis of various NGF components of *Crotalus adamanteus* on polyacrylamide gel at pH 4.3. (1) = Heavy molecular weight fraction; (2) = intermediate fraction A (see text); (3) = intermediate fraction B; (4) = low molecular weight fraction C; and (5) = low molecular weight component D.

The electrophoretic pattern at pH 4.3 on polyacrylamide gel of these different molecular forms of NGF obtained by gel filtration is shown in Fig. 5. As can be seen, A and B have identical patterns, each having two well-separated cathodic bands. The first excluded peak, however, contained only the more rapidly moving component plus several slowly migrating bands. The low molecular weight C fraction on the other band, had in common with the others only the faster migrating component, plus another more cathodic band. The low molecular weight fraction D exhibited only a single, very fast-moving component. When unstained gels of components A, B, C, and D were sliced and extracted with cold physiological saline, NGF activity could be recovered from the areas corresponding to each band, with no activity in other parts of the gel.

NGF from Bothrops jararaca venom

When one gram of venom from *Bothrops jararaca* was fractionated through Sephadex G-100 at pH 7.3 (Fig. 6), most of the NGF activity was recovered around the region of 40,000 molecular weight. The active fractions were pooled and applied to to DEAE column under the conditions described previously for the *C. adamanteus* venom. The major part of the activity was found in the first, unabsorbed peak. Some NGF activity under these conditions was, however, consistently found soon after the gradient began. Upon rechromatography of each peak using identical conditions, there was evidence of partial interconversion of one form into the other.

The pre-gradient peak was dialyzed against 50 m*M* acetate buffer pH 5.0 and applied to a CM-cellulose column. After initial washing with this starting buffer, a linear salt gradient from 0 to 1 *M* of NaCl was applied. As shown in Fig. 7, the activity was eluted in a rather broad peak. Esterase activity as measured by the hydrolysis of

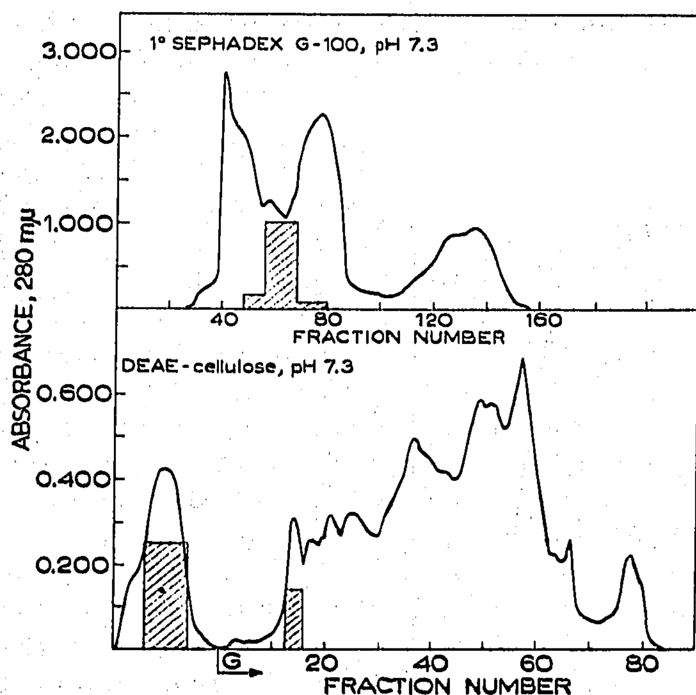

Fig. 6. Gel filtration and DEAE-cellulose chromatography of NGF from *Bothrops jararaca* venom. Hatched areas localize the NGF activity.

BAEE was also found in this peak, but could be separated from NGF upon rechromatography using a parabolic gradient from 0 to 0.5 *M* NaCl (pools 1 and 2). Successive rechromatography on CM-cellulose of both pools resulted in a further localization of the NGF activity. The most active fraction from both were finally pooled together and passed again over CM-cellulose. The activity was now recovered in one, sharp symmetrical peak. After concentration by lyophilization and subsequent dialysis, an analytical Sephadex G-100 chromatography (Fig. 7) revealed a pattern of protein and activity similar to that obtained for *Crotalus adamanteus*, the central peak having a molecular weight of approximately 30,000.

Similar results were obtained when the NGF fraction separated on DEAE-cellulose after the start of the gradient was applied on Sephadex G-100. Again NGF activity was found distributed in a broad area ranging from very high (greater than 100,000) to very low molecular weight forms. It is of interest that when the lowest molecular weight forms of NGF were pooled together and analyzed through a Sephadex G-25 column, they were found to have an elution volume sensibly greater than the void volume, and must therefore be of a molecular weight below 5,000.

DISCUSSION

LEVI-MONTALCINI first demonstrated that the nerve-growth-promoting activity elicited by some mouse tumors was due to a diffusable agent. In subsequent work, a nerve growth factor having similar biological properties was isolated in great quantities and identified as a protein molecule. The protein nature of the NGF was demonstrated by COHEN and repeatedly confirmed thereafter. The results of the present study

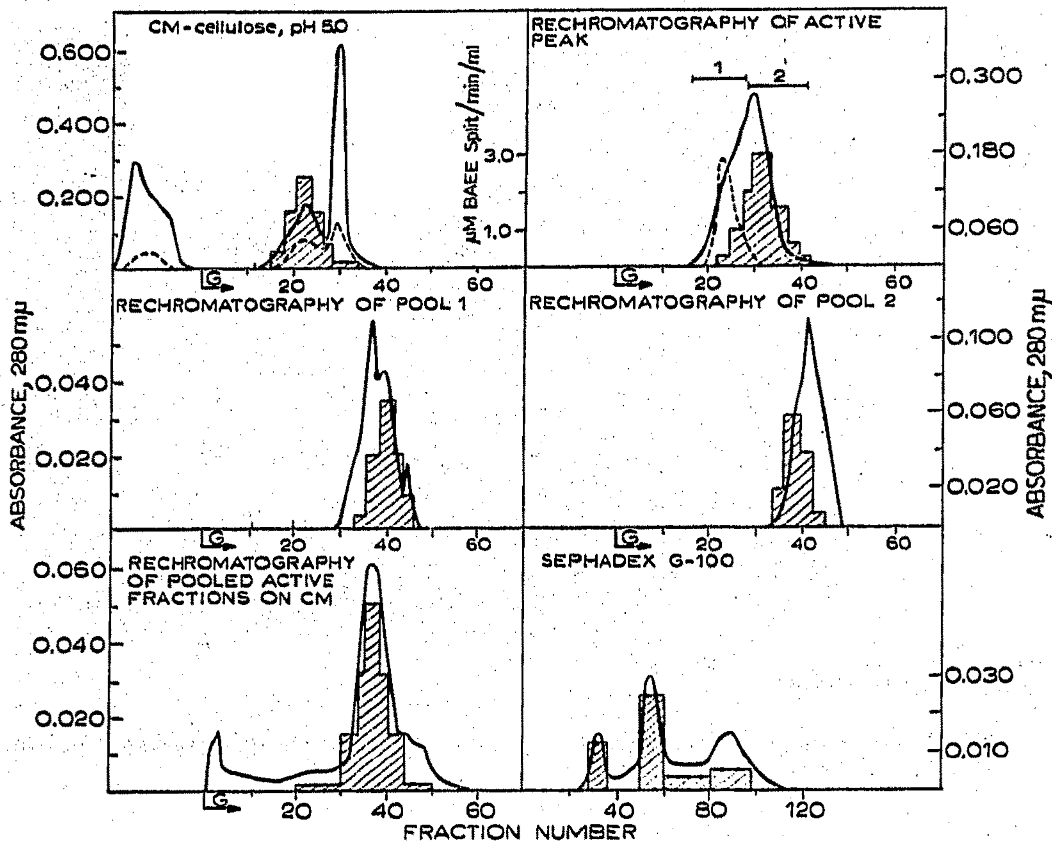

Fig. 7. Chromatography and rechromatography of *Bothrops jararaca* NGF. The first CM-cellulose chromatography was performed with a linear gradient of NaCl concentration from 0 to 1 *M* NaCl. Subsequent rechromatographies were performed on CM-cellulose columns using parabolic gradients of NaCl from 0 to 0.5 *M*. The Sephadex G-100 column was equilibrated with 50 m*M* Tris-HCl pH 7.3 + 0.100 *M* NaCl. Solid line, absorbance at 280 mμ. Dotted line, esteroprotease activity measured by hydrolysis of BAEE. Hatched areas, NGF activity.

demonstrated that snake venom NGF may exist in multiple molecular forms. Mild fractionation procedure such as gel filtration, revealed that there exists for each venom a predominant moiety of a given molecular weight value ranging in the species investigated from 60,000 to 20,000[4]. It should be pointed out that significant amounts of NGF activity are also found in other regions of the chromatogram corresponding to lower and higher molecular weights. The possibility that the various molecular forms of NGF could arise from an association-dissociation equilibrium of fundamental subunits rather than from unspecific interactions was investigated with purified preparations from *Crotalus adamanteus* and *Bothrops jararaca* venoms. In both cases there was evidence strongly in favor of this hypothesis. A highly purified and very active preparation from *Crotalus adamanteus* was resolved into two active components by gel filtration on Sephadex G-100. The second component undergoes further dissociation when processed on Sephadex G-25, giving rise to a form of molecular weight of approximately 5,000. Another purified preparation was resolved into three components on G-100, one of which was excluded from the column and therefore appears to be a large aggregate of NGF molecules. That two of the forms, with an $S_{20,w}$ of 2.53 and 1.56 respectively, had identical electrophoretic patterns consisting of two active

bands, could be attributed to the fact that each band represents a distinct active subunit or that each band represents a different state of aggregation of identical subunits.

The results obtained from *Bothrops jararaca* give evidence similarly for the existance of various molecular forms of NGF. Molecular heterogeneity was here evident from both chromatographic and gel filtration data. Two forms of NGF were in fact separated by DEAE chromatography. On rechromatography, each of these forms seemed to be moderately interconvertible to the other form. It is possible that this chromatographic behavior may result from the interaction of the NGF with other molecules, from conformational changes of the NGF, or from different states of aggregation of the NGF. One of these forms was further purified until it appeared chromatographically homogeneous. Nevertheless, this form was heterogeneous with respect to molecular weight when analyzed on Sephadex G-100, in a similar manner to the results obtained with *Crotalus adamenteus* NGF.

It is of interest that in both cases a fully active NGF of very low molecular weight, tentatively below 5,000, could be clearly demonstrated. This form may represent a fundamental NGF monomer or an NGF fragment produced during the various fractionation procedures, incomplete but still carrying biological activity. Immunochemical studies now in progress are expected to shed light on this problem.

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