Comparison of the Nerve Growth Factor Proteins from Cobra Venom (*Naja naja*) and Mouse Submaxillary Gland†

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ABSTRACT: The nerve growth factors (NGF's) isolated from cobra venom (*Naja naja*) and mouse submaxillary gland are closely related proteins. They are structurally similar in that about 60% of their amino acid residues are identical (Hogue-Angeletti, R. A., Frazier, W. A., Jacobs, J. W., Niall, H. D., and Bradshaw, R. A. (1976), *Biochemistry,* preceding paper in this issue). They are functionally similar in that they both elicit maximum neurite outgrowth from chick embryonic dorsal root ganglia at the same protein concentration. However, the extent of the response is not as great with *Naja naja* NGF. The cobra NGF has an affinity close to that of mouse NGF for a major proportion of the specific NGF receptors on cells from dissociated embryonic dorsal root ganglia. Despite these similarities there are differences which can be detected between the two proteins. High concentrations of *Naja naja* NGF will not displace approximately 20% of the binding of mouse NGF to specific NGF receptors. Moreover, *Naja naja* NGF shows limited cross-reactivity with antiserum to mouse NGF in a competition radioimmunoassay, consistent with the extent of its amino acid sequence homology with mouse NGF. *Naja naja* NGF does not interact with the α- and γ-subunits of 7S NGF to form a high molecular weight complex. In this behavior it resembles a modified form of mouse NGF which, like *Naja naja* NGF, lacks COOH-terminal arginine residues.

The two principal sources of nerve growth factor (NGF)[1] are the adult male mouse submaxillary gland (Cohen, 1960) and snake venoms (Cohen and Levi-Montalcini, 1956; Cohen, 1959). The NGF protein of the submaxillary gland is found in a high molecular weight complex, 7S NGF (Varon et al., 1967), and can be readily isolated after dissociation of the complex at acid pH. The preparation from the homogeneous complex contains predominantly two A chains (118 amino acids) (Mobley et al., 1974) and has been referred to as βNGF (Varon et al., 1968). Preparations isolated directly from the gland supernatant without prior purification of the 7S complex vary in chain composition depending on the method of preparation. The most common form, prepared as described by Bocchini and Angeletti (1969), contains approximately one A chain and one B chain (110 amino acids) and has been commonly referred to as 2.5S NGF. Both βNGF and 2.5S NGF are dimers (Angeletti et al., 1971; Greene et al., 1971) of molecular weight 26500, and the amino acid sequence of 2.5S NGF has been determined (Angeletti and Bradshaw, 1971; Angeletti et al., 1973a,b). Both the primary (Frazier et al., 1972) and three-dimensional (Frazier et al., 1973) structures of mouse NGF are similar to those of insulin and proinsulin.

The NGF protein from cobra venom (*Naja naja*) has been characterized previously (Angeletti, 1970) and its subunit structure and partial amino acid sequence have now been determined (Hogue-Angeletti et al., 1976). *Naja naja* NGF is a dimer of molecular weight similar to that of mouse NGF. The tentative amino acid sequence of *Naja naja* NGF indicates that the maximum possible number of identities between mouse NGF and *Naja naja* NGF is 64% of the amino acid residues. The two proteins appear to have comparable in vitro and in vivo biological activities with respect to their action on embryonic sensory and sympathetic neurons (Angeletti, 1970). The comparison of the mouse and *Naja naja* NGFs has now been extended, as reported here, to determine (a) the affinity of *Naja naja* NGF for the specific NGF receptors on embryonic dorsal root ganglion cells (Herrup and Shooter, 1973; Frazier et al., 1974), (b) the ability of *Naja naja* NGF to compete with mouse NGF for binding to antibody to mouse NGF, and (c) the ability of the cobra venom NGF to form a high molecular weight complex with the α- and γ-subunits of mouse 7S NGF.

Materials and Methods

NGF Proteins. Mouse 7S NGF was isolated by the procedure of Varon et al. (1967). The three subunits, βNGF, α, and γ, were isolated from 7S NGF as described by Smith et al. (1968). βNGF was stored, frozen, at concentrations of 1 to 3 mg/ml in 0.2% acetic acid. The α- and γ-subunits and 7S NGF were stored, frozen, at concentrations of 2 to 3 mg/ml in phosphate buffer (pH 6.7) and ionic strength

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[1] Abbreviations used are: NGF, nerve growth factor; bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Temed, *N,N,N',N'*-tetramethylethylethylenediamine; PBG, phosphate-buffered Gey's solution.

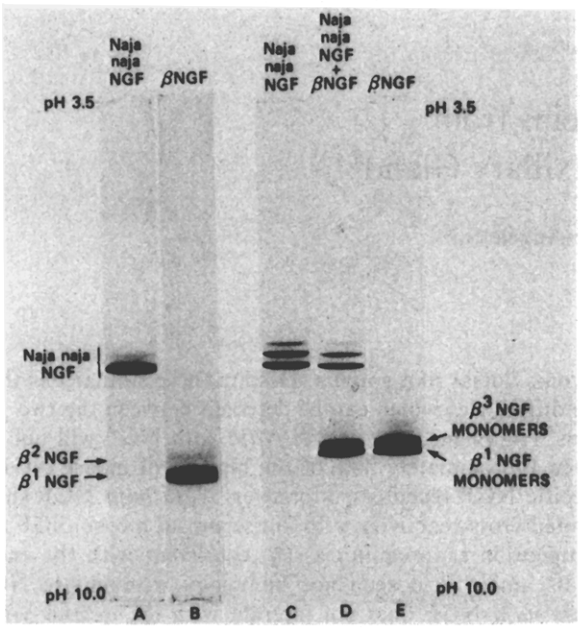

FIGURE 1: Isoelectric focusing of *Naja naja* NGF and βNGF in the absence and presence of urea. Analyses in gels A and B were carried out in the absence of urea: (A) 25 μg of *Naja naja* NGF; (B) 25 μg of βNGF; urea was present in the samples loaded onto gels C, D, and E as well as in the gels; (C) 50 μg of *Naja naja* NGF; (D) 25 μg of *Naja naja* NGF and 25 μg of βNGF; and (E) 50 μg of βNGF. β^1NGF is an NGF dimer with intact COOH-terminal arginine residues and β^2NGF is a dimer in which one chain lacks a COOH-terminal arginine residue. (β^3NGF, a dimer in which both chains lack these residues, was not detected in the βNGF preparation used in these analyses.) In the presence of urea β^1NGF dissociates into two β^1NGF monomers while β^2NGF dissociates into one β^1NGF monomer and one β^3NGF monomer.

0.05. Snake venom NGF was isolated from *Naja naja* venom by the method of Hogue-Angeletti et al. (1976) and lyophilized. Sequence data, ultracentrifugal studies, and the results from polyacrylamide gel electrophoresis at pH 9.5 or in the presence of sodium dodecyl sulfate indicate that the *Naja naja* NGF preparation used in this study was homogeneous (Hogue-Angeletti et al. 1976). For the experiments reported here 0.2% acetic acid was added to the lyophilized material to make solutions of concentrations from 1.4 to 2.0 mg/ml.

Chemicals. Ampholine (pH 3.5–10) was obtained from LKB-Produkter AB (Sweden), UltraPure urea from Schwarz/Mann, bis-tris[1] from General Biochemicals, Tes[1] from Calbiochem, and bovine plasma albumin from Armour Pharmaceutical Co.

Bioassay for NGF. The biological activites of βNGF and *Naja naja* NGF were measured using dorsal root ganglia from 8-day-old chick embryos grown on collagen coated cover slips in Gey's balanced salt as described by Herrup and Shooter (1973).

NGF Receptor Assay. The ability of *Naja naja* NGF to compete with [^{125}I]βNGF for binding to specific NGF receptors on cells from dissociated 8-day-old chick dorsal root ganglia was measured by the procedure of Herrup and Shooter (1973).

Immunodiffusion and Radioimmunoassay. Antiserum to βNGF was prepared in 8-week-old female New Zealand albino rabbits. Animals were injected initially with 2 mg of βNGF in complete Freund's adjuvant, boosted 1 month later with 1 mg of protein in the adjuvant, and bled 7 to 10 days later (Server and Shooter, manuscript in preparation). Immunodiffusion experiments used the Ouchterlony precedure (Ouchterlony, 1953). The radioimmunoassays were carried out using antiserum adsorbed to polystyrene tubes as described for the NGF system by Ishii and Shooter (1976).

Electrophoresis and Isoelectric Focusing in Acrylamide Gels. Electrophoresis in 7.5% acrylamide gels used a bis-tris–Tes system with a resolving pH of 7.05 at 25° as described by Server and Shooter (manuscript in preparation). After electrophoresis, gels were stained with 1% Acid Fast Green in 10% acetic acid and destained in the same solvent. Isoelectric focusing in 7.5% acrylamide gels followed the procedure of Perez-Polo and Shooter (1974) except that the riboflavin solution contained 6 mg/100 ml and the Temed[1] solution 0.8 ml/100 ml. Electrofocusing was carried out at 100 V for 1 hr and 200 V for 2 hr. After removal of ampholine by washing with 20% trichloroacetic acid, the gels were stained with 0.1% Naphthol Blue Black in 10% acetic acid and destained in the same solvent. The procedure for carrying out these analyses in the presence of 8 *M* urea has also been described (Server and Shooter, manuscript in preparation). The urea gels were washed, stained, and destained in the same way as the urea-free gels described above.

Results

Isoelectric Focusing Analyses. As noted in a previous study (Angeletti, 1970) *Naja naja* NGF has a lower isoelectric point than βNGF (Figure 1A,B). Like βNGF, *Naja naja* NGF showed a minor component of slightly lower isoelectric point, although the difference in isoelectric points betweeen the major and minor components was less in *Naja naja* NGF than in βNGF. The minor component in βNGF, β^2NGF, has been identified as an NGF dimer in which one of the NGF chains lacks its COOH-terminal arginine residue (Moore et al., 1974). This explanation cannot hold for *Naja naja* NGF because it does not have COOH-terminal arginine residues (Hogue-Angeletti et al., 1976). In the presence of 8 *M* urea, *Naja naja* NGF was separated into three components and βNGF into two (Figures 1C,D,E). In the case of βNGF, the component with the higher isoelectric point consists of single dissociated chains which retain their COOH-terminal arginine residues (β^1NGF monomers), while the component with the lower isoelectric point consists of NGF chains which lack this residue β^3NGF monomers) (Moore et al., 1974). Since the *Naja naja* NGF peptide chains do not show heterogeneity at either the NH_2 or COOH terminal in the sequence analyses, the multiple species observed in the isoelectric focusing analyses may have been generated by deamidation of asparagine and/or glutamine residues during gel filtration in 1 *M* acetic acid employed in the isolation procedure (Hogue-Angeletti et al., 1976).

Biological Activity and Specific Binding to Dorsal Root Ganglionic Cells. In the in vitro bioassay, βNGF elicited its maximal neurite outgrowth from embryonic dorsal root ganglia at a concentration of 10 ng/ml (Figure 2). *Naja naja* NGF showed a similar pattern of response with a peak response also at 10 ng/ml. The length and density of neurite outgrowth, however, were considerably lower with *Naja naja* NGF. More cell migration out of the ganglia was also noticed in these bioassays.

A further comparison of the biological activities of the two NGF's was made by measuring the ability of *Naja naja* NGF to compete with [^{125}I]βNGF for binding to the specific NGF receptors on cells from dissociated 8-day-old chick

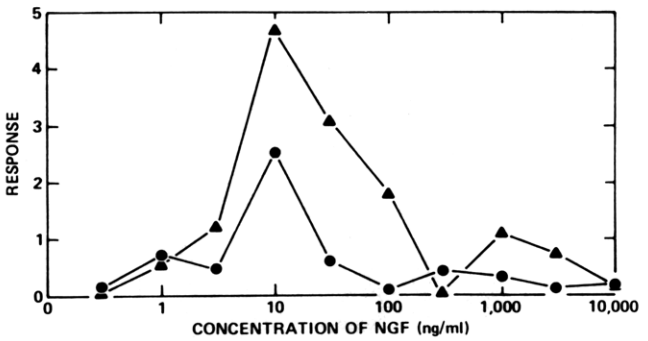

FIGURE 2: Bioassays of *Naja naja* NGF and βNGF. Response was measured in arbitrary units from 0 to 5, 0 indicating no response, 5 indicating maximal neurite outgrowth: mouse βNGF (▲); *Naja naja* NGF (●).

dorsal root ganglia. Cells were incubated either with [^{125}I]βNGF sufficient to saturate all the specific NGF receptors or with mixtures of this same amount of [^{125}I]βNGF plus increasing quantities of unlabeled *Naja naja* NGF. Specific binding was assayed by the procedure of Herrup and Shooter (1973). In a series of control experiments, unlabeled βNGF was used instead of the *Naja naja* NGF. The greatest amount of unlabeled βNGF added was 313 times the amount of [^{125}I]βNGF; the residual binding of the labeled βNGF under these conditions was the nonspecific binding of [^{125}I]βNGF. This nonspecific binding was 11% of the total binding and was subtracted from all points. The competition with unlabeled βNGF (Figure 3) confirmed that the native protein had the same affinity for the NGF receptors as [^{125}I]βNGF since the fraction of specifically bound [^{125}I]βNGF was equal, at all points, to the fraction of [^{125}I]βNGF in the protein mixture (Herrup and Shooter, 1973). The ability of *Naja naja* NGF to compete with [^{125}I]βNGF (Figure 3) appeared similar to that of βNGF at concentrations of *Naja naja* NGF up to about 30 ng/ml (50% displacement). Above this concentration, *Naja naja* NGF increasingly failed to compete for an equivalent fraction of the NGF receptor sites. At the highest concentrations of *Naja naja* NGF (a ratio of *Naja naja* NGF to [^{125}I]βNGF of 242:1) approximately 20% of the NGF receptors still bound [^{125}I]βNGF. This behavior suggests that *Naja naja* NGF does not bind to approximately 20% of the NGF receptors on the cells. The curve for *Naja naja* NGF was reproduced in two experiments of the type shown in Figure 3 and in one experiment in the presence of bovine plasma albumin where the nonspecific binding was reduced to 7% of the total binding.

The Ability of Naja naja to Cross-React with Antiserum to βNGF. While antiserum to βNGF gave a precipitin band with βNGF in the Ouchterlony assay, no such band was observed with *Naja naja* NGF at the same protein concentration (Figure 4) nor at a four-fold higher concentration. A similar result was reported by Angeletti (1971) who noted that the concentration of *Naja naja* NGF required for the formation of a precipitin band was at least 50 times the concentration of mouse NGF required. In the radioimmunoassay (Figure 5) *Naja naja* NGF showed very little ability to compete with [^{125}I]βNGF for binding to antibody to βNGF. This was in marked contrast to the competition for binding by native βNGF in the control assay. At most, *Naja naja* NGF displaced 10% of the labeled βNGF from the binding sites in the antiserum. By these two criteria *Naja naja* NGF shows only limited cross-reactivity with

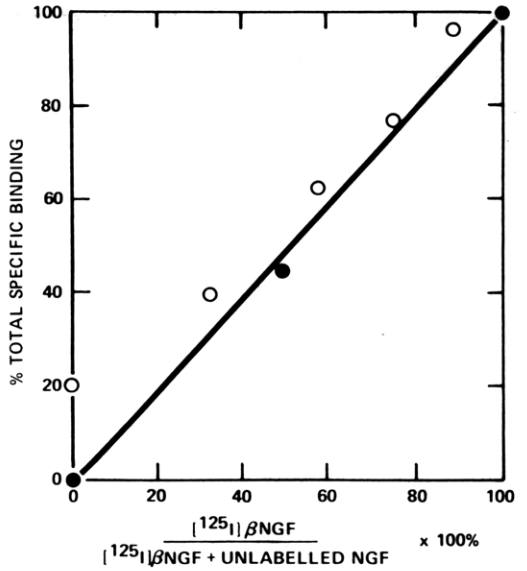

FIGURE 3: Ability of *Naja naja* NGF to compete with [^{125}I]βNGF for binding to specific NGF receptors on dorsal root ganglion cells. Cells (2.3×10^6) from dissociated 8-day-old chick dorsal root ganglia, suspended in 300 μl of PBG,[1] were incubated for 90 min at 25° with either 300 μl of PBG containing 18 ng of [^{125}I]βNGF (31.2×10^3 cpm per ng) or 300 μl of PBG containing 18 ng of [^{125}I]βNGF plus 2.7, 6.4, 12.9, 38.7, or 4350 ng of *Naja naja* NGF. In the control experiment the same number of cells in the same volume were incubated with 300 μl of PBG containing 18 ng of [^{125}I]βNGF plus 16.9 or 5640 ng of βNGF. The final concentration of [^{125}I]βNGF was always 30 ng/ml, sufficient to saturate the specific receptors. The percent of labeled βNGF in each incubation mixture was equal to: 18 ng of [^{125}I]βNGF/(18 ng of [^{125}I]βNGF + unlabeled NGF) × 100. After incubation, 100-μl samples were loaded on top of a two-step sucrose gradient in Beckman microfuge tubes and centrifuged as described by Herrup and Shooter (1973). The bottom 5 to 6 mm of each tube, containing the pelleted cells, was cut off and the tops and bottoms counted in a Nuclear-Chicago well type γ-scintillation counter. The amount of [^{125}I]βNGF bound to the cells was calculated as cpm bottom/(cpm bottom + cpm top) × ng of [^{125}I]βNGF in each tube and standardized to 10^5 cells. The percentage of total specific binding was calculated by dividing the difference between the [^{125}I]βNGF bound in a given mixture and that bound in the presence of excess unlabeled βNGF by the difference between the [^{125}I]βNGF bound in the absence of any added unlabeled NGF and that bound in the presence of excess unlabeled βNGF and multiplying by 100; mouse βNGF (●); *Naja naja* NGF (○).

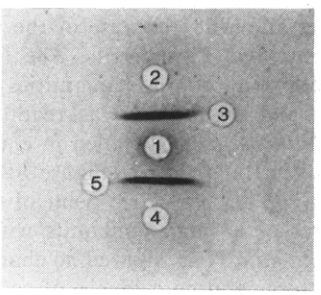

FIGURE 4: Immunodiffusion patterns of antiserum to βNGF with *Naja naja* NGF and βNGF: (well 1) 4.5 μl of antiserum to βNGF; (wells 2 and 4) 2.3 μg of βNGF; (wells 3 and 5) 2.3 μg of *Naja naja* NGF.

antibody to βNGF.

Attempt to Recombine Naja naja NGF with the α- and γ-Subunits of 7S NGF. One of the characteristic properties of βNGF is its ability to recombine with the α- and γ-subunits to form the 7S NGF complex, which does not occur when the COOH-terminal arginine residues are removed

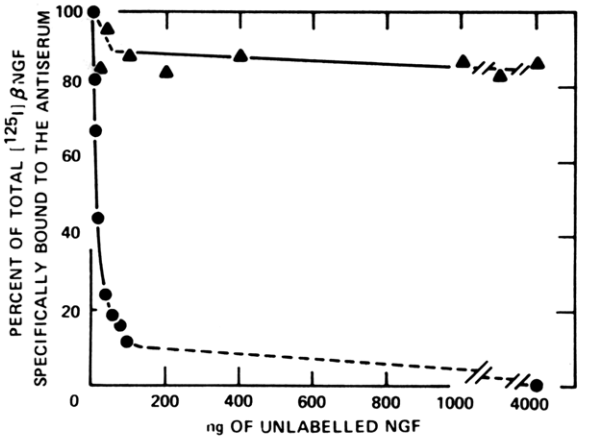

FIGURE 5: Ability of *Naja naja* NGF to compete with [^{125}I]βNGF for binding to antibody to βNGF as determined in a radioimmunoassay. Polystyrene tubes were coated with 0.3 ml of a 1:1000 dilution of the antiserum prepared against βNGF in 0.05 *M* NaCl, 0.05 *M* Tris (pH 7.4) and sodium azide (0.2 mg/ml). The antiserum solution was aspirated and the tubes washed twice with 0.05 *M* acetate buffer (pH 4.0). To the tubes were then added 100 μl of bovine serum albumin (1 mg/ml in the acetate buffer), 40 ng of [^{125}I]βNGF, and varying amounts of unlabeled βNGF (0, 5, 10, 20, 40, 60, 80, 100, and 4000 ng) or *Naja naja* NGF (20, 40, 100, 200, 400, 1000, 2000, and 4000 ng). The total volume was adjusted with the acetate buffer to 300 μl. After incubation at 4° for 20 hr the contents of the tubes were aspirated, the tubes washed once with the acetate buffer and twice with deionized water, and then counted in a Nuclear-Chicago well type γ-scintillation counter. Nonspecific binding of [^{125}I]βNGF (binding in the presence of excess unlabeled βNGF) was 7% of the total binding: mouse βNGF (●); *Naja naja* NGF (▲).

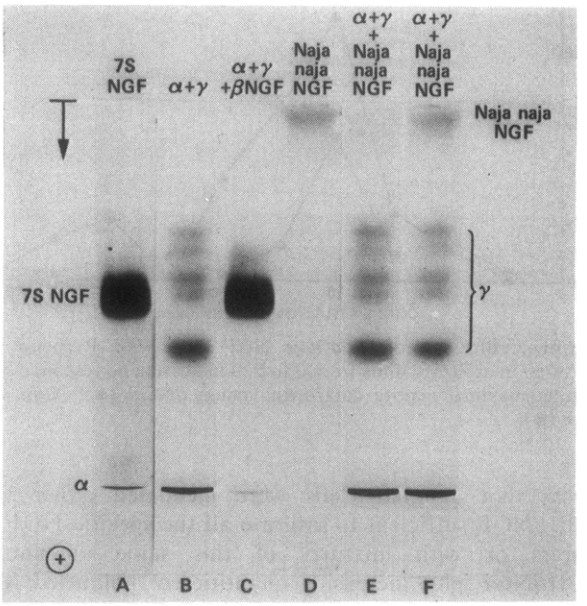

FIGURE 6: Attempted recombination of *Naja naja* NGF with the α- and γ-subunits of 7S NGF. Analyses were carried out in 7.5% acrylamide gels in the bis-tris–Tes system at pH 7.05 as described by Server and Shooter (manuscript in preparation). Protein samples were added to phosphate buffer (pH 6.7) and ionic strength 0.05 to give a final volume of 200 μl (final pH 6.4) and then incubated at 4° for 16 hr before being made 5% in sucrose and loaded onto the gels: (A) 100 μg of 7S NGF; (B) 35 μg of α-subunits and 45 μg of γ-subunits; (C) 35 μg of α-subunits, 45 μg of γ-subunits, and 30 μg of βNGF; (D) 30 μg of *Naja naja* NGF; (E) 35 μg of α-subunits, 45 μg of γ-subunits, and 15 μg of *Naja naja* NGF; (F) 35 μg of a α-subunits, 45 μg of γ-subunits, and 30 μg of *Naja naja* NGF.

from the two peptide chains of the mouse NGF dimer (Moore et al., 1974). It was of interest, therefore, to determine whether *Naja naja* NGF, a protein quite similar to mouse NGF, but not having COOH-terminal arginine residues, would take part in complex formation. The formation of 7S NGF or other complexes was followed by electrophoresis. In the bis-tris–Tes system, at pH 7.05, 7S NGF showed slight dissociation as judged by the appearance of a small amount of α-subunits, the latter co-migrating under these conditions (Figure 6A). This is in contrast to its behavior in the bis-tris–Tes system at pH 7.55 where it does not show dissociation (Smith et al., 1968). The γ-subunits showed five components (Figure 6B) rather than the usual three (Varon et al., 1968) because of the resolution of γ^1 and γ^2 each into two components. The formation of 7S NGF after adding appropriate quantities of βNGF to a mixture of α- and γ-subunits was readily demonstrated (Figure 6C). *Naja naja* NGF failed to enter the resolving gel in a sharp band at this pH (Figure 6D). This was expected since it has an isoelectric point of 6.75 (Angeletti, 1970). Mixtures of α- and γ-subunits with two different amounts of *Naja naja* NGF showed no change in their electrophoretic patterns and no evidence of complex formation (Figures 6E,F). No interaction of *Naja naja* NGF with either α- or γ-subunits themselves was found (data not shown).[2]

[2] It is of interest that Perez-Polo (1974) has recently isolated an NGF complex from *Crotalus adamantus* venom which is similar in structure to the $\alpha\beta$ complex formed between the βNGF and the α-subunits of 7S NGF. The subunits of the snake venom complex freely interchange with the corresponding subunits of 7S NGF. The difference in the binding domains of the *Crotalus adamantus* and *Naja naja* NGF's suggests that these proteins differ in certain of their structural properties.

Discussion

The data presented here confirm and extend earlier observations on the properties of *Naja naja* NGF. Its isoelectric point is significantly lower than that of mouse NGF (Angeletti, 1970). As noted above, the different species observed in the isoelectric focusing analyses of *Naja naja* NGF, in the presence and the absence of urea, may have resulted from different levels of deamidation of the asparagine and/or glutamine residues in the *Naja naja* NGF chains during purification.

Naja naja NGF elicits its maximal neurite outgrowth from embryonic chick ganglia at the same protein concentration as mouse NGF. It was a consistent finding, however, that less neurite outgrowth but more cell migration was observed in bioassays of *Naja naja* NGF than in those of mouse NGF. No explanation for these observations can be provided, at present, although they may be the biological manifestations of the differences in the structures of the two NGF proteins revealed in the sequence analysis (Hogue-Angeletti et al., 1976).

These structural differences may also account for the results of the competition assays with [^{125}I]βNGF in which *Naja naja* NGF was less effective in displacing [^{125}I]βNGF from dorsal root receptors. Eighty percent of the total receptors bound both mouse and cobra venom NGF indistinguishably. The proportion of those that did not, approximately 20% of the total, was significantly higher than the level of nonspecific binding in these experiments (11% in the presence of bovine plasma albumin, 7% in its absence) suggesting that this phenomenon is not an artifact of the experimental procedures. An explanation of the mo-

lecular basis for these observed differences in the interaction of βNGF and *N. naja* NGF with dorsal root ganglia is not possible from these experiments.

The results of the radioimmunoassay indicate that the ability of *Naja naja* NGF to compete with βNGF for antibody to βNGF is limited. These data are consistent with the approximately 60% amino acid sequence homology of the two proteins (Hogue-Angeletti et al., 1976). Prager and Wilson (1971) have shown, in a study of lysozyme molecules from different species, that proteins differing from each other by 40% or more in amino acid sequence tend to exhibit no cross-reactivity. The findings presented here on the degree of immunological relatedness of *Naja naja* NGF and βNGF with respect to antiserum to βNGF agree with the data of Angeletti (1971). As expected from the degree of identity in the sequences of the two proteins, the extent of immunological cross-reactivity of *Naja naja* NGF and mouse NGF is limited.

The recombination experiments show that *Naja naja* NGF does not interact with the γ-subunits of 7S NGF in either the absence or the presence of the α-subunits from this complex, analogous to the behavior of βNGF with both COOH-terminal arginine residues removed (Moore et al., 1974). The latter result supports the hypothesis (Angeletti and Bradshaw, 1971; Frazier et al., 1972; Moore et al., 1974) that βNGF peptide chains are derived from the longer pro-NGF chains by the action of an arginine esteropeptidase (the γ-subunits). According to this model, the active site of the γ-arginine esteropeptidase remains bound to the COOH-terminal arginine residue of the growth factor chain following the cleavage event. In the absence of the COOH-terminal arginine residue, the enzyme no longer recognizes the remaining βNGF chain as the product of its cleavage. The precursor hypothesis can be extended to include the synthesis of *Naja naja* NGF peptide chains. In the cobra, however, the action of the arginine esteropeptidase cleaving enzyme would be followed by a further proteolytic event to remove the newly formed COOH-terminal arginine residues by a carboxypeptidase B-like enzyme, as occurs in the conversion of proinsulin to insulin (Steiner et al., 1974). Arginine esteropeptidase activity, although extremely labile, has been detected in the venom of the *Naja naja* cobra (Perez-Polo, 1971). The processing enzyme may act as it does in the proinsulin to insulin system, within subcellular particles such as secretory granules (Steiner et al., 1974).

The 60% amino acid sequence homology of the NGF proteins from mouse and cobra venom indicates that the primary structure of NGF has been conserved through evolution (Hogue-Angeletti et al., 1976). Moreover, the similarities of these two proteins in their stimulation of neurite outgrowth and their affinity for NGF receptors on chick dorsal root ganglia cells suggest that, in particular, those regions of the NGF molecules which interact with the NGF receptor have been highly conserved. Some minor modifications must have occurred over the course of evolution since differences are observed between the two proteins in the qualitative aspects of their biological response and the concentration of protein needed to displace the binding of labeled mouse NGF in single cell suspensions of chick dorsal root ganglia. It is the structural and functional similarity of the two NGF proteins, however, which stands out in this report and the preceding one by Hogue-Angeletti et al. (1976).

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