Nerve Growth Factor: Multiple Dissociation Products in Homogenates of the Mouse Submandibular Gland. Purification and Molecular Properties of the Intact Undissociated Form of the Protein†

Michael Young,* Judith D. Saide, Richard A. Murphy, and Muriel H. Blanchard

ABSTRACT: Recent studies from this laboratory have shown that nerve growth factor (NGF) is secreted by many diverse types of cells in culture, including fibroblasts, L cells, muscle cells, and certain tumor cells. Further, studies of the molecular properties of cell-secreted NGF (Pantazis, N. J., et al. (1977) *Biochemistry 16,* 1525; Murphy, R. A., et al. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74, 4496) have revealed that this protein exhibits a much greater stability in solution than do previously recognized forms of the protein purified from mouse submandibular glands. This observation prompted us to reexamine the properties of NGF as it exists in fresh mouse submandibular gland extracts. Results indicate that NGF occurs as multiple (at least six) molecular species. Five of these species are unstable, and they dissociate in dilute solution at relatively high protein concentrations. The single stable and largest form of the protein has been purified. It is fully stable in the ng/mL range of concentration, and it has a molecular weight of 116 000. It seems likely that the five unstable species of NGF arise through degradation of the larger stable species in vivo and (or) in vitro. The properties of this protein are closely similar to the forms of NGF secreted by muscle and L cells in culture as well as the stable species of NGF which is naturally secreted in mouse saliva.

In 1958 Levi-Montalcini and her co-workers (1958) discovered that adult male mouse submandibular glands contain extraordinarily high concentrations of nerve growth factor (NGF),[1] and since that time several molecular forms of the protein have been described. Before proceeding to the purpose and results of the present study, it will be useful to summarize certain pertinent features of the various forms of NGF which have been recognized over the past 20 years.

(1) In 1960, Cohen (1960) first purified NGF from mouse submandibular glands. The protein exhibited potent nerve growth promoting activity in vitro, and it possessed a sedimentation coefficient of 4.3 S from which a molecular weight of 44 000 was estimated. To our knowledge, this species of NGF has not received further study since its original description by Cohen.

(2) Bocchini & Angeletti (1969) have purified salivary gland NGF in yet another form (molecular weight = 26 000). This species was given the name 2.5S NGF (referring to its sedimentation coefficient), and it is composed of two identical, noncovalently linked polypeptide chains, each of molecular weight 13 259 (Angeletti et al., 1973). The 2.5S NGF comprises a rapidly reversible monomer $\rightleftarrows$ dimer equilibrium system in solution with an association constant at neutral pH of $9.4 \times 10^6\ M^{-1}$. Consequently, at biologically active concentrations of the protein (about 1 ng/mL), solutions of 2.5S NGF consist virtually entirely of monomer (Young et al., 1976a).

(3) Over the past 10 years, Shooter & his colleagues have studied a third form of NGF. This protein is called 7S NGF; it has a molecular weight near 140 000 (Varon et al., 1967), and it is composed of three different kinds of subunits, termed α, β, and γ (Varon et al., 1968). These subunits have also been shown to display considerable heterogeneity (Smith et al., 1968). Only the β subunit is biologically active in promoting nerve fiber outgrowth; it is chemically closely similar to the 2.5S NGF discussed above. Because certain properties of the 7S NGF system are relevant to the studies to be described below, we shall discuss them in detail here.

Evidence from several sources indicates that 7S NGF is an unstable complex in solution. For example, in their original paper on the purification of this protein, Varon et al. (1967) noted that good recovery of NGF from one of their purification steps (DEAE-cellulose chromatography) was obtained only if the column was operated at an extraordinarily high flow rate (350 mL/h). At more normal flow rates, recovery was poor, and NGF activity appeared in many chromatographic fractions. Varon et al. (1967) attributed this behavior to progressive dissociation of the 7S complex during its elution from the column. Subsequent studies have revealed that 7S NGF is in equilibrium with its subunits at neutral pH and at relatively high protein concentrations (Greene et al., 1969; Smith et al., 1969). Moreover, two recent studies have confirmed earlier work on the dissociation of 7S NGF. For example, from sedimentation equilibrium measurements, Baker (1975) observed that 7S NGF begins to dissociate into its constituent polypeptide chains at neutral pH and at relatively high protein concentrations (0.2–1 mg/mL). Pantazis et al. (1977a) examined the dissociation behavior of 7S NGF at even lower total protein concentrations and found that at concentrations of 1 μg/mL this protein dissociates completely. Thus, all available evidence indicates that 7S NGF is unstable, and that it is fully dissociated at concentrations much higher than those required to display biological activity in the sensory ganglion assay system (Pantazis et al., 1977a).

(4) Recent studies in this laboratory have revealed that a variety of cells in culture secrete NGF (for a summary of the evidence, see Young et al., 1976b). Moreover, the mouse

† From the Laboratory of Physical Biochemistry and the Department of Medicine, Massachusetts General Hospital, and the Departments of Physiology and Anatomy, Harvard Medical School, Boston, Massachusetts 02114. *Received September 1, 1977.* This work was supported by National Institutes of Health Grant CA 17137 to M.Y., by National Institutes of Health Grant NINCDS-13674, and a grant from the Muscular Dystrophy Association of America to R.A.M. J.D.S. was supported by National Institutes of Health Postdoctoral Fellowship 32HL05070.

[1] Abbreviation used: NGF, nerve growth factor.

0006-2960/78/0417-1490$01.00/0

submandibular gland has now been shown to be an exocrine, not an endocrine, gland with respect to NGF (Murphy et al., 1977a), and extraordinarily large amounts of the factor are secreted in mouse saliva (Wallace & Partlow, 1976; Murphy et al., 1977a). These observations led us to examine the molecular properties of NGF in saliva as well as those of NGF secreted by cells in culture, with the following results.

Mouse L cells secrete a highly stable form of NGF whose molecular weight has been estimated to be on the order of 160 000 by gel filtration chromatography (Pantazis et al., 1977b). This protein contains as part of its structure a component (subunit) which is biologically, immunologically, and electrophoretically indistinguishable from the 26 000 molecular weight 2.5S NGF described under 2 above. Furthermore, L-cell NGF remains stable and does not dissociate even at concentrations as low as 3 ng/mL (Pantazis et al., 1977b). Still another form of cell-secreted NGF that has been studied is that produced by primary rat muscle cell cultures (Murphy et al., 1977b). Like L-cell NGF, this protein is also highly stable in dilute solution, and it exhibits a molecular weight near 150 000. Within experimental error, this number is not different from the molecular size of the NGF secreted by L cells.

The observation that both L-cell and muscle-cell secreted nerve growth factors are stable at very low concentrations, whereas both the purified 7S and 2.5S NGF gland species are not, was a puzzling finding, and it led us to examine the properties of NGF as it is secreted in saliva and to reexamine the properties of NGF as it exists in fresh submandibular gland extracts (Murphy et al., 1977c). Results demonstrated that saliva NGF exists in at least two forms. One of these is completely stable at high dilution, and it has a molecular weight of 114 000 as estimated by gel filtration chromatography; the other and smaller saliva form (molecular weight 13 000) arises from time dependent degradation of the larger species. Like saliva NGF, gland NGF also was found to exist in at least two molecular forms—one of high molecular weight and one or more species of lower molecular weight (Murphy et al., 1977c).

Taken together, all of the evidence presented above raised the possibility that the well-known purified forms of submandibular gland NGF (7S NGF and 2.5S NGF) might not be the forms of nerve growth factor which occur naturally in the submandibular gland and which are secreted in saliva under physiological conditions.

Consequently, in the study to be presented below, we have returned to the submandibular gland in order to try to resolve some of these problems. Results indicate that fresh submandibular gland homogenates contain multiple (at least six) molecular species of NGF which can be resolved by several chromatographic procedures. Furthermore, all but one of these species are appreciably unstable in dilute solution, and they dissociate to yield the single chain monomer (molecular weight 13 000), which is the subunit of 2.5S NGF. In contrast, only one of the species of gland NGF is stable in highly dilute solutions. We present a procedure for the complete purification of the single stable form of the protein, and we find that its properties are different from all of the submandibular gland forms of NGF which have been previously described. These results seem pertinent to further biological investigations of the function of NGF.

Experimental Procedures

Reagents. Double-glass-distilled H_2O was used for all solutions and all buffer salts were reagent grade. Sephadex, horse heart ferricytochrome *c*, ovalbumin, aldolase, bovine chymotrypsinogen A, bovine pancreatic ribonuclease A, and Dextran Blue 2000 were obtained from Pharmacia. Bovine serum albumin (crystalline) was purchased from Sigma, and ^3H_2O and $Na^{125}I$ (carrier free) from New England Nuclear. D_2O (100.0 mole percent) was obtained from Bio-Rad. DEAE-cellulose (DE-52, microgranular grade) was obtained from Whatman and hydroxylapatite (Bio-Gel HTP) from Bio-Rad.

Animals. All mice used in this study were adult male CD-1 outbred albino animals (Charles River Breeding Laboratories, Wilmington, Mass.).

Radioimmunoassay of NGF. 2.5S NGF was purified from male mouse submandibular glands by minor modifications (Oger et al., 1974) of the original method of Bocchini & Angeletti (1969). Preparations were shown to be electrophoretically homogeneous as previously described (Oger et al., 1974). Preparation of monospecific antibodies to 2.5S NGF (Oger et al., 1974) as well as the details of preparation of [^{125}I]NGF and the development and validation of the radioimmunoassay have been presented in detail elsewhere (Murphy et al., 1975; Young et al., 1977).

Biological Assays. Sensory ganglion bioassays of NGF were performed with 8-day chick embryo dorsal root ganglia as previously described (Murphy et al., 1975). All fractions which were evaluated by quantitative immunoassay for NGF were also measured for biological activity. For this purpose, all fractions separated chromatographically as described below were diluted so as to contain 1–10 ng/mL of protein and then examined for biological activity (Murphy et al., 1975).

Gel Electrophoresis. Samples were electrophoresed with 7.5% polyacrylamide gels at neutral pH at 4 °C. Both upper and lower reservoir buffers contained 0.05 M sodium phosphate, pH 7.0; protein solutions were dissolved in 0.01 M sodium phosphate, pH 7.0, containing 0.5 M sucrose and a trace of bromophenol blue. Electrophoresis was performed at a constant current of 8 mA/gel.

Sedimentation Studies. Sedimentation velocity and equilibrium measurements were performed with a Beckman Model E ultracentrifuge equipped with refractometric and absorption scanning optics. The optical systems were freshly realigned prior to these experiments by the procedures of Richards & Schachman (1959) and Schachman et al. (1962). The RITC unit of the instrument was calibrated with a National Bureau of Standards thermometer. Weight-average molecular weights were calculated from the equation:

$$M = \frac{2RT}{(1 - \bar{V}_2\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}x^2} \quad (1)$$

and values of $\mathrm{d} \ln c/\mathrm{d}x^2$ were obtained from the least-squares regression coefficients of plots of $\ln c$ vs. x^2. In all cases, the high speed meniscus depletion procedure of Yphantis (1964) was employed with 3-mm columns of protein solution.

The partial specific volume of NGF was measured with the H_2O–D_2O procedure of Edelstein & Schachman (1967) and the equation

$$\bar{V}_2 = \frac{k - R}{\rho_{D_2O} - R\rho_{H_2O}} \quad (2)$$

where

$$R = \frac{(\mathrm{d} \ln c/\mathrm{d}x^2)_{D_2O}}{(\mathrm{d} \ln c/\mathrm{d}x^2)_{H_2O}} \quad (3)$$

Here ρ_{D_2O} and ρ_{H_2O} are the solution densities and k is the ratio of the molecular weight of the deuterated protein to that of the protein H_2O. Protein solutions in H_2O or D_2O were dialyzed for 48 h against solvent to ensure complete deuterium exchange. The final D_2O concentration following dialysis was estimated to be greater than 99.8 mol %. A value of k = 1.0145

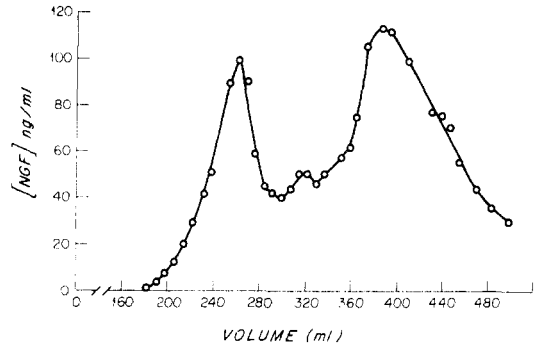

FIGURE 1: Sephadex G-200 chromatographic profile of NGF in a fresh submandibular gland extract. An extract of a pair of adult male mouse glands was prepared as described in the text. Column dimensions, 95 × 2.5 cm; solvent, 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL of bovine serum albumin, temperature, 4 °C. Solution applied (1.0 mL) contained 26.2 μg/mL of NGF as measured by radioimmunoassay. Fractions were measured by radioimmunoassay.

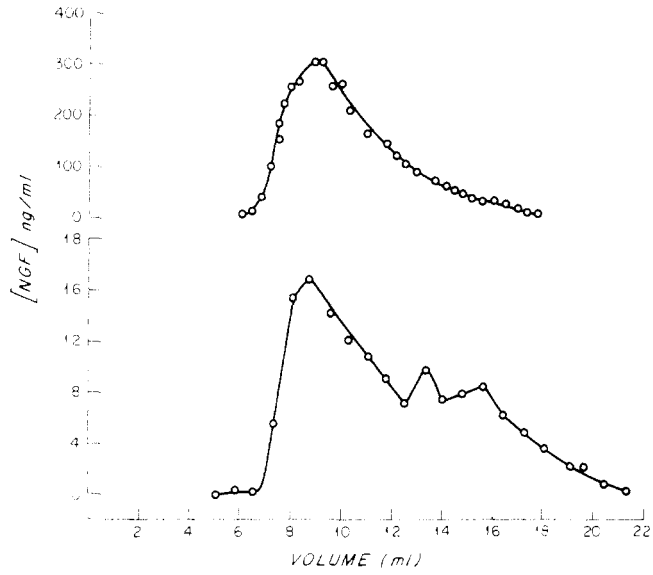

FIGURE 2: Sephadex G-200 chromatographic profiles of NGF in a fresh submandibular gland extract as a function of NGF concentration. Column dimensions, 1 × 23 cm; solvent, 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL bovine serum albumin; temperature, 4 °C. Top: 100 μL of 18 μg/mL immunoreactive NGF solution was applied. Bottom: 100 μL of 0.9 μg/mL immunoreactive NGF was applied. Fractions were measured by radioimmunoassay.

was calculated from the amino acid composition of NGF (see below) and this number is close to the value $k = 1.0155$ determined for a variety of proteins by direct measurement of deuterium exchange (Hvidt & Nielsen, 1966). Samples of protein dissolved in H_2O and D_2O were centrifuged simultaneously in a six-place rotor, and a multiplex system (Beckman) was used to isolate the images of the cells. Multiple scanner traces of each cell were taken after 24 and 48 h. Densities of D_2O and H_2O solvents were measured with a 10-mL Leach pycnometer in a constant temperature water bath (±0.01 °C). Where required for sedimentation coefficient calculations, solvent viscosities were determined with an Ostwald-type viscometer.

Protein Concentrations. Protein concentrations were measured by absorbance at $\lambda = 280$ nm. The extinction coefficient of the purified high molecular weight NGF to be described was determined by amino acid analyses (Beckman Model 121 analyzer) of the protein following 24-h hydrolysis with 6 N HCl in sealed, evacuated tubes. A value of 1.92 mL/(mg cm) was obtained.

Results

Throughout this study we have used a radioimmunoassay specific for pure 2.5S NGF to detect all forms of NGF in submandibular gland extracts which are immunochemically recognizable by antibody to 2.5S NGF. Thus, in what follows, when we refer to the concentration of NGF as measured by radioimmunoassay, what is meant is the apparent concentration of the protein based upon known concentrations of 2.5S NGF standards. This distinction between actual protein concentration and that measured immunologically is important since it cannot be assumed that larger molecules, which contain 2.5S NGF as part of their structure, react identically with 2.5S NGF antibodies. Furthermore, all results obtained from radioimmunoassay were checked with the sensory ganglion bioassay system.

First we consider the state of NGF as it exists in fresh gland extracts. Second, methods are presented for complete purification of the stable undissociated form of the protein. Finally, we describe some properties of the unstable dissociation products of NGF which are present in mouse submandibular gland extracts.

NGF in Fresh Submandibular Gland Extracts. Figure 1 presents a gel filtration profile of immunoreactive NGF in a fresh gland extract. For this experiment, two glands from a single adult male mouse were homogenized (ground-glass) in 5 mL of ice cold 0.1 M potassium phosphate, pH 7.0. Insoluble residue was removed by centrifugation at 40 000*g* for 15 min (4 °C), and 1.0 mL of the supernatant solution was applied to a large column of Sephadex G-200 operating at 4 °C. NGF was measured by radioimmunoassay and Figure 1 illustrates at least three distinct immunoreactive components. It should be emphasized (and it will be shown later) that the pattern presented in Figure 1 does not arise as a result of the use of glands from a single mouse. All individual mice examined, as well as pooled gland extracts obtained from 100 mice, yield profiles similar to that shown in Figure 1. However, the relative amounts of the three components depicted in Figure 1 do vary from animal to animal.

To study the properties of gland extracts as a function both of time following gland removal and of NGF concentration, smaller columns of Sephadex G-200 were employed. Figure 2 (top) illustrates the gel filtration profile of a fresh gland extract which contained a total of 18 μg/mL of immunoreactive NGF. In this case, a single peak was observed, although it will be seen that it is appreciably asymmetric. However, when the same gland extract used for Figure 2 (top) was diluted 20-fold to give a concentration of 0.9 μg/mL NGF, a clearly different gel-filtration pattern was obtained (Figure 2 (bottom)). The column used for these two experiments was the same, and the gland homogenate remained frozen until chromatographed. As shown in Figure 2 (bottom), considerable amounts of more slowly emerging forms of NGF appear when the gland extract is examined at higher dilutions. This behavior indicates the existence of a concentration-dependent dissociation reaction, in which a larger form of NGF is dissociating (initially upon dilution and probably as it migrates down the gel bed) to yield at least two smaller forms. The chromatographic behavior depicted in both Figures 1 and 2 is reminiscent of the properties of 7S NGF which also dissociates to yield smaller components as the protein concentration is lowered (Pantazis et al., 1977a).

To explore the possibility that a time-dependent degradation reaction (enzymic or otherwise) was responsible for the results shown in Figures 1 and 2, a solution of gland extract identical

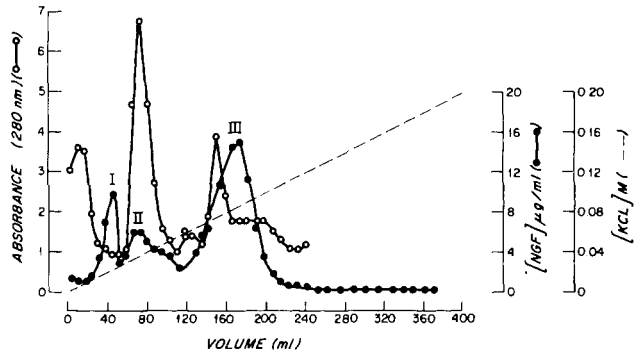

FIGURE 3: DEAE-cellulose chromatography of a submandibular gland extract. Glands from 100 adult male mice were homogenized with 55 mL of 0.025 M Tris-HCl, pH 8.0, as described in the text. Insoluble residue was removed by centrifugation and the supernatant solution was dialyzed against the same solvent at 4 °C. Column dimensions, 1 × 23 cm, packed with DE-52 under a nitrogen pressure of 8 psi and equilibrated at 4 °C with 0.025 M Tris-HCl, pH 8.0. Twenty-six milliliters of the above solution containing 3.7 mg/mL of immunoreactive NGF was applied to the column. A linear KCl gradient (volume 400 mL) was then begun from 0 to 0.2 M KCl at a flow rate of 9 mL/h. Fractions were measured by absorbance (O—O) (280 nm) and by radioimmunoassay for NGF (●—●).

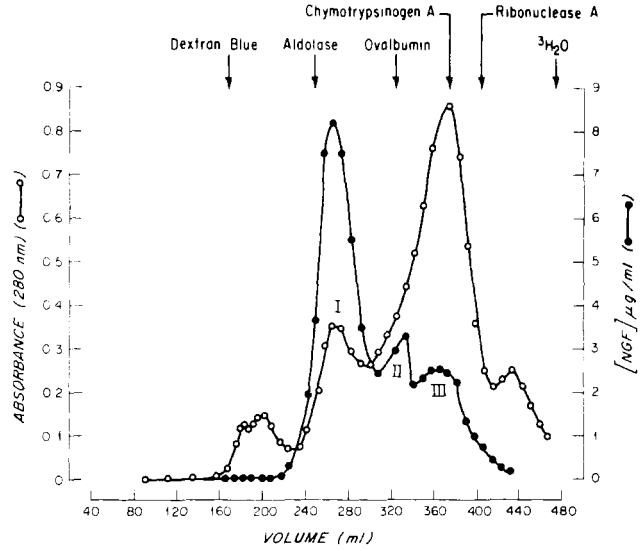

FIGURE 4: Sephadex G-200 chromatography of fraction III from the DE-52 column shown in Figure 3. Fraction III was desalted, lyophilized, and dissolved in 0.1 M potassium phosphate, pH 7.0. This solution was applied to a 2.5 × 96 cm column of Sephadex G-200 equilibrated with the same solvent at 4 °C. Flow rate, 26 mL/h. Fractions were measured for absorbance at 280 nm, (O—O), and by radioimmunoassay for NGF (●—●). The column was subsequently calibrated with the indicated marker proteins, each applied at a concentration of 10 mg/mL, and with dextran blue 2000 and ^3H_2O.

with that used for the chromatogram shown in Figure 2 (bottom) was incubated for 1 h at 25 °C and then applied to Sephadex. The resulting profile was virtually identical with that shown in Figure 2 (bottom). No further dissociation (or degradation) could be detected. The same result was obtained when a whole gland homogenate (without first removing the insoluble residue by centrifugation) was incubated for 1 h at 25 °C. Thus, it would appear that the multiple forms of NGF are initially present in gland extracts and that they are not produced by a time-dependent degradation reaction which can be detected in vitro.

Purification and Properties of the Intact Undissociated Form of Gland NGF. In an earlier study on the properties of NGF in submandibular gland extracts and that secreted in mouse saliva, it was observed that ion-exchange chromatography (DEAE-cellulose) served to isolate a high molecular weight form of the protein which remained stable even at high dilution (Murphy et al., 1977c). In what follows, we present a procedure for the complete purification of this species. In addition, some molecular properties of the NGF-containing by-products of this purification scheme will be considered later.

Submandibular glands from 100 adult male mice were excised and frozen with dry ice. After thawing they were homogenized in 55 mL of ice cold 0.025 M Tris-HCl, pH 8.0, with four 30-s bursts from a Servall Omni-mixer operating at full speed. This mixture was centrifuged for 15 min at 48 000 *g*, and the supernatant solution was removed and dialyzed for 18 h against 4 L of 0.025 M Tris-HCl, pH 8.0.

This solution was applied to a 1 × 23 cm column of DE-52, previously packed under pressure of 8 psi with N_2 and equilibrated with 0.025 M Tris-HCl, pH 8.0. After the column was loaded, a salt gradient was begun from 0 to 0.20 M KCl at a flow rate of 9 mL/h. Individual fractions were monitored by absorbance and by radioimmunoassay. Under these solvent conditions, 80–90% of the immunoreactive NGF remains bound to the resin prior to initiation of the salt gradient. Figure 3 illustrates the resulting gradient elution profile, and at least three zones of NGF immunoreactivity can be seen. Moreover, each of these zones (and no other) was strongly positive in the sensory ganglion bioassay system. They are labeled I, II, and III for further reference. By area analysis, 75% of the NGF applied to the column was recovered.

Fractions corresponding to component III were pooled, dialyzed exhaustively against 4 L of 0.001 M potassium phosphate, pH 7.0, and lyophilized. The dry powder was dissolved in 1.0 mL of 0.1 M potassium phosphate, pH 7.0, and applied to a 2.5 × 95 cm column of Sephadex G-200 equilibrated with the same solvent at 4 °C. Figure 4 illustrates the resulting chromatogram and three more peaks containing NGF-immunoreactivity are resolved. As shown in Figure 4, peak I emerges from the column behind aldolase (molecular weight 158 000), peak II near ovalbumin (molecular weight 45 000), and peak III close to chymotrypsinogen (molecular weight 26 000). In the concentration range 1–10 ng/mL, only peaks I, II, and III were biologically active. The material shown near the void volume, which did not contain NGF by immunoassay, was correspondingly inactive in the bioassay. The properties of peaks II and III will be considered later.

Fractions corresponding to peak I were pooled, dialyzed for 18 h against 4 L of 0.001 M potassium phosphate, pH 7.0, and lyophilized. The dry powder was redissolved in 3 mL of 0.01 M potassium phosphate, pH 7.0, and dialyzed thoroughly against this solvent at 4 °C. This solution was then applied to a 1.3 × 4 cm column of hydroxylapatite equilibrated with 0.01 M potassium phosphate, pH 7.0, and packed under atmospheric pressure. By radioimmunoassay, quantitative adsorption of NGF to the ion exchanger occurs at this stage. A linear gradient from 0.01 to 0.4 M potassium phosphate, pH 7.0, was begun at 4 °C, and Figure 5 illustrates the resulting chromatographic profile. The majority of the NGF-immunoreactive material emerges as a single zone coincident with absorbance at a phosphate concentration of 0.074 M. Fractions corresponding to the main NGF zone depicted in Figure 5 were pooled, dialyzed overnight against 1 L of 0.001 M potassium phosphate, pH 7.0, and lyophilized. The dry powder was redissolved in 2 mL of 0.1 M potassium phosphate, pH 7.0, and dialyzed thoroughly against this solvent.

Even at this stage of the procedure, this form of NGF is not completely homogeneous. For example, examination of the

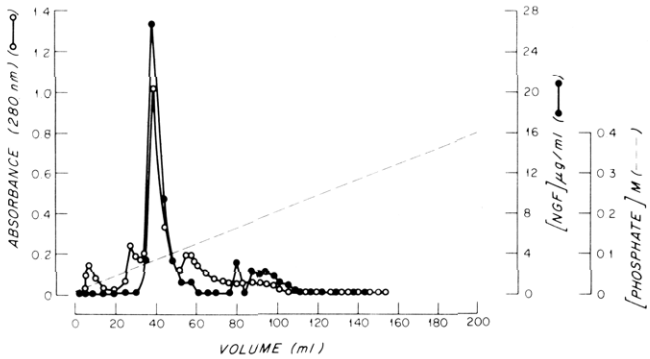

FIGURE 5: Hydroxylapatite chromatography of NGF from peak I shown in Figure 4. Fractions corresponding to peak I were pooled, desalted, and concentrated as described in the text. This solution was dialyzed against 0.01 M potassium phosphate, pH 7.0, and then applied to a 1.3 × 4 cm column of hydroxylapatite equilibrated with the same solvent at 4 °C. Following application of the sample (3.9 mL containing a total of 240 μg of immunoreactive NGF), a 200-mL linear gradient of potassium phosphate, pH 7.0, from 0.01 M to 0.4 M was begun. Flow rate, 15 mL/h. (○—○) Absorbance at 280 nm; (●—●) [NGF], measured by radioimmunoassay.

FIGURE 6: Gel electrophoretic pattern of NGF. Polyacrylamide gel electrophoresis was performed as described in the text. Two identical gels were run in parallel. After the tracking dye (bromophenol blue) had migrated to the base of the gels, current was stopped. One gel was stained with Coomassie Blue, and the other was divided into 1-mm serial slices. The individual slices were incubated overnight, each in 0.4 mL of a solution of 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL bovine serum albumin. Aliquots of these solutions were then measured for NGF by radioimmunoassay. Only two slices contained NGF, and these are indicated by the arrows. Ten micrograms of protein in a volume of 25 μL was applied to each gel at 4 °C.

preparation by velocity sedimentation revealed a small amount of slowly sedimenting material. To eliminate this component(s), a fourth chromatographic step (using a 2.5 × 95 cm column of Sephadex G-200 exactly like that used for Figure 4) is required. (The reason for this additional gel filtration step will be apparent when we consider the chromatographic properties of some of the dissociated, lower molecular weight forms of NGF.) Fractions from this second Sephadex G-200 column were monitored by absorbance at 280 nm and by radioimmunoassay. Most of the protein emerges at a position identical with that shown in Figure 4 (peak I), and a small

TABLE I: Apparent Partial Specific Volume of NGF.[a]

Run	Rotor speed	$\bar{V}_2$ (mL/g)
1	15 000	0.69_0
2	15 000	0.68_5
2	17 000	0.69_0
		0.68_8 (mean)

[a] Temperature was maintained close to 26 °C. The solvents were 0.1 M potassium phosphate, pH 7.0, dissolved in H_2O and in 99.8% D_2O. Each value represents the mean calculated from three individual scanner traces (λ = 280 nm) taken after 24 h at the given speed. In run 2 the rotor speed was subsequently increased to 17 000 rpm for an additional 24 h. Protein concentration was 70 μg/mL.

TABLE II: Molecular Weight of NGF.[a]

Run	Rotor speed	Solvent	Mol wt
1	15 000	H_2O	116 000
1	15 000	D_2O	123 000
1	17 000	H_2O	104 000
1	17 000	D_2O	114 000
2	15 000	H_2O	120 000
2	15 000	D_2O	127 000
2	17 000	H_2O	118 000
2	17 000	D_2O	125 000
3	15 000	H_2O	116 000
4	18 000	H_2O	127 000
		Mean:	116 000 ± 8000 (SD)

[a] Runs 1 and 2 were performed with absorption optics; runs 3 and 4 were with Rayleigh optics. The experimental conditions are given in the legend to Table I. Protein concentration, 70 μg/mL. Temperature, 26 °C; solvent, 0.1 M potassium phosphate, pH 7.0, dissolved either in H_2O or D_2O.

amount of lower molecular weight protein corresponding to peak II (Figure 4) can be seen and is readily eliminated. The total yield of protein after this final step is about 4 mg. By quantitative radioimmunoassay, this NGF species accounts for 60% of the total NGF present in crude gland extracts.

We turn now to the molecular properties of this high molecular weight form of NGF. After pooling and concentrating fractions from the final Sephadex G-200 column, the protein was dissolved in 0.1 M potassium phosphate, pH 7.0, and dialyzed versus this solvent. Sedimentation velocity studies were carried out at a rotor velocity of 60 000 rpm at 26 °C at protein concentrations over the range 1.7 to 0.048 mg/mL. A capillary-type synthetic boundary cell was employed for these studies, and only a single symmetric sedimenting boundary was seen. No slower or faster sedimenting material was detected. Values of the sedimentation coefficient were obtained with both the schlieren and absorption (λ = 230 nm) optical systems. The mean value is $s_{20,w} = 7.37 \pm 0.05$ S (standard deviation of the regression coefficient). Moreover, when this protein is examined by polyacrylamide gel electrophoresis, only a single sharp band is detected by staining the gel, and all of the NGF-immunoreactive material applied to the gel migrates coincident with this band as judged by radioimmunoassay of serial gel slices (see Figure 6).

Table I summarizes values of the partial specific volume of the protein determined with the H_2O–D_2O procedure. Values of $d \ln c/dx^2$ in the two solvents were determined by least-squares analyses of multiple scanner traces. In all cases, in both H_2O and D_2O solvents, plots of $\ln c$ vs. x^2 were strictly linear as would be expected for a pure protein. No curvature of these plots was detected, even at concentrations in the centrifuge cell

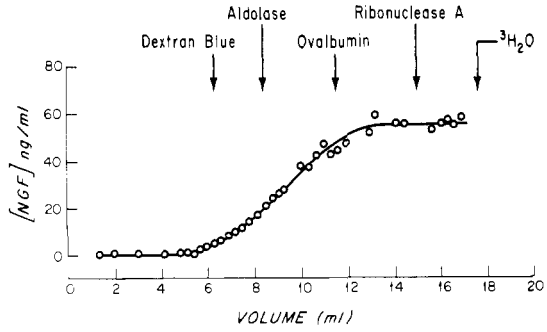

FIGURE 7: Frontal elution Sephadex G-200 profile of the 116 000 molecular weight form of NGF at low concentration. Eighteen milliliters of 47 ng/mL of immunoreactive NGF was applied to a 1 × 23 cm column equilibrated with 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL bovine serum albumin at 4 °C. Flow rate, 6 mL/h. The calibration proteins (100 μL each at a concentration of 10 mg/mL) and ^3H_2O and Blue Dextran 2000 were applied separately. Thirty-microliter fractions were collected and measured by radioimmunoassay.

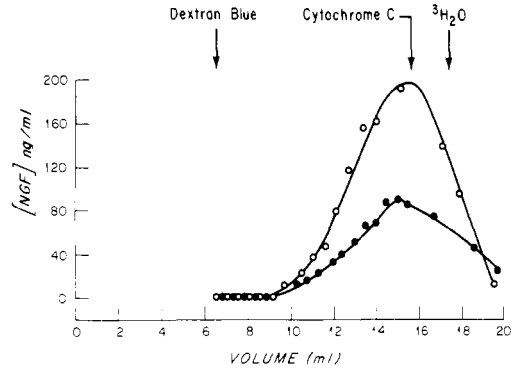

FIGURE 8: Sephadex G-200 gel filtration profiles of NGF fractions I and II shown in Figure 3. Fractions from peaks I and II (Figure 3) were pooled separately, desalted, concentrated, and dissolved in 0.1 M potassium phosphate, pH 7.0. These solutions (100 μL) were applied to a 1 × 23 cm column equilibrated with the same solvent containing 1 mg/mL bovine serum albumin at 4 °C. Cytochrome *c* (100 μL of 10 mg/mL), ^3H_2O, and Blue Dextran were applied separately. Fractions were measured by radioimmunoassay. (○—○) Fraction I; (●—●) fraction II.

as low as 40 μg/mL. As shown in Table I, values of $\bar{V}_2$ were measured in two separate experiments and at two different rotor velocities. A mean value of $\bar{V}_2$ = 0.688 mL/g was obtained.

Using the value of $\bar{V}_2$ from Table I, molecular weights were measured at sedimentation equilibrium at three different rotor speeds, in both H_2O and D_2O solvents, and with both absorption and Rayleigh optics. A mean value of 116 000 ± 8000 (SD) was obtained, and the data are presented in Table II.

One of the most interesting features of this 116 000 molecular weight form is its high stability at very low concentrations. As will be shown later, it is the only form of NGF which we have been able to detect in submandibular gland extracts which is stable. Figure 7 illustrates a typical gel filtration frontal elution profile of a solution of this protein at high dilution. For this chromatogram, a solution of NGF was diluted such that it contained only 50 ng/mL of immunoreactive material, and a large volume of this solution was applied to a 1 × 23 cm column of Sephadex G-200 equilibrated with 0.1 M potassium phosphate, pH 7.0, in order to establish a plateau region of protein concentration. Figure 7 reveals only a single symmetric component. The weight average partition coefficient (σ_w) of this species was calculated from the relation

$$\sigma_w = \frac{V_e - V_0}{V_i} \quad (4)$$

where V_e, V_0, and V_i are the elution volumes of the protein, the void volume, and the internal column volume, respectively. Proteins of known molecular weight were used to calibrate the chromatogram depicted in Figure 7 and, from plots of ln (mol wt) vs. σ_w for the calibration proteins, a value of 110 000 for the molecular weight of this species of NGF was estimated. This number is in good agreement with that measured (more accurately) by sedimentation equilibrium (Table II).

Properties of the Other Forms of NGF Present in Submandibular Gland Extracts. We now show that all other forms of NGF which can be detected immunochemically in gland extracts are unstable, and that at sufficiently low protein concentrations they all dissociate to yield a species whose molecular weight is closely similar to that of the 2.5S NGF monomer (molecular weight 13 000).

We begin by referring back to the DEAE-cellulose profile shown in Figure 3. It will be recalled that peak III from this column comprises the stable high molecular weight NGF whose purification has been described above. Peaks I and II from the same column were collected separately, dialyzed against 0.001 M potassium phosphate, pH 7.0, and lyophilized. The dry powders were dissolved in 0.1 M potassium phosphate, pH 7.0, and dialyzed against this solvent. Samples were applied separately to Sephadex G-200, and the results are shown in Figure 8. Both of these NGF fractions emerge from the column at a position close to that of cytochrome *c* (molecular weight 12 600) and with partition coefficients indistinguishable from those of the 2.5S NGF monomer (molecular weight 13 000) at comparable concentrations and under the same solvent conditions (Young et al., 1976a).

It will be recalled that peak III from DEAE-cellulose chromatography (Figure 3) gave rise to three fractions upon Sephadex G-200 (Figure 4). One of these is the stable 116 000 molecular weight form already described. We now turn to a study of the properties of the other two (peaks II and III, Figure 4). Fractions corresponding to peaks II and III (see Figure 4) were pooled together, dialyzed against 0.01 M potassium phosphate, pH 7.0, lyophilized, and redissolved in 10 mL of 0.01 M potassium phosphate, pH 7.0. Following dialysis against this solvent, the protein solution was applied to a 1.3 × 4 cm column of hydroxylapatite and a linear phosphate gradient was begun from 0.01 M to 0.4 M potassium phosphate, pH 7.0. The results are shown in Figure 9. All of the immunoreactive NGF applied to this column was initially bound to the exchanger, and three immunoreactive NGF species are resolved.[2]

Fractions corresponding to the three NGF zones shown in Figure 9 were combined, desalted by dialysis, lyophilized, and redissolved in 0.1 M potassium phosphate, pH 7.0. Sedimentation velocity studies of this material (schlieren optics, 60 000 rpm, 26 °C) revealed a single symmetric boundary with $s_{20,w}$ = 3.24 S.

[2] As noted in that portion of the text which describes purification of the 116 000 molecular weight form of NGF, a second gel-filtration step with Sephadex G-200 is required to obtain the pure protein. The reason for this can be seen by examination of the hydroxylapatite chromatogram shown in Figure 9. A large, non-NGF containing protein fraction emerges from this column at a phosphate concentration (0.074 M) identical with that of the elution position of the 116 000 molecular weight NGF (see Figure 5). Thus, the 116 000 molecular weight protein, as it was taken from the Sephadex G-200 column depicted in Figure 4 is variably contaminated with non-NGF protein arising from overlap of peak I (the 116 000 molecular weight NGF) with that of fraction II (Figure 4). A second chromatographic step with Sephadex G-200 following hydroxylpatite completely eliminates this material as described in the text.

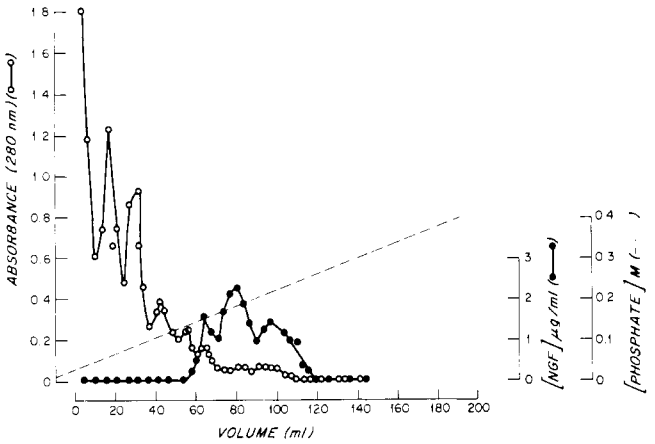

FIGURE 9: Hydroxylapatite chromatography of NGF obtained from fractions II and III shown in Figure 4. Fractions corresponding to peaks II and III shown in Figure 4 were pooled together, desalted, and concentrated as described in the text. After redissolution in and dialysis against 0.01 M potassium phosphate, pH 7.0, this solution (10 mL containing 30 μg/mL of immunoreactive NGF) was applied to a 1.3 × 4 cm column of hydroxylapatite equilibrated with the same solvent at 4 °C. A linear phosphate gradient (200 mL) was begun from 0.01 M to 0.4 M phosphate, pH 7.0, at a flow rate of 12 mL/h. Fractions were measured by radioimmunoassay (●—●) and by absorbance (○—○).

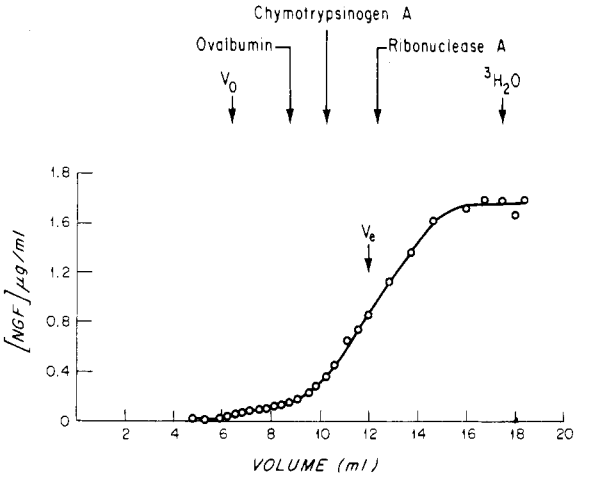

FIGURE 10: Sephadex G-75 frontal elution profile of 3.24 S NGF at low concentration. A concentrated solution of this protein was diluted with 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL bovine serum albumin, to yield a solution containing 1.8 μg/mL of immunoreactive NGF. Ten milliliters of this solution was applied to a 1 × 23 cm column of Sephadex G-75 equilibrated with the same solvent at 4 °C. Flow rate, 15 mL/h. ^3H_2O, dextran blue, and the calibration proteins (each at 10 mg/mL) were run separately. Fractions (35 μL each) were measured for NGF by radioimmunoassay. The elution volume of NGF is shown by the arrow (V_e).

It will be recalled that the material described in the preceding paragraph was first identified by gel filtration chromatography (see Figure 4) and that it emerged from the column as two peaks. One of these (peak III, Figure 4) appeared at a position indistinguishable from the elution volume of chymotrypsinogen and 2.5S NGF, both with molecular weights close to 26 000. The other (peak II, Figure 4) emerged close to the elution volume of ovalbumin (molecular weight 45 000). Yet when the sedimentation behavior of this NGF species is studied at much higher protein concentrations than those used for gel filtration, only a single boundary is present with $s_{20,w}$ = 3.24 S. This number is reminiscent of that measured by Cohen for his preparation of NGF rearly 20 years ago (Cohen, 1960). Cohen obtained a value for $s_{20,w}$ = 4.35 S, and from this he estimated that the molecular weight of his protein was about 44 000. While the details of these calculations are not given in Cohen's original paper, we have used the value $s_{20,w}$ = 3.24 S to estimate approximately the size of this form of NGF. For this purpose we have made the assumption that the friction factor and partial specific volume of 2.5S NGF (molecular weight 26 000, $s_{20,w}$ = 2.5 S) are close to that of 3.24 S NGF. Thus, based on the relationship (see, e.g., Schachman, 1959),

$$\frac{M_1}{M_2} = \left(\frac{S_1}{S_2}\right)^{3/2} \quad (5)$$

we estimate that the molecular weight of the 3.24S NGF species is about 40 000. The purpose of this calculation is only to emphasize the fact that the 3.24S NGF protein is not 2.5S NGF, and that it could be the form of the protein first isolated by Cohen (1960) but which has not been observed since his original description of it.

The following studies show that like the other smaller forms of NGF discussed in this section, the 3.24S NGF species is also unstable at low protein concentration. A solution of the 3.24S protein was diluted 2000-fold to give a concentration of 1.7 μg/mL of immunoreactive NGF, and a large volume of this solution was applied to a column of Sephadex G-75 which had been calibrated with proteins of known molecular weight. Results are shown in Figure 10. Only a single boundary of NGF was detected by radioimmunoassay, with a partition coefficient (σ_w = 0.48) which yields an estimated molecular weight of 19 000. This number is about half-way between the monomer and dimer molecular weights of 2.5S NGF (13 000 and 26 000 respectively), and it is in excellent agreement with that expected from the monomer ⇄ dimer reaction displayed by 2.5S NGF at comparable protein concentrations and under the same solvent conditions. For example, studies of the rapidly reversible monomer ⇄ dimer equilibrium system of 2.5S NGF have revealed that solutions of this protein consist of equal amounts of monomer and dimer at a total protein concentration of 1.4 μg/mL (Young et al., 1976a). In summary then, this NGF species possesses a sedimentation coefficient of 3.24 S when measured at the relatively high concentration of 2.9 mg/mL. At lower (but intermediate) concentrations, it partially dissociates to yield a 26 000 molecular weight species as judged by its gel filtration behavior (Figure 4), and, at even lower protein concentration, it further dissociates in a manner similar to that characteristic of the monomer ⇄ dimer equilibrium system of 2.5S NGF (Figure 10).

Finally, we should like to reemphasize the fact that all of the forms of NGF which have been identified by radioimmunoassay during this study are biologically active in the concentration range 1–10 ng/mL. After 18 h in culture, as described under Experimental Procedure, all forms produce characteristic neurite outgrowth from sensory ganglia. Furthermore, all chromatographic fractions which did not contain NGF by immunoassay were inactive in the bioassay system. Although the biologic assay is only qualitative, we have been unable to distinguish the several NGF species on the basis of their biological activity as tested in this system.

Discussion

The results presented above indicate that NGF exists in multiple molecular forms in fresh extracts of the mouse submandibular gland. Of the several species which have been detected, by both ion-exchange and gel filtration chromatography, all but one are unstable in dilute solution. Moreover, at sufficiently high dilution, these unstable species display gel

filtration properties which are similar to those exhibited by 2.5S NGF at comparable protein concentrations (Young et al., 1976a). One form of NGF has a sedimentation coefficient of 3.24 S at high concentration, and it undergoes dissociation to smaller species at very low protein concentrations. It is possible that this is the molecular form of NGF which was originally isolated from mouse submandibular glands by Cohen (1960).

Of the several forms of NGF present in adult male mouse submandibular gland extracts, we find only one that is stable at very high dilutions. This protein has a molecular weight near 116 000, and at very low protein concentrations, it remains intact (Figure 7). We turn now to a comparison of the properties of the 116 000 molecular weight protein with those of other known forms of NGF.

In their original paper on the purification of 7S NGF, Varon et al. (1967) observed that during DEAE-cellulose chromatography extremely high flow rates (on the order of 350 mL per h) were required to isolate 7S NGF. When lower flow rates were used, NGF recovery was poor, and activity was spread over many fractions. Varon et al. (1967) attributed this behavior to dissociation of the 7S NGF complex. Subsequent work from their laboratory confirmed the hypothesis that 7S NGF was in equilibrium with its subunits and that, unless the DEAE-cellulose step is carried out very quickly, the α subunits of 7S NGF are selectively removed from the protein complex by the exchange resin (see Perez-Polo et al., 1972, for a detailed summary of the evidence). Moreover, these observations on the instability and dissociation of 7S NGF have been confirmed by others (Baker, 1975; Pantazis et al., 1977a).

In contrast to these results with 7S NGF, isolation of the stable high molecular weight NGF reported here does not require the extraordinarily high flow rate (350 mL/h) employed by Varon et al. (1967). Rather, the DEAE-cellulose chromatographic step illustrated in Figure 3 typically utilizes a linear salt gradient at a flow rate of 9 mL/h—and even then, the high molecular weight NGF-containing zone which appears as peak III (Figure 3) is still contaminated with appreciable amounts of at least two lower molecular weight, unstable forms of NGF (see Figure 4). In this regard, it is of interest that Varon et al. (1967) have also reported that all of the NGF in gland homogenates is associated with the 7S species (see also Smith et al., 1968; Perez-Polo et al., 1972). It seems possible that this observation stemmed from the unusually high flow rates employed by these authors. When more normal flow rates are used (needed for high resolution by ion-exchange chromatography), multiple forms of NGF are resolved. From chromatographic studies of over a dozen individual adult male mouse gland extracts, as well as from an examination of the properties of NGF in pooled gland homogenates, each from 100 mice, we have never detected NGF as a single molecular form. In fact, at least six species of the protein can be readily identified (Figures 1–5 and 9). Consequently, the observation of Varon et al. (1967) that low ion-exchange flow rates gave rise to multiple fractions of NGF probably reflects the fact that these multiple forms are present as such in gland homogenates. Moreover, the results of Figure 3 reveal that these several species of NGF emerge from the anion-exchange resin within the range 0–0.1 M KCl when a linear gradient is employed. Varon et al. (1967) employed a step gradient of 0.08 M NaCl, and it could be that some or all of the NGF species coeluted together under these conditions. In this regard it should be noted that the yield of the purified 116 000 molecular weight NGF described here is considerably less than that obtained by Varon et al. (1967). From glands of one hundred mice, Varon et al. obtained 48 mg of 7S NGF. From the same number of animals, we recover only about 4 mg.

The major difference between 7S NGF and the 116 000 molecular weight protein described here lies in their greatly different stabilities. The 7S NGF begins to dissociate at relatively high protein concentrations (Baker, 1975), and it completely liberates its biologically active component as the protein solution is progressively diluted (Pantazis et al., 1977a). In contrast, the high molecular weight NGF species purified here is highly stable (Figure 7). Also, this is the only stable species which we have detected in fresh gland extracts, and it is also the largest in mass. The radioimmunoassay employed in these studies (see Experimental Procedures) can detect as little as 0.25 ng of immunoreactive NGF per mL, and, by this method together with gel filtration chromatography, we have never observed a species larger than the 116 000 molecular weight protein. Thus, if these is a still larger form, we have been unable to detect it in fresh gland extracts.

Two recent studies have shown that high concentrations of NGF are secreted in mouse saliva (Wallace & Partlow, 1976; Murphy et al., 1977a). Further, observations on the molecular properties of NGF in saliva have revealed the existence of at least two molecular weight classes of the protein (Murphy et al., 1977c). One of these has a molecular weight of 114 000 as estimated by gel filtration. The molecular weight of the other species corresponds to that of the monomer of 2.5S NGF (13 000). The relative amounts of these two proteins in saliva vary from animal to animal and the larger protein is slowly converted to the smaller one, presumably by proteases present in saliva (Murphy et al., 1977c). Although the reason for high concentrations of NGF in saliva is not known, it should be noted that the mass of the 116 000 molecular weight gland protein and that of the larger species in saliva are virtually identical. Thus, it would appear that the large, stable species in salivary gland extracts is the same form which is secreted in saliva.

The significance of the presence of multiple species of NGF in salivary gland extracts is not clear, but there are several possibilities. One is that the 116 000 molecular weight NGF is a precursor (i.e., in a pro-NGF sense) of the smaller forms. Yet the biological activity of this high molecular weight protein is indistinguishable from those of the dissociation products as tested in the sensory ganglion bioassay system. Further, there is no evidence for its rapid and specific enzymic conversion to a smaller form which is then destined for secretion by the submandibular gland. A second possibility is that the submandibular gland synthesizes de novo all of the different forms of NGF and that this process is directed by different genes. Such a metabolic process seems unlikely to us. Third, it could be that the submandibular glands are excretory (in a waste product sense) organs with respect to NGF. However, blood levels of mouse NGF do not rise following bilateral gland removal; i.e., if the glands were removing NGF from the circulation, concentrating it, and then excreting it into saliva, we would expect a significant rise in blood levels of the factor after removal of the glands (Murphy et al., 1977a). It could be that NGF is initially synthesized as the stable, high molecular weight form and that it is subsequently degraded enzymically as part of a process which serves to regulate NGF levels in glands and saliva. Finally, the unstable forms of NGF could arise from proteolysis, not occurring in vivo, but during extraction of the tissue. While we have been unable directly to demonstrate degradation in gland extracts, progressive breakdown of this form of the protein does not occur in saliva (Murphy et al., 1977c), and mouse submandibular glands do contain high levels of proteolytic enzymes (Sreebny, 1960). Failure to demonstrate degradation in gland extracts may

reflect the fact that cell-free preparations of mammalian tissues have often been found to lack certain properties characteristic of in vivo proteolysis (see Etlinger & Goldberg, 1977, and references therein). Another possibility is that the degradation enzymes act transiently when tissue is fractionated.

If degradation of the 116 000 molecular weight form of NGF is responsible for the presence of the less stable species in gland homogenates, then this would mean that the stable form is the one prepared for export from the submandibular gland into saliva. This hypothesis is consistent with the observation that the high molecular weight NGF described here has the same mass as the predominant form secreted in saliva (Murphy et al., 1977c). The hypothesis is also consistent with observations on the properties of the NGF secreted by cells in culture. Both mouse L cells (Pantazis et al., 1977b) and primary rat muscle cells (Murphy et al., 1977b) secrete high molecular weight (150 000–160 000) forms of NGF, and both are stable at very low protein concentrations.

The concept that the multiple dissociation forms of NGF are degradation products of the stable form may find its parallel in the known enzymic modifications of 2.5 S NGF which occur either prior to or during its purification. For example, the sequence studies of Angeletti et al. (1973) have shown that some of the polypeptide chains of 2.5S NGF lack the amino terminal octapeptide, and the work of Moore et al. (1974) has revealed the absence of some of the C-terminal arginine residues. Whether these, or other unrecognized, cleavage reactions are responsible for reducing the stability of the NGF species described here is not known.

Since the 116 000 molecular weight NGF species exhibits stability characteristics which are different from those of other forms of the protein which have been examined, it could be that this protein displays biological activities which have so far not been recognized. Although further work will be required to explore this possibility, studies with the stable NGF species described here may be useful in analyzing the physiological role of NGF—both that secreted by cells and by the mouse submandibular gland.

References

Angeletti, R. H., Hermodson, M. A., & Bradshaw, R. A. (1973) *Biochemistry 12,* 100.

Baker, M. E. (1975) *J. Biol. Chem. 250,* 1714.

Bocchini, V., & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. U.S.A. 64,* 787.

Cohen, S. (1960) *Proc. Natl. Acad. Sci. U.S.A. 46,* 302.

Edelstein, S. J., & Schachman, H. K. (1967) *J. Biol. Chem. 242,* 306.

Etlinger, J. D., & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74,* 54.

Greene, L. A., Shooter, E. M., & Varon, S. (1969) *Biochemistry 8,* 3735.

Hvidt, A., & Nielsen, S. O. (1966) *Adv. Protein Chem. 21,* 287.

Levi-Montalcini, R. (1958) in *Chemical Basis of Development* (McElroy, W. D., & Glass, B., Eds.) pp 646–664, Johns Hopkins Press, Baltimore, Md.

Moore, J. B., Mobley, W. C., & Shooter, E. M. (1974) *Biochemistry 13,* 833.

Murphy, R. A., Pantazis, N. J., Arnason, B. G. W., & Young, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72,* 1895.

Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2330.

Murphy, R. A., Singer, R. H., Saide, J. D., Pantazis, N. J., Blanchard, M. H., Byron, K. S., Arnason, B. G. W., & Young, M. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74,* 4496.

Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977c) *Proc. Natl. Acad. Sci. U.S.A. 74,* 2672.

Oger, J., Arnason, B. G. W., Pantazis, N. J., Lehrich, J., & Young, M. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71,* 155.

Pantazis, N. J., Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977a) *Biochemistry 16,* 1525.

Pantazis, N. J., Blanchard, M. H., Arnason, B. G. W., & Young, M. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74,* 1492.

Perez-Polo, J. R., Bamburg, J. R., DeJong, W. W. W., Straus, D., Baker, M. E., & Shooter, E. M. (1972) in *Nerve Growth Factor and its Antiserum* (Zaimis, E., Ed.) p 19, Athlone Press, London.

Richards, E. G., & Schachman, H. K. (1959) *J. Phys. Chem. 63,* 1578.

Schachman, H. K. (1959) *Ultracentrifugation in Biochemistry,* p 242, Academic Press, New York, N.Y.

Schachman, H. K., Gropper, L., Hanlon, S., & Putney, F. (1962) *Arch. Biochem. Biophys. 99,* 175.

Smith, A. P., Varon, S., & Shooter, E. M. (1968) *Biochemistry 7,* 3259.

Smith, A. P., Greene, L. A., Fisk, R. H., Varon, S., & Shooter, E. M. (1969) *Biochemistry 8,* 4918.

Sreebny, L. M. (1960) *Ann. N.Y. Acad. Sci. 85,* 182.

Varon, S., Nomura, J., & Shooter, E. M. (1967) *Biochemistry 6,* 2202.

Varon, S., Nomura, J., & Shooter, E. M. (1968) *Biochemistry 7,* 1296.

Wallace, L. J., & Partlow, L. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 4210.

Young, M., Saide, J. D., Murphy, R. A., & Arnason, B. G. W. (1976a) *J. Biol. Chem. 251,* 459.

Young, M., Murphy, R. A., Saide, J. D., Pantazis, N. J., Blanchard, M. H., & Arnason, B. G. W. (1976b) in *Surface Membrane Receptors* (Bradshaw, R. A., Frazier, W. A., Merrell, R. C., Gottlieb, D. I., Hogue-Angeletti, R. A. Eds.) pp 247–267, Plenum Press, New York, N.Y.

Young, M., Saide, J. H., & Blanchard, M. H. (1977) in *Methods of Hormone Radioimmunoassay* (Jaffe, B. M., Ed.) Academic Press, New York, N.Y. (in press).

Yphantis, D. A. (1964) *Biochemistry 3,* 297.