Dissociation of the 7S-Nerve Growth Factor Complex in Solution†

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ABSTRACT: Sedimentation and gel-filtration studies of mouse submandibular gland 7S-nerve growth factor (NGF) reveal that this complex dissociates to yield its components at concentrations much higher than those required to exhibit biological activity. Results further indicate that the α and γ protein components of the 7S-NGF complex probably play no role in its biological activity when tested in vitro. The dissociation behavior of 7S-NGF is quite different from the properties of very dilute solutions of the NGF secreted by mouse L cells and of that present in fresh, unpurified submandibular gland homogenates, since both of these proteins display high molecular weights at concentrations where 7S-NGF is fully dissociated. Thus, it could be that 7S-NGF is not the form in which NGF exists in the mouse submandibular gland.

Depending upon the isolation procedure used, nerve growth factor (NGF)[1] can be purified from mouse submandibular glands in two principal molecular forms. One of these has been given the name 7S-NGF (referring to its sedimentation coefficient) (Varon et al., 1967a,b; Varon et al., 1968) and the other has been designated 2.5S-NGF (Bocchini and Angeletti, 1969). These two species are interrelated, and in what follows we briefly describe their respective properties.

7S-NGF. This protein, with mass close to 140 000 g/mol, is a complex of three different molecular components which have been termed α, β, and γ (Varon et al., 1968). Of these, only the β component is responsible for the characteristic biological activity of NGF in promoting neurite outgrowth from ganglionic neurons in culture (Varon et al., 1967b, 1968). The chemical and biological roles of the α species are unknown. The γ component displays esterase activity upon certain synthetic arginine-containing substrates (Greene et al., 1968), although its function is also unknown. Outside the pH range 5–8, the 7S-NGF molecule dissociates to yield a mixture of its components; upon restoration of pH to neutrality, the three species reassociate to form the parent complex (Varon et al., 1967b, 1968).

2.5S-NGF. This biologically active species is virtually identical to the β component of 7S-NGF, and it is isolated from mouse submandibular glands by a chromatographic step at pH 5 (Bocchini and Angeletti, 1969) at which pH the 7S complex is fully dissociated. β- and 2.5S-NGF differ from each other only in that during isolation of the latter, limited proteolysis occurs at both the N- and C-terminal ends of the polypeptide chains (Angeletti et al., 1973; Moore et al., 1974). The complete primary structure of 2.5S-NGF has been solved, and it is composed of two noncovalently joined identical chains, each of molecular weight 13 259 (Angeletti et al., 1973).

Recent studies have shown that 2.5S-NGF comprises a rapidly reversible monomer $\rightleftarrows$ dimer equilibrium system (Young et al., 1976a). The association constant for this reaction at neutral pH is 9.4×10^6 M^{-1} and this means that at concentrations which are biologically active (about 1 ng/mL) solutions of the protein consist almost entirely of monomer. Consequently, in the ng/mL range of concentration the individual polypeptide chains of 2.5S-NGF are the biologically active species (Young et al., 1976a).

It is now known that nerve growth factor is secreted by a variety of both transformed and untransformed cells in culture (Oger et al., 1974; Young et al., 1975; Murphy et al., 1975; Young et al., 1976b). In the course of studies on the molecular properties of the factor produced by L cells in culture, we observed that the biologically active and immunologically reactive species exists in a high-molecular-weight form, even at concentrations approaching 1 ng/mL (Pantazis et al., 1977). It was this finding which led us to examine the molecular properties of 7S-NGF in dilute solution for comparison with the properties of L-cell NGF. Using high-speed sedimentation, as well as gel-filtration analyses, we find, that unlike L-cell-derived NGF, 7S-NGF at neutral pH begins to dissociate at concentrations much higher than those required for biological activity. At a total 7S-NGF concentration as high as 50 μg/mL, little if any original 7S complex is present, and, at protein concentrations near 0.8 μg/mL, the NGF monomer (molecular weight close to 13 000) is the predominant species. These observations indicate that 7S-NGF is a relatively unstable complex, and they raise certain questions regarding its biological role.

Experimental Procedures

Reagents. Double glass distilled H_2O was employed for all solutions and buffer salts were of reagent grade. Two 7S-NGF preparations were used throughout the study. One of these was a generous gift from the laboratory of Dr. Ralph A. Bradshaw. This preparation was constructed synthetically from the isolated α, β, and γ components and the resulting 7S-NSF complex was purified by gel filtration (Jeng et al., 1976). The other preparation of 7S-NGF was sent to us through the courtesy of Dr. J. R. Perez-Polo, and it was isolated directly from male mouse submandibular glands by the procedure of Varon et al. (1967a). In all of the studies reported here, both preparations of 7S-NGF behaved identically. Protein concentrations were measured both by the Lowry procedure (with

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[1] Abbreviations used are: NGF, nerve growth factor; EDTA, ethylenediaminetetraacetic acid.

bovine serum albumin as standard) and by absorption at λ = 280 nm. For optical density measurements, the specific absorbance of 7S-NGF was taken to be 0.9 mL/mg-cm (Smith, 1969). 2.5S-NGF was purified from adult male mouse submandibular glands by the method of Bocchini and Angeletti (1969) and all preparations were shown to be electrophoretically homogeneous in three solvent systems as previously described (Oger et al., 1974). Horse heart ferricytochrome *c* was obtained from Sigma and ovalbumin from Mann. ^3H_2O was a New England Nuclear product and blue dextran 2000 was obtained from Pharmacia. Pancreatic deoxyribonuclease I, ribonuclease A, and bovine serum albumin (three times recrystallized) were purchased from Sigma.

Sedimentation. Sedimentation velocity studies were performed with a Beckman, Model E, analytical ultracentrifuge equipped with an ultraviolet absorption electronic scanning optical system. Rotor temperature was calibrated with a National Bureau of Standards thermometer and the RTIC unit of the instrument.

All of the sedimentation studies employing the ultraviolet optical system of the centrifuge were conducted in two ways, as follows. For measurement of the sedimentation coefficient of 7S-NGF at concentrations on the order of 50 μg/mL (utilizing light of wavelength λ = 230 nm), a conventional 2° double-sector aluminum-filled epon centerpiece was employed with solvent in one sector and protein solution in the other.

For measurement of the sedimentation coefficient of the 7S-NGF complex at concentrations near 10 μg/mL (λ = 220 nm), two 7° double-sector cells were placed 180° apart in a two-place rotor. One of these cells contained solvent and the other solute. A multiplex assembly (Beckman) was then used to keep track of the photomultiplier pulses of the scanning system. This procedure, originally introduced by Schachman and Edelstein (1973), is designed to maximize the light levels striking the photocathode and it has been used successfully to study the monomer $\rightleftarrows$ dimer equilibrium of 2.5S-NGF (Young et al., 1976a).

Weight-average sedimentation coefficients were determined in two ways: first, by measuring the migration of the midpoint of the concentration vs. distance curves, and, second, from eq 1 (Schachman, 1959)

$$s = -\left(\frac{1}{2\omega^2 t}\right) \ln\left[\left(\frac{2\int_{x_m}^{x_p} cx\,dx}{x_p^2 c_0}\right) + \left(\frac{x_m^2}{x_p^2}\right)\right] \quad (1)$$

Here x_m and x_p are radial meniscus and plateau positions, respectively, c is the protein concentration at x, and c_0 is the initial concentration. It should be noted that the use of eq 1 is formally equivalent to measuring the rate of migration of the second moment of a gradient curve, and the sedimentation coefficient so obtained takes into account all sedimenting species. The integral of eq 1 was determined with Simpson's one-third rule.

Another method, originally devised by Tiselius et al. (1937; see also Yphantis and Waugh, 1956a,b), was used to evaluate (by net transport of solute) the sedimentation coefficient of only the β component as it exists in solutions of 7S-NGF. The basic feature of this method is that the sedimentation coefficient of a molecule can be determined by measuring its net transport across any given plane in the plateau region so long as the plateau region persists at this plane throughout the experiment. All that is required is the initial concentration of the component (c_0) and the concentration (c_t) remaining above the plane in the plateau after time t of centrifugation. If

$$\lambda = \frac{x_p^2}{x_p^2 - x_m^2} \quad (2)$$

then

$$s = \ln\left(\frac{\lambda}{c_t/c_0 - 1 + \lambda}\right)\left(2\int_0^t \omega^2 dt\right)^{-1} \quad (3)$$

In earlier studies on the monomer $\rightleftarrows$ dimer reaction of NGF using this procedure (Young et al., 1976a), the integral of eq 3 was determined by numerical integration of values of ω^2 estimated from the centrifuge tachometer and t was measured with a stop watch. To estimate this integral more accurately, the Schmitt output of the scanner multiplex system (Beckman Instruments, Inc.) was used to trigger a Model-L $\omega^2 t$ integrator assembly (Beckman). By this method, $\int_0^t \omega^2 dt$ was computed precisely during acceleration and deceleration of the rotor. A fixed partition cell (Beckman) was used for these studies and values of c_0 and c_t were measured by radioimmunoassay specific for the β component of 7S-NGF as previously described (Young et al., 1967a). The partial specific volume of 7S-NGF was taken to be 0.73 mL/g (Baker, 1975).

Gel-Filtration Studies. The chromatographic behavior of solutions of 7S-NGF was also studied utilizing columns of Sephadex G-75 (Pharmacia). To determine the weight-average partition coefficient (σ_w) of the β component of 7S-NGF, solutions of 7S-NGF were applied to solvent-equilibrated columns in such a way as to establish a plateau concentration of solute. Fractions were collected in preweighed plastic test tubes and the weight of the tube plus fraction provided an accurate measure of each fraction volume to within 50 μL. Solutions of cytochrome *c* and ovalbumin were used to establish the partition coefficients for these marker proteins, and the void (V_0) and internal (V_i) column volumes were determined with blue dextran and ^3H_2O, respectively.

Radioimmunoassays. Concentrations of the β component in dilute solutions of 7S-NGF were measured by radioimmunoassay, based upon pure mouse 2.5S-NGF standards. The limit of sensitivity of this assay is 0.5 ng/mL and the procedural details have been given elsewhere (Murphy et al., 1975).

Results

Figure 1 (top) presents three sedimentation velocity profiles of a 50 μg/mL solution of 7S-NGF at the times indicated after reaching a rotor speed of 48 000 rpm. As can be seen, two plateau regions corresponding to two distinct boundaries are beginning to emerge. Because of diffusion and insufficient resolution, we have been unable to measure the individual sedimentation coefficients of these boundaries. However, to estimate the weight-average sedimentation coefficient (taking together all sedimenting species), the second moment was determined from eq 1 (Table I). This procedure yields a value considerably less than 7 S ($s_{20,w}$ = 4.83S)[2]—a result which reflects appreciable dissociation of this form of NGF, even at concentrations as high as 50 μg/mL.

Upon further dilution of 7S-NGF solutions to a protein concentration of 10 μg/mL, values of $s_{20,w}$ fall to 3.6–3.8 S (see Table I). In light of the observation that only a single detectable boundary was observed in Figure 1(bottom), both the second moment and the conventional gradient midpoint methods were

[2] Actually, this value is an optical weight-average coefficient. Yet, since the specific absorbances of the individual α, β, and γ components of the 7S NGF complex are closely similar (Smith, 1969), we have taken the values obtained from eq 1 to represent weight-average numbers.

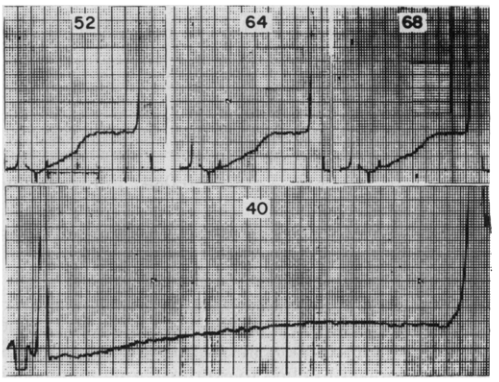

FIGURE 1: Velocity sedimentation patterns of 7S-NGF. Times (min) after reaching full speed are shown on the scanner traces. Temperature was maintained close to 25 °C. Solvent: 0.1 M potassium phosphate, pH 7.0. Top: 7S-NGF protein concentration, 50 μg/mL; λ = 230 nm; 48 000 rpm. Bottom: 7S-NGF protein concentration, 10 μg/mL; λ = 220 nm; 60 000 rpm.

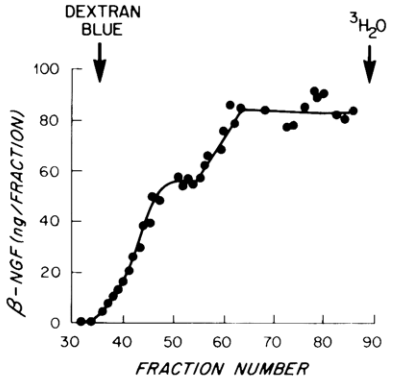

FIGURE 2: Frontal elution Sephadex G-75 profile of 7S-NGF at 50 μg/mL. Column volume was 5.2 mL and was equilibrated with 0.1 M potassium phosphate, 1 mg/mL bovine serum albumin, 0.01 EDTA, pH 7.0, at 4 °C. Sufficient protein solution (2.4 mL) was applied to the column to establish a plateau region. Fractions (50 μL) were measured for the β component by radioimmunoassay. V_0 and V_i were determined from a separate run.

TABLE I: Weight-Average Sedimentation Coefficients of 7S-NGF.[a]

Protein Concn (μg/mL)	Wavelength (nm)	Method[b]	$\bar{s}_{20,w}$(S)
50	230	2nd moment	4.83 ± 0.11
10	220	Midpoint	3.63 ± 0.15
10	220	2nd moment	3.85 ± 0.24
10		Transport	4.40 ± 0.05

[a] Experiments were conducted at 48 000 rpm (50 μg/mL) and 60 000 rpm (10 μg/mL). Temperature was maintained close to 25 °C. The solvent was 0.1 M potassium phosphate, pH 7.0. [b] Refers to use of the midpoint of the c vs. x scanner traces, the use of eq 1, or the transport of solute method (eq 3) for measuring $s_{20,w}$. Values represent $s_{20,w}$ ± the standard deviation of the regression coefficient.

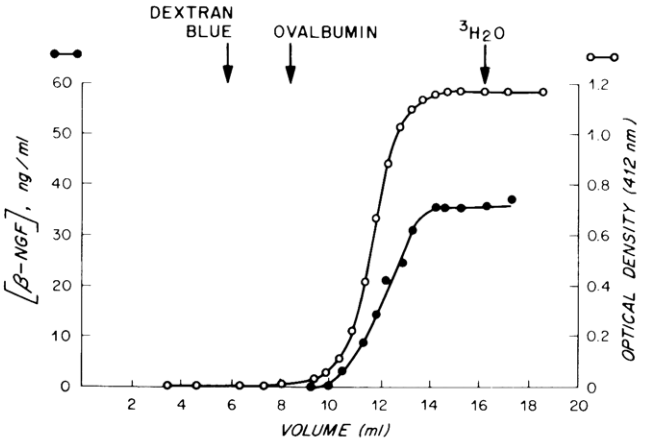

FIGURE 3: Frontal elution Sephadex G-75 profile of 7S-NGF at 0.76 μg/mL. Column volume was 17.3 mL (1 × 23 cm) equilibrated with 0.1 M potassium phosphate, 1 mg/mL bovine serum albumin, pH 7.0, at 4 °C. Ten milliliters of a solution containing 0.76 μg/mL 7S-NGF (●) and 1 mg/mL cytochrome c (○) was applied. Fractions (0.4 mL) were measured for β-NGF by radioimmunoassay and for cytochrome c by absorbance at 412 nm. Blue dextran, ovalbumin, and ^3H_2O were run separately.

used to measure $s_{20,w}$. Table I reveals that these two methods yield virtually identical numbers. Thus, under these conditions, 7S-NGF has undergone considerable dissociation at concentrations as high as 50 μg/mL, and, at 10 μg/mL, no component with $s_{20,w}$ greater than 3.6–3.8 S was observed. Furthermore, Table I also shows that when the sedimentation coefficient is measured by the transport method (specific for the β component), this biologically active component of 7S-NGF migrates with $s_{20,w}$ considerably less than 7 S.

To study the dissociation reaction(s) at lower total protein conentrations, we turned to frontal elution procedures using Sephadex G-75 gel filtration. It should be noted here that we are measuring the β component by radioimmunoassay to determine its elution behavior as it exists in solutions of 7S-NGF at a given total protein concentration.

Consistent with the sedimentation behavior at concentrations of 50 μg/mL 7S-NGF (Figure 1, top), Figure 2 also illustrates two species with different elution volumes. From plots of log (molecular weight) vs. the partition coefficients (σ_w) of proteins of known molecular weight (see below), we estimate that the mass of the heavier component illustrated in Figure 2 is on the order of 50 000 g/mol and that of the lighter 20 000 g/mol. It should be noted that these estimates are based upon the partition coefficients of known globular proteins, and we have no evidence that the complexes depicted in Figures 1 and 2 possess shapes similar to that of the calibration proteins. However, an increase in axial ratio would be expected to yield falsely high molecular weights, not falsely low values. Consequently, in agreement with the ultracentrifuge results of Figure 1 and Table I, gel-filtration studies also demonstrate appreciable dissociation of the 7S-NGF complex at a concentration of 50 μg/mL. Thus, the gel-filtration data of Figure 2 indicate that the biologically active nerve growth promoting component (β) no longer exists in a 7S complex at these concentrations.

Further dilution of solutions of 7S-NGF yielded the results shown in Figure 3, which illustrates the frontal elution profile of a solution of 7S-NGF at a concentration of 760 ng/mL. Cytochrome c and ovalbumin were used as reference proteins in these studies, and it is clear that the β-component of 7S-NGF now migrates in a *single zone* very close to the elution position of cytochrome c (molecular weight 12 400 g/mol). This number is close to the mass of the monomer of β-NGF (13 259 g/mol). Values for the elution volume (V_e) were calculated from the midpoint of the elution profiles illustrated in Figures 2 and 3 and used to calculate the weight-average partition coefficient (σ_w) from the relation

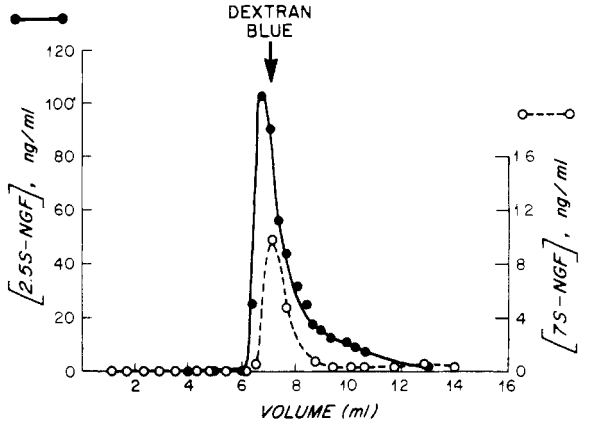

FIGURE 4: Sephadex G-25 elution profiles of 2.5S-NGF and 7S-NGF. Column dimensions 1 × 23 cm, equilibrated with 0.1 M potassium phosphate, 1 mg/mL bovine serum albumin, pH 7.0, at 4 °C. One hundred microliters of a 12 μg/mL solution of 7S-NGF (○) and 100 μL of a 5 μg/mL solution of 2.5S-NGF (●) were applied separately. Fractions (0.5 mL) were measured by radioimmunoassay. Blue dextran and ^3H_2O were applied in a separate run.

TABLE II: Weight-Average Partition Coefficients (σ_w) from Gel-Filtration Studies.[a]

c_0 (7S-NGF) (μg/mL)	c_0 (β Component) (μg/mL)	σ_w	
52[b]	1.6	0.084	(1st Boundary, Figure 2)
		0.27	(2nd Boundary, Figure 2)
0.76[c]	0.037	0.38	

[a] Initial concentrations (c_0) of 7S-NGF were determined by absorbance and of the β component by radioimmunoassay. Sufficient volume of solution was applied to establish a plateau concentration and individual fractions were measured by radioimmunoassay. In separate experiments, V_0 and V_i were determined with dextran blue 2000 and ^3H_2O, respectively. [b] Column volume, 5.2 mL; temperature 4 °C; solvent, 0.1 M potassium phosphate, 0.01 M EDTA, 1 mg/mL bovine serum albumin, pH 7.0; fraction volume, 50 μL. [c] Column volume, 17.3 mL; temperature 4 °C; solvent, 0.1 M potassium phosphate, 1 mg/mL bovine serum albumin, pH 7.0; fraction volume 0.35 mL.

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

where V_0 and V_i are the void and internal column volumes, respectively. The data are summarized in Table II.

To examine the possibility that the gel-filtration studies presented above arose from adsorption chromatography of the β-component of 7S-NGF upon cross-linked dextran, solutions of 2.5S-NGF as well as 7S-NGF were applied separately to a column of Sephadex G-25. As shown in Figure 4, both 2.5S-NGF and the β component of 7S-NGF emerge from the column at a position indistinguishable from the void volume—a result which eliminates the possibility of adsorption chromatography.

It will be appreciated from comparison of the data of Table II and that presented earlier (Young et al., 1976a) that, at a 7S-NGF concentration of 760 ng/mL, the β component exhibits a value of σ_w very close to that expected from the monomer ⇆ dimer reaction exhibited by 2.5S-NGF at comparable protein concentrations. Thus, if we make the assumption that the two polypeptide chains of the β dimer have completely dissociated from the α and the γ components at these low concentrations, then we can calculate the apparent dissociation constant (K) for the monomer ⇆ dimer equilibrium for comparison with that obtained earlier (Young et al., 1976a). If

$$\alpha = \frac{\sigma_w - \sigma_2}{\sigma_1 - \sigma_2} \quad (5)$$

where σ_1 and σ_2 are the partition coefficients for the pure monomer and dimer, respectively, then

$$K = \frac{1 - \alpha}{\alpha^2(c_0)} \quad (6)$$

where c_0 is the total initial protein concentration (Henn and Ackers, 1969). To estimate σ_1 and σ_2, a plot of ln V_e vs. ln (molecular weight) was constructed from values of V_e for ovalbumin and cytochrome c. By interpolation, σ_1 and σ_2 were evaluated from the monomer and dimer weights of NGF. When taken together with the data of Table II, where c_0 = 37 ng/mL (β component), this procedure yields $K = 1.1 \times 10^7$ M^{-1}. This number is virtually identical to the value $K = 9.4 \times 10^6$ M^{-1} determined earlier for the monomer ⇆ dimer equilibrium system of 2.5S-NGF (Young et al., 1976a). Thus, we infer that at these concentrations of 7S-NGF, not only is the β species fully dissociated from the α and γ components, but the two polypeptide chains of the β component itself are virtually completely dissociated from each other.

It has been reported that Zn^{2+} ions bind tightly to 7S-NGF with a dissociation constant = 10^{-11} M and that Zn^{2+} stabilizes the 7S-NGF structure (Pattison and Dunn, 1975; Au and Dunn, 1976). As isolated from mouse glands, Pattison and Dunn (1975) found 1-2 molecules of Zn^{2+} per molecule of protein. Yet the sedimentation studies summarized in Table I were performed with 7S-NGF solutions in the absence of a chelating reagent, and the gel filtration profile of Figure 2 was obtained with a solution containing 0.01 M EDTA (whose stability constant with Zn^{2+} is on the order of 3×10^{16} (Vallee and Wacker, 1970)). In view of the similarity of the results derived from Table I and Figure 2, it would appear that the stability of the 7S-NGF complex is not under the control of Zn^{2+}—at least at a protein concentration of 50 μg/mL and below.

As noted in the introduction, NGF secreted by L cells in culture exists as a high-molecular-weight complex (mol wt 160 000) even at concentrations of 1 ng/mL—at which concentration 7S-NGF is virtually fully dissociated. Since both L cell NGF and 7S-NGF are derived from the mouse and since their stabilities in solution are quite different, we have studied in a series of experiments the gel-filtration behavior of NGF in fresh submandibular-gland homogenates as follows. In each case, the submandibular glands of a single adult male Swiss mouse were excised and homogenized and a clear supernatant solution was obtained by centrifugation at 5000g for 15 min. This solution was then diluted by a factor of 1×10^4 to yield a β-NGF concentration on the order of 30 ng/mL by radioimmunoassay. Figure 5 presents a representative frontal elution profile of this solution on a column of Sephadex G-75. As shown in Figure 5, this material, quite unlike 7S-NGF at comparable concentrations, emerges from the column at a position indistinguishable from the void volume.

To minimize the possibility that NGF might be tightly bound to nucleic acids in gland homogenates, a solution identical to that described above was treated with 50 μg/mL each of ribonuclease A and deoxyribonuclease I for 2 h at 4 °C. (Analyses by trichloroacetic acid precipitation revealed that this treatment eliminated high-molecular-weight nucleic

acids.) Figure 5 illustrates the zonal elution profile of this preparation and again a single high-molecular-weight component was detected by radioimmunoassay.

It should be emphasized that these results do not establish whether the α and γ components of 7S-NGF are part of the high-molecular-weight complex depicted in Figure 5, nor do they permit any conclusions to be drawn about the molecular form of NGF as it exists in the gland in vivo. What the results of Figure 5 do reveal is that submandibular-gland homogenates contain a macromolecule which has a significantly higher stability in solution than purified 7S-NGF.

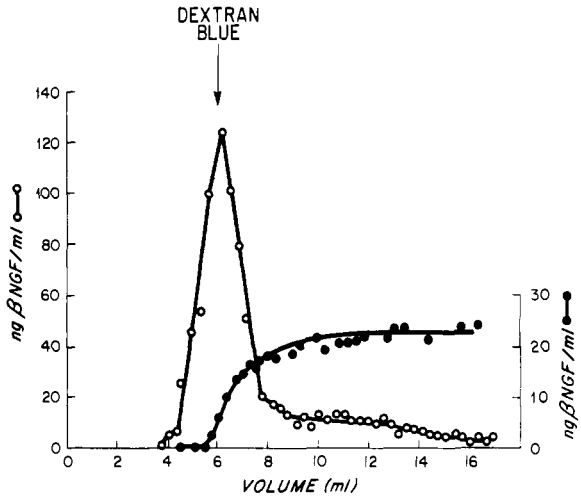

FIGURE 5: Sephadex G-75 elution profiles of NGF in fresh mouse submandibular-gland homogenates. The submandibular glands of a single mouse were homogenized, and the supernatant solution was diluted to give a β-NGF concentration of 30 ng/mL, as determined by radioimmunoassay. A sufficient volume of this solution was applied to the column to yield a plateau concentration (●). In a separate experiment (○) a gland homogenate was treated with 50 μg/mL each of deoxyribonuclease and ribonuclease for 2 h at 4 °C. This solution was then diluted to yield a β-NGF concentration of 3.7 μg/mL and 50 μL of this was applied to the column. Blue dextran was run separately. Column dimensions, 1 × 23 cm, equilibrated with 0.1 M potassium phosphate, 1 mg/mL bovine serum albumin, pH 7.0. Fractions were measured by radioimmunoassay.

Discussion

Taken together, the results presented above indicate that the 7S-NGF complex undergoes appreciable dissociation into its component polypeptide chains even at relatively high protein concentrations (in the μg/mL range). At protein concentrations in the ng/mL range, the biologically active β component, like 2.5S-NGF (Young et al., 1976a), is virtually completely dissociated into its constituent polypeptide chains. In an earlier sedimentation equilibrium study, Baker (1975) observed no dissociation of 7S-NGF at pH 6.8 at protein concentrations as low as 0.2 mg/mL and some dissociation between pH 7.4–7.8 at protein concentrations below 1 mg/mL. Thus, present evidence indicates that 7S-NGF is a relatively unstable complex, even at neutral pH values.

Measurements of the monomer $\leftrightarrows$ dimer equilibrium of pure 2.5S-NGF solutions indicate that the biological activity of this protein, as assayed in vitro, is mediated by its monomeric form (Young et al., 1976a). The present work reveals that the same is true of the β component in dilute solutions of 7S-NGF—a finding which raises a question as to the functional and structural significance of the 7S complex. From dorsal-root ganglion bioassays, Varon et al. (1967b, 1968) concluded that all three components of 7S-NGF are needed for full biological activity and that the β component alone accounts for only 25% of the activity of 7S-NGF preparations. In light of the present studies which demonstrate that the β component is completely dissociated from the 7S-NGF complex at concentrations far higher than those required for biological activity in vitro (Greene, 1974), it is difficult to see how the other components of the 7S species could enhance biological activity. On the other hand, it is possible that the dissociated α and γ components could increase biological activity of the β species by interacting independently with separate cellular binding sites. Yet several lines of evidence, as follows, suggest that this is not the case. Shooter and his colleagues (Moore et al., 1974; Perez-Polo and Shooter, 1975) have shown: (1) that proteolytic removal of both C-terminal arginine residues of β-NGF does not alter biological activity of the molecule, (2) that this modified protein is no longer able to form the 7S-complex, and (3) that the α and γ components do not increase its biological activity. However, the present study reveals that dilution alone is sufficient to cause complete dissociation of 7S-NGF. Consequently, we are unable to reconcile our findings with the concept (Perez-Polo and Shooter, 1975) that the α and γ components enhance the activity of β-NGF. In this regard, it should be noted that the findings of the present study are in complete agreement with the work of Levi-Montalcini and her colleagues (Zanini et al., 1968) who were unable to demonstrate any biologic effect of the α and γ components upon NGF activity.

There are at least three other possibilities which could serve to explain the origin and role of 7S-NGF. One is that it could be a storage form of NGF within the mouse submandibular gland. Second, in light of the enzymatic activity of the γ component (Greene et al., 1968), it could be that 7S-NGF is a by-product of, or plays a role in, the conversion of a larger pro-NGF to NGF. (At present, there is no direct evidence for such a precursor.) Finally, it remains possible that the 7S-NGF complex does not exist in the mouse salivary gland, but that it arises during the course of isolation and purification. In view of the strikingly different chromatographic behavior of 7S-NGF and the NGF in submandibular gland homogenates shown in Figure 5, this possibility deserves further study.

One last point concerning the monomer $\leftrightarrows$ dimer equilibrium system of β-NGF should be mentioned. The gel-filtration results of the present work indicate that the association constant for the two polypeptide chains of the β component of 7S-NGF ($K = 1.1 \times 10^7\ M^{-1}$) is virtually identical to that measured previously using homogeneous solutions containing only 2.5S-NGF ($K = 9.4 \times 10^6\ M^{-1}$) (Young et al., 1976a). These association constants do not agree with that reported by Moore and Shooter (1975) ($K = 3.3 \times 10^9\ M^{-1}$). However, these authors did not measure directly the equilibrium constant for the reaction; rather, they calculated it from forward (association) and reverse (dissociation) rate constants. Careful study of their paper will reveal that the forward rate constant was obtained from a subunit exchange reaction which was analyzed by isoelectric focusing. Yet, this technique, because of the wide pH gradient employed (pH 3–10) as well as the time required, would be expected to upset significantly the equilibrium state. Furthermore, the reverse rate constant was not measured at all—it was assumed, based upon the premise that this parameter is strictly diffusion controlled. Thus, the value for the association constant calculated by Moore and Shooter (1975) cannot be taken to a number obtained by direct measurement under true equilibrium conditions.

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References

Angeletti, R. H., and Bradshaw, R. A. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2417.

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

Au, A. M.-J., and Dunn, M. F. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1685, abstract.

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

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

Greene, L. A. (1974), *Neurobiology 4,* 286.

Greene, L. A., Shooter, E. M., and Varon, S. (1968), *Proc. Natl. Acad. Sci. U.S.A. 60,* 1383.

Henn, S. W., and Ackers, G. K. (1969), *Biochemistry 8,* 3829.

Jeng, I., Andres, R. Y., and Bradshaw, R. A. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1684, abstract.

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

Moore, J. B., Jr., and Shooter, E. M. (1975), *Neurobiology 5,* 369.

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

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

Pantazis, N. J., Arnason, B. G. W., and Young, M. (1977), *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Pattison, S. E., and Dunn, M. F. (1975), *Biochemistry 14,* 2733.

Perez-Polo, J. R., and Shooter, E. M. (1975), *Neurobiology 5,* 329.

Schachman, H. K. (1959), in Ultracentrifugation in Biochemistry, New York, N.Y., Academic Press, p 87.

Schachman, H. K., and Edelstein, S. J. (1973), *Methods Enzymol. 27,* 3.

Smith, A. P. (1969), Doctoral Dissertation, Stanford University, Palo Alto, Calif.

Tiselius, A., Pederson, K. O., and Svedberg, T. (1937), *Nature (London) 140,* 848.

Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5,* 134.

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

Varon, S., Nomura, J., and Shooter, E. M. (1967b), *Proc. Natl. Acad. Sci. U.S.A. 57,* 1782.

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

Young, M., Oger, J., Blanchard, M. H., Asdourian, H., Amos, H., and Arnason, B. G. W. (1975), *Science 187,* 361.

Young, M., Murphy, R. A., Saide, J. D., Pantazis, N. J., Blanchard, M. H., and Arnason, B. G. W. (1976b), in Surface Membrane Receptors, Bradshaw, R. A., Ed., New York, N.Y., Plenum Press, pp 247–267.

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

Yphantis, D. A., and Waugh, D. F. (1956a), *J. Phys. Chem. 60,* 623.

Yphantis, D. A., and Waugh, D. F. (1956b), *J. Phys. Chem. 60,* 630.

Zanini, A., Angeletti, P. U., and Levi-Montalcini, R. (1968), *Proc. Natl. Acad. Sci. U.S.A. 61,* 835.