Articles

Comparison of 7S Nerve Growth Factor and Nerve Growth Factor I from Mouse Submandibular Glands†

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ABSTRACT: 7S nerve growth factor (7S NGF) and nerve growth factor I (NGF_I) are NGF-containing protein complexes isolated from mouse submandibular glands by different protocols, and reports suggest that the molecules differ chemically. In this study, we compared the molecular properties and subunit compositions of the two proteins. Purified 7S NGF and NGF_I electrophoresed to identical positions on polyacrylamide gels in nondissociating buffers, with electrophoretic mobilities indistinguishable from that of unpurified NGF in salivary gland extracts. Ultraviolet absorption curves were identical, and sedimentation coefficients were similar (7.3 ± 0.25 S for 7S NGF; 7.2 ± 0.2 S for NGF_I) as determined by sedimentation velocity analysis. By sedimentation equilibrium analysis, molecular weights of 135 000–140 000 were obtained for both complexes at protein concentrations in the centrifuge cell >85 μg/mL; when protein concentrations within the centrifuge cell ranged from ~30 to 100 μg/mL at equilibrium, both complexes dissociated. Molecular weight values determined by gel filtration on Bio-Gel P300 and Sephadex G200 resins were similar for both proteins, and the values determined on Sephadex agreed with those obtained by ultracentrifugation. The subunit compositions of the complexes were also similar as determined by nonequilibrium isoelectric focusing, NGF_I being composed of proteins that migrated to positions identical with those of the α, β, and γ subunits of 7S NGF. Furthermore, the stoichiometry of the subunits was similar in the two complexes as determined by radioimmunoassays to each of the subunits and by densitometric analysis of electrophoretic gels. Both methods showed that the complexes contain approximately 2 mol of the α and γ subunits per mole of β-NGF. By all the criteria we tested, 7S NGF and NGF_I were indistinguishable proteins.

Two protocols have been published for purifying the high molecular weight form of mouse salivary gland nerve growth factor (NGF),[1] but there is disagreement as to whether or not the procedures generate the same product. Varon et al. (1967) first isolated high molecular weight NGF and named it 7S NGF because of its sedimentation coefficient in sucrose gradients. 7S NGF has a molecular weight of approximately 137 000 (Baker, 1975) and is made up of three different noncovalently linked subunits (α, β, and γ) of similar size (M_w ~ 26 000) [reviewed by Bradshaw (1978), Green & Shooter (1980), and Thoenen & Barde (1980)]. Nerve growth-promoting activity resides in the β-NGF subunit, which is a dimer made from identical polypeptide chains, each of molecular weight 13 250. The γ subunit is an arginine-specific protease of the serine type that cleaves synthetic substrates when separated from the 7S NGF complex (Greene et al., 1969). The α subunit is structurally similar to the γ protein (greater than 80% identity in amino acid sequence) (Ronne et al., 1984) but is enzymatically inactive (Isackson & Bradshaw, 1984). Physiological functions for the α and γ subunits have been uncovered. The stoichiometry of the complex has not been determined directly, but Server & Shooter (1977) estimated from molecular weight measurements of each subunit relative to the entire complex that 7S NGF contains 2 mol each of the α and γ subunits and 1 mol of β-NGF. 7S NGF also contains 1–2 mol of Zn(II) per mole, which enhances the stability of the molecule in solution (Pattison & Dunn, 1975).

A second protocol for isolating the high molecular weight form of mouse salivary gland NGF was developed as a result of a series of studies that suggested that 7S NGF may not be the form in which NGF is actually stored in the salivary gland. The stability of 7S NGF was found to be lower than that of a high molecular weight form of NGF of unknown composition identified in unfractionated salivary gland extracts (Pantazis et al., 1977) and in saliva (Murphy et al., 1977). Subsequently, Young et al. (1978) developed a new protocol for isolating NGF and reported that the stability of the isolation product was similar to that of the high molecular weight NGF in

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[1] Abbreviations: NGF, nerve growth factor; NGF_I, nerve growth factor I; RIA, radioimmunoassay; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Tes, 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid; Bis-Tris, [bis(2-hydroxyethyl)-amino]tris(hydroxymethyl)methane; OD, optical density.

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salivary gland extracts and saliva. The subunit composition of this molecule, called NGF_I, was not determined, but it was shown subsequently to contain a protein electrophoretically indistinguishable from β-NGF, 1 mol of a subunit with enzyme activity identical with that of the γ subunit of 7S NGF, and 1 mol of Zn(II) per mole of protein (Orenstein et al., 1978; Young & Koroly, 1980). The molecular weight of NGF_I was determined to be 116 000 by sedimentation equilibrium and by gel filtration (Young et al., 1978).

In this study, we directly compared the molecular properties and subunit compositions of 7S NGF and NGF_I. By the criteria tested, the proteins were indistinguishable, suggesting that the two isolation procedures yield the same form of the NGF complex.

Materials and Methods

Reagents. All reagents were of the highest grade available and, unless otherwise indicated, were purchased from Sigma. Acrylamide, bis(acrylamide), and Bio-Gel P300 gel filtration resin were purchased from Bio-Rad. Sephadex and protein molecular weight standards used for gel filtration columns were obtained from Pharmacia. Ampholines were purchased from LKB.

Protein Purification. 7S NGF and NGF_I were isolated from submandibular glands of adult male mice according to the procedures of Varon et al. (1967) and Young et al. (1978), respectively. Modifications were made in each procedure as outlined under Results. Eluates from ion-exchange and gel filtration columns were monitored spectrophotometrically either directly or after dilution at 280 nm on a Gilford 250 spectrophotometer and by a radioimmunoassay (RIA) utilizing antibodies raised against the 2.5S form of the β-NGF subunit (Young et al., 1977). The RIA detects free β-NGF as well as β-NGF that is part of the high molecular weight NGF complex.

Following the final columns in the purification protocols for 7S NGF and NGF_I, the proteins were dialyzed extensively at 4 °C against 0.01 M ammonium acetate, and aliquots containing 1 mg of protein were lyophilized in plastic tubes and stored at −18 °C. Protein concentrations were determined by the procedure of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

In one series of experiments (electrophoresis on acetic acid–urea containing gels) we used a sample of 7S NGF kindly provided by Dr. Robert Stach.

The 2.5S NGF form of β-NGF was purified by the method of Mobley et al. (1976) as modified by Watson et al. (1985), and the individual subunits of 7S NGF were purified by the procedures of Smith et al. (1968).

Amino Acid Analysis. For some experiments, protein concentrations were determined by amino acid composition. Acid hydrolysates were prepared by heating samples with 5.7 N HCl for 24 h at 110 °C in vacuo. Spectrofluorometric amino analyses following precolumn derivitization with *o*-phthaldialdehyde were performed by a modification of the procedure of Jones et al. (1981).

Gel Electrophoresis. Isoelectric focusing gels were prepared as described by Stach et al. (1976), as modified by Pantazis (1983).

Polyacrylamide tube gels (10 × 0.6 cm) were prepared and electrophoresed in potassium phosphate buffer according to previously published methods (Young et al., 1978) or in the Bis-Tris–Tes system (Server & Shooter, 1976). Gels were stained overnight in 0.25% Coomassie blue in 9% acetic acid and 45% methanol and destained in 7.5% acetic acid and 5% methanol.

Acetic acid–urea gels were run according to the methods of Panyim & Chalkley (1969) and stained and destained by the methods described for tube gels. Dried gels were scanned with an Helena Quick Scan densitometer.

Some gels were analyzed by RIA for 2.5S NGF. Following electrophoresis, 0.2-cm gel slices were placed in separate tubes containing 1 mL of elution buffer (1.0 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mg/mL bovine serum albumin), and protein was eluted overnight at 4 °C. Aliquots were sampled for RIA.

Radioimmunoassay. Radioimmunoassays were carried out for 2.5S NGF according to the procedures of Young et al. (1977) and for the α, β, and γ subunits of 7S NGF by the methods of Pantazis (1983). Cross-reactivity between the α and γ subunits was approximately 10% in the RIAs (Pantazis, 1983).

Analytical Ultracentrifugation. Sedimentation experiments were performed at 5–10 °C in a Spinco Model E analytical ultracentrifuge equipped with an ultraviolet absorption electronic scanning optical system. Rotor temperature was calibrated by using a National Bureau of Standards thermometer with the RTIC unit of the instrument. Samples for analysis were diluted from stock solutions of 7S NGF or NGF_I (0.5–1.0 mg/mL protein) that were prepared by dissolving lyophilized samples in cold 0.1 M potassium phosphate buffer, pH 7.0, followed by overnight dialysis at 4 °C against the same buffer. A capillary type synthetic boundary double-sector cell with sapphire windows and 12-mm path length was used for sedimentation velocity studies. A double-sector cell with sapphire windows and 12-mm path length was used for sedimentation equilibrium studies. Protein samples (50 μL) were layered onto a fluorocarbon bed for sedimentation equilibrium analysis, and sedimentation was carried out for a minimum of 24 h. Equilibrium was considered established when no changes in sequential scan patterns were observed over a 12-h period.

Protein concentrations for sedimentation equilibrium studies were determined from UV absorption scans at equilibrium by using a standard curve (absorption vs. concentration) calculated for the UV optics of the centrifuge. The standard curve was established by measuring the initial absorption (before sedimentation redistribution occurred) of NGF loaded into the centrifuge cell at different concentrations.

Sedimentation coefficients and apparent molecular weights were determined by linear regression analysis of linear $\ln r/r_0$ vs. t and $\ln c$ vs. r^2 curves, respectively. Apparent molecular weights were calculated from the first derivative of a least-squares power fit of nonlinear $\ln c$ vs. r^2 curves.

Ultraviolet Absorption Spectroscopy. Ultraviolet absorption spectroscopy was performed with a Beckman scanning spectrophotometer. Lyophilized protein samples were dissolved in distilled deionized water and scanned from 300 to 230 nm. Correction for scatter was determined from extrapolation to absorption wavelengths of ln OD vs. ln λ curves ($\lambda > 320$ nm) (Englander & Epstein, 1957). $E^{1\%}_{1cm}$ vs. λ curves was calculated by using protein concentrations determined by the Lowry method with BSA as standard.

Gel Filtration. Columns (0.9 × 52 cm) of Sephadex G-200 and Bio-Gel P-300 were equilibrated at 4 °C in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL bovine serum albumin. Column effluents were collected in preweighed test tubes, and elution volumes were determined by weight. The void and total column volumes were determined with blue dextran and ^3H_2O, respectively. The columns were calibrated in separate runs with ferritin, catalase, aldolase, BSA, egg albumin, chymotrypsinogen, and cytochrome *c*. For calibra-

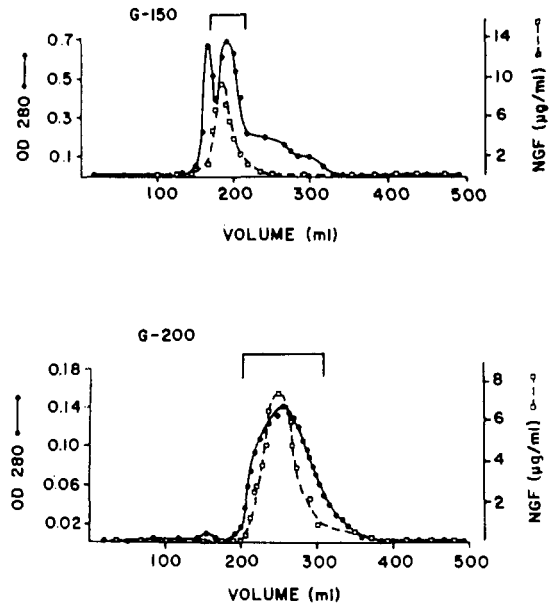

FIGURE 1: Elution profiles of 7S NGF on gel filtration columns (95 × 2.5 cm). Column fractions were monitored spectrophotometrically and sampled for analysis in the RIA for 2.5S NGF. (Top) Elution profile of 7S NGF from the final Sephadex G-150 column in the isolation protocol of Varon et al. (1967). The sample was eluted in 50 mM Tris-HCl (pH 7.4) at a flow rate of 1.8 cm/h. Fraction volumes averaged 3.1 mL. Fractions under the bracket were pooled and concentrated to ~4 mL by pressure filtration in an Amicon Micro-Ultrafiltration system equipped with a UM 0.05 filter. (Bottom) Concentrate obtained from the column in the top panel applied to a column of Sephadex G-200 equilibrated in 50 mM Tris-HCl, pH 7.4. Flow rate was 27 mL/h; fraction volumes averaged 3.95 mL. Fractions indicated by the bracket were pooled and concentrated.

tion, 0.2 mL of solutions containing 20 mg/mL protein standards was applied to the column, and the effluent was analyzed spectrophotometrically. The elution volumes of NGF were determined by sampling effluent fractions in the NGF RIA. Molecular weights were calculated from linear regression analysis of plots of log molecular weights vs. V_e/V_0 where V_e is the elution volume of the calibration proteins and V_0 is the column void volume.

Results

Protein Purification. We set out to isolate 7S NGF and NGF_I precisely as indicated in the original protocols (Varon et al., 1967; Young et al., 1978) to ensure the authenticity of the proteins. It was necessary, however, to modify both procedures. The product in three different preparations of 7S NGF eluted from the final gel filtration column (Sephadex G-150) in one of three overlapping protein peaks (Figure 1, top). To improve separation of the peaks, we pooled and concentrated the NGF-containing fractions and reapplied the sample to an additional column of Sephadex G-200 (Figure 1, bottom). NGF emerged in a single peak that was symmetrical by RIA analysis but asymmetrical by optical density measurements, suggesting some degree of protein heterogeneity.

Two modifications were made in the Young et al. (1978) procedure for isolating NGF_I. In the initial step, we applied the salivary gland homogenate to the DE-52 anion-exchange column and washed the column extensively with buffer. Only when the optical density of the effluent approached zero was the salt gradient initiated. As a result, NGF emerged in a distinct peak that could be identified by optical density measurements. In the original procedure, the salt gradient was begun soon after the gland extract was applied to the

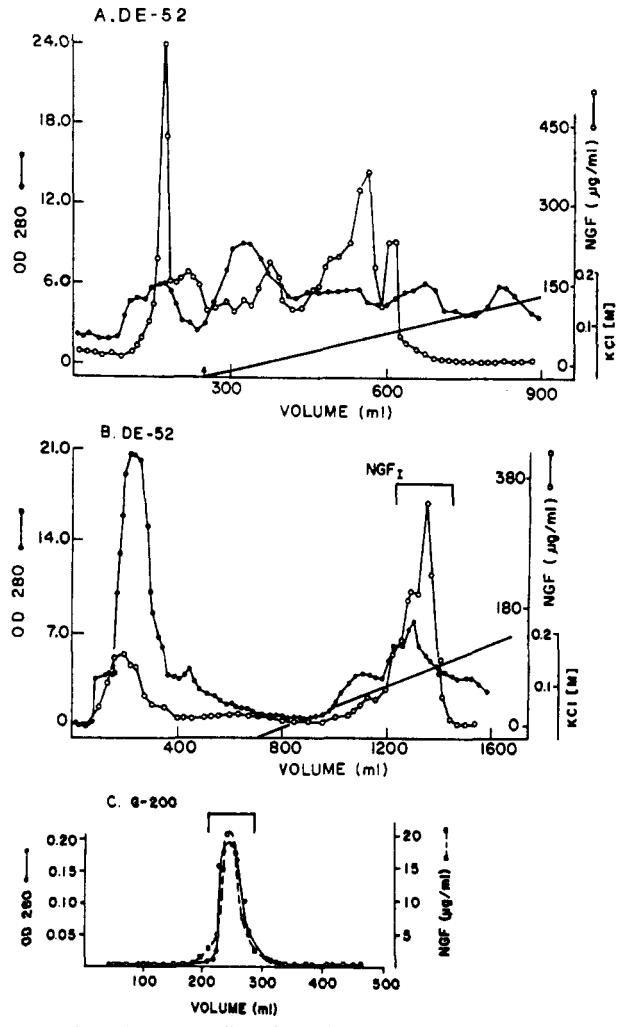

FIGURE 2: Elution profiles from ion-exchange and gel filtration columns in the isolation protocol for NGF_I. Column fractions were analyzed for 2.5S NGF by RIA and monitored spectrophotometrically. (A) Salivary gland extracts chromatographed over a DE-52 anion-exchange column (26 × 2.5 cm) according to the procedure of Young et al. (1978). Supernatants from gland homogenates (150 mL in 25 mM Tris, pH 8.0) were applied to the column, and the column was rinsed in 135 mL of buffer and the HCl gradient initiated. Flow rate ranged from 17 mL/h (during sample loading) to 42 mL/h once the gradient was initiated. Fraction volumes averaged 4.21 mL. (B) Conditions were identical with those in (A) except that the gradient was begun after the column was washed with approximately 550 mL of buffer until optical density approached zero. Fractions under the bracket were pooled. (C) Elution profile of NGF_I on the final gel filtration column (Sephadex G-200; 95 × 2.5 cm) in the procedure. Flow rate was 10.8 mL/h; fraction volumes averaged 4.1 mL. Fractions indicated by the bracket were pooled and concentrated.

column; NGF eluted in the midst of a broad protein peak and could be identified with precision only when the effluent fractions were assayed by RIA (compare parts A and B of Figure 2). In a second modification, NGF was eluted from the hydroxylapatite column (the third chromatographic step in the procedure) by extensive washing with 0.1 M potassium phosphate buffer, pH 7.0. The original protocol recommends a phosphate buffer gradient. With or without these modifications, NGF_I (four preparations) emerged from the final Sephadex G-200 column in a peak that was symmetrical by both RIA and OD measurements (Figure 2C). These modifications simplify the procedure and obviate the need of identifying NGF by specific methods such as RIA or bioassay.

Gel Electrophoresis in Nondissociating Buffers. The electrophoretic mobilities of purified 7S NGF and NGF_I were compared on polyacrylamide gels in nondenaturing solvents.

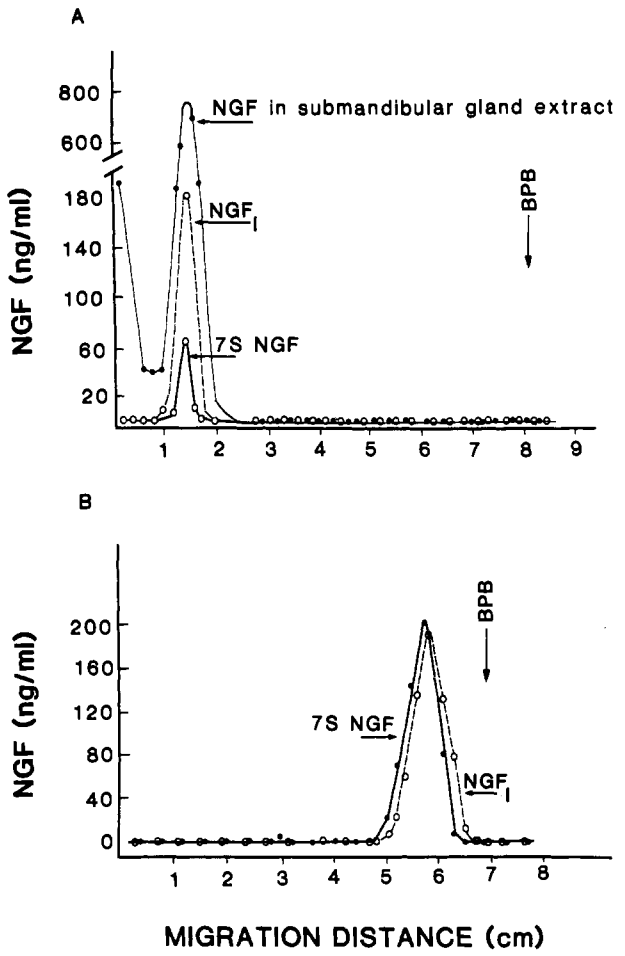

FIGURE 3: Electrophoretic profiles of NGF on polyacrylamide tube gels (10 × 0.6 cm) in nondenaturing buffers. (A) Lyophilized samples of NGF_I and 7S NGF (1 mg of protein) were dissolved in 1 mL of cold 50 mM potassium phosphate buffer (pH 7.0). The tissue extract was prepared by homogenizing submandibular glands from a male mouse in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) followed by centrifugation at 48000*g*. The supernatant (10 mg/mL protein) was collected. Then 25 μL of each sample was applied to the gels and electrophoresed at room temperature at a current of 5 mA/gel. Following electrophoresis, gels were sliced into 2-mm sections and incubated overnight with shaking in separate tubes containing 1.0 mL of potassium phosphate buffer (pH 7.0) with 1 mg/mL BSA. The eluates were assayed for β-NGF by 2.5S NGF RIA. (B) Samples (0.5 mL) of NGF_I (50 μg/mL) and 7S NGF (40 μg/mL) were applied to polyacrylamide tube gels and run in the Bis-Tris–Tes system. The resolving gel contained 5% acrylamide and the stacking gel, 2.5%. Following electrophoresis, proteins were eluted from gel slices as described in (A). β-NGF concentrations were determined by radioimmunoassay.

Figure 3A shows that the proteins migrate identically on 7.5% polyacrylamide gels in potassium phosphate buffer, pH 7.0. The positions of the proteins are identical with that of the major peak of immunoreactive NGF in unfractionated salivary gland extracts. 7S NGF and NGF_I also migrated to identical positions when electrophoresed on 7.5% gels in Bis-Tris–TES buffer (Figure 3B).

Ultraviolet Absorption Spectroscopy. The UV extinction curves for 7S NGF and for NGF_I are identical. Both molecules have absorbance optima at 277 nm, absorbance minima at 248 nm, and a shelf at 288 nm. Extinction coefficients ($E^{1\%}_{1cm}$) at 280 nm were calculated to be 13.0 for both proteins.

Analytical Ultracentrifugation. A single sedimenting boundary was observed for both 7S NGF and NGF_I at protein concentrations of 45 and 200 μg/mL. Identical sedimentation coefficients were observed with mean values ($s_{20,w}$) of 7.3 ± 0.2 S for 7S NGF (n = 3) and 7.2 ± 0.2 S for NGF_I (n = 3) tested at 200 μg/mL; at 45 μg/mL, the sedimentation coefficients were 7.2 ± 0.1 S for NGF_I (n = 3) and 7.1 ± 0.2 S for 7S NGF (n = 3).

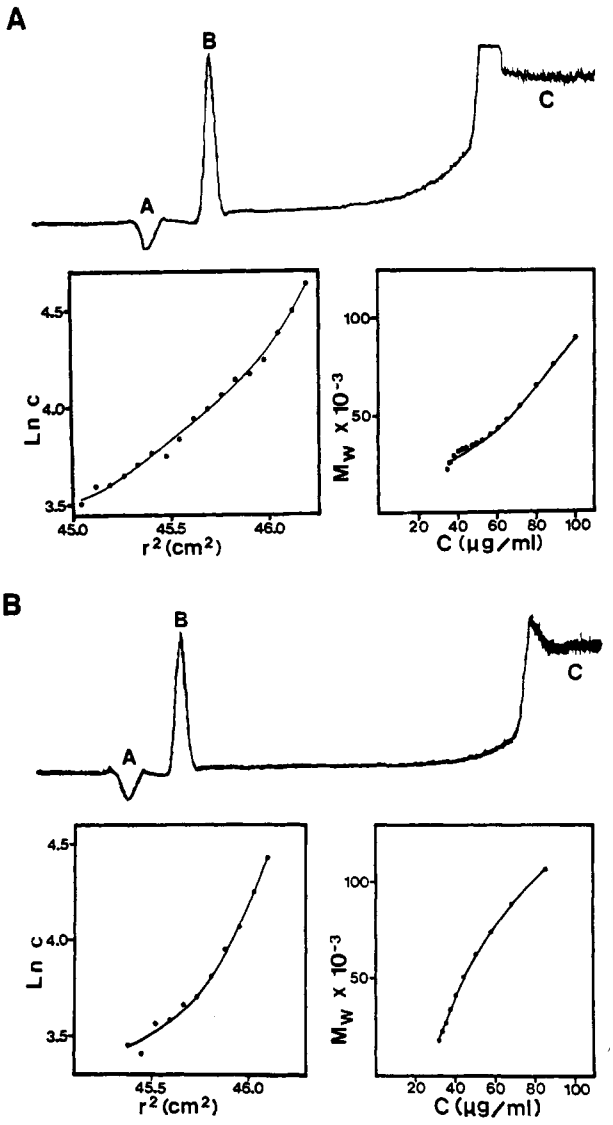

FIGURE 4: Sedimentation equilibrium centrifugation of 7S NGF and NGF_I at low protein concentrations. The ln c vs. r^2 curves for both proteins are nonlinear, indicating protein dissociation. By use of a least-squares power fit for each ln c vs. r^2 curve, the apparent average molecular weights as a function of concentration were calculated from the instantaneous slopes. The corresponding ultraviolet absorption scans at equilibrium are shown for each protein. In each panel, A is the reference meniscus; B, the solution meniscus; and C, the fluorocarbon bed. Panel A: NGF_I at equilibrium concentrations in the centrifuge cell ranging from 30 to 90 μg/mL. Panel B: 7S NGF at equilibrium concentrations in the centrifuge cell ranging from 33 to 100 μg/mL. c = concentration (μg/mL); r = distance from centrifuge axis of rotation (cm); M_w = weight-average molecular weight.

Two sedimentation equilibrium studies were carried out. In the first series, protein concentrations in the centrifuge cell at equilibrium ranged from 86 to 233 μg/mL for NGF_I and from 115 to 400 μg/mL for 7S NGF. In both cases, the ln c vs. r^2 curves were linear (correlation coefficients = 1.00), giving weight-average molecular weights of 140 000 ± 6000 for NGF_I and 135 000 ± 6000 for 7S NGF.

In the second series, equilibrium protein concentrations were reduced to a range of 30–90 μg/mL for NGF_I and 33–100 μg/mL for 7S NGF. The ln c vs. r^2 curves were nonlinear for both NGF_I (Figure 4A) and 7S NGF (Figure 4B), with

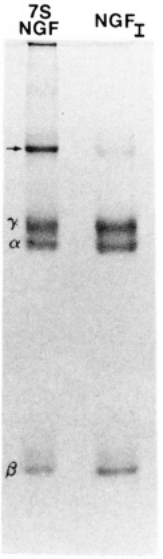

FIGURE 5: Electrophoresis of 7S NGF and NGF_I on acetic acid–urea gels. The migration positions of the α, β, and γ subunits are indicated. In both complexes an additional unidentified band (arrow) migrating more slowly than the major subunits was evident. The band accounted for 6.8 ± 0.3% of the absorbance in NGF_I and 18.2 ± 1.5% in 7S NGF. A small amount of material not entering the gel was also evident in both preparations, again more prominent in 7S NGF.

estimated apparent molecular weights ranging from 20 000 to 110 000. Under these conditions of analysis, therefore, both NGF complexes apparently undergo dissociation. The shape of the M_r vs. c curves for NGF_I and 7S NGF differ, probably as a result of different loading concentrations and protein purity.

Gel Filtration Analysis. 7S NGF and NGF_I were analyzed by gel filtration on columns of Sephadex G-200 and Bio-Gel P-300 resins. The average molecular weights for both proteins determined from elution volumes are higher on Bio-Gel P-300 than on Sephadex G-200 resins, for reasons unclear. Nonetheless, molecular weight values for 7S NGF and NGF_I were indistinguishable on both resins. On Bio-Gel P-300, values for 7S NGF (n = 5) averaged 170 000 ± 12 000 and for NGF_I (n = 5), 164 000 ± 13 000. On Sephadex G-200, 7S NGF (n = 4) eluted with an average molecular weight of 133 000 ± 10 000 and NGF_I (n = 3) with a molecular weight of 134 000 ± 2000. Values obtained on Sephadex were in agreement with the molecular weight values determined by analytical ultracentrifugation.

Subunit Analysis. Polyacrylamide gel electrophoresis and isoelectric focusing were done to compare the subunit compositions of 7S NGF and NGF_I. The electrophoretic profiles of the two proteins were similar on acetic acid–urea gels, which separate the subunits on the basis of size and charge (Figure 5). In this gel system, an unidentified band migrating more slowly than the complex subunits was also evident, and was more abundant in 7S NGF. On isoelectric focusing gels, NGF_I was found to contain molecules with isoelectric points indistinguishable from those of the α, β, and γ subunits of 7S NGF (Figure 6). It should be noted that, in two preparations of NGF_I tested, the complex contained three species of the γ subunit, while five γ species were detected in 7S NGF.

Stoichiometric Analysis. To quantitate the relative proportion of each of the major components in 7S NGF and NGF_I, we analyzed acetic acid–urea gels by densitometry following electrophoresis. Concentrations determined by amino acid analysis of the purified subunits of 7S NGF were run as standards. By this method, the molar ratios for the α, β, and γ subunits in 7S NGF were determined to be 1.85:1:2.07, and in NGF_I, 1.86:1:2.01, values that are indistinguishable (Table I).

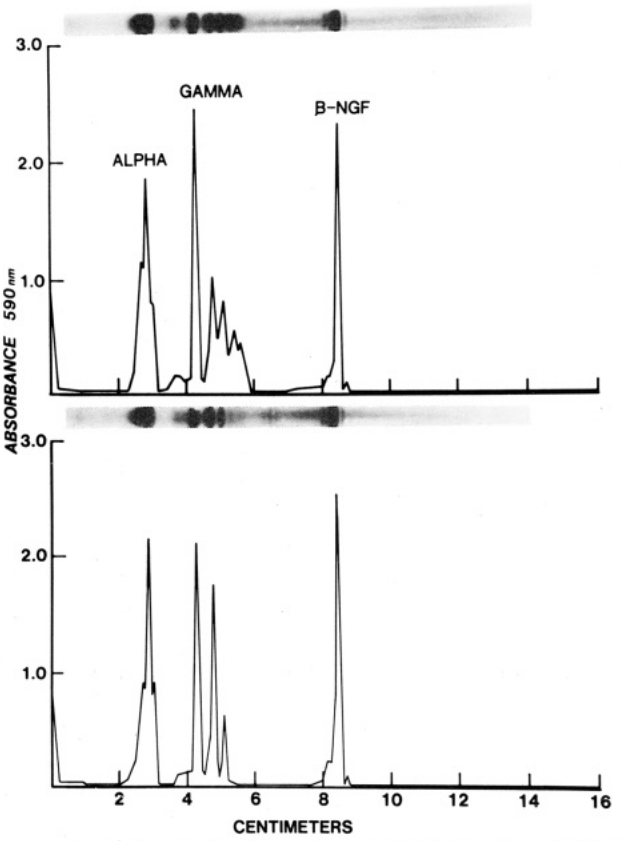

FIGURE 6: Isoelectric focusing gels of 7S NGF (top) and NGF_I (bottom). A sample of 100 μg for each protein was electrophoresed at 100 V for 1 h and 200 V for 2.5 h. Companion gels containing the purified subunits of 7S NGF were run as standards. The profiles for the two complexes are similar, differing only in the number of γ subunits. NGF_I contains three γ subunits, whereas 7S NGF contained five.

Table I: Quantitation by Densitometry of the α, β, and γ Subunits of 7S NGF and NGF_I on Acetic Acid–Urea Gels[a]

	α:β	γ:β
7S NGF (n = 7)	1.85 ± 0.03	2.07 ± 0.05
NGF_I (n = 10)	1.86 ± 0.04	2.01 ± 0.04

[a] 7S NGF and NGF_I (4–9 μg) were electrophoresed in separate wells on acetic acid–urea gels (see Figure 6). Four concentrations of the 2.5S form of β-NGF (0.5–2.0 μg) and α and γ subunits (1.0–4.0 μg) were electrophoresed in companion wells on the same gels. (Concentrations of the purified subunits were determined by amino acid analysis.) Gels were stained, destained, and dried, and all lanes were scanned on a densitometer. Standard curves (absorbance vs. concentration) constructed from the protein standards (r^2 = 0.91–1.00) were used to determine the relative amount of each subunit in the two NGF complexes. Values indicate the mean (±SEM) of molar ratios determined in four gels containing seven samples of 7S NGF and ten samples of NGF_I. Molar ratios were determined by using molecular weights of 26 000 for α (Stach et al., 1980), 26 518 for β (Angeletti et al., 1973), and 26 000 for γ (Stach et al., 1976).

The proteins were also analyzed by radioimmunoassays specific for the α, β, and γ subunits. The molar ratios (Table II) for the α, β, and γ subunits are 2.2:1:1.8 in 7S NGF and 2.3:1:2.1 in NGF_I. Within experimental error, these values are indistinguishable and comparable to those obtained by densitometry.

Discussion

Biochemical and hydrodynamic data in this study suggest that 7S NGF and NGF_I are the same isolation product.

Table II: Molar Ratios of the α, β, and γ Subunits of 7S NGF and NGF_I Determined by RIA[a]

	α:β	γ:β
7S NGF	2.2 ± 0.2	1.8 ± 0.2
NGF_I	2.3 ± 0.2	2.1 ± 0.1

[a] 7S NGF and NGF_I were diluted to 0.1 M potassium phosphate buffer, pH 7.0, to protein concentrations yielding 1–50 ng of the subunits per milliliter. Concentrations of the α, β, and γ subunits in four dilutions of the protein complexes were measured in triplicate and normalized to the concentration of β-NGF at the same dilution. Values represent mean (±SEM) of the molar ratios determined at each dilution. Molecular weight values used for calculating molar ratios are indicated in the legend to Table I.

Despite minor differences in purity and subunit composition, the preparations were nearly identical by all the criteria we tested.

No differences between 7S NGF and NGF_I were detected either by gel electrophoresis or by isoelectric focusing. The molecules migrated identically on polyacrylamide gels in nondenaturing solvents to positions indistinguishable from that of the major peak of NGF activity in unfractionated salivary gland extracts. The proteins' subunit compositions were also similar when analyzed by electrophoresis on acetic acid–urea gels and by isoelectric focusing, which showed that NGF_I contained three species of the γ subunit while five γ proteins were detected in 7S NGF. These differences are not likely to be significant. 7S NGF is known to contain three to five forms of the γ protein when analyzed by isoelectric focusing (Smith et al., 1968; Stach et al., 1976; Thomas et al., 1981), and the relative amount of each species varies from one preparation to another. The different forms appear to arise as a result of posttranslational modifications in the parent molecule. Modifications in the γ proteins could well have occurred during the additional gel filtration step that we, as well as others (Au & Dunn, 1977), have found necessary in the purification protocol for 7S NGF.

RIA data show that 7S NGF and NGF_I contain the α, β, and γ subunits in approximately the same molar ratios. The values we determined experimentally are in agreement with the estimate made by Server & Shooter (1977) that 7S NGF contains 2 mol each of the α and γ subunits per mole of β-NGF. Caution should be exercised in interpreting these results, however, since we have not determined the concentration in solution at which 7S NGF and NGF_I fully dissociate. Therefore, at protein concentrations tested in RIA (1–50 ng of each subunit per milliliter), a proportion of the complexes may remain intact, limiting the access of antibodies to the individual NGF subunits. Nonetheless, under our conditions of analysis, the subunit ratios that were obtained for 7S NGF and NGF_I were identical. The values were also similar to those obtained by densitometry of NGF_I and 7S NGF electrophoresed on acetic acid–urea gels. The values we obtained do not agree with the estimate of Young & Koroly (1980) that NGF_I contains 1 subunit/mol of the γ subunit, an estimate based upon diisopropyl fluorophosphate binding to the active site serine within NGF_I.

In previous sedimentation equilibrium studies, Baker (1975) reported a molecular weight for 7S NGF of 137 000 ± 7000 while Young et al. (1978) reported a molecular weight of 116 000 ± 8000 for NGF_I. The latter group calculated molecular weight by using a value for partial specific volume ($\bar{V}$) of 0.69 estimated by the H_2O–D_2O procedure of Edelstein & Schachman (1967), whereas Baker used a value for $\bar{V}$ of 0.73 based on amino acid composition. A value for $\bar{V}$ of 0.73 would raise the molecular weight determined by Young et al. from 116 000 to 134 000, in good agreement with the value obtained by Baker. Thus the apparent discrepancy in reported molecular weights based on sedimentation equilibrium ultracentrifugation for 7S NGF and NGF_I may simply reflect the method of determination of $\bar{V}$. In our hands, NGF_I and 7S NGF had identical ultraviolet absorption curves, and the amino acid compositions of the complexes based on a single 24-h hydrolysis (data not shown) were nearly identical. Consequently, we selected a $\bar{V}$ of 0.73, as calculated by Baker, and determined molecular weight values for 7S NGF and NGF_I to be 135 000 ± 6000 and 140 000 ± 6000, respectively. These values are identical within experimental error and were indistinguishable from those obtained by gel filtration analysis on Sephadex resins.

NGF is known to dissociate in solution, but there is disagreement as to the concentration at which dissociation begins to occur. By sedimentation velocity analysis, we found that both 7S NGF and NGF_I were undissociated at concentrations in the centrifuge cell of 45 μg/mL, in agreement with the results of Young et al. (1978) obtained for NGF_I. By similar methods, however, Pantazis et al. (1977) and Palmer & Neet (1980) showed that 7S NGF dissociates at concentrations of 45 and 33 μg/mL, respectively. Using sedimentation equilibrium analysis, we observed dissociation of both complexes when equilibrium concentrations were limited in the centrifuge cell to below 90–100 μg/mL, while Young et al. (1978) reported that NGF_I is stable over a concentration range from 48 to 1700 μg/mL. There are several differences between these studies that could account for the discrepancies, including the use of different methods of determining protein concentration, temperatures, and pH. Furthermore, zinc concentration, which was not determined in this study, is an additional and important variable. 7S NGF and NGF_I both contain Zn, and there is ample evidence that the amount of zinc affects the stability of both proteins (Pattison & Dunn, 1975; Bothwell & Shooter, 1978; Young & Koroly, 1980). Bothwell & Shooter (1978) found that the dissociation of 7S NGF may occur over a wide range of protein concentration depending on the zinc concentration. Other variables that could alter the stability of the proteins include methods of isolation, protein purity, and the conditions under which the proteins are stored. Nonetheless, our studies suggest that the stabilities of the two proteins in solution are similar when the molecules are examined under identical experimental conditions.

Although the two purification protocols yield identical products, the procedure of Young et al. (1978), as modified in this study, may be preferable to that of Varon et al. (1967) for isolating 7S NGF. The Varon et al. procedure utilizes unusually high flow rates (350 mL/h) on the anion-exchange column and requires an additional gel filtration column to separate completely 7S NGF from other salivary gland proteins. In contrast, the Young et al. procedure does not require high flow rates on anion-exchange columns. Furthermore, in three of four NGF_I preparations, the final Sephadex G-200 column (the fourth column step in the procedure) did not increase the purity of the molecular appreciably and could have been omitted, thereby shortening the procedure considerably. It should be noted, however, that the protein yields reported in the literature are approximately 10-fold higher for the Varon et al. (1967) procedure as compared to those for the Young et al. (1978) procedure. In our hands, there were no differences between the two, both procedures generating approximately 6 mg of product from salivary glands obtained from 100 mice. The additional Sephadex column we used in the Varon et al. (1967) procedure may reduce yields considerably. It should be noted that Stach et al. (1977) have published a

rapid method for the isolation of 7S NGF in which the yields are much greater (70–80 mg from 100 mice) than for either of these two other procedures.

Registry No. Nerve growth factor, 9061-61-4.

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Intrinsic Uncoupling of Mitochondrial Proton Pumps. 1. Non-Ohmic Conductance Cannot Account for the Nonlinear Dependence of Static Head Respiration on $\Delta\tilde{\mu}_H$

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ABSTRACT: The passive membrane conductance L_H^l of rat liver mitochondria has been measured and compared with the quantity $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ (n = H^+/e stoichiometry; J_e^{sh} = rate of electron transfer in static head) over a $\Delta\tilde{\mu}_H$ range. The two curves approach each other only in the lower part of the range, while they sharply diverge at large values of $\Delta\tilde{\mu}_H$. Thus $nJ_e^{sh}/\Delta\tilde{\mu}_H^{sh}$ cannot be considered to be a measure of L_H^l in the upper $\Delta\tilde{\mu}_H$ region. Only a fraction of the static head electron flow is accounted for by futile proton cycling via leaks. Contaminating open membrane fragments or completely leaky mitochondria can be responsible for only a small part of the residual rate of oxygen consumption. We conclude that a large part of static head respiration must have yet another cause and propose it to be intrinsic uncoupling of the respiratory chain enzymes.

The relationship between the electron-transfer rate J_e^{sh} and the output force for the redox H^+ pumps $\Delta\tilde{\mu}_H^{sh}$[1] in static head (state 4) mitochondria has been the object of considerable interest in the past ten years (Nicholls, 1974, 1977; Sorgato

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