Multiple Forms of the Nerve Growth Factor Protein and Its Subunits*

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ABSTRACT: Of the three types of subunits produced by the acid or alkaline dissociation of the 7S species of the mouse nerve growth factor protein, two of them display heterogeneity on electrophoresis. The α subunits contain three major and one minor component and the γ subunits three components. Since each subunit component has a molecular weight around 30,000, they are not all derived from a single 7S nerve growth factor protein. Both individual α and γ subunits are separable by ion-exchange chromatography and remain stable after separation. Recombination of any one α and any one γ subunit with the biologically active β subunit produces a 7S species with the physicochemical properties and increased biological activity characteristic of the original preparation of the nerve growth factor protein. The 7S species produced from a common γ but differing α subunits show small differences in electrophoretic mobility which reflect the mobility differences between the α subunits. When a common α but differing γ subunits are used in the recombination, the resultant 7S species have the same mobility. Dissociation at either acid or alkaline pH of the 7S species formed from individual α and γ subunits produces only those subunits used in the initial recombination. These results suggest that the nerve growth factor protein preparation contains multiple forms of the 7S species all with the same general subunit composition but differing in the types of subunit they contain.

In agreement with this hypothesis, the α subunit composition of the nerve growth factor protein is not constant across its migrating zone on electrophoresis or during elution from ion-exchange resin but shows a continuous change from species containing predominantly the α subunit of lowest mobility to those with α subunits of higher mobility. The finding that the γ subunit composition also varies in the same way suggests that the multiple forms of the nerve growth factor protein may be in equilibrium.

The isolation from the adult male mouse submaxillary gland of a high molecular weight form of the nerve growth factor protein has recently been reported (Varon *et al.*, 1967a). Like a lower molecular weight nerve growth factor preparation previously described (Cohen, 1960) the new protein produces significant increases in the size of the superior cervical ganglia when injected into young mice and *in vitro* stimulates fiber outgrowth from explanted sympathetic or sensory ganglia. Although the new protein has the same biological potency as the earlier preparations, it is obtained in much higher yield and accounts for some 2% of the soluble protein of the submaxillary gland. The high molecular weight form (or 7S species) of the nerve growth factor protein is quite stable, but only within the narrow pH limits of 5 and 8. Outside this range, it undergoes a reversible dissociation which ultimately results in subunits (2.5S species) of approximately 30,000 molecular weight (Varon *et al.*, 1967b, 1968). Three different types of subunits can be separated by ion-exchange chromatography initiated at acid pH, one group (α subunits) being relatively acidic, the second (γ subunits) of intermediate charge properties, and the third (β subunits) basic. The basic, β subunits are the only ones showing biological activity in the *in vitro* assay but account, at best, for about one-quarter the activity of the original 7S protein and, unlike the latter, rapidly lose activity with time. Varon *et al.* (1967b, 1968) also showed that the three subunits recombine spontaneously at neutral pH to reform the original 7S species with an accompanying increase in biological activity. This recombination process is specific, requiring all three subunits. The products of the interaction of β with either α or γ subunits alone differ from the 7S species by having both smaller sizes and lower biological activity.

Upon electrophoresis, the α subunits show four, and the γ subunits three, protein components. All of these components take part, with apparently equal activity, in the recombination with β subunits. It is clear, however, from the molecular weights of the subunits (about 30,000) and the final complex (about 140,000) that not all these components can be represented within a single 7S molecule. This problem has been examined, as reported here, by isolating the individual α and γ subunits and characterizing the products of their recombination with the β subunit. The results of these experi-

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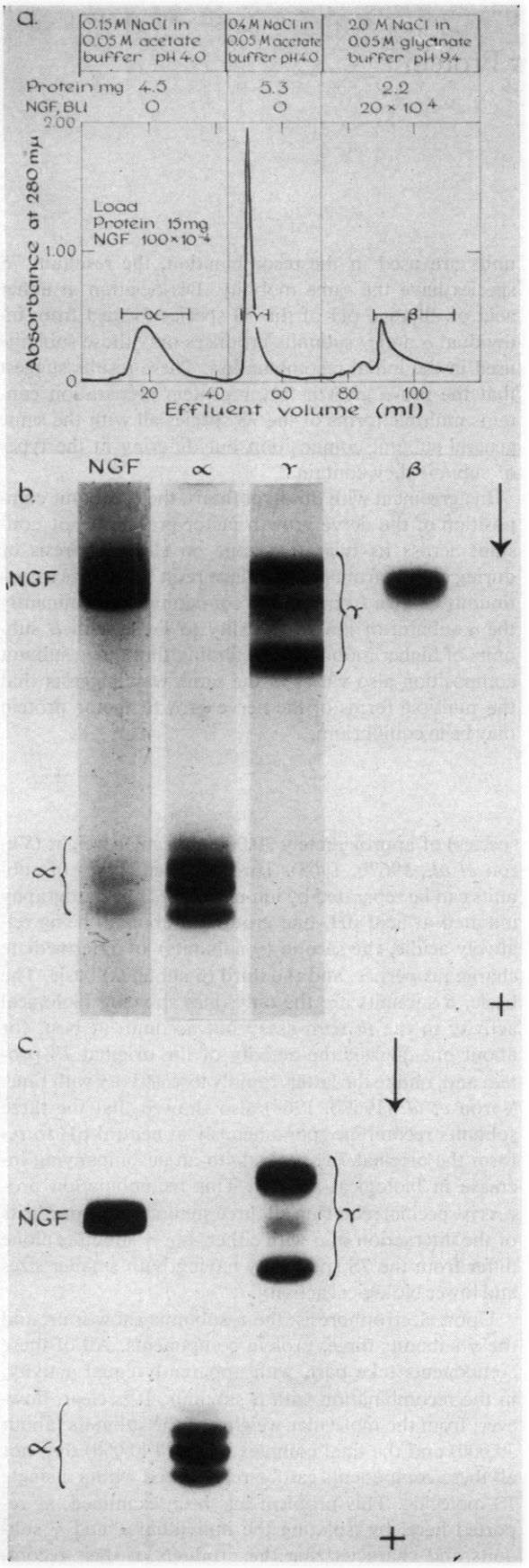


ments lead to the conclusion that preparations of the nerve growth factor protein from mouse submaxillary gland are mixtures of 7S species all with the same biological activity and general composition but containing different forms of the α and γ subunits.

Materials and Methods

The Nerve Growth Factor Protein. The 7S species was isolated by the methods previously described (Varon *et al.*, 1967a) and routinely characterized by its electrophoretic properties, sedimentation characteristics, and biological activity. The α, β, and γ subunits were isolated by a modification of the original method (Varon *et al.*, 1968) (see Results).

Ion-exchange chromatography was carried out on CM- and DEAE-cellulose (Whatman CM32, DE11, and DE32) precycled in acid and base according to the manufacturers' instructions and then equilibrated with the appropriate buffer before and after packing the column. Linear sucrose gradients were generated by the procedure described by Britten and Roberts (1960). Eluates were scanned at 230 or 280 mμ in a Zeiss PMQII spectrophotometer and the protein content of selected pools determined by the method of Lowry *et al.* (1951). Nerve growth factor activity was measured by the standard bioassay *in vitro* (Levi-Montalcini *et al.*, 1954). Assays were done in at least triplicate and often quintuplicate sets. Comparative assays were performed on the same day by one operator using a given set of reagents; these experiments were repeated several times.

Electrophoresis in acrylamide gel was carried out in four different buffer systems, the pH values of which are specified at 25°. One was the Tris–glycine system (Davis, 1964) of resolving pH 9.7. A second was the Tris–tricinate system, resolving pH 8.3, described earlier (Varon *et al.*, 1968). Another, a continuous sodium glycinate system (Varon *et al.*, 1968), was modified to operate at the higher pH of 10.3. Resolving gels were made by mixing, in the ratio 1:1:2, solutions A′ (7.2 ml of 2

FIGURE 1: The separation and characterization of the nerve growth factor protein. (a) Chromatographic separation of the subunits on CM-cellulose. Solution (1 ml) containing 12 mg of nerve growth factor protein was equilibrated by dialysis against 0.05 M acetate buffer (pH 4.0), containing 0.15 M NaCl, and applied to a 0.5 × 10 cm column of CM-cellulose (Whatman CM32) equilibrated with the same buffer. Column eluted in a stepwise fashion with the solvents indicated in the figure. Flow rate 17 ml/hr, fraction size 0.8 ml, absorbance measured at 280 mμ. (b) Identification of subunits by acrylamide gel electrophoresis. The nerve growth factor protein (80 μg) and α and γ subunits (50 μg each) analyzed in the Tris–tricinate system (pH 8.3); migration time 2.25 hr. The β subunits analyzed in the sodium glycinate system (pH 10.3); migration time 2 hr. Stained with Naphthol Blue Black. (c) As for b, but omitting the β-subunit analysis, with migration in the bistris–tes system (pH 7.55). Migration time 1 hr.

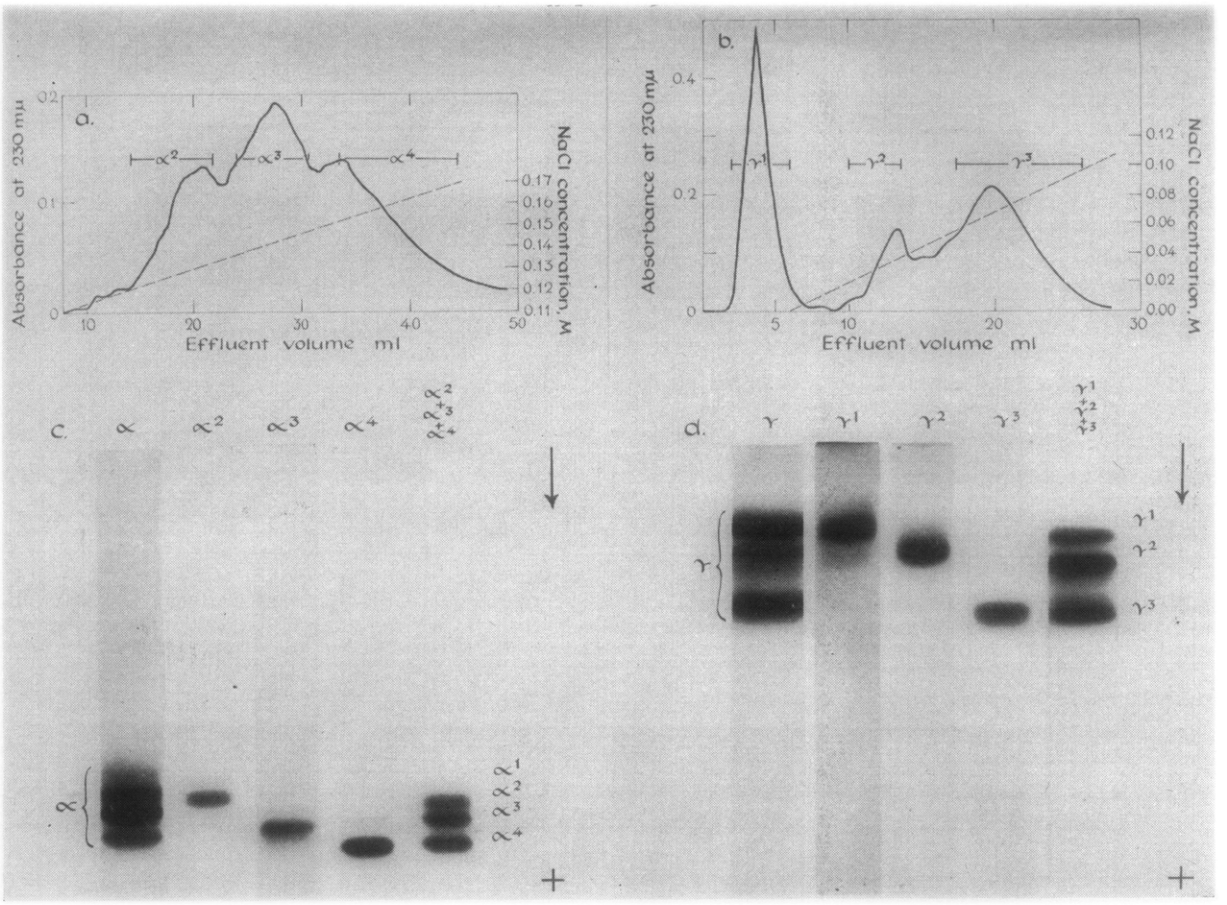


FIGURE 2: Separation and characterization of individual α and γ subunits. (a) Chromatographic separation of individual α subunits on DEAE-cellulose. 1.5 mg of α subunits in 100 μl of 0.05 M Tris-Cl buffer (pH 7.4) applied to a 0.5 × 25 cm column of DEAE-cellulose (Whatman DE32) equilibrated in 0.05 M Tris-Cl buffer (pH 7.4) containing 0.11 M NaCl. Column eluted with linear gradient between 0.11 and 0.17 M NaCl in 0.05 M Tris-Cl buffer (pH 7.4). Flow rate 5 ml/hr, fraction size 0.2 ml, absorbance measured at 230 mμ. (b) Chromatographic separation of individual γ subunits on DEAE-cellulose. γ subunits (1.5 mg) in 300 μl of 0.05 M Tris-Cl buffer (pH 7.4) applied to 0.5 × 10 cm column of DEAE-cellulose (Whatman DE11) equilibrated in 0.05 M Tris-Cl buffer (pH 7.4). Column eluted with 0.05 M Tris-Cl buffer (pH 7.4) until γ^1 subunit emerged and then with a linear gradient between 0 and 0.15 M NaCl in 0.05 M Tris-Cl buffer (pH 7.4). Flow rate 5 ml/hr, fraction size 0.2 ml, absorbance measured at 230 mμ. (c and d) Electrophoretic analysis in acrylamide gel of the fractions pooled as indicated in a and b. Original α or γ subunit (50 μg) shown on the left in c and d, respectively. Approximately 30 μg of protein applied to each gel for the analysis of the pooled fractions. The analysis on the right in c and d are of mixtures of 30 μg of each of the α and each of the γ subunits, respectively. Analysis in the Tris–tricine system (pH 8.3); migration time 2.25 hr; stained with Naphthol Blue Black.

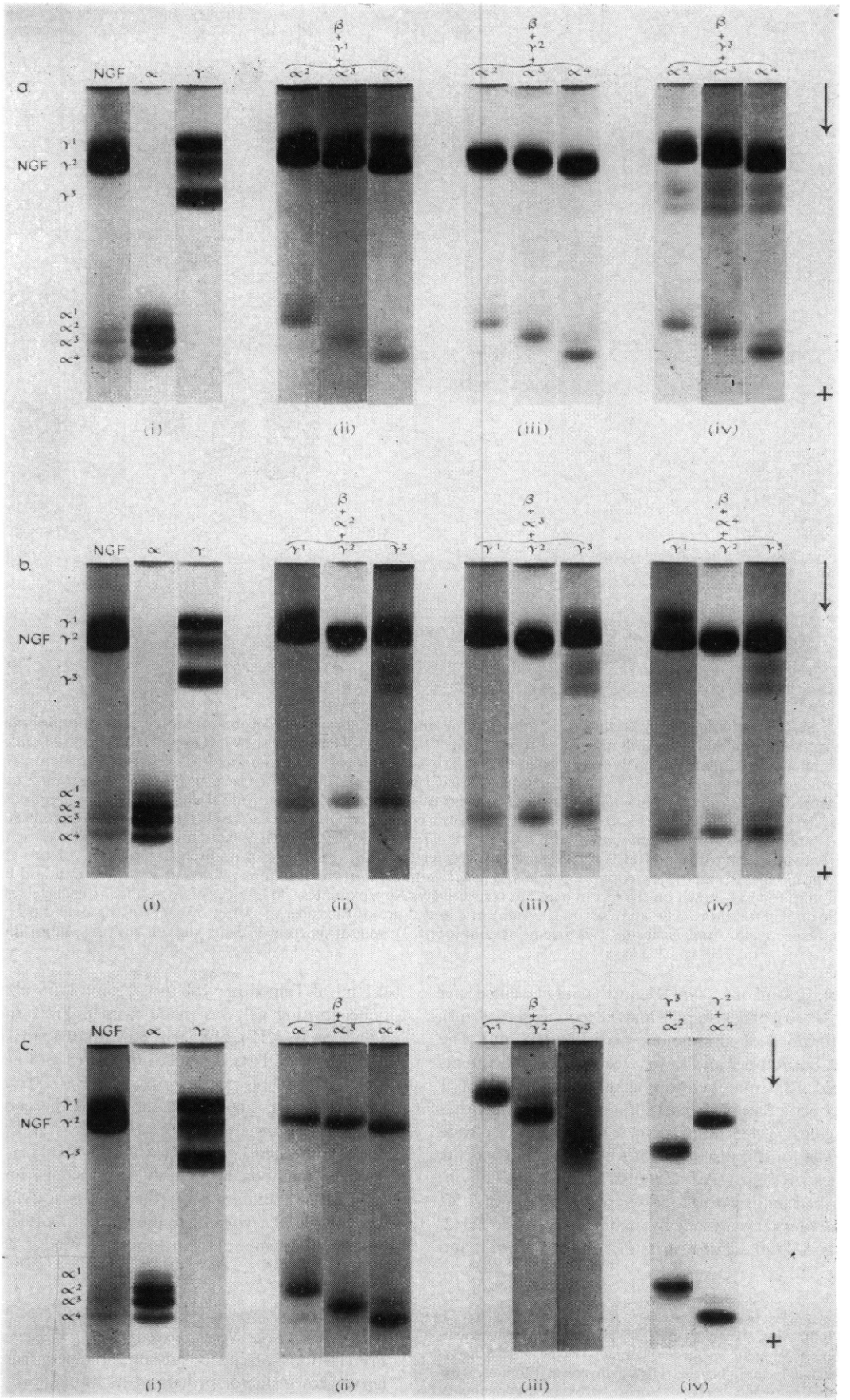
M glycine, 12.0 ml of 1 N NaOH, and 0.4 ml of Temed[1] per 100 ml), C (30 g of acrylamide and 0.8 g of bis per 100 ml), and P (0.025 g of ammonium persulfate/100 ml). The buffer in both upper and lower electrode compartments contained 18.0 ml of 2 M glycine and 300 ml of 1 N NaOH per l. Samples up to 300 μl in volume were dialyzed against a 1:5 dilution in water of the electrode buffer. The fourth, discontinuous buffer system was the bistris–tes system (A. Crambach and T. Jovin, unpublished data) with the lower resolving pH of 7.55. The resolving gel was made by mixing, in the ratio 1:1:2, soultions A'' (20.2 g of bistris, 19.5 ml of 1 N HCl, and 0.1 ml of Temed per 100 ml), C, and P (as above). The concentrating gel was made from a 1:2:1 mixture of solutions B (4.51 g of bistris, 20.9 ml of 1 M H_3PO_4, and and 0.1 ml of Temed per 100 ml), D (5 g of acrylamide and 1.25 g of bis per 100 ml), and E (0.002 g of riboflavin/100 ml). The upper buffer had the composition 9.38 g of bistris and 10.1 g of tes per l. and the lower buffer contained 12.5 g of bistris and 50 ml of 1 N HCl/l.

Other methods have been described earlier (Varon *et al.*, 1968). Buffers other than those described above were prepared according to the data of Datta and Gryzbowski (1961) and pH values are specified at 25°.

Results

Isolation of the Nerve Growth Factor Protein Subunits. Preparations of the β subunit obtained from the 7S nerve growth factor protein at its ultimate (β subunits) or penultimate (β_D subunits) purification stage had the

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: Temed, *N,N,N',N'*-tetramethylethylenediamine; bis, *N,N'*-methylenebisacrylamide; Tricine, tris(hydroxymethyl)methylglycine; bistris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; tes, *N*-tris(hydroxymethy)lmethyl-2-aminoethanesulfonic acid.


a.
NGF
α
γ
β + γ1 + α2 α3 α4
β + γ2 + α2 α3 α4
β + γ3 + α2 α3 α4
γ1
γ2
γ3
α1
α2
α3
α4
(i)
(ii)
(iii)
(iv)
b.
β + α2 + γ1 γ2 γ3
β + α3 + γ1 γ2 γ3
β + α4 + γ1 γ2 γ3
c.
γ3 + α2
γ2 + α4

same physical and recombination characteristics, but their biological activity differed in terms of yield and stability, the β_D subunits being superior in both instances (Varon *et al.*, 1968). The method for isolating subunits from the nerve growth factor protein has therefore been modified in attempts to reduce these differences. In the new procedure (Figure 1a), purified 7S nerve growth factor was dissociated by dialysis against 0.05 M acetate buffer (pH 4.0) containing 0.15 M NaCl and applied to a CM-cellulose column equilibrated with the same solvent, a condition where α subunits do not bind to the resin and can thus be eluted directly. The γ subunits were then collected by increasing the salt concentration to 0.4 M at the same pH. Finally the β subunits were eluted when the pH of the column was changed rapidly to pH 9.4 with a sodium glycinate buffer containing 2 M NaCl. The over-all yield, measured directly on the eluates, was approximately 20% of the load for the biological activity and 80% for the protein. Of the recovered protein, about equal amounts were collected in the α and the γ subunits, and half that amount in the β subunit. Both the activity and the protein recovered in the β subunits were, however, significantly higher than with the previous, slower fractionation procedure.

The electrophoretic patterns of the 7S protein, the α subunits, and γ subunits are shown in Figure 1 as observed on acrylamide gel at pH 8.3 (Figure 1b) and 7.55 (Figure 1c). The β subunits did not migrate into the gel at such pH's and were analyzed in the pH 10.3 system (Figure 1b, right). The nerve growth factor protein appeared as a single band at the lower pH with no evidence of dissociation; at pH 8.3, however, that is, above its upper pH stability limit, trace amounts of α subunits could be observed as the result of the partial dissociation of the nerve growth factor protein. At all pH values, as noted previously (Varon *et al.*, 1968), the α subunits showed three major and one minor component. Similarly, the γ subunits resolved into three components. (In each group, the individual components will be identified by superscripts, starting from the one with the lowest mobility.) Only one zone was observed for the β subunits migrating in the high pH–gel system. The composition of the α-subunit group was relatively constant from preparation to preparation and on storage in the cold for at least 1 month. That of the γ group was more variable between preparations and showed changes after storage for as little as 1 week, with a decrease in the amount of the γ^1 subunit and the appearance of an additional protein zone between the γ^2 and the γ^3 subunits (*e.g.*, Figure 5a).

Isolation of the Individual α and γ Subunits. The individual subunits within each group were resolved by chromatography on DEAE-cellulose. For the α subunits a reasonable but not complete separation was achieved with a linear gradient of salt in Tris-Cl buffer (pH 7.4) (Figure 2a) and eluate fractions could be selected in which the α^2, α^3, and α^4 subunits, respectively, were the major components (Figure 2c). No attempt was made to isolate the minor α^1 subunit although an enriched preparation could probably be obtained from the shoulder preceding the first major (α^2 subunit) peak. The isolated subunits were stable on storage and mixtures of the three approximated the original α-subunit preparation (Figure 2c).

The individual γ subunits were isolated in a similar manner (Figure 2b). Because of the greater differences in net charge among them, a more complete separation was achieved (Figure 2d) than in the case of the α subunits. Again the isolated γ^1, γ^2, and γ^3 subunits were stable for periods up to 1 week and mixtures gave electrophoretic patterns similar to that of the γ-subunit preparation (Figure 2d).

Recombination Experiments with the Individual α and γ Subunits. The ability of individual α and γ subunits to regenerate nerve growth factor protein when mixed with the β subunit was examined by analyzing the products of such mixtures on acrylamide gels. The tris–tricinate system at pH 8.3 was chosen because the nerve growth factor protein appears as a single, easily recognizable band and because, as already noted, the slight dissociation of the 7S protein taking place at such pH offers the possibility of characterizing, in the same electrophoretic experiment, the recombination product and some of the subunits resulting from its redissociation. A single α and a single γ subunit were added to β subunits on a 1:1:1 basis by protein. All the subunits were in 0.05 M Tris-Cl buffer (pH 7.4) and the mixtures were applied to the gels within 1 hr after mixing.

In each instance (Figure 3a,b), the major product of the recombination had a mobility close to that of the original nerve growth factor protein. Furthermore, only one α-subunit zone could be detected at the high mobility end of the gel, and it corresponded to the particular α subunit used in the individual experiment. The α bands were presumably due to excess α subunits in the recombination mixture as well as α subunits released by slight dissociation of the regenerated nerve growth factor protein. Excess γ subunits were observed on gesl loaded with γ^1-containing mixtures, but not on gels with γ^2-containing mixtures since this γ subunit has about

FIGURE 3: The recombination of the individual α and γ subunits with the β subunit. Recombination experiments were carried out at 0° by adding 25 μg of the appropriate α and γ subunit to an equal amount of β subunits, all subunits being in 0.05 M Tris-Cl buffer (pH 7.4). The resultant solutions were loaded onto the acrylamide gels within 1 hr of mixing. (a) The three analyses in block i are of 75 μg of nerve growth factor, 50 μg of α subunits, and 50 μg of γ subunits, respectively. In block ii are shown the analyses of the recombination of β and γ^1 with α^2, α^3, and α^4 subunits, respectively; block iii, the products using β and γ^2 with α^2, α^3, and α^4 subunits and block iv the products using β and γ^3 with α^2, α^3, and α^4 subunits, respectively. (b) Block i continues the same controls as in a,i. In blocks ii–iv the analyses shown in a,ii–iv, have been rearranged so that each block now contains the products of the recombination of β with one α but varying γ subunits. (c) Block i contains the same controls as in a,i. Block ii shows the products of the recombination of 25 μg of β with 25 μg of the α^2, α^3, and α^4 subunits; block iii, those of β with γ^1, γ^2, and γ^3 subunits and block iv those of two combinations of an α with γ subunit in the same proportions. Analyses in Tris - tricinate system (pH 8.3). Migration time 2.25 hr. Stained with Naphthol Blue Black.

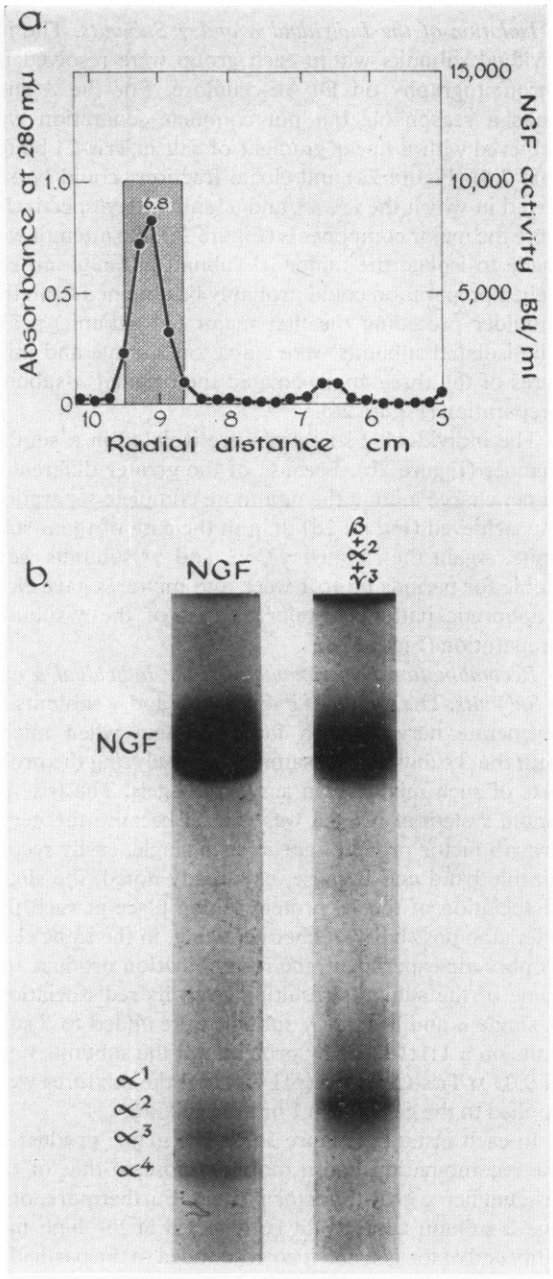


FIGURE 4: Characterization of the product of a recombination of an individual α and an individual γ subunit with β subunits. (a) 400 μg each of α^2, γ^3, and β subunits in 0.05 M Tris-Cl buffer (pH 7.4) mixed, the volume reduced to 100–200 μl by pressure dialysis, and the resultant solution layered on top of a 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4). Sedimentation was carried out in a SB 405 rotor (International B-60 centrifuge) at 60,000 rpm for 13 hr at 5°. Fractions (3 drops) collected from bottom of tube and their absorbance measured at 280 mμ. A pool corresponding to the 6.8S peak was dialyzed against 0.05 M Tris-Cl buffer (pH 7.4) and 0.1 ml of it used for measurement of biological activity. (b) Acrylamide gel electrophoresis of the 6.8S pool; 100 μl of the 6.8S pool containing approximately 50 μg of protein (right), 80 μg of nerve growth factor protein (left), analyzed in the Tris–tricine system (pH 8.3). Migration time 2.25 hr. Stained with Naphthol Blue Black.

the same mobility as the nerve growth factor protein itself. With γ^3-containing mixtures, the excess γ^3 subunit produced its characteristic band, which was accompanied in all cases by a second, higher mobility zone.

When any one particular γ subunit (Figure 3a) was recombined with each of the three different α^2, α^3, and α^4 subunits (as well as β subunits), the major recombination products banded with slight but significant increases in mobility paralleling the mobility differences among their α precursors. This is best seen, for example, by using the excess γ^1 or γ^3 bands as internal markers against which to compare the positions of the major band. In contrast (Figure 3b), recombination between one α subunit and differing γ subunits yielded products of uniform mobility, as clearly observed when the single α band, and the origin at the top of the resolving gel, are used as mobility markers. Thus, the mobility or net charge of the product of the recombination reflects that of the particular α subunit but not that of the particular γ subunit used as precursors.

The relevant controls for these recombination experiments are shown in Figure 3c. Individual α and γ subunits did not interact in the absence of the β subunits; this was observed earlier for the α- and γ-subunit groups (Varon *et al.*, 1968). The α subunits interacted with β subunits, even in the absence of γ subunits, to give proteins whose mobilities again reflected that of the particular α subunit used in the reaction. Although the new species had mobilities similar to the nerve growth factor protein they were distinguished from the latter by their lower biological activity and lower sedimentation coefficients on sucrose gradients ($s_{20,w}$ of 4.5 S for the $\alpha\beta$ species as contrasted with the value of 6.8 S for the $\alpha\beta\gamma$ complex). These were also the characteristics of the $\alpha\beta$ species produced when the whole α-subunit fraction was used (Varon *et al.*, 1968). Of the γ subunits, only the γ^3 component appeared to interact with β subunits under the conditions used in this investigation. The result was a mixture of components (Figure 3c), the fastest migrating of which corresponded to the new minor zone noted in the $\alpha\beta\gamma$ recombinations using the γ^3 subunit (Figure 3a,b).

Characterization of the Major Product of the Recombination. The products of the recombination experiments, after concentration by pressure dialysis, were analyzed by sucrose gradient sedimentation. Typically, as seen in Figure 4a, the main component sedimented with an $s_{20,w}$ around 6.8 S, the value found for the nerve growth factor protein under these conditions (Varon *et al.*, 1968). A smaller peak with an $s_{20,w}$ of 2.5 S presumably represented excess unreacted subunits. The protein in the 6.8 S position showed, on electrophoresis at pH 8.3, one major zone with the mobility of the nerve growth factor protein, together with one zone in the α-subunit region corresponding to the particular α subunit used in the recombination. The analysis, for example, of a recombination product from α^2, γ^3, and β subunits showed only the α^2 subunit (Figure 4b). Complete dissociation and identification of the subunits of the 6.8S material from the recombination experiments were achieved in two ways. Electrophoresis in the Tris–glycinate system at pH 9.7 allowed the direct identification on the acrylamide gel of the α and γ subunits resulting from an alkaline dissociation. Alternatively,

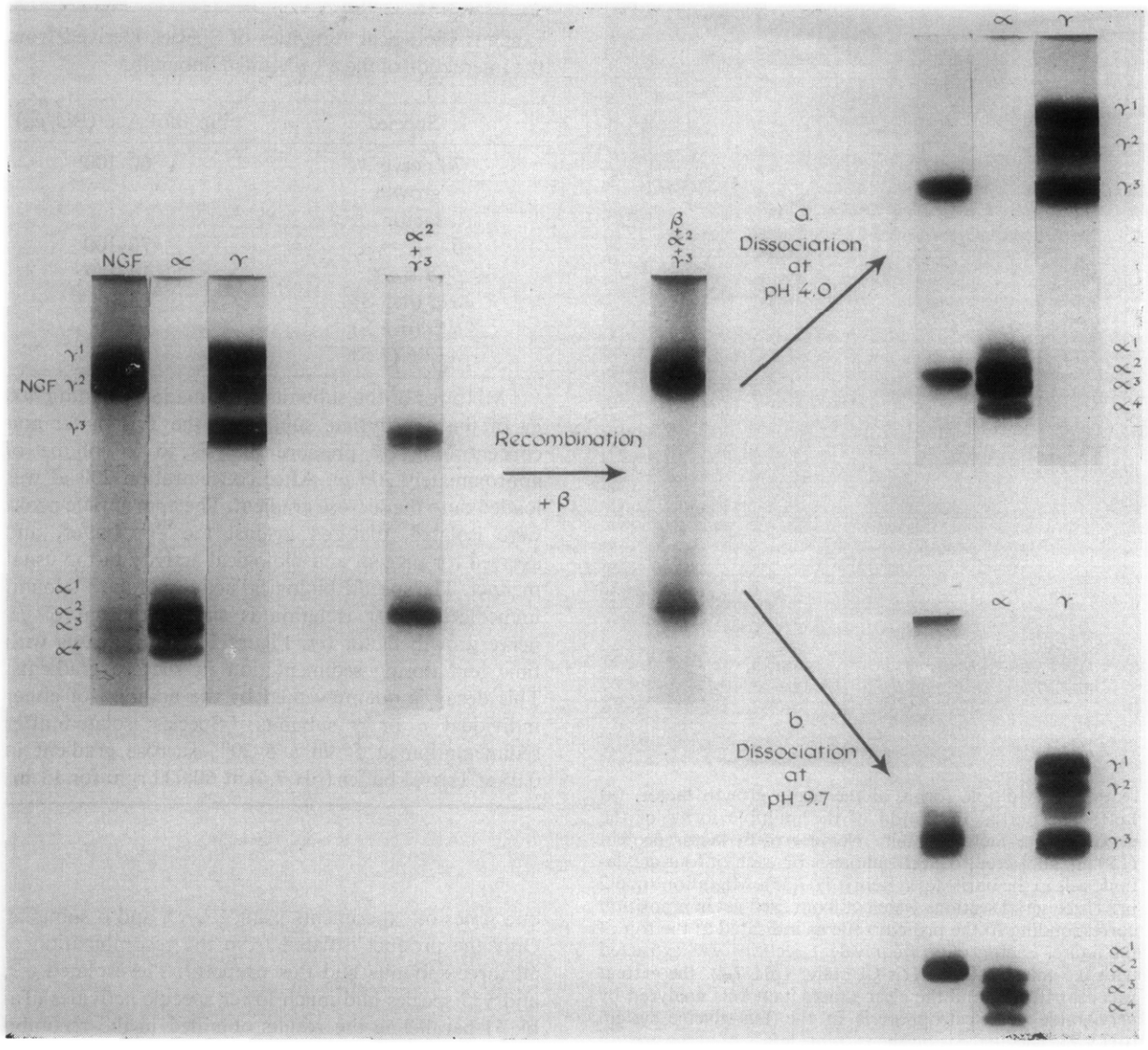


FIGURE 5: Characterization of the subunits of the recombination product formed from α^2, γ^3, and β subunits. Thet hree analyses on the left are of 80 μg of nerve growth factor protein, 50 μg of α, and 50 μg of γ subunits and serve as standards for comparison. The next analysis shows the α^2 and γ^3 subunits (30 μg of each) used in the recombination. Recombinations were made with 400 μg each of α^2, γ^3, and β subunits and the product isolated from the 6.8S peak in the sucrose gradient as shown in Figure 4a. The analysis of this product was the same as in Figure 4b and is shown above after the arrow depicting Recombination. The product was dissociated in two ways. (a) The 6.8S pool fraction containing approximately 300 μg of protein was dialyzed against 0.05 M acetate buffer (pH 4.0) containing 0.40 M NaCl, applied to a 0.5 × 5.0 cm column of CM-cellulose (Whatman CM32) equilibrated with this buffer, and eluted with the same buffer. Flow rate was 5 ml/hr, fraction size 0.1 ml. The α and γ subunits emerged in a single protein peak between 0.8 and 1.5 ml; this pool was concentrated by pressure dialysis against 0.05 M Tris-Cl buffer (pH 7.4). An aliquot of the concentrated pool containing 60 μg of protein was analyzed in the Tris–tricine system (pH 8.3) Controls of 50 μg of α and 50 μg of γ subunits were analyzed in the same system. (b) An aliquot of the 6.8S pool containing 60 μg of protein was analyzed directly by electrophoresis in the Tris–glycinate system at pH 9.7. Controls of 50 μg of α and 50 μg of γ subunits were analyzed in the same system.

the subunits were isolated at acid pH on CM-cellulose. For this the 6.8S material was dialyzed against 0.05 M acetate buffer (pH 4.0) containing 0.4 M NaCl and applied to a CM-cellulose column equilibrated with the same buffer. Subsequent elution with this buffer gave a single protein peak which contained both α and γ subunits, and these were identified by electrophoresis at pH 8.3. A representative experiment using the recombiation of α^2, γ^3, and β subunits is shown in Figure 5. Dissociation at either pH 4.0 or 9.7 of the major recombination product isolated from the sucrose gradient showed that only the α^2 and γ^3 subunits used for the recombination were recovered (Figure 5). Analysis of the recombination product in the continuous sodium glycinate system at pH 10.3 confirmed the presence of the β subunits in the recombination products. The characterization by the above methods of the recombination product and of the subunits it contained was carried out for products made with each α subunit and each γ subunit. All the recombination products had the physical characteristics of the nerve growth factor protein and each, on dissociation, produced only those subunits

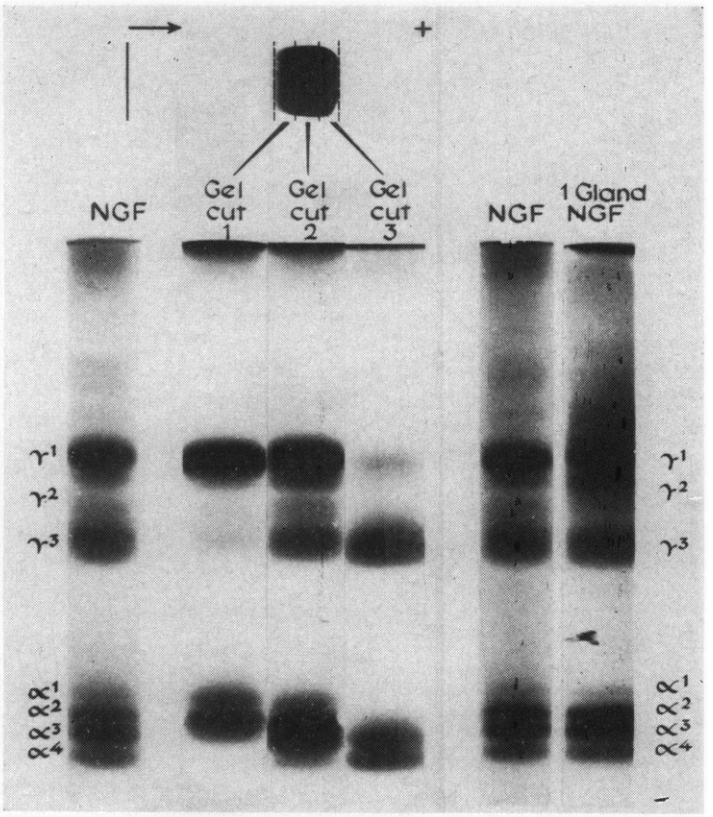


FIGURE 6: Multiple forms of the nerve growth factor. (a) Left: The partial resolution of the multiple forms of the nerve growth factor protein. Nerve growth factor protein (150 μg) subjected to electrophoresis on each of four acrylamide gels in the bitris–tes system (pH 7.55). Migration time 2 hr. Three serial sections were cut from each gel in a position corresponding to the protein zone as indicated at the top of the figure. Each group of equivalent sections was extracted with 0.5 ml of 0.01 M Tris-Cl buffer (pH 7.4), the extract was centrifuged, and the clear supernatant was analyzed by acrylamide gel electrophoresis in the Tris–glycine system (pH 9.7). The analysis on the extreme left is of nerve growth factor protein (80 μg); the remaining analyses are of the protein extracted serially from the three sections. Migration time in the pH 9.7 system 2.25 hr; stained with Naphthol Blue Black. (b) Right: Multiple forms of the nerve growth factor protein in individual mice. One lobe from a submaxillary gland of a Swiss albino, 50–60-day-old male mouse, homogenized in 1.5 ml of H_2O and lyophilized. Residue suspended in 0.2 ml of 0.05 M Tris-Cl buffer (pH 7.4) and applied to a 0.9 × 100 cm column of Sephadex G-100, equilibrated in the same buffer. A pool containing the nerve growth factor protein was collected between 34 and 37 ml of eluent buffer and 300 μl from this pool was further purified on an acrylamide gel in the bistris–tes system (pH 7.55). Migration time was 2 hr. The section of the gel corresponding to the whole nerve growth factor protein (determined from a parallel control gel stained with Naphthol Blue Black) was extracted with 0.5 ml of 0.01 M Tris-Cl buffer (pH 7.4) and centrifuged, and the clear supernatant was analyzed in acrylamide gel in the Tris–glycine system (pH 9.7). Migration time 2.25 hr; stained wth Naphthol Blue Black. Standard nerve growth factor protein from 100 glands (left) and preparation from one lobe (right).

TABLE I: Biological Activities of Species Derived from the Interaction of the α^2, γ^3, and β Subunits.[a]

Species	Sp Biol Act. (BU/μg)
7S nerve growth factor	60–100
β	70–100
β (2.5 S)[b]	14
$\alpha^2\beta$ (4.5 S)[b]	5
$\gamma^3\beta$ (6.6 S)[b]	5
$\alpha^2\gamma^3\beta$ (7 S)[b]	60

[a] Mixtures of the subunits were made by adding 400 μg of the appropriate subunit in the Tris buffer and concentrating by pressure dialysis to a volume of approximately 200 μl. After concentration 200 μl was loaded onto the sucrose gradient. The appropriate peaks were isolated, dialyzed against the Tris buffer, and assayed for protein and biological activity in the usual manner. The specific biological activity of the β subunit immediately after isolation is similar to that of 7S nerve growth factor (*cf.* Figure 1) but decreases with time and during sedimentation in sucrose gradients. This decay is not prevented by the addition of either individual α or γ subunits. [b] Species isolated after sedimentation at 5° in a 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4) at 60,000 rpm for 13 hr.

used in the initial recombination reaction. Finally all the 6.8S proteins from these reactions elicited the same level of response in the bioassay as did the original nerve growth factor protein. Table I gives typical results for one series of experiments using α^2, γ^3, and β subunits. Only the product isolated from the recombination of all three subunits had this property. The isolated $\alpha^2\beta$ and $\gamma^3\beta$ species had much lower specific activities (Table I) paralleling the results obtained in the recombination reactions with the unfractionated α and γ subunits (Varon *et al.*, 1968).

Occurrence of Multiple Forms in the Undissociated 7S Nerve Growth Factor Protein. Since many different $\alpha\beta\gamma$ forms of the nerve growth factor protein can be made by recombination of appropriate subunits the question arises as to whether the original preparation itself contains these multiple forms of the 7S species. The slight difference in electrophoretic mobility observed among 7S proteins formed from different α subunits can be used to reexamine the homogeneity of the original preparation because, if present, species containing α^1 subunits will be concentrated on the low and those containing α^4 subunits on the high mobility side, respectively, of the single migrating nerve growth factor protein zone. This was tested by subjecting a nerve growth factor preparation to electrophoresis at pH 7.55 (that is, within its pH stability limits) and taking serial sections from the low to the high mobility side of the protein zone (Figure 6a). The proteins from the individual acrylamide gel slices were extracted with 0.01 M Tris-Cl buffer (pH 7.4) and analyzed by a second electrophoresis, this time at pH 9.7, to characterize their dissociation products. Figure 6a shows that the nerve growth factor

protein eluted from the low mobility side of the first protein zone did contain more of the α^2 than of the α^3 or α^4 subunits, and correspondingly that the α^4 subunits predominated on the high mobility side. This finding of a gradient of α subunits which parallels their known effect on the mobilities of the 7S species is thus evidence in favor of multiple forms of the 7S species in the original preparation. Unexpectedly the above analyses showed a similar bias with respect to the γ subunits, γ^1 subunits appearing preferentially on the low and γ^3 subunits on the high mobility side.

Since the three fractionation steps used in the isolation of 7S nerve growth factor involve techniques usually considered to be mild, the final preparation should reflect the composition of the nerve growth factor protein as it exists in the homogenate of the gland. However, the chromatographic step on DEAE-cellulose was unusual in requiring high flow rates (Varon *et al.*, 1967a), a requirement now known to be related to the dissociation equilibrium of the 7S species (J. Nomura, S. Varon, and E. M. Shooter, unpublished data). Therefore, the subunit composition of nerve growth factor protein prepared by a modified procedure omitting the chromatographic step was also analyzed by use of the high pH electrophoretic technique. Crude gland extracts were filtered on a G-100 Sephadex column (the first step of the standard procedure) and the active fractions concentrated and subjected to electrophoresis at pH 7.55. A zone corresponding to the known position of the 7S species was cut from the gel and the protein extracted and analyzed at pH 9.7. It showed the same typical composition of α and γ subunits as the standard 7S preparation. The same experiment was carried out with the crude extract from one lobe of the submaxillary gland from a single mouse, with essentially the same result (Figure 6b).

Discussion

The Multiple Forms of the 7S Nerve Growth Factor. The experiments described in the preceding section demonstrate clearly that the heterogeneity characterizing the α- and the γ-subunit groups is not an artifact of the electrophoretic procedure. The same patterns of α and γ subunits are seen in a variety of buffers with pH values between 7.0 and 9.7. More significantly, in the same pH range, the individual subunits, isolated by ion-exchange chromatography, remain stable and migrate essentially as single components (minor bands being reasonably attributed to preexisting contamination with subunits of closest mobility). The recombination experiments using individual subunits have demonstrated that the individual subunits retain their identities during the formation and subsequent dissociation of their 7S complexes, ruling out the possibility that the multiple forms of the subunits are merely artifacts of the dissociation procedures. The question whether the multiplicity of the subunits could have risen from a common α or γ precursor present in a single type of 7S nerve growth factor protein was answered negatively by the demonstration of a gradient of α subunits from the low to the high mobility sides of a 7S protein zone. A similar gradient of α subunits can be observed when purified nerve growth factor protein is rechromatographed on DEAE-cellulose under appropriate conditions (J. Nomura, S. Varon, and E. M. Shooter, unpublished data). The analysis of the nerve growth factor protein prepared by a modified procedure from a single lobe of a single mouse submaxillary gland indicates that the multiplicity of the subunits is neither induced by some of the purification steps nor related to a heterogeneity in the source tissue. Finally, the recombination experiments with the individual subunits showed that 7S species containing single types of α or of γ subunits are typical, biologically active nerve growth factor proteins. At present, therefore, the most likely explanation for the occurrence of multiple α and γ subunits in preparations of 7S nerve growth factor is that they are all constituent subunits of multiple forms of nerve growth factor protein as it is extracted from the gland into the homogenate. The nature of the differences among the α or among the γ subunits remains to be investigated. It may be noted that, although the differences in the mobilities of the α subunits (or in their net charges, since they are of the same size) are reflected in similar, though smaller, differences in the mobilities of the various 7S species (and of the $\alpha\beta$ complexes), this is not so for the γ subunits. Whatever groups account for the relatively large differences in net charges among the γ subunits are therefore hidden when they are assembled into the 7S complex.

The Specificity of the Recombination of α, γ, and β Subunits. The 7S $\alpha\gamma\beta$ complexes are the only forms in which nerve growth factor activity is present in the gland extract at neutral pH (Varon *et al.*, 1967). The other three forms, β, $\alpha\beta$, and $\gamma\beta$, can be derived from 7S nerve growth factor by a variety of treatments. However, although the β subunits will combine with either the α or the γ subunits, it is only when they are used together with both that the biological activity of the resulting complex is higher and more stable than that of the β subunits alone. This increase in both activity and stability has been confirmed in the present studies for all the 7S species obtained from individual α and γ subunits. Whether the higher activity of the 7S complex derives from a synergistic action of the three subunits on the responsive cells, an enhancement of the intrinsic activity of the β subunit by the other two, or a protective or stabilizing action of the α and γ subunits on the β protein remains completely open at the moment. The high degree of specificity already noted in recombination experiments using the α and γ groups of subunits (Varon *et al.*, 1968) has been confirmed in the experiments using the individual subunits and by the demonstration that all the 7S species so obtained have the correct qualitative subunit composition. That the interaction of these subunits is specific has been further stressed by the recent finding (Greene *et al.*, 1968) of an enzymatic activity residing with the γ subunits, the properties of which are also significantly altered by their recombination into 7S complexes.

Proteins with Nerve Growth Factor Activity. Previous work in this laboratory (Varon *et al.*, 1967a,b, 1968) has demonstrated that the nerve growth factor activity of the male mouse submaxillary gland can be associated

with at least four proteins of different size which can be designated in general terms as β, $\alpha\beta$, $\beta\gamma$, and $\alpha\beta\gamma$ with sedimentation coefficients of approximately 2.5, 4.5, 6.5, and 7 S, respectively. At least two of them, $\alpha\beta\gamma$ and $\alpha\beta$, can exist in multiple forms as shown in the present report. The nerve growth factor proteins obtained by earlier procedures from mouse glands (Cohen, 1960) and from snake venoms (Cohen, 1959) possibly correspond to the $\alpha\beta$ and β proteins, respectively. A more recent report (Angeletti *et al.*, 1967) confirms, by Sephadex filtration studies, the occurrence of nerve growth factor activity in proteins of different sizes derived from mouse gland extracts and snake venoms, as well as from mouse saliva.

Schenkein and Bueker (1964) have reported that mouse nerve growth factor protein, prepared essentially by Cohen's method except for the omission of the chromatographic steps at acid pH, could be split by electrophoresis or chromatography at pH 7.4 into three components, two of which appeared essential for the biological activity; one of these, furthermore, was dialyzable. No further information has appeared on these preparations and the available data do not permit a correlation of these components to the other known nerve growth factor proteins. Recently, another paper from the same laboratory (Schenkein *et al.*, 1968) described a mouse gland preparation which elicits fiber outgrowth in the standard bioassay system at levels dramatically lower than with any previous proteins. The authors referred to it as nerve growth factor but distinguished between two nerve growth factors A and B. It is not clear whether their distinction pertained to two different types of activity exhibited by the same compound in different concentration ranges or to two distinct molecules endowed with different biological activities. Nor is it clear whether the detection of activity at the extreme dilutions actually depended upon the removal from the preparation of an enzyme whose activity was observed in less purified submaxillary materials.

Clearly, the classical definition of the nerve growth factor as a protein which selectively stimulates the growth of sympathetic and embryonic sensory ganglia needs to be reevaluated. There appear to be two possible types of nerve growth factor *activity* (Schenkein *et al.*'s A and B?), there are at least four types of nerve growth factor *proteins* (β, $\alpha\beta$, $\beta\gamma$, and the parent $\alpha\beta\gamma$ complex), and some of these, as shown in this paper, may exist in a number of multiple forms similar in size but not in the detail of their α and/or γ subunits. It remains an open question whether the entity which ultimately interacts with the responsive cell (the actual nerve growth *factor*) is the parent complex, one of the derivative forms, or an even smaller molecule released from them.

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