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Reversible Dissociation of the Mouse Nerve Growth Factor Protein into Different Subunits*

Silvio Varon, Junichi Nomura,† and E. M. Shooter

ABSTRACT: Nerve growth factor activity occurs in the crude extract of the adult male mouse submaxillary gland and can be isolated from it as a protein of approximately 140,000 mol wt. This communication describes the effects of acid and alkaline pH's on the size, net charge, biological activity, and stability of the factor protein. The large molecule is stable only between pH 5 and 8. Outside this pH range, it dissociates reversibly into smaller species with no, or considerably lower, activity. The products of the complete dissociation are three groups of subunits, one acidic (α), one basic (β), and the third intermediate in net charge (γ), all three having molecular weights of approximately 30,000. The three groups of subunits have been isolated by chromatography on CM-cellulose at low pH and each displays heterogeneity on electrophoresis. Only the β subunit elicits a nerve growth factor type of response in the standard bioassay. It accounts for 25% or less of the original activity and is markedly unstable. However, the original highly active nerve growth factor protein is spontaneously reconstituted when the three subunits are mixed at neutral pH. All three types of subunits are needed for this regeneration; α and γ subunits, while not reacting with each other, combine separately with the β subunits but the resulting complexes have the same low activity and instability as the isolated β subunit itself. Possible biological roles of the α and γ subunits are discussed.

The nerve growth factor, discovered and extensively studied by Levi-Montalcini and her colleagues (Levi-Montalcini, 1966), selectively stimulates the growth of sympathetic and embryonic sensory spinal ganglia. The factor is a protein which can be isolated from snake venom (Cohen, 1959) and the adult male mouse submaxillary gland (Cohen, 1960). The isolation of a new high molecular weight form of the nerve growth factor protein from the mouse submaxillary gland has recently been described (Varon *et al.*, 1967a). This method of isolation differs from the original procedures in using only three fractionation steps, two involving gel filtration on Sephadex and the third ion-exchange chromatography on DEAE-cellulose, and in maintaining the pH close to neutrality. The new nerve growth factor species has a molecular weight of approximately 140,000 in contrast to the lower values reported earlier (Cohen, 1959, 1960), and is obtained in considerably higher yield. Varon *et al.* (1967a) also showed that the differences in molecular weight between new and older preparations arose from the differences in the methods of isolation, in particular the use in the older method of a chromatographic separation initiated at acid pH. If the new high molecular weight form was subjected to this treatment the biological activity was found to decrease considerably and to be now

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TABLE I: Effect of pH on the Activity of the Nerve Growth Factor Protein.[a]

	Original	After Dialysis					
pH	7.4	7.4	7.4/7.4	3.8	3.8/7.4	9.7	9.7/7.4
Activity (BU/ml $\times 10^{-3}$)	188	183	184	60	167	57	175

[a] Samples (0.5-ml volume) of nerve growth factor protein solutions (5 mg/ml) dialyzed against 0.05 M Tris-Cl buffer (pH 7.4), 0.05 M acetate buffer (pH 3.8), or 0.05 M sodium glycinate buffer (pH 9.7) and subsequently against the Tris-Cl buffer (pH 7.4) for 20 hr. Activity assayed in quintuplicate after the first and second dialyses.

associated with a protein of approximately 30,000 mol wt, the remaining protein eluted from the column being inactive in the usual bioassay system. Subsequent investigations, reported here, have shown that the decrease in both molecular weight and biological activity results from a reversible dissociation of the high molecular weight nerve growth factor induced by low pH and that the resulting subunits although similar in size differ sufficiently in net charge to be readily separated by ion-exchange chromatography into three groups. The isolated subunits retain the ability to spontaneously recombine with one another to re-form the original highly active nerve growth factor protein. A brief account of part of this work has already been given (Varon *et al.*, 1967b).

Materials and Methods

Isolation of the Nerve Growth Factor Protein. Batches of 100 submaxillary glands from adult male mice were used to isolate the new form of the nerve growth factor by the method previously described in detail (Varon *et al.*, 1967a). The method comprises a gel filtration on Sephadex G-100, chromatography on DEAE-cellulose, and gel filtration on Sephadex G-150. The final eluate (G-150 fraction) was concentrated to about 15–20 mg of protein/ml, filtered on Millipore, and stored sterile in the cold. Most experiments were carried out with the final G-150 fraction, 95–100% of which is made up by the nerve growth factor protein. Other experiments were run with the DEAE fraction (0.08 M NaCl eluate) that is the penultimate stage of the isolation procedure where the nerve growth factor accounts for about 70% of the total protein.

Biological activity was measured in tissue culture as described by Levi-Montalcini *et al.* (1954). Results are expressed as the total number of biological units (BU) of a sample. This was obtained by multiplying volumes by biological activities in biological units per milliliter, 1 BU/ml being arbitrarily defined as the nerve growth factor concentration in an assay system showing an optimal (4+) fiber response.

Sedimentation analyses in either the Spinco Model E or in sucrose gradients were as previously outlined (Varon *et al.*, 1967a). Viscosities of the various buffers used in the sedimentation experiments were measured in a Cannon–Fenske Routine viscometer (size 25) and densities in a micropycnometer (approximate volume 200 μl) at 25 ± 0.01°. Buffers of ionic strength 0.05, unless otherwise stated, were prepared according to the data given by Datta and Gryzbowski (1961) and pH values are specified at 25°.

Electrophoresis in acrylamide gel used two new buffer systems, one continuous at pH 9.9 and the other a discontinuous system in which the protein zones were resolved at pH 8.3 (T. Jovin and A. Crambach, unpublished data). For the continuous system the resolving gel was made by mixing solutions A′, C, and P in the ratio of 1:1:2. These solutions had the following composition: (A′) 6.3 ml of 2 M glycine, 6.0 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) 0.2 g of ammonium persulfate per 100 ml. No concentrating gel was used and the upper and lower electrode compartment buffers contained 15.8 ml of 2 M glycine and 15.0 ml of 1 N NaOH per l. Samples were dialyzed against a 1:5 dilution of the electrode buffer. Between 50 and 100 μg of protein was layered above the gel in a volume not exceeding 300 μl. Sucrose was added to a final concentration of 5 g per 100 ml for stability.

The resolving gel in the discontinuous system was made by mixing A″, C, and P solutions in the ratio of 1:1:2, their composition being: (A″) 4.26 g of Tris, 33.6 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 solutions B, D, and E in the ratio of 1:2:1, their composition being (B) 2.23 g of Tris, 14.0 ml of 1 M H_3PO_4, 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 per 100 ml. The upper buffer had the following composition: 4.0 g of Tris and 4.22 g of tricine per l.; the lower buffer was 12.1 g of Tris and 50 ml of 1 N HCl per l. Using 0.3 ml of concentrating gel, satisfactory resolution was obtained with 50–100 μg of protein in volumes up to 400 μl. Concentrated protein solutions were diluted with a 1:5 dilution of solution B omitting the Temed while dilute protein solutions were dialyzed against the latter buffer before electrophoresis. A third continuous phosphate system at pH 7.0 has already been described (Varon *et al.*, 1967a).

[1] Abbreviations used in this paper that are not defined in *Biochemistry* 5, 1445 (1966) are: Temed, *N,N,N′,N′*-tetramethylethylenediamine; Bis, *N,N′*-methylenebisacrylamide; Tricine, tris(hydroxymethyl)methylglycine.

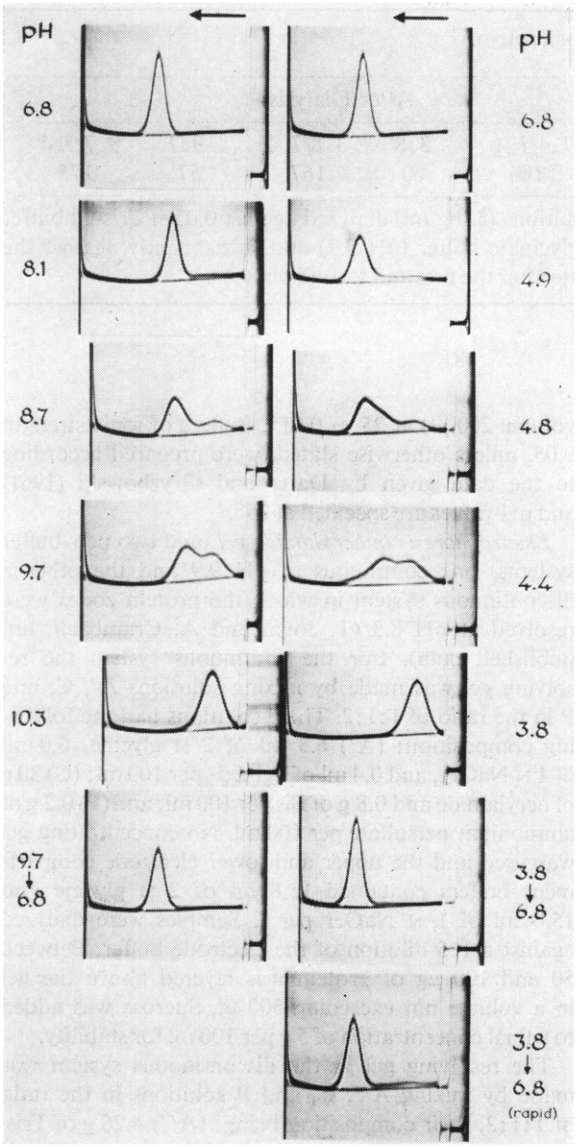

FIGURE 1: Reversible dissociation of the nerve growth factor protein at extremes of pH. Nerve growth factor protein solutions dialyzed against appropriate buffer solutions of ionic strength 0.05 and adjusted after dialysis to a concentration of 10 mg/ml. For the two pH reversal experiments, protein solutions were dialyzed first to pH 3.8 or 9.7 and then subsequently (in the same dialysis sac) for 24 hr to pH 6.8. For the experiment recorded in the bottom line 500 μl of a 14-mg/ml solution of nerve growth factor protein in 0.05 M acetate buffer was added to 200 μl of 1 M Tris-Cl buffer (pH 7.4); the final pH was 6.82. Sedimentation in double-sector cells at 59,780 rpm and 5°. Photographs taken 96 min after reaching speed. Diaphragm angle 60 or 65°.

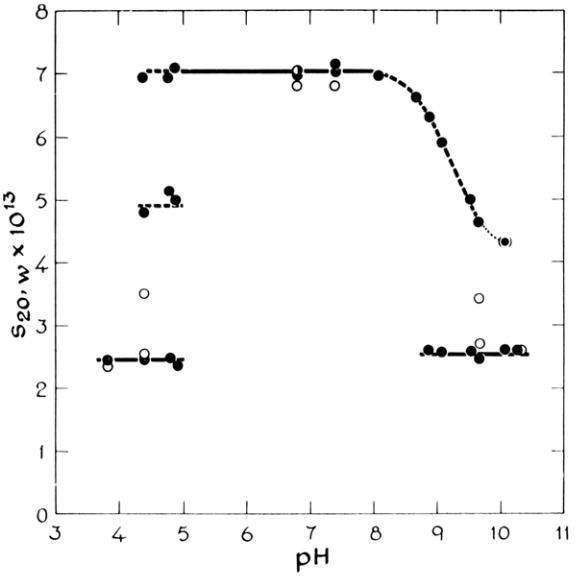

FIGURE 2: Sedimentation properties of the nerve growth factor protein and its subunits as a function of pH. Experimental details given in Figure 1. (●) Ambient pH; (◑) after exposure to pH 3.8, 9.7, or 10.3; (○) sucrose density gradient values.

Results

The Effect of pH on the Biological Activity of the Nerve Growth Factor. Aliquots of solutions of nerve growth factor protein were dialyzed at pH 7.4, 3.8, and 9.7, respectively, samples were taken for assay, and the remaining solutions were all redialyzed to pH 7.4 for further assay. Each sample was assayed in five complete replicates and the results of a typical experiment are shown in Table I. The recovery of protein was greater than 90% in each instance but because the biological assays are not sensitive to small variations no corrections for the changes in protein concentration during dialysis were made. No loss of activity was observed after dialysis at pH 7.4. In contrast, after dialysis to pH 3.8 or 9.4, the total activity decreased by approximately two-thirds. Subsequent dialysis to pH 7.4 of the acid- or alkaline-exposed solutions raised their activity back to the original levels.

To rule out the possibility that extreme pH values adversely affected the assay system, ganglia were preexposed to the pH 3.8, 7.4, or 9.7 buffers at concentrations corresponding to those used in the serial assay of the pH-treated factor, and then used to assay a known amount of untreated nerve growth factor protein. The responses of the ganglia were the same in all cases. These results therefore indicate that exposure to extreme pH values decreases the activity of the nerve growth factor protein and that the activity can be recovered when the protein is returned to neutral pH.

pH-Dependent Reversible Dissociation of the Nerve Growth Factor Protein. The physical properties of nerve growth factor protein after dialysis against buffers of various pH values were examined in sedimentation velocity experiments (Figure 1). The sedimentation coefficients of the various species as a function of pH are plotted in Figure 2. The final product of the new isolation procedure (Varon *et al.*, 1967a) sediments at neutral pH as one main component of $s_{20,w}$ = 7.1 S and shows between 1 and 5% of a slower sedimenting inactive material. The major component, however, was found to be stable only in the relatively narrow pH range between 5 and 8.

At pH values between 5.0 and 4.4 two other components were noted, having lower sedimentation co-

efficients of around 4.9 and 2.5 S, respectively. Both appeared as soon as the lower pH-stability limit of the 7.1S component was reached and increased in amount with progressively lower pH. At pH values lower than 4.2 where the 7.1S species could no longer be detected, the 4.9S component decreased while the smaller species increased in amount so that by pH 3.8 only the latter remained. The dissociation of the nerve growth factor protein at acid pH thus produces two species with lower but different sedimentation coefficients, the intermediate species itself finally dissociating at pH 3.8 into subunits with an $s_{20,w}$ of 2.5 S. The dissociation at acid pH was almost completely reversible if the pH was changed by dialysis to neutrality and 80–90% reversible if neutralization was achieved rapidly by direct addition of concentrated buffer (Figure 1). The recovery of protein after dissociation and reassociation was greater than 90%.

At pH 10.3, the same complete dissociation of the 7.1S protein into subunits of $s_{20,w}$ = 2.5 S was noted. Components with this sedimentation coefficient appeared as soon as the upper pH-stability limit of 8.0 was exceeded. However, the intermediate stages of the dissociation appeared to be more complex than at acid pH. The dissociation at acid pH involved only the three species of $s_{20,w}$ = 7.1 (the original protein), 4.9, and 2.5 S and all three were present simultaneously over a wide range of pH. The dissociation at alkaline pH was instead characterized by a progressive decrease in the $s_{20,w}$ of the main protein component from the original value of 7.1 S at pH 8.0 to 4.65 S at pH 9.7, and at all pH's only one species was observed beside the 2.5S component. Above pH 9.7, the intermediate species decreased in amount, while the smaller 2.5S species increased to become the only component left at pH 10.3. Furthermore, the dissociation at alkaline pH was less reversible than at acid pH. After complete dissociation (pH 10.3) only 60% of the protein reverted to the high molecular weight form on subsequent dialysis to neutral pH (see Figure 3d). More complete reversibility could only be observed (Figure 1) from lower pH values, *e.g.*, 9.7, where the initial dissociation was itself incomplete. The $s_{20,w}$ for the re-formed nerve growth factor protein, whether dissociated at acid or alkaline pH, was indistinguishable from that of the original, undissociated protein (Figure 2).

Sucrose density gradient experiments (Figure 3) were used to determine how the changes in biological and physical properties were correlated. As with the sedimentation velocity experiments, 2.3–2.6S subunits appeared as soon as the pH-stability limits were exceeded, and complete dissociation into such subunits was observed at pH 3.8 or 10.3. Intermediate species appeared at intermediate pH values, although at any given pH (*e.g.*, Figure 3b,f) their sedimentation coefficients were lower than in the corresponding sedimentation velocity experiments (Figure 2). The amount of biological activity recovered in gradient experiments where dissociation of the original protein was observable was always lower than in the controls and was about 25% of the initial level at pH values where none of the original nerve growth factor remained. At intermediate

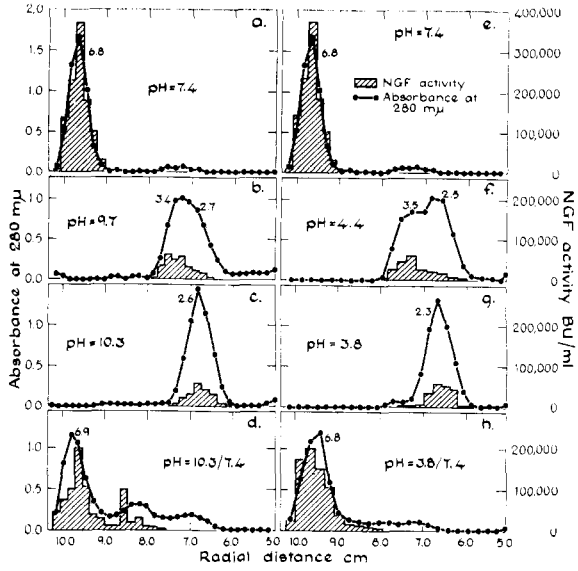

FIGURE 3: Reversible dissociation of the nerve growth factor protein at extremes of pH. Samples (75 μl) of a 20-mg/ml solution of nerve growth factor protein dialyzed against buffer of appropriate pH, mixed with 25 μl of dansylated bovine serum albumin, and centrifuged in SB-405 rotor for 15 hr at 60,000 rpm at 5° in a 5–20% sucrose gradient in the same buffer. (a and e) 0.05 M Tris-Cl buffer (pH 7.4); (b) 0.05 M sodium glycinate buffer (pH 9.7); (c) 0.05 M sodium glycinate buffer (pH 10.3); (d) after dialysis as in c and further dialysis for 20 hr against 0.05 M Tris-Cl buffer (pH 7.4); (f) 0.05 M sodium acetate buffer (pH 4.4); (g) 0.05 M sodium acetate buffer (pH 3.8); (h) dialysis as in g and further dialysis for 20 hr against 0.05 M Tris-Cl buffer (pH 7.4). Contents of each tube collected in three-drop fractions and assayed for absorbance and nerve growth factor activity in the usual manner. Figures given alongside the protein peaks are $s_{20,w}$ values in Svedbergs.

pH values, the activity was mainly associated with the heavier of the two new species (Figure 3b,f). At extremes of pH, the activity was found with the 2.3–2.6S subunits (Figure 3c,g). After exposure to pH 3.8 and subsequent dialysis back to pH 7.4 (Figure 3h), the biological activity was again found with the 7S protein (that is the high molecular weight protein with a $s_{20,w}$ of 7.1 S in analytical sedimentation and 6.8–6.9 S in sucrose gradient sedimentation), and the total amount of activity recovered approached the level of the undissociated nerve growth factor protein. About 10% of inactive protein still sedimented in the subunit range. After exposure to pH 10.3 and redialysis to neutral pH (Figure 3d), sizeable amounts of the two components of $s_{20,w}$ lower than 7 S could still be detected and the recovery of activity associated with the 7S species was less complete. A small but significant amount of nerve growth factor activity remained with the intermediate, 4.7S component. The reversibility of the incomplete dissociation from pH 9.7, in agreement with the results obtained in both dialysis and sedimentation velocity experiments, was the same as from the extreme acid pH.

Isolation of the Nerve Growth Factor Subunits by Ion-Exchange Chromatography. Electrophoresis of dissociated nerve growth factor protein on acrylamide gel showed that the subunits differed considerably in net

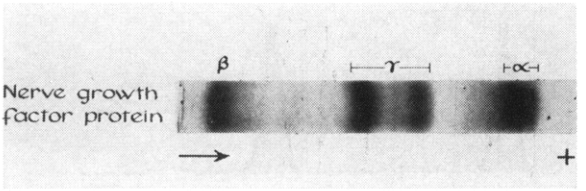

FIGURE 4: The subunits of the nerve growth factor protein. Acrylamide gel electrophoresis of 80 μg of nerve growth factor protein in sodium glycinate buffer, pH 9.9. Migration time 2 hr; stained with Naphthol Blue Black.

charge (Figure 4). Three groups were distinguished: one group (α subunits) migrated rapidly to the anode, a second (β subunits) comprised a single diffuse band of low mobility, and a third group (γ subunits) showed two bands with intermediate mobility at this pH.

The α, β, and γ groups of subunits were readily separated by chromatography on CM-cellulose. The starting material was the dilute G-150 fraction obtained in the last step of the nerve growth factor purification (Varon *et al.*, 1967a). After adjustment to pH 6.0 it was found that this material was completely adsorbed to CM-cellulose equilibrated at acid pH and was also dissociated during the process. Subsequent stepwise elution with three solvents, the first at acid pH, the second at neutral pH, and the last at neutral pH but higher salt concentration (Figure 5a), produced three fractions which were identified by acrylamide gel electrophoresis as the α, γ, and β subunits, respectively, in order of elution. A fourth fraction, eluted from the column at neutral pH and high salt concentration, was similar electrophoretically to the β subunits (Figure 6d). The heterogeneity of the α and γ subunits, grossly observed at pH 9.9, was more clearly seen in the electrophoretic analyses at pH 8.3 where α subunits (Figure 6a) showed three major and one minor zone and γ subunits (Figure 6b) three zones. The same patterns were observed at pH 7. The β subunits were too basic to migrate to the anode at these lower pH values; at pH 9.9 (Figure 6c) they moved as one major sharp band with a slower minor component. β-Subunit preparations occasionally showed an additional minor band whose higher mobility suggested that it was a contamination from the γ-subunit fraction.

The amounts of α- and γ-subunit protein obtained by this procedure after concentration of the eluates were approximately equal (Figure 5a), but that of the β-subunit protein considerably lower. Only the β subunits had biological activity in the standard bioassay system, the activity measured directly on the eluate being about 10% of that loaded on the column. The activity was unstable in this eluate. A 20-fold concentration by pressure dialysis made the activity more stable, although 30–50% of it was lost in the process.

The separation procedure was also applied to the DEAE fraction, the penultimate stage in the purification of the nerve growth factor protein. With this material, loaded without prior concentration, an additional fraction was eluted with the pH 4.4 buffer alone and four other fractions using the solvents

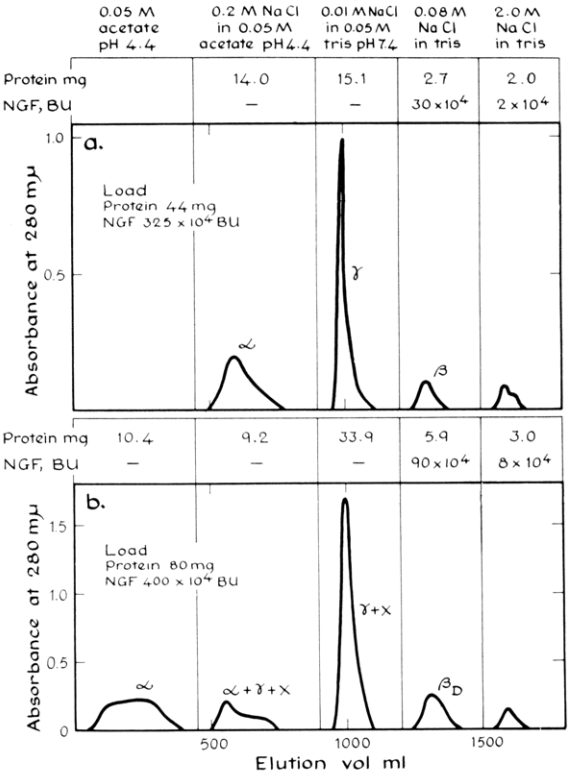

FIGURE 5: Chromatographic separation of the nerve growth factor subunits. (a) Dilute nerve growth factor protein solution (45 ml) (eluate from final G-150 Sephadex column) adjusted to pH 6.0 with 0.5 M acetate buffer (pH 4.4) and applied to 2.4 × 20 cm column of CM-cellulose (Whatman CM 11) equilibrated with 0.05 M acetate buffer (pH 4.4). Column eluted stepwise with solvents given in the figure. Flow rate 320 ml/hr. Nerve growth factor activity determined directly on eluates from column, protein content after concentration by pressure dialysis. Identification of α, β, and γ subunits made by electrophoresis as shown in Figure 6a–d. (b) Dilute DEAE fraction (600 ml) (eluate from DEAE-cellulose column) adjusted to pH 6.0 with 0.5 M acetate buffer (pH 4.4) and applied to and eluted from CM-cellulose column as described in a. Composition of various fractions determined by electrophoresis as shown in Figure 6e–i. X denotes extra protein zones seen in fraction from DEAE material but not from purified nerve growth factor protein.

described for the first chromatogram (Figure 5b). The first fraction contained the α subunits (Figure 6e), the fourth and fifth contained β subunits (Figure 6h,i), while the two intermediate fractions showed all of the characteristic bands of the γ subunits together with additional zones, presumably corresponding to the impurities in the DEAE fraction (Figure 6f,g). The difference in elution behavior of the α subunits from the two columns (Figure 5a,b) stems from the pH increase induced by the large volume in which the DEAE fraction was loaded. The amounts of α and γ subunits recovered from the DEAE material were also approximately equal, if allowances are made for the impurities in the γ-subunit fractions. The amount of β_D-subunit protein (the suffix D is used to indicate that the source of these subunits is the DEAE fraction) was again considerably lower than that of the other subunits, but the yield in biological activity

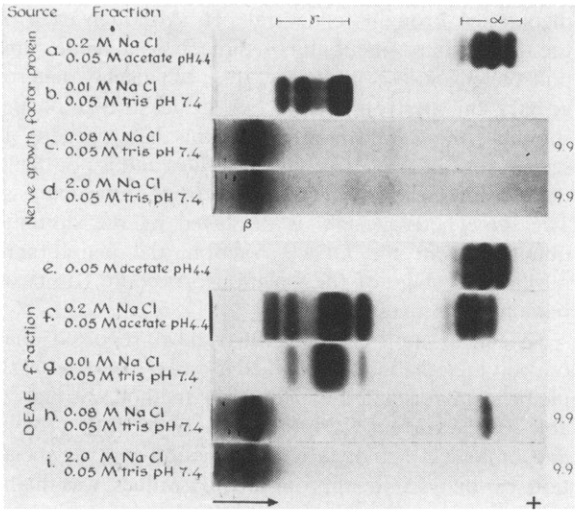

FIGURE 6: Electrophoretic analysis of the nerve growth factor subunits. (a–d) Chromatographic fractions obtained from acid-dissociated nerve growth factor protein (Figure 5a). (e–i) Chromatographic fractions obtained from acid dissociation of DEAE fraction (Figure 5b). Analysis in 7.5% acrylamide gel in Tris–tricinate buffer system (pH 8.3) except where indicated at the right-hand side of the gel pattern by the figure 9.9. In these experiments the sodium glycinate buffer system (pH 9.9) was used. Migration time 2.25 hr at pH 8.3 or 3 hr at pH 9.9; stained with Naphthol Blue Black.

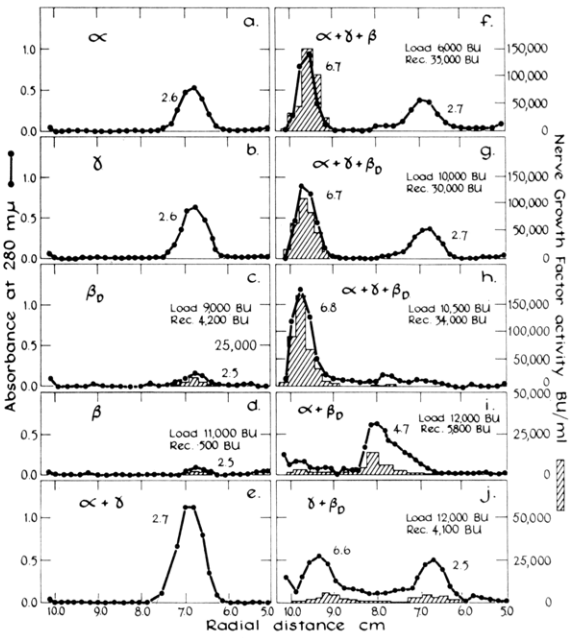

FIGURE 7: Sedimentation properties of the isolated and recombined nerve growth factor subunits. Sedimentation for 15 hr at 60,000 rpm in SB-405 rotor at 5°; 5–20% sucrose gradient in 0.05 M Tris-Cl buffer (pH 7.4). Each sample (between 75 and 350 μl) and dansylated bovine serum albumin (25 μl) were loaded on top of the gradient. Subunits were isolated from nerve growth factor protein by the method described in Figure 5a. The β_D subunit was isolated from the DEAE fraction (Figure 5b). (a) 300 μg of α subunits; (b) 300 μg of γ subunits; (c) 100 μg of β_D subunits; (d) 130 μg of β subunits; (e) 300 μg of α plus 300 μg of γ subunits; (f) 300 μg of α plus 300 μg of γ plus 60 μg of β subunits; (g) 300 μg of α plus 300 μg of γ plus 100 μg of β_D subunits; (h) 120 μg of α plus 120 μg of γ plus 120 μg of β_D subunits; (i) 300 μg of α plus 150 μg of β_D subunits; and (j) 600 μg of γ plus 150 μg of β_D subunits.

measured directly on the eluate was now 25% of the original load. The biological activity of β_D subunits was partially stabilized by concentration. Its electrophoretic analysis (Figure 6h) showed the same major and minor bands seen in the β subunits (Figure 6c).

The Molecular and Recombination Properties of the Isolated Subunits. All the subunits resulting from the complete dissociation of the nerve growth factor protein at extremes of pH have similar sedimentation coefficients in the range 2.4–2.65 S. As judged by sucrose gradient sedimentation this is also true for the subunits at neutral pH (Figure 7a–d), indicating that each group once isolated has no tendency to reaggregate. Although the β subunits prepared from the purified nerve growth factor and the DEAE fraction, respectively, showed the same sedimentation properties, they differed considerably in terms of the stability of their biological activity on sucrose gradients. Only 5% of the activity was recovered after sedimentation of the β subunits, in contrast to approximately 45% from the β_D subunits. The activity of both types of β subunit was, however, stabilized when they were mixed with α and γ subunits at neutral pH (Figure 7f,g). In fact, the combination of all three subunits resulted in a marked increase in total activity, comparable to that observed when acid- or alkaline-dissociated nerve growth factor subunits were allowed to reassociate at neutral pH (Table I). The relative increase in activity was greater for the β than the β_D subunits. In both instances sizeable amounts of protein with the sedimentation characteristics of the original nerve growth factor protein were formed, although some subunit protein remained. In these experiments the three types of subunits were mixed in the proportions in which they had been isolated. When the amounts of α and γ subunits were lowered to approach that of the β subunits, about the same amount of high molecular weight protein and a similar increase in biological activity were noted but very little low molecular weight material remained (Figure 7h).

The specific activity of the regenerated high molecular weight compound (80 BU/μg) was identical with that of the original nerve growth factor, as also were the electrophoretic characteristics of this material (Figure 8). The gel filtration behavior on Sephadex G-100 of the original and the reconstituted proteins were also the same (Varon *et al.*, 1967b). The product of the interaction of the three different types of subunits was therefore, as judged by a number of criteria, identical with the nerve growth factor protein. The recombination process was also followed by electrophoresis on acrylamide gel (Figure 8). The addition of an excess of β_D subunit to a mixture containing equal quantities of α and γ subunits regenerated nerve growth factor protein and used up all the γ subunits in the process. At the pH of these analyses the original nerve growth factor is slightly dissociated, as evidenced by the appearance of the α subunits, and the regenerated nerve growth factor showed exactly the same behavior.

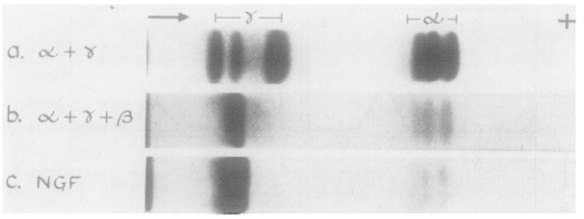

FIGURE 8: Recombination of the nerve growth factor protein subunits. (a) Mixture of 30 μg of α and 30 μg of γ subunits. (b) Mixture of 10 μg of α, 10 μg of γ, and 40 μg of β subunits. α and γ subunits initially in 0.05 M Tris-Cl buffer (pH 7.4), β subunits in a 1:5 dilution of solution B of the tricine system (see Methods). Appropriate volumes of each subunit mixed at 0° and loaded onto gel within 30 min of mixing. (c) 50 μg of nerve growth factor protein. Analysis in 7.5% acrylamide gels in Tris–tricinate buffer system (pH 8.3). Migration time 2.25 hr; stained with Naphthol Blue Black.

In contrast to the recombination of the three subunits, interaction of any pair of subunits did not regenerate the specific nerve growth factor protein. The α and γ subunits did not interact at all at neutral pH; both sedimentation (Figure 7e) and electrophoretic analysis (Figure 8a) showed a simple mixture of the two. The α and β_D subunits interacted partially to form a new species with an $s_{20,w}$ of 4.7 S but the interaction did not prevent the typical loss of activity of the β_D subunit on the gradient (Figure 7i). A species with an $s_{20,w}$ close to that of the nerve growth factor was formed in the partial interaction of the γ and β_D subunits, but again the activity of the β_D subunit in the complex was unstable (Figure 7j).

Discussion

The Nerve Growth Factor Protein Subunits. The first soluble form of the mouse nerve growth factor detected in the crude gland extract (Varon *et al.*, 1967a) is a protein with a sedimentation coefficient of 7.1 S or a molecular weight of about 140,000 (the 7S species). It can be isolated in this form by a relatively simple procedure carried out at or near neutral pH. The latter is a necessary requirement because the 7S species is only stable between pH 5 and 8. At acid pH the nerve growth factor protein undergoes a reversible dissociation into a number of subunits whose sedimentation coefficients lie in the range 2.4–2.6 S corresponding to molecular weights of approximately 30,000. The subunits produced at alkaline pH have the same $s_{20,w}$, display essentially the same electrophoretic properties, and show the same distribution of biological activity as those isolated from an acid-dissociated system. The final products of the pH-induced dissociation appear therefore to be the same irrespective of the pH at which they are produced. The incomplete reversibility of the dissociation at alkaline pH suggests that under these conditions one or more of the subunits are denatured. The one subunit which is relatively unstable and perhaps the most readily denatured is the biologically active β subunit. Since the sedimentation behavior of the subunits is the same at neutral as at acid or alkaline pH, it follows that none of them form homopolymers when brought to neutral pH. Although each of the three groups of subunits sediments as a single component in sucrose gradients, they all display heterogeneity on electrophoresis. For at least two of the subunits, the α and γ subunits, this heterogeneity is seen at two different alkaline pH values and is not likely therefore to be an artifact of the electrophoretic analysis. The same heterogeneity is displayed by the subunits obtained from the DEAE fraction, the penultimate purification stage of the isolation procedure. Its basis remains to be investigated.

Schenkein and Bueker (1964) have reported that one of their subunits obtained from a nerve growth factor protein isolated by a different method was dialyzable. In the current work the recovery of both protein and biological activity after dissociation and reassociation by dialysis to appropriate pH values was high, suggesting, in agreement with the known molecular size, that none of the α, β, or γ subunits was lost during dialysis. Whether these subunits can be dissociated further by other agents has not yet been ascertained.

The three subunits[2] recombine into a protein which has been characterized as the high molecular weight nerve growth factor protein by its sedimentation, electrophoretic, and gel filtration properties as well as by its specific activity and stability. The recombination occurs spontaneously at neutral pH and is highly specific, requiring all three subunits, no two subunits being sufficient to regenerate the nerve growth factor protein. Specificity and spontaneity of the recombination are similar to those shown by other protein systems, notably hemoglobin (Huehns and Shooter, 1962) and aspartate transcarbamylase (Gerhard and Schachman, 1965), and provide most convincing evidence for the identification of the high molecular weight species as a functional form of the nerve growth factor. Also, the specific interactions of the subunits of the nerve growth factor stabilize the biological activity, another parallelism to the hemoglobin system.

Biological Activity of the Nerve Growth Factor and Its Subunits. Only one of the three groups of isolated subunits, the β subunits, displays activity in the standard bioassay system. The total activity eluted at neutral pH from the CM-cellulose column in the β_D subunits is approximately equal to that measured in the unfractionated mixture of all three subunits produced at acid or alkaline pH and accounts for only about one-quarter of that in the untreated nerve growth factor protein. This decrease is not due to an incomplete collection of the β_D subunits from the column, since it is reversed *after* isolation of the β_D subunits when α and γ subunits are added to them. The β subunits, therefore, elicit a significantly lower response in the assay system when supplied in the isolated form rather than as part of the

[2] The term "subunit" is used throughout for the α, β, and γ species on the simplest assumption that they are direct products, as well as precursors, of the nerve growth factor protein. However, it must be kept in mind that, if the 2.5S species are themselves aggregates of smaller units, the latter may appear as intermediates in the breakdown and/or the assembly of the high molecular weight species.

whole nerve growth factor molecule. In attempting to interpret this result it must be remembered that the complexity of the bioassay system is such that the final measured response, the outgrowth of fibers, may depend on more than the intrinsic activity of the molecule being tested. One interpretation would be that the intrinsic activity of the β subunit is modified by its interaction with the other two, in other words that the α and γ subunits act as regulatory subunits as defined by Gerhard and Schachman (1965) for aspartate transcarbamylase. Another interpretation, however, is that β subunits retain after dissociation the same intrinsic activity, but fewer of them reach the responsive cells, for example, because the others denature or become sequestered elsewhere in the assay system. That α and γ subunits are relevant to the stability of the β subunits is supported by the observation that solutions of the latter, in contrast to those of the original protein, yield a precipitate and lose biological activity on standing.

The Nerve Growth Factor Protein. The molecular weights of the undissociated protein and its subunits are only known to a first approximation, so it is not possible to determine the number of subunits in the nerve growth factor protein from these data. The probable range, however, lies between four and six. Neither can the relative contributions of the three subunits to the high molecular weight species be deduced directly from the protein yields of the isolated subunits after concentration, since the over-all protein recoveries varied between 65 and 80%. The actual amounts recovered were 32, 34, and 6% of the load for α, γ, and β subunits, respectively. These figures would reflect the subunit composition of the nerve growth factor protein only if the same proportion of each subunit was lost during their isolation. On the other hand, it is conceivable that the protein loss was almost entirely incurred by the β subunits, in view of the massive losses in biological activity observed after the chromatographic and concentration steps, as well as of the noted tendency of the β subunits to precipitate with time. Moreover, the recombination experiments showed that the amount of subunits left unrecombined was least when the three subunits were mixed in equal quantities. It is therefore possible that the nerve growth factor protein contains approximately equimolar proportions of the three types of subunits.

The Dissociation of the Nerve Growth Factor Protein. The sedimentation patterns at intermediate stages of the dissociation differ at acid and alkaline pH. In both instances, individual subunits are split off as soon as the pH-stability range is exceeded, as evidenced by the appearance of a 2.5S component. However, it need not be the same subunit which is initially observed at acid and at alkaline pH. If, for example, γ subunits were liberated first at acid pH, the remaining complex would be of the $\alpha\beta$ type, which is known, from the recombination experiments, to be stable, have an intermediate $s_{20,w}$ value, and have low activity. The one intermediate seen in the acid dissociation displays all three such features. Beyond the alkaline stability limit, it is the α subunits and not the γ subunits which are detected in the electrophoretic analysis (Figure 8c). The remaining complex would therefore be of the $\beta\gamma$ type. The recombination experiments show only a $\beta\gamma$ complex sedimenting close to the undissociated protein, and thus no stable intermediate species should be observed in the first stages of the alkaline dissociation, a conclusion which agrees with the available data. While the analysis of the system is complicated by the number of possible components, the rapid reversibility, and the noted $s_{20,w}$ differences in sucrose gradient and sedimentation velocity experiments, the preceding observations open at least some experimental approaches for the understanding of the mechanism of the dissociation.

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