Seaman, G. V. F., and Heard, D. H. (1960), *J. Gen. Physiol. 44*, 251.
Springer, G. F., Nagai, Y., and Tegtmeyer, H. (1966), *Biochemistry 5*, 3254.
Tanaka, K., Bertolini, M., and Pigman, W. (1964), *Biochem. Biophys. Res. Commun. 16*, 404.
Uhlenbruck, G., and Weber, P. (1963), *Z. Immunit. Allergieforsch. 126*, 325.
Warren, L., (1959), *J. Biol. Chem. 234*, 1971.
Werner, I., and Odin, L. (1952), *Acta Soc. Med. Upsalien. 57*, 230.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 279.

The Isolation of the Mouse Nerve Growth Factor Protein in a High Molecular Weight Form*

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

ABSTRACT: The nerve growth factor is a protein which specifically stimulates the growth of sympathetic and embryonic sensory ganglia. This communication describes the isolation of the nerve growth factor protein from the adult male mouse salivary gland in a new high molecular weight form. The new nerve growth factor form has been purified 40-fold from homogenates of the gland by a procedure involving only gel filtration on Sephadex G-100, DEAE-cellulose chromatography, and a second gel filtration on Sephadex G-150. In the final product the nerve growth factor activity is associated, as judged by a number of physical criteria, with a single protein component comprising over 95% of the total protein of the fraction. It represents 2% of the soluble protein of the gland and 80% of the expressed activity of the gland homogenate. The molecular weight of this new nerve growth factor species is approximately 140,000. Chromatography on CM-cellulose at acid pH separates an active subunit(s) whose molecular weight is approximately 30,000, grossly in the range of the nerve growth factor products isolated by the older procedure.

For the past 10 years, Levi-Montalcini (1966) and her group have been investigating a protein factor which selectively enhances the growth of sympathetic and embryonic sensory ganglia. The factor is present in the sympathetic ganglia themselves (Levi-Montalcini and Angeletti, 1961; Winick and Greenberg, 1965) and appears to be essential to their preservation (Levi-Montalcini and Angeletti, 1963). Biochemical studies (Cohen, 1959; Angeletti *et al.*, 1964; Liuzzi *et al.*, 1965; Angeletti *et al.*, 1965) have shown that this nerve growth factor enhances, in the responsive ganglia, oxidation of glucose, the incorporation of acetate into lipids, and the synthesis of both protein and RNA and that the stimulation of RNA synthesis occurs independently from, and before, that of protein synthesis (Angeletti *et al.*, 1965).

Little work on the nerve growth factor protein has been reported since the earlier studies (Cohen, 1957, 1959, 1960; Levi-Montalcini *et al.*, 1965). The factor has been purified from snake venom (Cohen, 1959) and adult male mouse submaxillary gland (Cohen, 1960). The two preparations are very similar in their biological properties, but differ in their behavior on ion-exchange chromatography, in specific activity, and in sedimentation properties with sedimentation coefficients of 2.2 and 4.33 S and estimated molecular weights of 20,000 and 44,000, respectively (Cohen, 1959, 1960). It is possible that these are two different protein factors present in snake venom and the mouse submaxillary gland, respectively, but having the same biological activity. On the other hand, as Cohen (1960) pointed out, the difference in molecular weight might simply reflect differences in the isolation procedures, for example, the use of a step involving urea for the material of lower molecular weight. It has been subsequently observed (S. Varon, unpublished data) that gel filtration of crude salivary extracts on Sephadex G-100 satisfactorily separated the nerve growth factor from other biologically active materials such as the epithelial (Cohen, 1964) and the mesenchymal growth factors (Gandini-Attardi *et al.*, 1965). Two other observations were made at the same time. The nerve growth factor emerged from the Sephadex column in a position indicative of a higher molecular

* From the Department of Genetics and Lt. Joseph P. Kennedy, Jr. Laboratories for Molecular Medicine, Stanford University School of Medicine, Palo Alto, California. *Received March 10, 1967.* This research was supported by funds from the Lt. Joseph P. Kennedy, Jr. Foundation, by a U. S. Public Health Service research grant from the National Institute of Neurological Diseases and Blindness (NB-04270) and Training Grant GM-295, and by a United Cerebral Palsy Foundation research grant (R-178-64).

† On leave from the Department of Neuropsychiatry, Kyoto University Faculty of Medicine, Kyoto, Japan.

weight than those previously reported and, furthermore, the total activity recovered from the column was considerably higher than that of the crude extract applied to it. These observations have focused attention on the molecular complexity of the nerve growth factor protein, knowledge of which could be of considerable help in clarifying the important role this factor plays in nervous tissues, particularly during development. An extension of the gel filtration studies has led, as shown in this communication, to the development of a new procedure for isolating the nerve growth factor in considerably higher yield. This nerve growth factor differs from the previous preparation in having a high molecular weight. A lower molecular weight nerve growth factor species can be obtained from the high molecular weight product by ion-exchange chromatography at acid pH, indicating that the new nerve growth factor species is complex but dissociates at low pH.

Materials and Methods

Submaxillary glands were dissected from 50- to 60-day-old male Swiss albino mice and stored frozen until required. Sephadex G-100, G-150, and G-200 were obtained from Pharmacia. They were hydrated for 5 days, defined through six cycles, and finally suspended in 0.05 M Tris·Cl buffer (pH 7.4, at 25°) prior to being packed to columns. The columns were equilibrated overnight in the cold with the Tris buffer and operated at flow rates of 30–100 ml/hr. DEAE- and CM-cellulose were Whatman products. They were cycled through 0.5 N HCl and 0.5 N NaOH according to the manufacturer's instructions before being equilibrated with the appropriate buffer. Effluents from these columns were continuously scanned at 280 mμ with a G.M.E. (Gilson Medical Electronics) absorption meter and collected fractionally. The absorbance of individual fractions, or selected pools, was determined at 260 and 280 mμ in a Zeiss PMQ II spectrophotometer. In some experiments protein was also determined by the method of Lowry *et al.* (1951).

Tissue Culture Assay for Nerve Growth Factor Activity. Aliquots of the gland homogenate, supernatant, and the various fractions were submitted to Levi-Montalcini's biological test for nerve growth factor activity (Levi-Montalcini *et al.*, 1954). Sensory ganglia from 8-day-old chick embryos were explanted in Maximov slides with a plasma clot consisting of one-third plasma (rooster), one-third Eagle's MEM medium with 1:10 diluted thrombin (1000 U/ml of saline, Topical, Parke Davis and Co.), and one-third of the nerve growth factor sample serially diluted in Gey's balanced salt solution. The cultures were examined after an 18-hr incubation at 37°. The responding ganglia were graded according to the size and morphology of their fiber outgrowth. For convenience in the evaluation of yields and purification ratios, the results were expressed as biological units per milliliter of sample, 1 BU/ml being arbitrarily defined as the nerve growth factor concentration in an assay system showing an optimal (4+) fiber response. Thus, the activity of a sample is calculated as the reciprocal of the dilution factor applied to achieve the 4+ effect. This definition of nerve growth factor activity, while convenient in practice, rests on the assumption that none of the contaminants present in the different samples under test either affects the activity of nerve growth factor or interferes with the responsiveness of the ganglia; it will be shown in the Experimental Section that such an assumption may not always be accurate. The extent of purification achieved during the various stages of the isolation procedure has been evaluated, as in earlier work (Cohen, 1960), by estimating the amount of protein for maximal activity. The specific activity is expressed as nanograms of protein per biological unit.

HOMOGENATE (5%, w/v) of 100 submaxillary glands in water was centrifuged at 50,000*g* for 30 min to give SUPERNATANT. The latter, approximately 360 ml in volume, was lyophilized and the powder was taken up in 20 ml of 0.05 M Tris·Cl buffer (pH 7.4).

↓

Lyophilized SUPERNATANT was loaded on two G-100 Sephadex columns (125 × 4 cm), equilibrated with the Tris·Cl buffer, and eluted with the same buffer. The initial flow rate was 100 ml/hr. Approximately 400 ml of effluent was collected between maximum of first peak and the first trough on the absorbance curve to give G-100 FRACTION (Figure 2a).

↓

G-100 FRACTION was loaded onto two DEAE-cellulose columns (17 × 2.5 cm) equilibrated with the Tris·Cl buffer. The flow rate was approximately 350 ml/hr. Each column was washed with 300 ml of 0.01 M NaCl in the same buffer and eluted with 350 ml of 0.08 M NaCl in the same buffer to give DEAE FRACTION (Figure 2b). The combined DEAE FRACTIONS were concentrated by pressure dialysis against the Tris buffer to about 4 ml.

↓

Sucrose was added to concentrated DEAE FRACTION to a final concentration of 5 g/100 ml and the mixture was loaded onto a G-150 Sephadex column (95 × 2.5 cm) equilibrated with the Tris·Cl buffer and eluted with the same buffer. The flow rate was 20 ml/hr. G-150 FRACTION (Figure 2c), approximately 40 ml in volume, was concentrated by pressure dialysis against the Tris buffer to about 2 ml.

FIGURE 1: Flow sheet for the isolation of nerve growth factor.

Electrophoresis. Starch gel electrophoresis was carried out at 4° in 0.04 M phosphate buffer (pH 7.4) (Gammack *et al.*, 1960). Polyacrylamide disk electrophoresis followed the methods of Davis (1964) and Ornstein (1964) but used a phosphate buffer, pH 7.0, and ionic strength 0.015. The resolving gel was made by mixing the following solutions in the ratio 1:1:2: (A) 6.2 ml of 0.5 M KH_2PO_4, 2.9 ml of 0.5 N NaOH, 0.2 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/100 ml. No concentrating gel was used. Upper and low buffers were prepared by making a mixture of 15.5 ml of 0.5 M KH_2PO_4 and 7.25 ml of 1 N NaOH up to 1 l. Samples were diluted to a volume of 50 or 100 μl with a 1:5 dilu-

[1] Abbreviations used: Temed, *N,N,N',N'*-tetramethylethylenediamine; Bis, *N,N'*-methylenebisacrylamide.

TABLE I: Protein and Nerve Growth Factor Content of Various Fractions Derived from Mouse Submaxillary Gland.[a]

Fraction	Protein		Nerve Growth Factor		Sp Act. (ng of protein/BU)
	Total (mg)	% of Original	Total (BU × 10^{-6})	% of Original	
		A. Current Procedure			
Homogenate[b]	2520	100	4.7	100	537
Supernatant	1590	63	5.2	110	307
G-100 fraction	292	11.5	6.0	128	48
DEAE fraction	72	2.9	4.3	91	17
G-150 fraction	48	1.9	3.8	81	13
		B. Cohen Preparation			
Homogenate[c]	2400	100	1.6	100	1500
Final product	4	0.2	0.27	17	15

[a] Starting material for preparation was glands from 100 mice, 50–60-day old, wet weight 16.5 g. The fractions were isolated as described in text. Results are the mean of 30 experiments through to the DEAE fraction and 12 experiments including the G-150 fraction. The comparative data for the Cohen (1960) preparation have been recalculated from his data to be in line with the scale of the present method. [b] Only A and B groups of mice (see Table II) were used. [c] Note that in spite of equal amounts of protein the nerve growth factor activity of Cohen's homogenate was one-third that of present homogenates.

tion of the buffer and sucrose was added to a final concentration of 5 g/100 ml.

Sedimentation velocity experiments were performed at 5° in the Spinco Model E with a double-sector cell. Sedimentation in 5–20% linear sucrose gradients was carried out also at 5° using the procedure of Martin and Ames (1961). The sample (75 μl in volume) was mixed with 25 μl of a 1-mg/ml solution of dansylated bovine serum albumin (Weber, 1952) and layered on top of the gradient poured in a 4-ml polypropylene tube from the SB 405 rotor (International Equipment Co.). After centrifugation for 15 hr at 60,000 rpm and 5°, the contents of the tube were collected dropwise from the bottom, each fraction consisting of 3 drops, and the absorbance at 280 mμ and the nerve growth factor activity of the fractions were determined. $s_{20,w}$ values were calculated using a value of 4.3 for the $s_{20,w}$ of the serum albumin marker.

Results

Isolation of the Higher Molecular Weight Form of Nerve Growth Factor. The procedure for the isolation of the higher molecular weight nerve growth factor species is summarized in Figure 1. The concentrated supernatant from 100 submaxillary glands was divided into two and each half was fractionated by gel filtration and DEAE chromatography. The two active DEAE fractions were combined and concentrated for the final step, the gel filtration on G-150 Sephadex.

Gel Filtration of the Supernatant. The protein pattern (Figure 2a) showed three major peaks. The absorbance curve at 280 mμ grossly paralleled the protein curve except in peak I and the minor peaks IV and V. An absorbance curve at 260 mμ (not shown in Figure 2a) indicated that a relatively large amount of nucleic acid material was present in these peaks. Nerve growth factor activity was detected between the tail of the turbid region in peak I and the beginning of the hemoglobin in peak II. In routine experiments the nerve growth factor containing fraction was collected by reference to the absorbance curve at 280 mμ alone (G pool in Figure 2a); it comprised about 20% of the soluble protein of the supernatant. The over-all activity of the G-100 fraction whether calculated from the nerve growth factor content of the individual fractions or measured directly on the pool was significantly higher than the apparent

TABLE II: Activity Differences in the Initial Steps of Nerve Growth Factor Isolation from Different Batches of Mouse Salivary Glands.[a]

	Wet Wt of 100 Glands (g)	Nerve Growth Factor Act. (BU/mg wet weight × 10^{-3})		
		Homogenate	Supernatant	G-100 Fraction
A	15.8	200	200	460
B	18.8	410	370	430
C	17.2	420	390	170

[a] Fractions prepared from glands of 50–60-day-old mice as described in text and Table I.

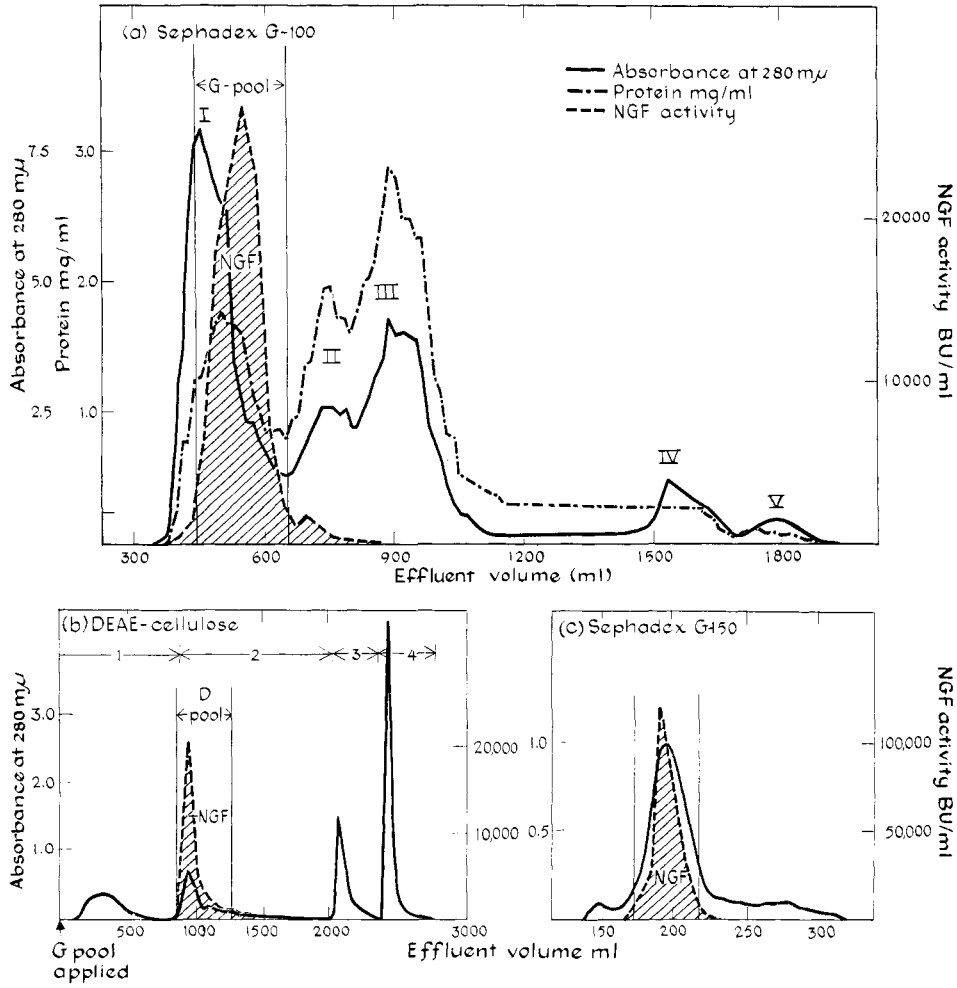

FIGURE 2: Flow diagram of the isolation of nerve growth factor. (a) Gel filtration of supernatant on Sephadex G-100. (b) Chromatography of G-100 fraction on DEAE-cellulose. (c) Gel filtration of DEAE fraction on Sephadex G-150. Experimental details given in flow sheet in Figure 1. Solvents for elution of DEAE-cellulose column in b were (1) 0.01 M NaCl, (2) 0.08 M NaCl, (3) 0.30 M NaCl, and (4) 2.0 M NaCl, all in 0.05 M Tris · Cl buffer (pH 7.4).

nerve growth factor content of the gland homogenate (Table I). This increase in activity may be due to the removal of a material interfering with the response of the ganglia in the biological assay or, more specifically, of an inhibitor of nerve growth factor. Considerable variation was found for different groups of mice, most groups falling into one of the three groups shown in Table II. In group A, homogenates had a low activity similar to those reported in the old procedure (Cohen, 1960), but a twofold increase was brought about by gel filtration. In group B the activity expressed in the homogenate was already twice as high as in group A and no increase occurred after gel filtration. Group C had the same high activity in homogenates as group B but the activity fell sharply after gel filtration and during subsequent fractionation steps. The fact that all groups show high activity at some stage argues against differences in the intrinsic nerve growth factor content of the glands and suggests, rather, the involvement of other gland constituents or some, as yet unidentified, change in molecular properties. The nerve growth factor activity at this stage of the fractionation was unstable and decayed at a significant rate. Concentration by pressure dialysis or by lyophilization resulted in a variable but always sizeable loss of activity, thereby limiting the choice of the next purification step. Recycling on Sephadex G-200, for example, while removing about 80% of the inert protein of the G-100 fraction yielded only 20–30% of the nerve growth factor activity, much of the activity being lost during the required concentration of the G-100 fraction.

DEAE-Cellulose Chromatography. A satisfactory second step in the fractionation, which could be applied directly to the G-100 fraction without any intermediate manipulation, was found to be ion-exchange chromatography on DEAE-cellulose. It was noted that the nerve growth factor activity of the G-100 fraction was completely adsorbed to a DEAE-cellulose column equilibrated with the 0.05 M Tris·Cl buffer (pH 7.4) used in the preceding gel filtration. About 70% of the nerve

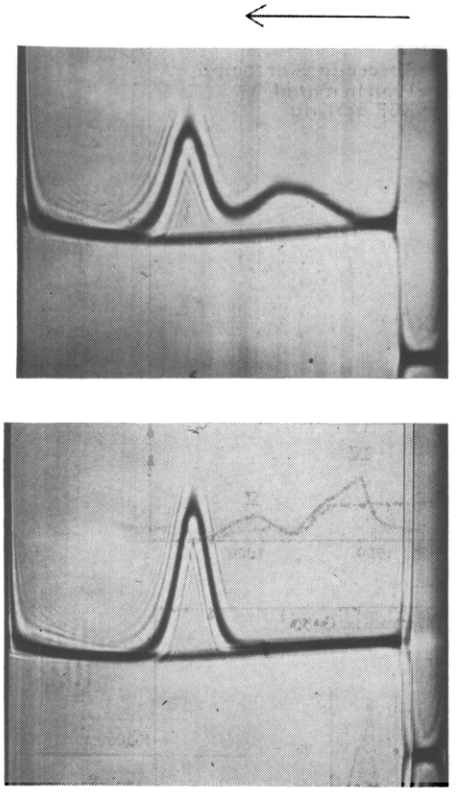

FIGURE 3: Sedimentation velocity patterns of DEAE (top) and G-150 (bottom) fractions. Sedimentation at 59,780 rpm in Spinco Model E; temperature, 5°; buffer, 0.05 M Tris·Cl (pH 7.4); and protein concentration, 10 mg/ml. Photographs taken 96 min after reaching speed.

growth factor activity and 25% of the protein content of the G-100 fraction was collected in the 0.08 M NaCl eluate as indicated in Figure 2b by the vertical lines defining the DEAE fraction (D pool), another 10% being present in the slowly eluted tail of the main zone. It was found that a high flow rate was critical for such results. Continued elution with NaCl concentrations higher than 0.08 M produced further protein peaks, an additional 5% of the nerve growth factor activity being sometimes detected in the 0.3 M NaCl eluate. The DEAE fraction was concentrated by pressure dialysis with practically no loss of activity and after concentration did not lose activity over a period of several weeks at 3°. It had a specific activity of 17 ng of protein/BU, approximately the same as that of the most purified material previously described, but was obtained with about an 18-fold increase in yield (Table I).

In sedimentation velocity experiments, the DEAE fraction showed one major and two minor components (Figure 3). Only the species with the highest sedimentation coefficient had nerve growth factor activity (Figure 4c) and within the limits of the biological assay all the activity loaded on the sucrose gradient was recovered with this component. Electrophoresis of the DEAE fraction on acrylamide gel at pH 7.0 (Figure 5a) or starch gel at pH 7.4 (Figure 5b) also showed one major zone with minor protein contaminants. The nerve growth factor activity migrated with this major zone on acrylamide gel (Figure 5a) and again all the activity loaded on the gel was recovered in this one component. By these criteria, the nerve growth factor activity is associated with the major component of the DEAE fraction, which comprises approximately 70% of the total protein.

Gel Filtration on Sephadex G-150. The impurities in the DEAE fraction were considerably decreased by gel filtration on Sephadex G-150. The elution pattern showed one major protein component with which the nerve growth factor activity was associated (Figure 2c). An appropriate selection of effluent fractions provided a G-150 fraction with about 66% of the protein and 90% of the activity of the DEAE fraction, the activity remaining stable after concentration for at least 2 weeks at 3°. The specific activity of the G-150 fraction was slightly better than that of the DEAE fraction (Table I). Sedimentation experiments showed that the G-150 fraction contained less than 5% of low molecular weight inert protein (Figures 3 and 4d) and that the nerve growth factor activity sedimented with the main component (Figure 4d). The sedimentation coefficient found for this component in the sucrose gradient experiments was in good agreement with the value of 7.1 S determined in the analytical ultracentrifuge. Only one migrating zone was seen in the G-150 fraction when analyzed by electrophoresis in acrylamide gel at pH 7.0 (Figure 5a) or starch gel at pH 7.4 (Figure 5b). The nerve growth factor activity again migrated with the single protein zone in the acrylamide gel (Figure 5a).

The nerve growth factor activity in the G-100 and DEAE fractions sedimented at the same rate as in the G-150 fraction, as also did a major part of the activity in the original supernatant (Figure 4). Acrylamide gel analyses also confirmed that the nerve growth factor component had the same electrophoretic mobility in the G-100, DEAE, and the final G-150 fraction.

Nerve Growth Factor Species of Different Size. Because of the difference in the molecular weights of the nerve growth factor isolated by the current and the older procedures each fractionation stage in the latter was investigated to determine where the change in molecular size occurred. Using gel filtration on Sephadex G-100 as an index of size, it was found that streptomycin, alcohol, and ammonium sulfate treatment of the gland supernatant did not affect the molecular weight of nerve growth factor, although they did cause considerable loss of activity. The remaining step in the previous method not used in the current procedure was chromatography on CM-cellulose at acid pH and this was, therefore, investigated. In agreement with the earlier results it was found that all the nerve growth factor activity in the dilute DEAE fraction was adsorbed onto CM-cellulose at pH 4.4. It could be recovered in discrete fractions either by elution with increasing concentration of NaCl or by a combination of increased salt and pH. However, such fractions contained only 30–40% of the nerve growth factor activity and 10% or less of the pro-

tein loaded onto the column. The remaining protein eluted from these columns was inactive. The active CM fractions lost activity rapidly if kept in the dilute form, but a 20-fold concentration by pressure dialysis greatly improved their stability. The specific activity of these fractions was three or four times better than that of the original DEAE fraction. Sedimentation in sucrose gradients showed that the active CM fractions differed strikingly in molecular size from the high molecular weight nerve growth factor (Figure 4e). The activity and optical density peaks coincided and had an $s_{20,w}$ of 2.6 corresponding to a molecular weight of approximately 30,000.

Discussion

The procedure outlined above differs in a number of ways from the original method for isolating nerve growth factor from the mouse submaxillary gland (Cohen, 1960). Only three fractionation steps are required to isolate a fraction in which the nerve growth factor activity is associated, as judged by a number of physical criteria, with a single protein component which accounts for more than 95% of the total protein of the fraction. The whole procedure takes about 3 days to be completed, and this time could be reduced if a more efficient method of concentrating the protein fractions were employed. While the specific activity of the new product is similar to that described by Cohen (1960), the higher yields of the new procedure now make it feasible to undertake an extensive chemical and physicochemical characterization of the nerve growth factor protein. It should be noted that injection of the G-100 fraction, containing the high molecular weight nerve growth factor species, into newborn mice resulted (S. Varon, unpublished data) in a four- to sixfold increase in the size of the superior cervical ganglia, which is one of the characteristic *in vivo* effects of the original nerve growth factor preparation (Levi-Montalcini and Booker, 1960). The G-100 fraction showed none of the toxicity reported for the crude gland extract or partially purified fractions prepared by the older method. The toxic material itself is left in the supernatant which emerges from the G-100 column in peak III (Figure 2a).

The major difference between the new and the old nerve growth factor product lies, however, in their physical properties. The sedimentation coefficient of 7.1 S obtained for the new nerve growth factor species corresponds to an approximate molecular weight of

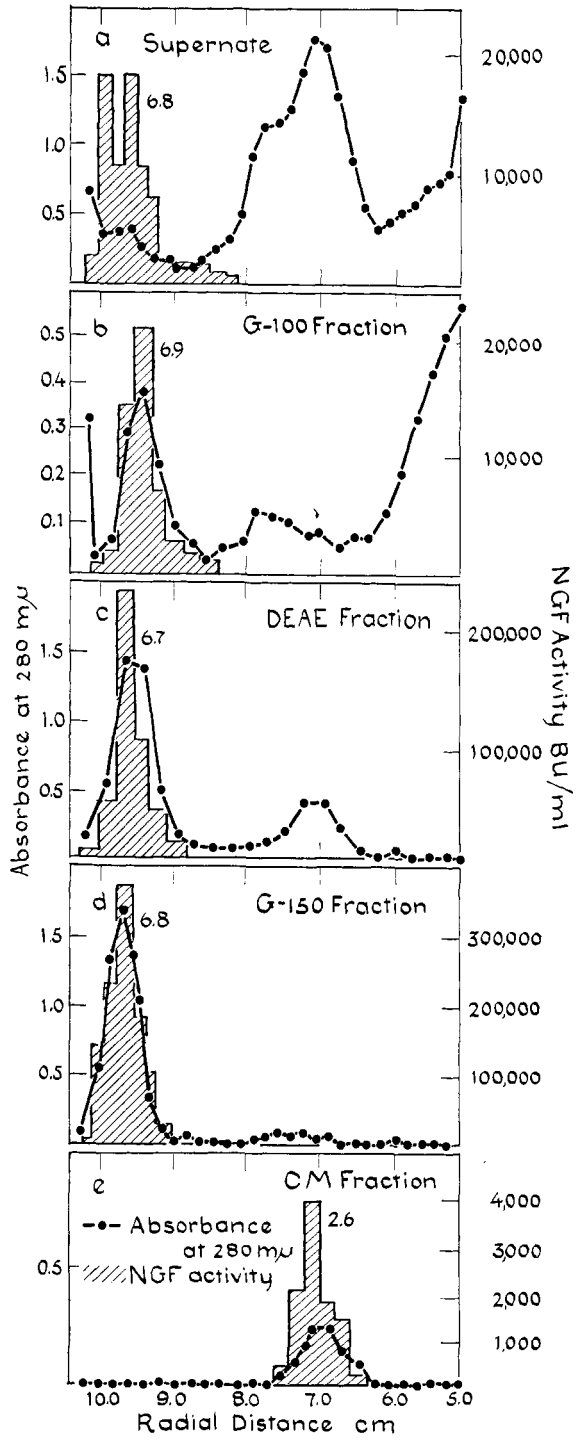

FIGURE 4 (right): Sedimentation of fractions from mouse salivary gland homogenate in sucrose gradients. Sedimentation for 15 hr at 60,000 rpm in SB 405 rotor at 5°; 5–20% linear sucrose gradient in 0.05 M Tris·Cl buffer (pH 7.4). Each fraction (75 μl) and dansylated bovine serum albumin (25 μl) were loaded on top of gradient. (a) Supernatant concentrated fivefold. (b) G-100 fraction concentrated eightfold. (c) DEAE fraction concentrated 200-fold. (d) G-150 fraction concentrated 20-fold. (e) CM-fraction prepared by adsorbing DEAE fraction onto a CM-cellulose column (20 × 2.5 cm) equilibrated with 0.05 M sodium acetate buffer (pH 4.4), washing column with 300 ml of 0.2 M NaCl and 500 ml of 0.01 M NaCl in 0.05 M Tris·Cl buffer (pH 7.4), and eluting the active fraction with 0.08 M NaCl in the Tris buffer. This fraction concentrated 20-fold.

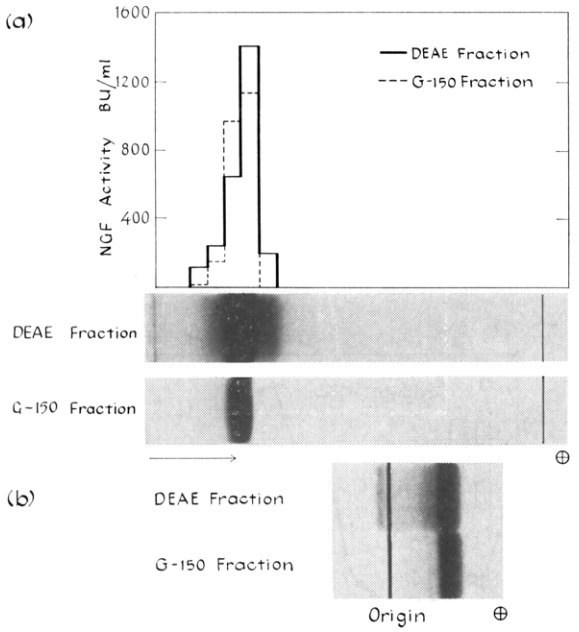

FIGURE 5: Analysis of DEAE and G-150 fractions by acrylamide and starch gel electrophoresis. (a) Acrylamide gel electrophoresis in 7.5% gels in phosphate buffer (pH 7.0) of ionic strength 0.015. Migration time was 3 hr at 2 ma/tube; 3°. Gels stained in 0.1% Naphthol Blue Black in 10% acetic acid. Line at anode end of gel indicates position of dye band. Gels for nerve growth factor assay cut into 2-mm slices. Each slice was homogenized in 1.0 ml of phosphate buffer (pH 7.0), a second 1.0 ml of buffer being used to wash out homogenizer. Combined homogenate and wash were centrifuged for 5 min at 1000*g* and the supernatant was assayed for nerve growth activity as described in Methods. All extraction procedures were carried out at 0°. (b) Starch gel electrophoresis was in 0.04 M phosphate buffer (pH 7.4); migration time 4 hr. Gel sliced and stained in 1% Naphthol Blue Black in methanol–acetic acid–water (60:10:40, v/v). Excess dye was removed with the same solvent. DEAE and G-150 fractions were at a concentration of 20 mg/ml.

140,000 and the sucrose gradient analyses of the various nerve growth factor containing fractions (Figure 4) show that this molecular weight species is present, in large part, in the gland extract and is not modified in the subsequent steps of the isolation procedure. At no stage was any nerve growth factor activity found with proteins of molecular weight as low as the 44,000 or 20,000 values reported for the earlier nerve growth factor products. A part of the nerve growth factor activity in the gland supernatant apparently sedimented slightly faster than the material of 7.1 S; this will be further investigated since it may point to either the natural occurrence of another nerve growth factor species of somewhat higher molecular weight or to the association of nerve growth factor with other gland constituents. A nerve growth factor species whose size is grossly within the range of the previously described nerve growth factor products from snake venom and the mouse submaxillary gland can be obtained from the high molecular weight form by a chromatographic separation which starts at acid pH. The remaining proteins separated on this column are inactive in the usual nerve growth factor bioassay system. Preliminary experiments have shown (Varon *et al.*, 1967) that these phenomena are the result of a dissociation of the high molecular weight nerve growth factor species induced by the low pH and of the subsequent separation on the ion-exchange column of active from inactive subunits. The ability to separate the subunits by ion-exchange chromatography points to a significant difference in net charge between them.

Two unusual features were noticed during the development of the isolation procedure. Good recovery of the nerve growth factor activity from the DEAE-cellulose in discrete fractions was only obtained at very high flow rates. At more normal flow rates nerve growth factor activity was eluted from the column over many fractions and with poor recovery. Secondly, although the first gel filtration on Sephadex G-100 removed proteins which are eluted with or later than hemoglobin, after the DEAE-cellulose chromatography significant amounts of such proteins reappeared and a second gel filtration was required to isolate the final nerve growth factor product. It is possible that these two phenomena are related to the dissociation of the high molecular weight form of nerve growth factor.

The work described here shows a complexity in the nerve growth factor molecule which may be of considerable importance in terms of its biological activity. For example, it raises the question as to what is the ultimate size of the nerve growth factor molecule interacting with the responsive ganglia and what is the relevance of the remaining portion(s) of the larger molecule.

Acknowledgments

We are indebted to Charles Raiborn, Jr., and Judith Timmins for their skilled assistance. One of us (S. V.) gratefully acknowledges his debt to Dr. Levi-Montalcini for her generous and stimulating support toward the initiation of this investigation.

References

Angeletti, P. U., Gandini-Attardi, D., Toschi, G., Salvi, M. L., and Levi-Montalcini, R. (1965), *Biochim. Biophys. Acta 95*, 111.

Angeletti, P. U., Liuzzi, A., Levi-Montalcini, R., and Gandini-Attardi, D. (1964), *Biochim. Biophys. Acta 90*, 445.

Cohen, S. (1957), *Cancer Res. 17*, 15.

Cohen, S. (1959), *J. Biol. Chem. 234*, 1129.

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

Cohen, S. (1964), *Natl. Cancer Inst. Monograph 13*, 13.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Gammack, D. B., Huehns, E. R., Shooter, E. M., and Gerald, P. S. (1960), *J. Mol. Biol. 2*, 372.

Gandini-Attardi, D., Levi-Montalcini, R., Wenger,

B. S., and Angeletti, P. U. (1965), *Science 150*, 1307.
Levi-Montalcini, R. (1966), *Harvey Lecture Ser. 60*, 217.
Levi-Montalcini, R., and Angeletti, P. U. (1961), *Quart. Rev. Biol. 36*, 99.
Levi-Montalcini, R., and Angeletti, P. U. (1963), *Dev. Biol. 7*, 653.
Levi-Montalcini, R., and Booker, B. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 373.
Levi-Montalcini, R., Meyer, H., and Hamburger, V. (1954), *Cancer Res. 14*, 49.
Levi-Montalcini, R., Tentori, L., Vivaldi, G., Angeletti, P. U., and Marini-Bettolo, G. B. (1965), *Gazz. Chim. Ital. 95*, 333.
Liuzzi, A., Angeletti, P. U., and Levi-Montalcini, R. (1965), *J. Neurochem. 12*, 705.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Varon, S., Nomura, J., and Shooter, E. M. (1967), *Proc. Natl. Acad. Sci. U. S.* (in press).
Weber, G. (1952), *Biochem. J. 51*, 155.
Winick, M., and Greenberg, R. (1965), *Pediatrics 35*, 221.