*THE NERVE GROWTH FACTOR: PURIFICATION AS A 30,000-MOLECULAR-WEIGHT PROTEIN**

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Abstract.—The nerve growth factor protein was purified over 100-fold from adult mouse salivary glands. The first step was a gel filtration on Sephadex G-100 at pH 7.5 of the aqueous gland extract. After gel filtration, most of the NGF activity was eluted in the 80,000–90,000-molecular-weight region (G-100 pool). The G-100 pool was dialyzed at pH 5.0 and fractionated by CM52 cellulose chromatography at pH 5.0. Recovery from CM52 cellulose columns was quantitative for protein and ranged 80–100 per cent for the nerve growth factor activity; the latter was almost completely carried by a protein which did not show any heterogeneity when examined by several analytical tests. The purified nerve growth factor showed an $s_{20,w} = 2.43$, a $D_{20,w} = 7.30$ and a 30,000 molecular weight. The over-all recovery was about 45 per cent.

The nerve growth factor is a protein which selectively stimulates the growth and differentiation of sympathetic and embryonic sensory nerve cells.[1, 2] It was first observed in certain mouse sarcomas and later found at much higher levels in other sources, such as snake venom ($s_{20} = 2.2$)[3–5] and submaxillary glands of adult mice ($s_{20} = 4.33$).[6–8]

The isolation from mouse submaxillary glands of a nerve growth factor form of 140,000 mol wt was recently reported.[9, 10] This high-moleculer-weight nerve growth factor was mostly inactivated upon exposure to pH below 5.0 and above 8.0; inactivation was accompanied by dissociation into acidic, basic, and neutral components, called α-, β-, and γ-subunits, respectively. Once separated by chromatography on carboxymethyl (CM) cellulose, only the β subunit displayed nerve growth factor activity, which, however, accounted for only 25 per cent of the amount loaded on the column. Recovery of the total nerve growth factor activity and reconstitution of the 140,000-molecular-weight protein were obtained by mixing the three components at neutral pH.[11, 12]

Studies from this laboratory did not support these findings.[2, 13] A procedure is here described for the purification of the nerve growth factor in which pH was kept between 7.5 and 5.0, i.e., within values reported[11, 12] as not affecting either the activity or the molecular size of the nerve growth factor. The purified nerve growth factor showed a 30,000 mol wt, accounted for full recovery of biological activity, and did not require any other protein component for maximal response.

Materials and Methods.—Submaxillary glands were excised from adult albino mice (25–30 gm body weight), freed of fat and connective tissue, and stored frozen at −20° until used. The usual amount was 10 gm of glands (wet weight) from 100 mice.

Sephadex G-100 (Uppsala, Sweden) columns were calibrated as indicated by Andrews.[14] DE52 cellulose and CM52 cellulose (Whatman, London) were used as anion exchanger and cation exchanger, respectively. Fractions from gel filtration or ion exchange chro-

matography were checked for protein by UV absorption at 280 mμ using a Zeiss model II spectrophotometer. Protein content in selected pools was estimated by Lowry's method,[15] using bovine serum albumin as a standard.

The antiserum (As) to the 30,000-molecular-weight nerve growth factor was prepared by injecting into rabbits the purified protein in complete Freund's adjuvant. Antiserum to the 140,000-mol wt nerve growth factor[9, 10] was a commercial preparation of the Wellcome Co. (England).

With a Shandon apparatus, 7.5 per cent polyacrylamide gel disc electrophoresis was performed as indicated by Davis.[16] Gel systems used were at pH 4.3,[17] pH 6.6,[18] pH 7.0,[10] and pH 9.6.[16] Gels were stained with 1% Amido-Schwarz in 7.5% acetic acid.

Ultracentrifuge studies were performed with a Spinco model E analytical ultracentrifuge at 20°. In all the analyses, the nerve growth factor was exhaustively dialyzed against 5×10^{-2} *M* acetate buffer pH 5.0 containing 1×10^{-1} *M* NaCl. Sedimentation velocity measurements were carried out in a double sector cell at 56,100 rpm at different protein concentrations. Diffusion measurements were performed at 8,000 rpm in a double sector cell with preformed boundary; the diffusion coefficient was calculated by the "height-area" method.[19] Sedimentation and diffusion data were corrected to standard conditions in water at 20° and then used for calculating the molecular weight.[19] The molecular weight of the purified nerve growth factor was also determined in a double sector cell by the meniscus depletion method of Yphantis[20] using Rayleigh interference optics at 35,600 rpm rotor speed. Photographic plates were analyzed with a Nikon two-dimensional microcomparator.

The nerve growth factor activity was assayed as described by Levi-Montalcini and coworkers.[21] One BU was referred to as the amount of the nerve growth factor required to give a 4+ response in the tissue culture system. Since 0.05 ml of the diluted nerve growth factor samples was used for the activity assay, the number of BU in 1 ml of the *original* sample tested (BU/ml) was 20 times the dilution factor used to achieve the 4 + response. The specific activity was expressed as BU/μg.

Results.—Preparation of gland extract: 40–50 gm of mouse submaxillary glands were suspended in 3 volumes of ice-cold distilled water and homogenized for two minutes in a Waring Blendor and for one additional minute in a Potter homogenizer. The homogenate was centrifuged for 30 minutes at 13,000 rpm and the supernate diluted with 0.5 volume of ice-cold distilled water. One volume of 0.2 *M* streptomycin sulfate (Squibb) in 1×10^{-1} *M* Tris-HCl buffer pH 7.5 was slowly added to 9 volumes of extract.[8] The mixture was allowed to stand in ice for 30 minutes and then centrifuged as above. The clear red supernate was lyophilized to dryness.

Gel filtration on Sephadex G-100: The powder was dissolved in 5×10^{-2} *M* Tris-HCl buffer pH 7.5 containing 5×10^{-4} *M* ethylenediamine tetraacetate (EDTA). The concentrated extract was applied on two Sephadex G-100 columns equilibrated with the same buffer. A typical fractionation is reported in Figure 1. The nerve growth factor highest specific activity ranging 70–80 BU/μg, was constantly found in peak II whose elution volume accounted for a 80,000–90,000 mol wt. Proteins collected in this peak were about 8 per cent and recovery for the nerve growth factor activity ranged 60–75 per cent of the amount loaded. Six per cent proteins and 2–6 per cent nerve growth factor activity (spec. act. 1–5 BU/μg) were eluted with peak I, usually turbid, which emerged almost at the void volume. It must be mentioned that omitting addition of streptomycin to the crude extract resulted in a poor separation between peaks I and II.[10, 22] Eight per cent proteins and 5–15 per cent nerve growth

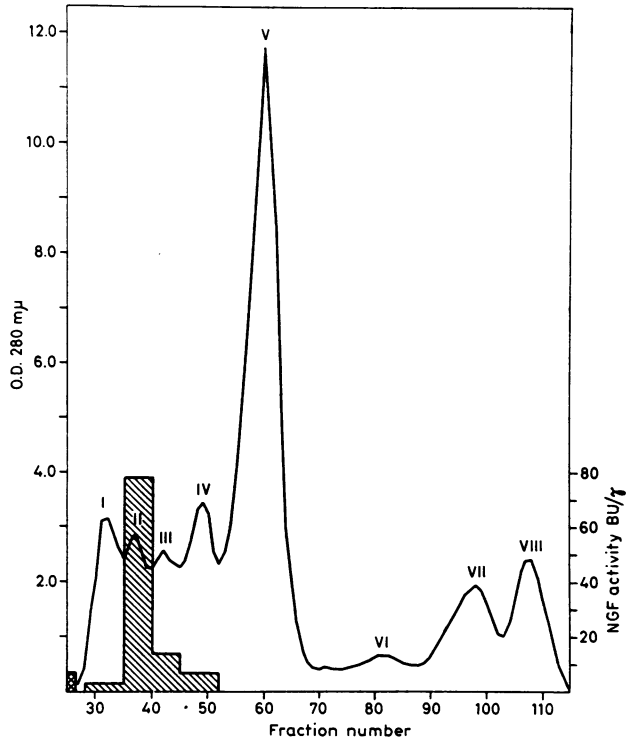


FIG. 1.—Gel filtration of mouse salivary gland extract. 6.2 ml of extract (298 mg/ml proteins) were loaded on Sephadex G-100 column (2.7 × 143 cm) equilibrated with 5×10^{-2} *M* Tris HCl buffer pH 7.5 containing 5×10^{-4} *M* EDTA. Void volume 254 ml. Flow rate was 1.5 ml/cm²/hr, fraction volume 7–8 ml. Hatched area represents the spec. act. of the nerve growth factor; the small bar on the left shows the specific activity of the sample applied.

factor activity (spec. act. 5–20 BU/μg) were collected in peak III, which sometimes appeared as a shoulder. Twelve per cent proteins and 7 per cent nerve growth factor activity (spec. act. 3–8 BU/μg) were recovered in the red-colored peak IV. The recovery of proteins as well as of nerve growth factor activity from G-100 columns was quantitative. In routine experiments only fractions from peak II and peak III were further processed. As judged from several preparations, the pool of these fractions (G-100 pool) accounted for 70–90 per cent of the nerve growth factor activity and about 15 per cent of the proteins loaded.

Ion-exchange chromatography of the G-100 pool: In the early stages of the present study the second step for the purification of the nerve growth factor was an anionic exchange chromatography of the G-100 pool on DE52 cellulose equilibrated with 5×10^{-2} *M* Tris-HCl buffer pH 7.5. Seventy to eighty per cent of the nerve growth factor activity and 25 per cent of proteins were not bound to the resin (DE52 pool). The DE52 pool was dialyzed against 5×10^{-2} *M* acetate buffer pH 5.0 and then applied on a CM52 cellulose column equilibrated with the same buffer. Later it was found more convenient to omit the DE52 cellulose chromatography. The G-100 pool was dialyzed overnight against 5×10^{-2} *M* acetate buffer pH 5.0; the precipitate formed was removed by centrifugation and the supernate was loaded on a CM52 cellulose column equilibrated with 5×10^{-2} *M* acetate buffer pH 5.0. The chromatographic separation is shown in Figure 2. Recovery was quantitative for proteins and

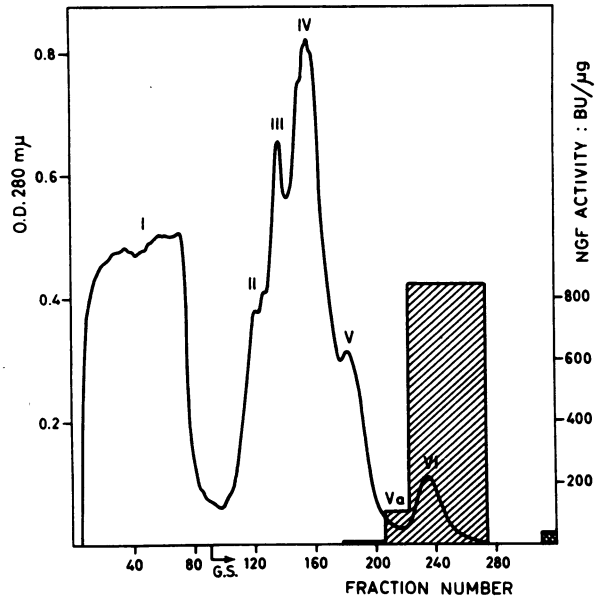


FIG. 2.—Chromatography of the G-100 pool on CM52-cellulose at pH 5.0. 300 mg of the G-100 pool dialyzed at pH 5.0 carrying 11.0 × 10^6 BU were loaded on a 1.5 × 8 cm column equilibrated with 5 × 10^{-2} *M* acetate buffer pH 5.0. Elution by parabolic salt gradient 0 to 1.0 *M* NaCl (total volume 750 ml). Flow rate 25 ml/hr; fraction volume 3–4 ml. See Table 1 for recovery data. Other details as indicated under Fig. 1.

ranged 80–100 per cent for the nerve growth factor activity (Table 1). About 35 per cent of the proteins were collected in the excluded peak I. Proteins bound to the resin were eluted by increasing NaCl concentration, keeping the pH unchanged at 5.0. Most of the nerve growth factor activity and 3 per cent of proteins loaded were recovered in peak VI, which was eluted at 0.5 *M* NaCl. The nerve growth factor specific activity in peak VI was about 850 BU/μg; values up to 1500 were occasionally calculated. Injection of peak VI into newborn mice did not produce any toxic effect, whereas it did produce the usual increase in size of sympathetic ganglia previously described.[1, 2] As recovered from CM52 cellulose columns the nerve growth factor rapidly lost activity. Stability was greatly improved by concentration; samples at 2–10 mg/ml stored at 0° in 5 × 10^{-2} *M* acetate buffer pH 5.0 containing 1 × 10^{-1} *M* NaCl remained fully active for several weeks. The over-all procedure for the purification of the nerve growth factor is summarized in Table 2.

Purity tests and physical properties of the nerve growth factor: The nerve growth factor collected from the central region of peak VI from CM52 cellulose columns was concentrated by pressure dialysis and used for purity tests and chemicophysical studies. The OD 280/260 absorption ratio was 1.54, in agreement with that reported by Cohen.[8] On immunodiffusion a single precipitin band was obtained when the antiserum was reacted with our purified nerve growth factor (mol wt 30,000) over a wide range of protein concentration and at various pH values. The antiserum to our purified nerve growth factor gave likewise a single precipitin band when tested with crude salivary gland extract or with the G-100 pool (Fig. 3*A*). In contrast, the Wellcome antiserum (which was quoted by the manufacturer as prepared against the large molecular weight nerve growth factor of Varon and co-workers[9, 10]) gave several precipitin bands

TABLE 1. *Protein and nerve growth factor activity recovery in the different fractions from the CM52-cellulose chromatography shown in Figure 2.*

Fraction	Protein		Nerve Growth Factor Activity		
	Total (mg)	Recovery (%)	BU/μg	Total (BU × 10^{-3})	Recovery (%)
I	100	33.3	1.5	150	1.4
II	21.3	7.1	2.1	45	0.4
III	41.4	13.8	0.9	38	0.3
IV	100.4	33.4	1.34	135	1.2
V	20.0	6.7	9.8	196	1.8
Va	3.0	1.0	110	332	3.0
VI	9.5	3.2	862	8,188	74.4
Total	295.6	98.5		9,084	82.5

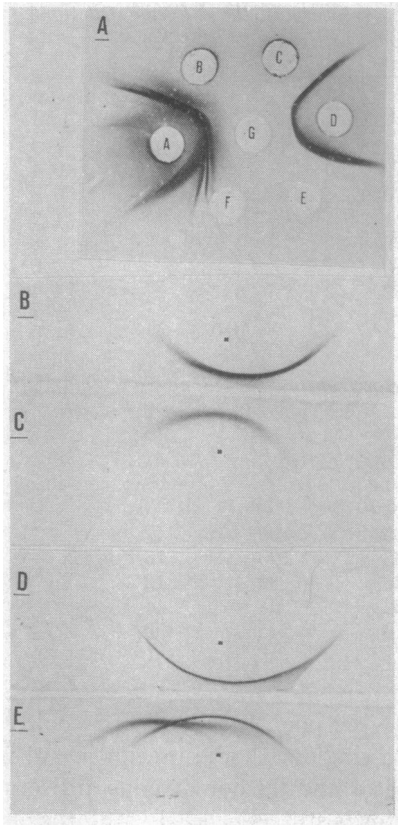
TABLE 2. *The purification of the nerve growth factor from mouse submaxillary gland.*

Fraction	Protein		Biological Activity		Nerve growth factor spec. act. (BU/μg)	Purification
	Total (mg)	(%)	Total (BU × 10^{-6})	(%)		
Homogenate	5,000	100	35	100	7	1
Extract on G-100	3,500	70	35	100	10	1
G-100 pool pH 7.5	670	13	28	80	42	6
G-100 pool pH 5.0	500	10	22	64	44	6
CM52 peak VI	19	0.38	16	45	850	120

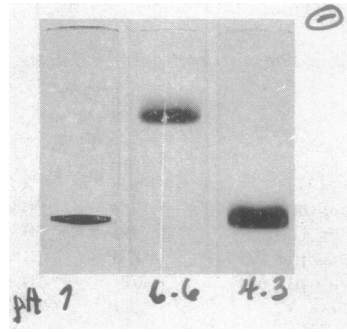
The data were derived from 43 different preparations.

when tested against the crude extract, the G-100 pool, and the DE52 pool (Fig. 3*A*). On immunoelectrophoresis (pH 8.3) a single cathodic precipitin arc was obtained with our purified nerve growth factor and the homologous antiserum. The DE52 pool, where the nerve growth factor is still associated with other protein components, still gave a single precipitin arc near the origin or in a slight anodic position (Fig. 3*B* and *C*). With the Wellcome antiserum, at least three different antigenic components were clearly demonstrated in the DE52 pool but only one was evident in the purified nerve growth factor (Fig. 3*D* and *E*). On disc polyacrylamide gel electrophoresis at pH 9.6, the purified nerve growth factor did not enter the gel matrix; at pH 7.0, 6.6, and 4.3, one single band was observed which migrated toward the cathode; no other components could be detected even after loading up to 100 μg of protein (Fig. 4). Preliminary data from electrofocusing experiments[23] indicated as 9.3 the isoelectric point of the nerve growth factor.

The sedimentation velocity coefficient $s_{20,w}$ was measured at 15, 12, 9, 6, and 4.5 mg/ml nerve growth factor. A single symmetrical peak was in all cases observed (Fig. 5) with no evidence for any faster or slower sedimenting material. The $s_{20,w}$ did not change appreciably with protein concentration and the general average was 2.43 ± 0.05 (standard deviation), which is lower than the value of 4.33 reported by Cohen.[8] The diffusion coefficient $D_{20,w}$, measured at 6 mg/ml nerve growth factor, was calculated as 7.30. The molecular weight calculated from sedimentation and diffusion data was 32,000, which was very close to the 30,000 value determined by the meniscus depletion method of Yphantis.[20] Once the equilibrium was attained, no heterogeneity was detectable (Fig. 6).



(*Left*) FIG. 3.—(*A*) Immunodiffusion patterns with nerve growth factor at different steps of purification (*A*, Wellcome antiserum; *B*, DE52 pool; *C*, purified nerve growth factor; *D*, nerve growth factor antiserum; *E* and *F*, gland extract; *G*, G-100 pool); (*B*) immunoelectrophoretic pattern of purified nerve growth factor with its antiserum; (*C*) DE52 pool; (*D*) immunoelectrophoretic pattern of purified nerve growth factor with Wellcome antiserum; (*E*) DE52 pool.



(*Above*) FIG. 4.—Disc electrophoresis of the purified nerve growth factor at different pH. From left to right: pH 7.0 (75 μg), pH 6.6 (30 μg), pH 4.3 (50 μg). Migration time 1.5 to 3 hr at 3 ma/tube, 4°. Anode on the top. See *Materials and Methods* for details.

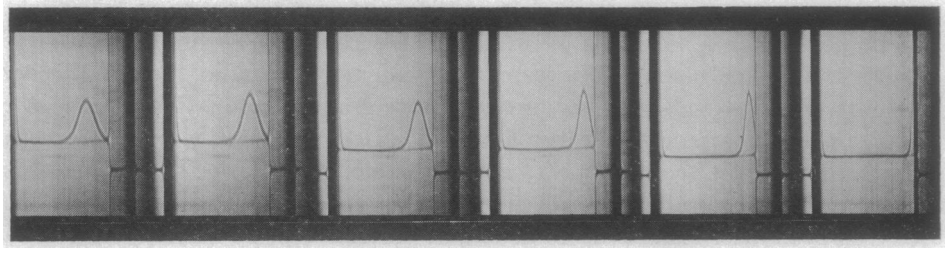


FIG. 5.—Sedimentation velocity pattern of the purified nerve growth factor, at 56,100 rpm, nerve growth factor concentration 12 mg/ml; first picture was taken within 1 min of reaching speed, others at 16-min intervals. Bar angle was 60°, 55°, or 50°. See *Materials and Methods* for details.

Discussion.—The nerve growth factor protein was purified over 100-fold from submaxillary glands of adult mice. The initial steps of purification, i.e., preparation of gland extract and gel filtration on Sephadex G-100, were similar to those described by Varon and co-workers[9, 10] with the only modification that streptomycin sulfate was added to the supernate of gland homogenate. The addition of streptomycin, while not affecting the biological activity, notably improved the separation of the nerve growth factor on gel filtration (Fig. 1) and allowed a more precise evaluation of the molecular size of the native nerve growth factor. After

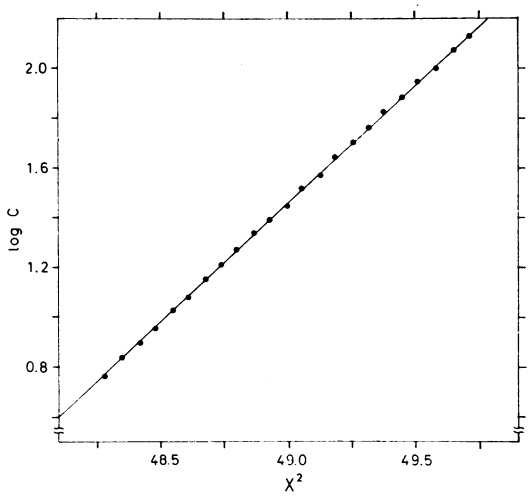


FIG. 6.—Equilibrium sedimentation of the purified nerve growth factor by Yphantis method.[20] C is the concentration in arbitrary units, X is the distance from the axis of the rotor. Nerve growth factor concentration 370 μg/ml. See *Materials and Methods* for details.

gel filtration of the gland extract at pH 7.5 on Sephadex G-100, most of the nerve growth factor activity was constantly collected in an elution zone indicative of an 80,000–90,000 mol wt. This was also confirmed after refractionation on Sephadex G-100 of the G-100 pool. It is likely that the higher 140,000 mol wt reported[9, 10] for the native nerve growth factor depended on unsatisfactory fractionation, probably due to the somewhat different conditions employed.

Lowering the pH of the G-100 pool from 7.5 to 5.0 caused a 20–25 per cent loss of both protein and biological activity. Most of the activity lost was recovered in the precipitate after this was resuspended in 5×10^{-2} Tris HCl buffer 7.5. The loss of the nerve growth factor activity could be reduced somewhat by lowering the protein concentration before dialysis to about 1 mg/ml. The CM52 cellulose chromatography at pH 5.0 (Fig. 2) accounted for a 20-fold purification. It has already been mentioned that recovery of biological activity from CM52 cellulose columns was almost quantitative (80–100%); it should be stressed that at least 80 per cent of the activity recovered was associated with a single protein (see Table 1 and Figs. 3, 4, 5, and 6); therefore, this protein can be considered as solely responsible for the nerve growth factor activity.

Varon and co-workers[9–12] reported recently the isolation from mouse submaxillary glands of a partially active protein, called β-subunit, which had chemicophysical properties very similar to those of the nerve growth factor protein isolated by us. This β-subunit, however, differs strikingly from our nerve growth factor protein since it accounted for only 10–25 per cent recovery of biological activity and required other protein components, namely, α- and γ-subunits, to be fully active. These results did not find confirmation during the course of the present investigation. In addition, the data reported in Table 2 show that at each step the degree of purification of the nerve growth factor is proportional to the amount of proteins removed. If the 30,000 mol wt purified nerve growth factor required other protein components for maximal response, this proportionality could not be observed.

The nerve growth factor purified by the procedure outlined above showed a 30,000 mol wt. Since the native nerve growth factor showed an 80,000–90,000 mol wt, it is evident that a splitting of the molecule occurred during the course of purification. Preliminary data from gel filtration experiments indicate that a partial splitting of the native nerve growth factor can occur even at pH 7.5 during chromatography of the G-100 pool on DE52 cellulose; while after dialysis at pH 5.0 of the G-100 pool, the biological activity is still associated with a relatively higher molecular weight protein. Therefore the CM52 cellulose chromatography at pH 5.0 is the only step which completely converts the native nerve growth factor into the 30,000-mol wt form. The attempts made to reconstitute the high molecular weight nerve growth factor from a unit 30,000 mol wt by bringing back the pH to 7.5 were unsuccessful. These results, however, cannot be considered conclusive. The chemicophysical similarities between the β-subunit of Varon and co-workers[11, 12] and our purified nerve growth factor have already been mentioned. The same authors reported the ability of the β-subunit to combine with α- and γ-subunits to form high-molecular-weight proteins. The possibility that our nerve growth factor behaves in a similar manner cannot be excluded.

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