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Nerve Growth Factor Synthesized by Mouse Fibroblast Cells in Culture: Absence of α and γ Subunits†

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ABSTRACT: Nerve growth factor (NGF) is found in high concentrations in the mouse salivary gland. However, this gland is unique since salivary glands from other animals have only trace amounts of NGF. In the mouse gland, two high molecular weight forms of NGF have been reported, 7S-NGF [Varon, S., Nomura, J., & Shooter, E. M. (1967) *Biochemistry 6*, 2202-2209] and NGF_1 [Young, M., Saide, J. D., Murphy, R. A., & Blanchard, M. H. (1978) *Biochemistry 17*, 1490-1498]. 7S-NGF is comprised of three noncovalently associated subunits: β-NGF, which is the biologically active subunit, α subunit, and γ subunit. A similar subunit composition is seen with NGF_1 (unpublished work with R. A. Murphy). Since the mouse salivary gland is unique with regard to its synthesis of NGF, the following question arises. Do other sources of NGF produce either 7S-NGF or NGF_1? Mouse fibroblast cells (L_{929}) in culture synthesize and secrete into their feeding medium (conditioned medium) a β-NGF-like molecule [Pantazis, N. J., Blanchard, M. H., Arnason, B. G. W., & Young, M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 1492-1496]. These cells therefore provided the opportunity to examine the molecular nature of NGF produced by a nonsalivary gland source. In this study, it was determined by radioimmunoassay that neither the α nor the γ subunit is present in fibroblast cell conditioned medium. Since α- and γ-proteins are present in both 7S-NGF and NGF_1, this indicates that neither of the salivary gland forms of NGF are produced by the mouse fibroblast cell.

The biochemical and biological properties of nerve growth factor (NGF)[1] in the mouse submandibular gland have been studied extensively. Two high molecular weight forms of NGF called 7S-NGF (Varon et al., 1967) and NGF_1 (Young et al., 1978) have been isolated from this tissue by using different purification protocols. The 7S-NGF complex (molecular weight, M_r, 130000) contains three noncovalently associated protein subunits called α, γ, and β-NGF. Only the β-NGF subunit is capable of producing the NGF biological response which is stimulation of neurite outgrowth from sensory and sympathetic ganglia. β-NGF elicits neurite formation at concentrations of 10 ng/mL. The α subunit has no known function. It has been suggested that the γ subunit is involved in cleavage of a pro-β-NGF precursor to produce β-NGF (Berger & Shooter, 1977).

The α, γ, and β-NGF subunits of 7S-NGF are noncovalently associated and will dissociate from one another when the 7S-NGF complex is diluted to biologically active concentrations (Pantazis et al., 1977b). This observation has been corroborated by the demonstration that 7S-NGF must dissociate in order for the β-NGF molecule to bind to NGF receptors (Harris-Warrick et al., 1980). The binding of β-NGF to its receptors is probably the initial step in the NGF biological response.

Recent isoelectric focusing experiments with NGF_1 (M_r 116000) indicate that this complex contains proteins with isoelectric points similar to those found in 7S-NGF (unpublished work with R. A. Murphy). These electrophoretic results suggest that NGF_1, like 7S-NGF, contains α, γ, and β-NGF.

Both 7S-NGF and NGF_1 are purified from the mouse submandibular gland. However, this source of NGF is unique in that most other animals do not have high concentrations of NGF in their salivary glands (Levi-Montalcini & Angeletti, 1968). Since NGF is known to play a vital role in neuronal development and maintenance in many species, where does the NGF come from? There is the possibility that several different types of tissues synthesize small quantities of NGF to maintain the peripheral nervous systems in the animal. Trace amounts of NGF have been reported in several tissues (Bueker et al., 1960; Levi-Montalcini et al., 1954;) however, the NGF in these tissues has not been biochemically characterized because of the low concentrations. Further support for the concept of multisite synthesis of NGF is the fact that several different cell types in culture synthesize NGF (Oger et al., 1974; Young et al., 1975; Longo & Penhoet, 1974; Murphy et al., 1975, 1977a,b; Pantazis et al., 1977a) and

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[1] Abbreviations: NGF, nerve growth factor; LCM, L cell conditioned medium; BSA, bovine serum albumin; M_r, molecular weight; EDTA, ethylenediaminetetraacetic acid; DE-52, diethylaminoethylcellulose; CM-52, carboxymethylcellulose; IEF gels, isoelectric focusing gels; RIA, radioimmunoassay; D-MEM, Dulbecco's modified Eagle's medium; IgG, γ-globulin.

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secrete it into their feeding medium (conditioned medium). Since large quantities of conditioned medium can be obtained from roller bottle cultures, it is possible to examine NGF synthesized by a nonsalivary gland source (mouse fibroblasts) and to compare it to the NGF molecules reported in the mouse salivary gland.

Biochemical studies (Pantazis et al., 1977a) with conditioned medium from a mouse fibroblast cell line, L_{929}, indicated that these cells synthesized a β-NGF-like molecule. The molecular weight of mouse submandibular gland β-NGF is 26000 (Angeletti & Bradshaw, 1971). However, in fibroblast cell conditioned medium, two β-NGF-containing molecules were detected by β-NGF radioimmunoassay (Murphy et al., 1979b). These results are further substantiated in this paper. One of these molecules had a molecular weight of >300000; the other had a molecular weight of approximately 50000. It is possible that β-NGF synthesized by the fibroblast cells is bound to other cellular component(s) yielding molecules of higher molecular weight. The subunit composition of these NGF molecules produced by fibroblast cells has not as yet been established. However, the molecular weights of the fibroblast NGF molecules do not correspond to the weights of either 7S-NGF (M_r 130000) or NGF_1 (M_r 116000). This fact suggests that fibroblast cells do not produce either 7S-NGF or NGF_1.

Are there other differences between the submandibular gland forms of NGF and the fibroblast NGF molecules which would further substantiate the hypothesis that fibroblast cells do not produce either 7S-NGF or NGF_1? For example, does either of the fibroblast NGF molecules contain α or γ subunits? Radioimmunoassays specific for these proteins were established to answer this question. These assays revealed that the α- and γ-proteins are *not* present in fibroblast cell conditioned medium and furthermore these proteins are *not* part of the fibroblast NGF molecules found in cell-conditioned medium. The fibroblast NGF molecules appear to be new forms of NGF which are different from both 7S-NGF and NGF_1. A preliminary report of the work has been presented (Pantazis, 1982).

Materials and Methods

Purification of Subunit Proteins. Two *different* purification procedures were utilized to obtain each of the three subunits, α, γ, and β-NGF. In one purification protocol, 7S-NGF was purified to homogeneity by using the procedures of Varon et al. (1967). The last Sephadex G-150 (Pharmacia) column was repeated since more than one protein peak was observed by absorbance measurements at 280 nm. This additional step resulted in a homogeneous protein preparation. To obtain α, γ, and β-NGF, the 7S-NGF was acid dissociated, and α, γ, and β-NGF were isolated according to the procedure of Smith et al. (1968).

In an alternative purification scheme α, γ, and β-NGF were isolated directly from the submandibular gland without first isolating 7S-NGF. β-NGF, or more correctly 2.5S-NGF,[2] was purified by methods described by Bocchini & Angeletti (1969). The procedures of Jeng et al. (1979) were utilized to obtain α- and y-proteins from the unused fractions of the Bocchini and Angeletti purification protocol. However, several additional steps were added to the procedure. For example, the α-protein was further purified on diethylaminoethylcellulose (DE-52) columns, while the γ-protein was run on two additional ion-exchange columns, carboxymethylcellulose (CM-52) followed by DE-52.

Both purification methods for α, γ, and β-NGF yielded biochemically similar proteins which behaved identically when tested in the radioimmunoassays. The fact that two preparations of subunit protein, prepared by different protocols, were indistinguishable in the radioimmunoassay confirms that these assays are highly specific for their appropriate subunit.

All liquid chromatography columns were run at 4 °C. Absorbancies (280 nm) of column elution fractions were determined on a Gilford Model 250 spectrophotometer.

Protein concentrations were determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) for the standard concentrations.

Isoelectric Focusing Gels. Isoelectric focusing gels as described by Stach et al. (1977) were utilized to determine the purity of all protein preparations. After electrophoresis, gels were fixed overnight in 20% trichloroacetic acid. The gels were then stained overnight in a solution containing 0.04% Coomassie Brilliant Blue R-250, 0.5% copper sulfate (to reduce background staining), 10% acetic acid, and 27% ethanol (Righetti & Drysdale, 1974). The gels were destained in a 12% ethanol, 7% acetic acid, and 0.5% copper sulfate solution with two or three changes of the solution. The final destaining solution had no copper sulfate, containing only 12% ethanol and 7% acetic acid.

Production of Antisera. Female New Zealand white rabbits (5–6 lb) were immunized according to the following protocol. Approximately 100 μg of protein was emulsified in complete Freund's adjuvant and injected into both thighs and rear foot pads. After 4–6 weeks, the rabbits were boosted by thigh injection of 100 μg of protein in incomplete Freund's adjuvant. In a similar fashion, rabbits were boosted 3 more times at 2-week intervals. Rabbits were bled periodically via the ear vein in order to test the serum titer. The final bleed was achieved by cardiac puncture. Serum was stored at −70 °C.

The γ-globulin (IgG) fraction was purified from antiserum by precipitation with ammonium sulfate (50%) followed by chromatography on DE-52.

Agar plates for double immunodiffusion (Ouchterlony plates) were prepared from a solution of 1% agarose (Baker) in 0.01 M barbital buffer, pH 8.6, with 3% (w/v) polyethylene glycol 6000 and 0.04% sodium azide.

Establishing the Radioimmunoassays (RIAs). All protein subunits were iodinated by using a Chloramine-T oxidation method (Hunter & Greenwood, 1962). For separation of radioactively labeled protein from free ^{125}I, either gel filtration or ion-exchange chromatography was utilized, depending on the subunit. The ^{125}I-labeled β-NGF was separated on Bio-Rad Ag 1-X8 columns (1–2 mL) equilibrated in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL BSA. β-NGF does not bind to this column. Sephadex G-10 (Pharmacia) columns (2–3 mL) equilibrated in 0.1 M potassium phosphate, pH 7.0, and 1 mg/mL BSA were utilized to separate labeled α-protein or labeled γ-protein from free ^{125}I. Both α and γ eluted in the void volumes of these columns.

The radioimmunoassays for α, γ, or β-NGF were set up by utilizing a protocol similar to that previously described (Murphy et al., 1979a) for the epidermal growth factor radioimmunoassay. Standard curves were generated by logit transformation (Rodbard et al., 1969). Most of the data was obtained by using unpurified antisera in the RIA; however,

[2] The name 2.5S-NGF is given to the NGF purified directly from the submandibular gland without first isolating 7S-NGF. The 2.5S-NGF and β-NGF are very similar molecules except for some proteolytic cleavages which occur more frequently in the 2.5S-NGF preparation (Angeletti et al., 1973a,b; Moore et al., 1974). The term β-NGF will be used for the most part in this paper in order to avoid confusion.

later work with purified IgG produced equivalent results.

Production of L Cell Conditioned Medium (LCM). The mouse fibroblast cell line L_{929} was purchased from the American Type Culture Collection (560th passage) and was initially grown in 75-cm^2 tissue culture flasks with Dulbecco's modified Eagle's medium (D-MEM, Gibco), 4500 mg of glucose/L, 10% bovine or calf serum (Sterile Systems), 100 units of penicillin/mL (Gibco), and 100 μg of streptomycin/mL (Gibco), supplemented with additional L-glutamine, 292 μg/mL (Gibco). The cells were placed in a humidified, 5% CO_2 environment at 37 °C. For subculturing, cells were removed with a solution consisting of 500 μg/mL trypsin and 200 μg/mL ethylenediaminetetraacetic acid in Hanks' balanced salt solution, without calcium or magnesium. After the cell line was well established, the cells were transferred to plastic roller bottles (850 cm^2) and grown in the D-MEM described above. When the cells reached confluence, they were placed in D-MEM without serum overnight in order to wash out the serum. The next day, the cells were refed D-MEM without serum and were left in this medium for 7 days. On the seventh day, the L cell conditioned medium (LCM) was removed, and the cells were fed fresh D-MEM without serum. Although the cells do not grow in serum-free medium, for the most part they remain attached to the roller bottle and can be maintained in serum-free medium for a considerable period of time. There is gradual loss of cells with time; however, it was possible to cycle cells through 12 changes of medium (12 weeks) before cells were placed back into serum-containing medium to reinitiate growth.

The LCM was centrifuged at 6800*g* for 10 min at 4 °C to remove particulate material. The medium was dialyzed exhaustively (at least three changes of solution) against 0.01 M ammonium acetate, pH 7.0. The dialyzed LCM was frozen and lyophilized, and the freeze-dried powder was stored at −20 °C until use.

Results

Purity of Subunit Proteins. The purity of the subunit proteins was determined on isoelectric focusing (IEF) gels. Each of the three subunit proteins, α, γ, and β-NGF, is known to display heterogeneity on IEF gels caused by minor proteolytic cleavages (Smith et al., 1968; Angeletti et al., 1973a,b; Moore et al., 1974; Stach et al., 1976, 1980). Similar results were seen here. IEF gels of the two α preparations are shown in Figure 1 (gels 2 and 3). Both of these proteins migrate to similar positions and reveal an identical electrophoretic pattern on these gels. Furthermore, both of the pure α preparations migrate identically with the α subunit seen in a sample of 7S-NGF[3] (Figure 1, compare gels 2 and 3 with gel 1). The heterogeneity of the α subunit seen here is similar to that observed by several workers (Smith et al., 1968; Stach et al., 1980). No other bands were seen, and there was no contamination of the α-protein by either the γ or the β-NGF subunit.

Like the α subunit, the γ-protein was also purified from mouse submandibular gland by two independent procedures described under Materials and Methods. One purification procedure isolated γ from pure 7S-NGF (shown in Figure 1, gel 4). Several bands can be seen, and their pattern is characteristic of the γ subunit (Stach et al., 1976; Server & Shooter, 1976). The multiple bands seen for the γ subunit

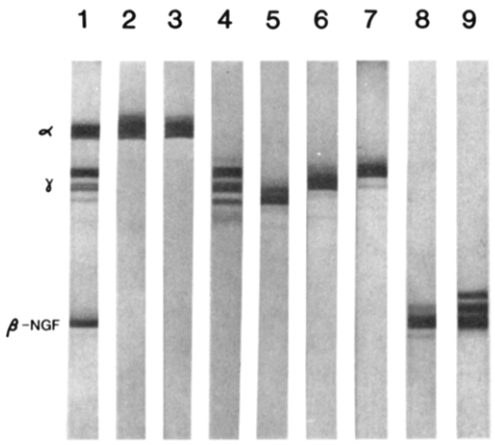

FIGURE 1: Isoelectric focusing polyacrylamide gels of α, γ, and β-NGF subunits. Tube gels of 5-mm diameter (10% acrylamide, pH 3.5–10) were prepared and run as described under Materials and Methods. The following samples were loaded: gel 1, 100 μg of 7S-NGF, purified by modification of the procedure of Varon et al. (1967); gel 2, 49 μg of α, purified from 7S-NGF (Smith et al., 1968); gel 3, 99 μg of α, purified by modification of the procedure of Jeng et al. (1979); gel 4, 31 μg of γ, purified from 7S-NGF (Smith et al., 1968); gel 5, 126 μg of γ^1, purified by modification of the procedure of Jeng et al. (1979); gel 6, 122 μg of γ^2, purified by modification of the procedure of Jeng et al. (1979); gel 7, 108 μg of γ^3, purified by modification of the procedure of Jeng et al. (1979); gel 8, 40 μg of β-NGF, purified from 7S-NGF (Smith et al., 1968); gel 9, 71 μg of 2.5S-NGF, purified by the method of Bocchini & Angeletti (1969).

are possibly due to the fact that γ can occur in a two- or three-chain form and proteolysis is also believed to occur (Thomas et al., 1981; Burton & Shooter, 1981). If γ as seen in Figure 1 (gel 4) is compared to a sample of 7S-NGF (Figure 1, gel 1), it is evident that the γ subunits match up identically. In both cases, the γ subunits migrate to a similar position, and identical banding patterns are seen.

Three proteins (γ^1, γ^2, and γ^3) were obtained from the final ion-exchange (DE-52) column (see Materials and Methods) by using an alternative isolation method for the γ subunit. The DE-52 column separates the individual γ-proteins. When γ^1, γ^2, and γ^3 are run on IEF gels (Figure 1, gels 5, 6, and 7, respectively), they migrate to positions which are characteristic for the γ subunit. There was no contamination of any of the γ preparations with α subunit or β-NGF.

β-NGF and 2.5S-NGF were also compared on IEF gels (Figure 1, gels 8 and 9, respectively). β-NGF revealed a single major band, indicating that isolating this subunit from pure 7S-NGF results in little proteolytic cleavage. 2.5S-NGF showed its characteristic three-band pattern caused by limited proteolysis (Angeletti et al., 1973a,b; Moore et al., 1974). Comparison of β-NGF (gel 8) and 2.5S-NGF (gel 9) with the β-NGF subunit of 7S-NGF (gel 1) reveals identical migration. Neither β-NGF nor 2.5S-NGF was contaminated with other proteins, specifically α and γ subunit.

Specificity of Antiserum. The specificities of the three antisera were determined by three methods: (1) immunodiffusion; (2) precipitation of ^{125}I-labeled antigen; (3) RIA detection of antigen on IEF polyacrylamide gels.

Rabbit immune serum was tested on immunodiffusion plates, and the results are shown in Figure 2. Each antiserum produced a precipitin line only with the protein against which the rabbit was immunized. For example, α antiserum only precipitated α subunit. There was no cross-reaction among the antisera; α antiserum did not produce a precipitin line with either the γ or the β-NGF subunit. Normal serum obtained from each rabbit prior to immunization did not produce any precipitin bands in immunodiffusion tests (not shown). In

[3] 7S-NGF contains α, γ, and β-NGF subunits which are associated in a noncovalent complex. The complex is not stable at acid pH and will dissociate into its component subunits in this gel system which utilizes acidic buffers.

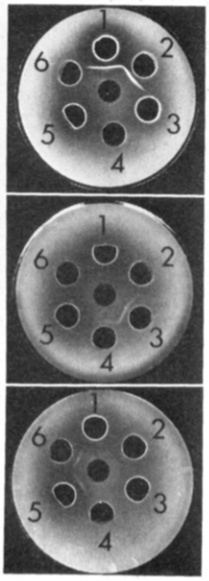

FIGURE 2: Immunodiffusion of purified 7S-NGF subunits and subunit antisera. Plates were prepared from 1% agarose in 0.01 M barbital buffer, pH 8.6, 3% (w/v) polyethylene glycol 6000, and 0.04% sodium azide. Purified 7S-NGF subunit was placed in the center well as follows: top panel, α (20 μL of 43 μg/mL); middle panel, β-NGF (20 μL of 65 μg/mL); bottom panel, γ subunit (20 μL of 13 μg/mL). The surrounding wells received subunit antiserum, and the pattern is identical in each panel. Wells 1 and 2 contained, respectively, undiluted anti-α antiserum and a 1:1 dilution of anti-α antiserum (20 μL in each well). Wells 3 and 4 received, respectively, undiluted anti-β-NGF antiserum and a 1:1 dilution of anti-β-NGF antiserum (20 μL in each well). Wells 5 and 6 contained, respectively, undiluted anti-γ antiserum and a 1:1 dilution of anti-γ antiserum (20 μL in each well). Plates were allowed to sit overnight at room temperature to develop precipitin lines.

summary, immunodiffusion indicates that each of the three antisera precipitates only its corresponding antigen, and there was no evidence of cross-reactivity among the three antisera.

The cross-reactivity of the α, γ, and β-NGF antibodies was further tested by precipitation of radioactively (^{125}I) labeled antigen. The results as shown in Table I indicate that each antibody precipitated its corresponding antigen to the greatest extent (40–60%). Only nonspecific background precipitation (6–11%) was seen between antibody and either of the other two subunits. These results further substantiate the fact that each antibody specifically recognizes and precipitates its corresponding antigen.

The third test of antisera specificity involved running all three proteins (α, γ, and β-NGF) together on IEF gels and establishing if each RIA measures only its corresponding antigen. Two equal aliquots of pure 7S-NGF were run on two separate IEF gels. One of the two gels was stained and is shown at the top of Figure 3. The relative migration values for all three protein subunits were determined from this gel (Table II). The second gel was sliced, and protein was eluted from each slice into an elution buffer. The positions of α, γ, and β-NGF were determined by radioimmunoassays of the elution buffers, and the data are plotted in Figure 3 (bottom panel). The relative migration value was calculated for each peak. As shown in Table II, the relative migration values show excellent agreement between the stained gel and sliced gel. Each RIA detects only its appropriate antigen, and there was no cross-reactivity among the assays.

Studies with L Cell Conditioned Medium (LCM). Previous work (Pantazis et al., 1977a) has established the presence of a β-NGF-like molecule in LCM. Samples of LCM were examined in the α- and γ-RIA in order to determine if α and

Table I: Binding of ^{125}I-Labeled α, γ, and β-NGF by Their Respective Antibodies[a]

final dilution of antibody	anti-α antibody	anti-β-NGF antibody	anti-γ antibody
	Percent Bound of ^{125}I-α		
1:2500	62	4	11
1:5000	47	4	7
1:10000	29	1	3
1:20000	16	2	1
1:40000	8	1	1
	Percent Bound of ^{125}I-β-NGF		
1:2500	5	47	6
1:5000	2	17	6
1:10000	4	9	4
1:20000	5	6	5
1:40000	3	4	1
	Percent Bound of ^{125}I-γ		
1:2500	9	0	38
1:5000	5	0	25
1:10000	1	0	14
1:20000	0	0	6
1:40000	0	0	5

[a] The α, γ, and β-NGF proteins were labeled with ^{125}I as previously described under Materials and Methods. Purified antibodies (γ-globulin purification described under Materials and Methods) against α, γ, or β-NGF were diluted in 0.1 M potassium phosphate, pH 7.0. The following reagents were added to a series of tubes: 0.6 mL of 0.1 M potassium phosphate, pH 7.0; 0.1 mL of 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL BSA; 0.1 mL of ^{125}I-labeled protein diluted in 0.15 mg/mL nonimmune rabbit IgG, 10 mg/mL BSA, and 0.1 M EDTA; 0.1 mL of diluted antibody. Samples in which no binding occurred (zero binding tubes) were set up as described above, except the antibody was replaced with 0.1 mL of 0.1 M potassium phosphate, pH 7.0. All samples were done in duplicate, and tubes were kept overnight at 4 °C. The next day they received 0.1 mL of goat anti-rabbit IgG antiserum (diluted 1:10 in 0.1 M potassium phosphate, pH 7.0). After 4 h at 4 °C, the tubes were centrifuged at 3000g for 20 min, 4 °C. An aliquot (200 μL) of the supernatant was removed from each sample, placed in a tube, and counted in a gamma counter. The percent bound was calculated by the following equation: % bound = (average cpm zero binding tubes − average cpm sample tubes)/average cpm zero binding tubes.

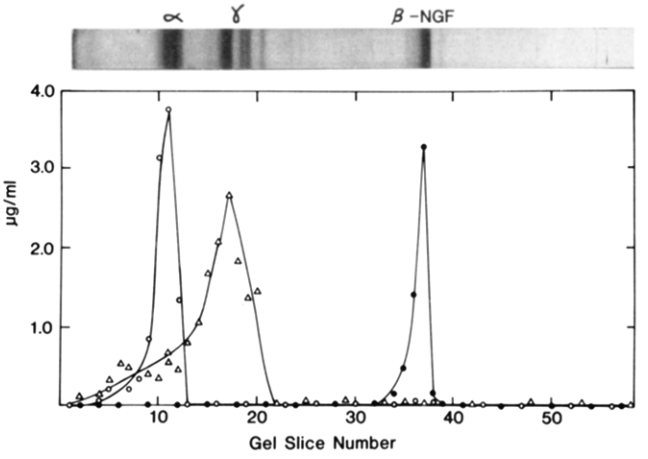

FIGURE 3: Isoelectric focusing and radioimmunoassay of 7S-NGF. Isoelectric focusing gels (10% acrylamide, pH 3.5–10) were prepared in 5-mm glass tubes as described under Materials and Methods. Two gels were each loaded with 100 μg of 7S-NGF and run. One of the gels was stained with Coomassie Blue and destained as described under Materials and Methods. This gel is shown overlayed across the top of this figure. The second gel was not stained but was sliced, and each slice was placed in 1 mL of 0.1 M potassium phosphate buffer containing 1 mg/mL BSA. Protein was eluted from the gel slices overnight. The elution buffer for each slice was assayed for α, γ, and β-NGF by RIA. (○) α subunit; (△) γ subunit; (●) β-NGF.

Table II: Relative Migration (R_f) Values for α, γ, and β-NGF on Isoelectric Focusing Gels[a]

	R_f	
	stained gel	RIA of sliced gel
α	0.15–0.18	0.16–0.21
γ	0.25–0.32	0.24–0.35
β-NGF	0.60–0.61	0.60–0.64

[a] The R_f values for the two isoelectric focusing gels run in Figures 3 were calculated as follows. Since the α- and γ-proteins are heterogeneous, the R_f value was not calculated for each band, but instead, the ranges for the α-proteins and γ-proteins were determined. As an example, with the stained gel, the distance from the top of the gel to the top of the first α band was measured, and this distance was divided by the total length of the gel. Then the distance from the top of the gel to the bottom of the lowest α band was measured and divided by the gel length. These values for the range of α-proteins are shown above, under the stained gel column. The R_f values were also established from the RIA data derived from the sliced gel in Figure 3. The first and last slices which contained concentrations of α-protein above background were determined, and both of these slice numbers were divided by the total number of gel slices. This R_f range is reported under the RIA column.

γ were also present. A sample of conditioned medium was concentrated by freeze-drying and dissolving the dried powder in a reduced volume. This sample was dialyzed extensively *prior* to freeze-drying in order to remove salts but was not dialyzed further after the dried powder was reconstituted in 0.1 M ammonium acetate. The RIA data are shown in Table III under L Cell Conditioned Medium (Undialyzed). The results indicate that there is no α- or γ-protein present in LCM. The very low levels of α and γ seen in Table III are most likely due to nonspecific background in the RIAs. In contrast, considerable amounts of β-NGF (811 ng/mL) can be detected in this sample.

The sample of LCM used in the above work was not dialyzed after the lyophilized powder was reconstituted in buffer. Since the reconstituted LCM was concentrated, the possibility existed that residual salts might be interfering with the assays. Therefore, samples of LCM were dialyzed after being reconstituted. Another change was also made: a buffer consisting of 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL BSA (as a carrier) was used. As shown in Table III, the overall result was the same. The β-NGF protein was present (713 ng/mL) whereas the α- and γ-proteins were not. The results were unaffected by changing the buffer or adding carrier protein (BSA) or by additional dialysis.

For comparison, pure 7S-NGF was assayed in each RIA, and the results are shown in Table IV. With 7S-NGF, all three subunits were readily detectable, and the concentrations of α and γ exceeded that of β-NGF. In summary, these results show that two out of the three subunit components of 7S-NGF (α and γ) are undetectable in LCM, indicating that 7S-NGF is *not* present in medium conditioned by mouse fibroblasts.

One possible explanation for the lack of α and γ subunits is that LCM may contain substances which inhibit the α- and γ-RIAs, preventing these assays from detecting these proteins. To test this idea, known quantities of pure α- and γ-proteins were added to LCM and assayed in their respective RIAs. As a control, identical concentrations of these proteins were added to buffer. The results shown in Table V indicate that LCM has no effect upon either the α- or the γ-RIA. The α-RIA detected identical amounts of α-protein both in LCM and in buffer; there was no interference with the α-RIA by LCM (Table V). Similar results were obtained with the γ-RIA

Table III: Radioimmunoassay Determination of α, γ, and β-NGF in L Cell Conditioned Medium

dilution	β-NGF (ng/mL)	α (ng/mL)	γ (ng/mL)
L Cell Conditioned Medium (Undialyzed)[a]			
undiluted	too high	16	11
	too high	30	11
	too high	21	12
1/5	972	undetectable	undetectable
	850		
	1026		
1/10	794	undetectable	undetectable
	836		
	891		
1/20	861	undetectable	undetectable
	800		
	824		
1/40	689	undetectable	undetectable
	612		
	578		
	811 ± 38[c]	22 ± 4[c]	11 ± 0.3[c]
L Cell Conditioned Medium (Dialyzed)[b]			
undiluted	too high	0.3	2.7
		0.6	5.0
		1.3	4.0
		2.5	9.0
		2.5	11.3
		0	8.3
		0	21.2
		0	20.1
1/10	725		
	922		
	906		
1/20	802		
	816		
	788		
1/40	656		
	604		
	548		
1/80	528		
	656		
	600		
	713 ± 39[c]	0.9 ± 0.4[c]	10.2 ± 2.5[c]

[a] A sample of LCM (1300 mL) was dialyzed extensively against 0.01 M ammonium acetate, pH 7.0. The sample was then freeze-dried, and the powder was dissolved in 2.4 mL of 0.1 M ammonium acetate, pH 7.0. Undissolved material was removed by centrifugation (two runs at 29000*g* for 30 min each, 4 °C). The sample was split into three equal aliquots and kept frozen until it was assayed by RIA. For RIA, serial dilutions (1/5, 1/10, 1/20, and 1/40) of the LCM sample were made in 0.1 M ammonium acetate, pH 7.0, and the dilutions were assayed in all three RIAs. In each RIA, the four serial dilutions and the undiluted, original sample were assayed in triplicate. Diluted samples were multiplied by the appropriate dilution factor in order to normalize all the data. For example, the RIA values of a sample diluted 1/5 were multiplied by 5. The mean and standard error of the mean were calculated. [b] A sample of LCM (4200 mL) was dialyzed against 0.01 M ammonium acetate, pH 7.0, and freeze-dried. The dried powder was dissolved in 7.5 mL of 0.1 M potassium phosphate and 1 mg/mL BSA, pH 7.0, and dialyzed against this buffer overnight. For β-NGF RIA, four serial dilutions (1/10, 1/20, 1/40, and 1/80) were made of the LCM sample. These dilutions were made in 0.1 M potassium phosphate and 1 mg/mL BSA, pH 7.0, and they were assayed in the β-NGF RIA in triplicate. For the determination of α and γ, the LCM sample was not diluted; instead, varying amounts of LCM were added to the assays (total of eight determinations). Diluted samples were multiplied by a dilution factor. The mean and standard error of the mean were calculated. [c] Mean values.

(Table V). Since there was no loss of either α- or γ-protein when they were added to samples of LCM, it would suggest that substantial proteolysis of the α- and γ-proteins does not occur in samples of LCM.

Table IV: Radioimmunoassay Determination of α, γ, and β-NGF in 7S-NGF[a]

dilution	β-NGF (μg/mL)	α (μg/mL)	γ (μg/mL)
1/200	24.41	65.60	57.01
	25.99	52.74	50.12
	29.15	61.94	42.31
1/400	36.67	75.68	67.75
	37.50	86.58	75.94
	38.69	68.28	73.80
1/800	35.70	66.75	96.83
	36.07	79.69	99.69
	36.67	71.11	99.94
1/1600	39.72	36.20	123.03
	35.78	42.03	118.61
	35.58	41.50	126.83
1/3200	30.12	23.39	131.67
	33.82	47.66	115.66
	33.55	38.17	129.97
1/6400			120.44
			95.84
			87.66
	33.96 ± 1.17[b]	57.16 ± 4.75[b]	94.17 ± 6.59[b]

[a] Samples of pure 7S-NGF were diluted in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/mL BSA. Diluted samples were assayed in the three radioimmunoassays, and all values were multiplied by a dilution factor. The means and standard error of the means were calculated. [b] Mean values.

Table V: Addition of α or γ Subunit to L Cell Conditioned Medium (LCM)[a]

α + LCM	3.86 ± 0.24 (15)	α + buffer	3.69 ± 0.29 (15)
γ + LCM	2.06 ± 0.31 (14)	γ + buffer	2.37 ± 0.40 (15)

[a] A 50-μL aliquot of purified α or γ subunit was added to a sample (300 μL) of L cell conditioned medium which was prepared by methods similar to those described in Table III [LCM (undialyzed)]. Dilutions were made in 0.1 M potassium phosphate, pH 7.0, and 1 mg/mL bovine serum albumin, and samples were assayed in the α- or γ-RIA. As a control, 50 μL of α or γ subunit was added to 300 μL of buffer (0.1 M ammonium acetate, pH 7.0), and dilutions were made in the phosphate-BSA buffer. In each case, the mean of several RIA determinations is reported. The number of determinations is in parentheses.

Studies of LCM on Gel Filtration Columns. A sample of LCM was loaded onto a calibrated Sephadex G-200 column. The concentration of each subunit (α, γ, and β-NGF) in the elution fractions was determined by RIA, and the results are shown in Figure 4. The β-NGF profile shows two peaks. The first has a molecular weight greater than 300 000; the second peak is broad, and the molecular weight was estimated from 40 000–70 000. For simplicity, this second peak will be called the M_r 50 000 molecule. This two-peak elution pattern for β-NGF in LCM was previously observed (Murphy et al., 1979b) in nondenaturing buffers.

As mentioned in the introduction, the β-NGF synthesized by L cells may be associated with other components to yield higher molecular weight complexes (M_r >300 000 and M_r 50 000). Further study on the subunit composition of these two molecules is necessary to verify this possibility. However, one conclusion that can be made is that neither of the L cell molecules (M_r >300 000 or M_r 50 000) is similar in molecular weight to 7S-NGF (M_r 130 000) or NGF_1 (M_r 116 000). Because of these differences in molecular weight, it appears that neither 7S-NGF nor NGF_1 is present in LCM.

When the elution fractions from the Sephadex G-200 column (Figure 4) were assayed for α- and γ-proteins, neither was detected. These results agree with previous experiments (Table III) in which α and γ were not detected in unpurified samples of L cell conditioned medium. Therefore, the components to which L cell β-NGF binds are immunologically quite different from the α and γ subunits. This evidence further substantiates the fact that neither 7S-NGF nor NGF_1 is present in LCM.

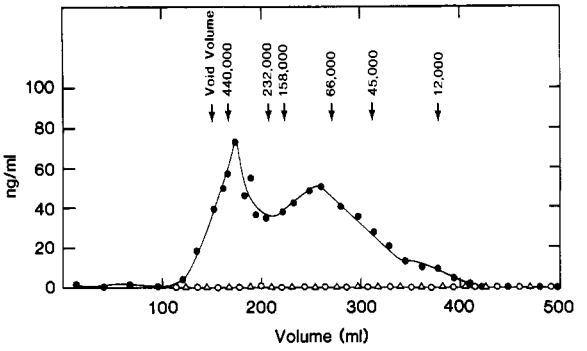

FIGURE 4: Gel filtration of L cell conditioned medium on Sephadex G-200. L cell conditioned medium (5.4 L) was dialyzed against 0.01 M ammonium acetate and freeze-dried. The dried powder was dissolved in 15 mL of 0.1 M potassium phosphate, pH 7.0, and centrifuged to remove undissolved material. The sample was loaded onto a Sephadex G-200 column (90 × 2.5 cm) equilibrated in 0.1 M potassium phosphate. The concentrations of α, γ, and β-NGF in the elution fractions were determined by radioimmunoassay. On separate runs, the column was calibrated with purified proteins of known molecular weights. (○) α; (△) γ; (●) β-NGF.

One final point concerning the gel filtration columns should be made. In Figure 4, the concentration of β-NGF in both of the peaks is below 100 ng/mL. In contrast, 7S-NGF would have dissociated into its component subunits at this concentration (Pantazis et al., 1977b; Harris-Warrick et al., 1980). This difference in stability between the L cell molecules and 7S-NGF is an additional property in which these molecules differ.

Discussion

Two forms of nerve growth factor, 7S-NGF and NGF_1, have been identified in the mouse submandibular gland. One of the primary reasons for undertaking this study was to determine if 7S-NGF or NGF_1 is produced by a *nonsalivary gland* source. Several experimental observations reported in this paper indicate that neither of these molecules is made by mouse fibroblast cells in culture. For example, specific RIAs detected only the β-NGF subunit in samples of conditioned medium, but they did not detect α- or γ-protein, both of which are constituents of 7S-NGF and NGF_1. Furthermore, gel filtration studies of LCM did not identify a molecule with a molecular weight similar to that of either 7S-NGF or NGF_1. Gel filtration work did reveal two β-NGF-containing molecules (M_r >300 000 and M_r 50 000) detected by β-NGF RIA. Neither one of these molecules contained α or γ components. In summary, the forms of NGF made by the mouse salivary gland are *not* produced by mouse fibroblast cells, and it appears as though two *new* forms of NGF are made by the fibroblasts.

Although NGF is vital for the development and maintenance of the sensory and sympathetic nervous systems, the source of NGF for most animals, and possibly the mouse also, has not been established. The role of NGF in the mouse submandibular gland is not understood. Several facts (Levi-Montalcini & Angeletti, 1968) suggest that this gland is not the source of NGF in the mouse. For example, removal of the gland has no detrimental effect on the sympathetic nervous system. The salivary glands of other animals do not have high concentrations of NGF. NGF appears in high concentrations in the mouse salivary gland when the animal reaches puberty. However, the target tissues for NGF (sensory and sympathetic

nervous systems) are fully developed by this time. In addition, male salivary glands have much higher NGF concentrations than the female; however, there is no obvious difference in their sympathetic nervous systems. Finally, the mouse submandibular gland is apparently exocrine rather than endocrine, secreting NGF into saliva but not into serum (Murphy et al., 1977c,d, 1980). The function of NGF in mouse saliva is not known.

Since no universal site of NGF synthesis has been established, where does NGF come from? The possibility exists that several types of tissues in the animal produce *very low* concentrations of NGF to locally supply and maintain the peripheral nervous system. To date, the experimental evidence for this concept of multisite synthesis of NGF is indirect. For example, NGF can be retrogradely transported (Thoenen & Barde, 1980) from the nerve terminal back to the nerve soma. This suggests a possible mechanism whereby NGF produced in peripheral tissues can maintain the regional nerve supply.

Attempts to measure NGF in various tissue have yielded conflicting results. Using biological assays, some investigators have detected NGF in several tissues (Bueker et al., 1960; Levi-Montalcini et al., 1954), whereas others have reported negative results (Harper et al., 1976, 1980a). Radioimmunoassays have also proven to be contradictory. Several reports measured significant levels of NGF in various tissues (Johnson et al., 1971; Hendry, 1972; Walker et al., 1979). However, questions about the specificity of NGF antisera have arisen (Carstairs et al., 1977) as well as the accuracy of certain NGF radioimmunoassay procedures (Suda et al., 1978).

If NGF is produced by various tissues in the animal, it is more than likely that the concentrations would be small. These very low concentrations of NGF and possibly the inability to measure them accurately may account for some of the discrepancies noted above. NGF research has been ongoing for approximately 30 years, yet no unequivocal and universal source has been established for this protein. It would appear unlikely that future investigations will find a unique site of NGF production of high concentration.

Further indirect evidence for the multisite synthesis of NGF by various tissues arises from the observation that numerous cell types in culture synthesize NGF (Oger et al., 1974; Young et al., 1975; Longo & Penhoet, 1974; Murphy et al., 1975, 1977a,b; Pantazis et al., 1977a). Cells in culture often mimic in vivo situations, and results obtained in vitro can often be applied to in vivo circumstances. The fact that numerous cell types in culture produce NGF does not prove, by any means, that the corresponding tissues in the animal synthesize NGF, although this observation does support that possibility. In light of the fact that there is no established site of NGF synthesis, the idea that numerous tissues in the animal produce low concentrations of NGF to maintain the peripheral nervous system is a very plausible one.

Ebendal et al. (1980) have shown that when target tissue, such as the rat iris, is denervated detectable levels of NGF can be found in the target tissue, whereas in the innervated tissue no NGF was detected. Reinnervation of the iris results in loss of detectable NGF. Several possible explanations exist for this apparent increase in NGF following denervation. For example, the tissue may be synthesizing more NGF, possibly because negative feedback provided by the nerve has been eliminated. Alternatively, since NGF may be removed from the target tissue by retrograde transport occurring in the nerve axon, denervation may result in an accumulation of NGF in the target tissue because it is not being continually removed. In this case, there may be no net increase in NGF synthesis. A third possibility is that cell culture conditions somehow stimulate cells to produce NGF, which they would not normally synthesize in the animal. In the work of Ebendal et al. (1980), this is an unlikely explanation since increases in NGF levels were also observed in situ following denervation.

Harper et al. (1980a,b) have also examined this question of NGF synthesis by several different types of tissues in the animal. They determined NGF concentrations in tissue explants maintained in vitro. For comparison, they homogenized tissues freshly excised from the animal, and these were the in vivo samples. Since NGF was detected only in the in vitro samples, they concluded that cell culture conditions may be stimulating NGF production. However, in this case, like the studies with the iris mentioned above, it is also possible that NGF synthesis is higher in vitro than in vivo not because cell culture conditions have nonspecifically turned it on, but because negative feedback provided by the nerve has been removed in denervated tissue. An alternative explanation is that loss of NGF by retrograde transport has been eliminated, allowing for the accumulation of NGF.

In summary, current available information neither adequately proves nor disproves the hypothesis of multisite synthesis of NGF. Cells in culture may produce forms of NGF which may be very similar to the native NGF found in the animal. If one considers the fact that only mice, and probably one species of rat (Darling & Shooter, 1982), have high concentrations of NGF in their salivary glands, are the forms of NGF found in these tissues any more representative of the true biochemical and biological nature of NGF? This important question is presently unanswered.

Although it appears as though both salivary gland cells in vivo and fibroblast cells in vitro synthesize a biochemically and biologically similar β-NGF protein (Pantazis et al., 1977a), the proteins with which β-NGF apparently associates are different in each case. In the salivary gland, β-NGF is bound to α- and γ-proteins. With the L cell fibroblasts, the nature of the binding protein(s) is unknown although RIAs indicate that they are *not* immunologically similar to either α- or γ-protein. It is of interest that preliminary studies in this laboratory using another mouse cell line, S-180, indicate that both α- and γ-proteins were also undetectable in S-180 cell conditioned medium (studies with In Sook Kim).

What are the functions of the proteins with which β-NGF associates? To date, the α-protein has no defined role, while γ-protein is believed to cleave a β-NGF precursor, producing the 13 000 molecular weight monomer subunit of β-NGF (Berger & Shooter, 1977). If the L cell does not have γ-protein, how is any putative NGF precursor cleaved? It would appear that γ is not the only protein capable of cleaving an NGF precursor. Another possibility, which is being studied, is whether or not γ is present *within* the L cells and remains intracellularly while only β-NGF is secreted into the conditioned medium. For examination of this problem, cell homogenates will be concentrated and partially purified. It would seem unlikely that α and γ would remain intracellularly since studies with the salivary gland indicate that all three proteins (α, γ, and β-NGF) are secreted into saliva (Murphy et al., 1977d; Burton et al., 1978).

The biochemical and biological properties of the M_r >300 000 and M_r 50 000 molecules are under investigation. The M_r 50 000 molecule is active in the NGF bioassay, while the M_r >300 000 molecule is only weakly positive. Better bioassay results may be obtained after partial purification. Gel filtration studies in denaturing buffers indicate that both molecules contain a M_r 13 000 protein chain which is detected

by β-NGF RIA. For comparison, β-NGF produced in the mouse submandibular gland is a noncovalent dimer containing two identical chains of 13 259 molecular weight (Angeletti & Bradshaw, 1971; Greene et al., 1971). It appears, therefore, that both molecules produced by fibroblast cells in vitro contain a β-NGF subunit. The properties of the other components of these *new* fibroblast NGF molecules are being studied.

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