Proc. Natl. Acad. Sci. USA
Vol. 74, No. 4, pp. 1492–1496, April 1977
Biochemistry

Molecular properties of the nerve growth factor secreted by L cells*

(growth factors/mouse submandibular gland/fibroblasts)

NICHOLAS J. PANTAZIS, MURIEL H. BLANCHARD, BARRY G. W. ARNASON, AND MICHAEL YOUNG†

Departments of Biological Chemistry, Medicine, and Neurology, Harvard Medical School and the Massachusetts General Hospital, Boston, Massachusetts 02134

Communicated by Paul C. Zamecnik, January 27, 1977

ABSTRACT **The molecular size and stability of the nerve growth factor (NGF) secreted in culture by L cells have been studied by sedimentation and gel filtration chromatography. Results indicate that L cell NGF has a molecular weight of 160,000. It contains as part of its structure a protein component that is biologically, immunologically, and electrophoretically indistinguishable from the biologically active factor purified from mouse submandibular glands. However, unlike pure gland NGF, L cell NGF is highly stable in solution, and this finding indicates that L cell NGF is a form of the factor different from that previously described.**

The biological activity of nerve growth factor (NGF) in stimulating neurite outgrowth from sensory and sympathetic ganglia in culture is a property of a 26,000 molecular weight protein isolated from male mouse submandibular glands (2). The protein is present in high concentrations in these glands (for reasons unknown) and can be isolated directly from them (2). Determination of the primary structure of this biologically active species reveals that it is composed of two noncovalently linked polypeptide chains, each of molecular weight 13,259 (3, 4). It has been given the name β-NGF.

Depending upon the purification scheme used, β-NGF can also be isolated from mouse submandibular glands as part of a much larger complex called 7S-NGF (molecular weight, about 140,000) (5). This macromolecule contains the β species noncovalently linked to other protein components whose biological and chemical functions are not known. It also is not known in what form NGF exists in the salivary gland, nor what its function is there. However, evidence has been presented to indicate that NGF is synthesized by the gland (6–9).

In 1974, mouse L cells in culture were shown to synthesize and to secrete a biologically active nerve growth-promoting factor that could not be distinguished immunologically from pure mouse submandibular gland NGF (10). Since that time, various transformed as well as primary cells have also been found to secrete biologically active NGF in culture (refs. 10–14; for reviews, see refs. 15 and 16).

In the present study, we have measured several physicochemical properties of mouse L cell NGF for comparison with those of NGF (both 7S and β-NGF) purified from mouse submandibular glands. For purposes of this comparison, it is pertinent to consider some recent information on the behavior of β-NGF and 7S-NGF in solution.

β-NGF. As noted above, this species is a dimer of identical chains. However, solutions of the protein at neutral pH comprise a rapidly reversible monomer–dimer equilibrium system with an apparent association constant of $9.4 \times 10^6\ M^{-1}$ (17). At a total protein concentration of 1.4 μg/ml, solutions of the protein contain an equal mixture of monomer and dimer; at concentrations considerably higher than those required to display biological activity (about 10 ng/ml), solutions consist virtually entirely of monomer. Consequently, at concentrations conventionally used in the *in vitro* ganglion bioassay system, the individual polypeptide chains of β-NGF are the biologically active species (17).

7S-NGF. At concentrations on the order of 1 mg/ml and greater, this protein exhibits a sedimentation coefficient of 7 S and a molecular weight close to 140,000 (5, 18, 19), and it contains β-NGF as well as two other protein species that have been termed the α and γ components (19). However, this complex (α,β,γ) is appreciably unstable in solution. At total 7S-NGF concentrations as high as 50 μg/ml, considerable dissociation of the complex occurs. At concentrations on the order of 1 μg/ml, the β component of 7S-NGF is completely dissociated from the α and γ species (20).

As will be seen from the results presented below, L cell NGF (both intracellular and that secreted in culture) exists in a high-molecular-weight form, even at concentrations as low as 1 ng/ml (at which level both β-NGF and 7S-NGF are fully dissociated). L cell NGF also contains a biologically active component that is immunologically similar to mouse submandibular gland β-NGF. Taken together, the evidence indicates that L cell NGF may be the form secreted by the many different types of cells discussed above.

MATERIALS AND METHODS

Reagents. Doubly glass-distilled water was used for all solutions, and all buffer salts were reagent grade. Sephadex and dextran blue 2000 were obtained from Pharmacia; ovalbumin and human IgG were from Schwarz/Mann; bovine serum albumin (three times recrystallized) and cytochrome *c* were from Sigma; Eagle's minimal essential medium was from Flow Laboratories; fetal calf serum was from Gibco; and ^3H_2O and $Na^{125}I$ were from New England Nuclear. NGF was isolated from adult male mouse submandibular glands by the method of Bocchini and Angeletti (2). Preparations were shown to be electrophoretically homogeneous as previously described (10). Protein concentrations were measured by the Lowry procedure.

Cell Cultures. Mouse L-929 cells (subline L_2) were grown to confluence, in 75-cm^2 Falcon tissue culture flasks, in Eagle's minimal essential medium containing gentamicin (50 μg/ml) (Squibb) and 10% (vol/vol) heat-inactivated fetal calf serum (humidified atmosphere; 5% CO_2/95% air, 37°). Medium was changed twice per week. To prepare culture fluids for assay, serum-containing medium was removed, and the cells were washed three times with 10 ml of unsupplemented medium. Cells were then incubated for 4 days in 10 ml of medium without serum. Over a period of 4 days, considerable amounts of NGF are secreted into the medium (10). The serum-free medium was removed from cells, dialyzed thoroughly against

Abbreviation: NGF, nerve growth factor.

* A preliminary account of some of this work has been presented (1). It forms part of the dissertation of N.J.P. to be submitted to Harvard University in partial fulfillment of the requirements for the Ph.D. degree.

† To whom correspondence should be addressed.

0.01 M ammonium acetate at 4°, and lyophilized. The dry residue was redissolved in a small volume of 0.1 M potassium phosphate (pH 7.0) and dialyzed against this solvent at 4°. For biological assays, aliquots were dialyzed against Eagle's minimal essential medium.

Radioimmunoassays. Radioimmunoassays of L cell culture medium were performed with monospecific antisera to the biologically active 26,000 molecular weight NGF (β-NGF) isolated and purified from mouse salivary glands as described above. Preparation of antisera, and ^{125}I-labeled NGF and the immunoassay details are given elsewhere (10, 12).

Electrophoresis. Polyacrylamide gel electrophoresis was performed with the 6 M urea/acetic acid system of Panyim and Chalkley (21).

Biological Assays of NGF. These were performed with 8-day-old chick embryo sensory ganglia as previously described (12).

Gel Filtration. Gel filtration studies utilized 1 × 23 cm columns of either G-75 or G-200 Sephadex. Columns were equilibrated with 0.1 M potassium phosphate containing bovine serum albumin at 1 mg/ml, and they were operated at a flow rate of 3–5 ml/hr at 4°. In most experiments, small volumes (50–100 μl) of solution were applied to the column to establish a zonal elution profile. In others, a large volume of dilute solution was applied to create a frontal elution profile for the purpose of measuring partition coefficients. Fractions were collected in preweighed glass or plastic tubes and the weight of fraction plus tube provided a measure of the elution volume to within 50 μl. NGF concentration in the fractions was measured by radioimmunoassay.

Sedimentation. Sedimentation coefficients were measured with a Beckman model E ultracentrifuge. The method used, originally devised by Tiselius *et al.* (ref. 22; see also refs 23 and 24), derives the sedimentation coefficient of a molecule from a knowledge of its net transport across a fixed plane of the centrifuge cell during the period of centrifugation. A fixed partition cell (Beckman) was used for these studies, together with a piece of Whatman no. 50 filter paper cut to fit the partition and thus isolate the upper and lower cell compartments at the end of the experiment. Concentrations of NGF in the initial solution (c_0) and above the separation partition after time t of centrifugation (c_t) were measured in triplicate by radioimmunoassay.

If

$$\lambda = x_p^2/(x_p^2 - x_m^2) \qquad [1]$$

in which x_m and x_p are the radial meniscus and separation partition positions, respectively, then

$$s = \left[ln \frac{\lambda}{(c_t/c_0) - 1 + \lambda} \right] \left[2 \int_0^t \omega^2 \, dt \right]^{-1} \qquad [2]$$

The integral of Eq. 2 was accurately computed by coupling the multiplex scanner assembly of the centrifuge with a $\omega^2 t$ integrator system (Beckman model L). A partial specific volume of 0.73 ml/g was assumed for calculation of $s_{20,w}$. This method has been used successfully to study the monomer–dimer equilibrium of β-NGF (17) as well as the dissociation reactions of 7S-NGF (20).

RESULTS

Fig. 1 illustrates the G-75 Sephadex gel filtration behavior of L cell NGF and β-NGF. The latter protein emerged as expected from studies (17, 20) on the monomer–dimer equilibrium of β-NGF. In contrast, L cell NGF is of much higher molecular weight and emerges at a position virtually indistinguishable

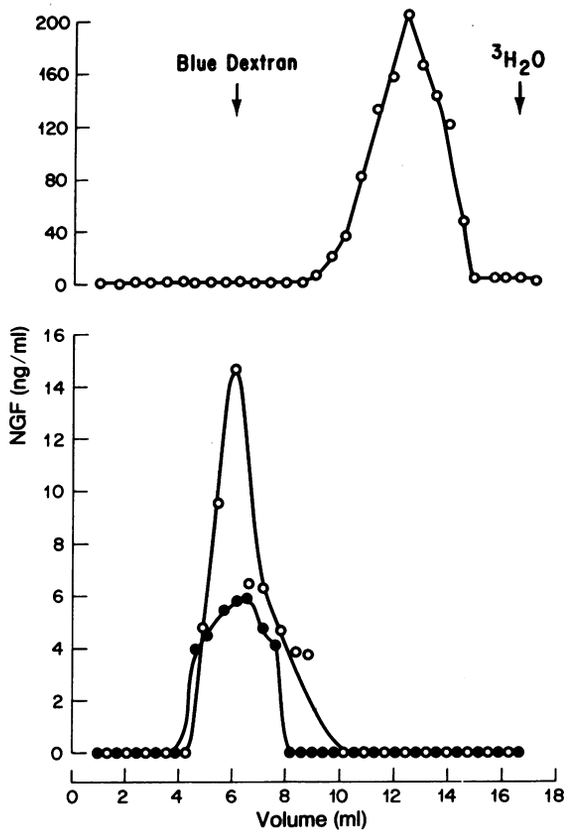

FIG. 1. Comparison of Sephadex G-75 chromatographic properties of β-NGF (submandibular) (*Upper*) and L cell NGF (*Lower*). Column dimensions, 1 × 23 cm; flow rate, 5 ml/hr; temperature, 4°. *Upper*, Column loaded with 100 μl of β-NGF, 9.8 μg/ml in 0.1 M potassium phosphate (pH 7.0) containing bovine serum albumin (1 mg/ml). *Lower*, Column loaded with 90 μl of secreted L cell NGF (○), 370 ng/ml in 0.1 M potassium phosphate (pH 7.0) (no added bovine serum albumin), or 150 μl of intracellular L cell NGF (●), 110 ng/ml prepared by homogenizing 4 × 10^8 cells with a ground-glass homogenizer in 0.4 ml of 0.1 M potassium phosphate (pH 7.0) containing bovine serum albumin 1 mg/ml. Elution solvent was 0.1 M potassium phosphate, pH 7.0/serum albumin, 1 mg/ml except when albumin was omitted.

from the void volume of the column. Fig. 1 also shows that intracellular NGF, prepared by mechanical disruption of L cells as described in the legend, chromatographed as a high-molecular-weight species. To ensure that biological activity migrated with the immunoreactive material secreted by L cells, sensory ganglion assays were performed with the peak fraction shown in Fig. 1, and strongly positive results were obtained.

The results in Fig. 1 were obtained with a solution of L cell NGF that had been extensively manipulated and concentrated by lyophilization. To ensure that these procedures did not artificially change the gel filtration behavior of the protein, a large volume of freshly harvested culture fluid was applied directly to a column of Sephadex G-75 to establish a plateau region of concentration. The resulting frontal elution profile (Fig. 2) revealed that, under these conditions, L cell NGF also emerged near the void volume (5.5 ml).

To study the possibility that L cell NGF is simply a nonspecific complex containing the biologically active, immunoreactive β component, an excess (200 ng) of pure salivary gland NGF was added to approximately 40 ng of L cell NGF in a total volume of 75 μl. This mixture was applied to a column of Sephadex G-75. Both the β-NGF and L cell NGF emerged from the column as expected (Fig. 3). These results indicate that L

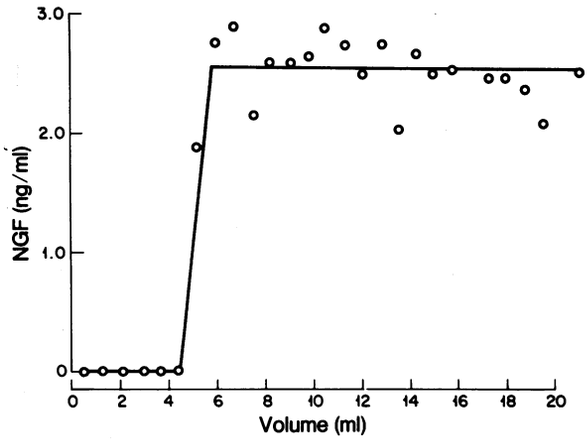

FIG. 2. Frontal elution profile of fresh, unconcentrated L cell culture medium. Sephadex G-75 column, 1 × 23 cm; solvent, 0.01 M ammonium acetate, pH 7.0/bovine serum albumin, 1 mg/ml; 20 ml containing 3.7 ng of L cell NGF per ml loaded on column; temperature, 4°.

cell NGF does not bind submandibular gland β-NGF when the latter is added in excess. Two possibilities could account for this finding. Either the binding sites of L cell NGF for the β component are saturated—which would favor the existence of a highly stable, specific complex—or submandibular gland NGF is in some way chemically different from NGF in the L cell complex and therefore cannot interact with it. However, evidence to be presented later indicates that the β component of L cell NGF cannot be distinguished electrophoretically from gland β NGF.

For approximate measurement of the molecular size of L cell NGF, concentrated culture fluids were examined both by Sephadex G-200 gel filtration and by ultracentrifugation. Within the limits of experimental error for this method at these low protein concentrations, the data of Table 1 give a mean $s_{20,w}$ of 8.9 S. If L cell NGF is a globular protein, these values would correspond to a molecular weight of about 200,000. However, in view of the several assumptions required to estimate mass from $s_{20,w}$ alone, we consider this number to be only approximate. Consequently, for another (and probably more accurate) value for molecular weight, the partition coefficient of L cell NGF was determined by gel filtration on columns of Sephadex G-200 calibrated with several proteins of known molecular weight. From plots of partition coefficient versus logarithm of

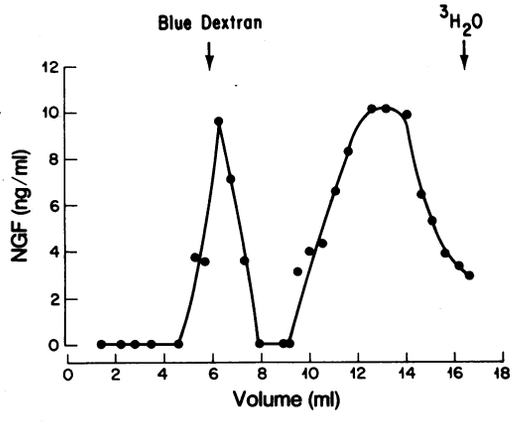

FIG. 3. Gel filtration properties of L cell NGF solution containing mouse submandibular gland β-NGF. Column and solvent as in Fig. 1. β-NGF (200 ng) plus L cell NGF (40 ng) in a volume of 75 μl were loaded. Dextran blue and ^3H_2O were run separately.

Table 1. Sedimentation velocity studies of L cell NGF

c_0 (ng/ml)	c_t/c_0	$s_{20,w}$ (S)
52	0.422	9.7
68	0.441	9.9
34	0.361	7.1

Two different preparations of concentrated L cell culture medium were dissolved in 0.1 M potassium phosphate, pH 7.0, containing bovine serum albumin (1 mg/ml) and dialyzed against 0.1 M potassium phosphate, pH 7.0. Movement of the serum albumin boundary was followed with the *Schlieren* optical system, and the centrifuge was operated at 60,000 rpm until this boundary had migrated ⅓ of the distance between the meniscus and the separation partition. Values for c_0 and c_t (Eq. 2) were measured by radioimmunoassay in triplicate. Rotor temperature was maintained close to 25°.

molecular weight for the marker proteins, the data of Fig. 4 yield a molecular weight of 160,000 for L cell NGF.

The following studies revealed that, when L cell NGF was treated with denaturing solvents, the complex dissociated to yield a protein that is closely similar to the β-NGF from mouse salivary glands. Fig. 5 *upper* presents the G-75 Sephadex profile of β-NGF dissolved in 5 M guanidine·HCl and Fig. 5 *lower* illustrates the elution pattern of L cell NGF in the same solvent. Both β-NGF and the major component of L cell NGF have the same partition coefficient. The minor peak shown in Fig. 5 *lower* remains unexplained.

To compare L cell and β-NGF in another denaturing system, both proteins were examined by gel electrophoresis with the urea/acetic acid procedure of Panyim and Chalkley (21). After electrophoresis, the gels were sliced and each slice was eluted with buffer and analyzed by radioimmunoassay (Fig. 6). Within experimental error, both β-NGF and the immunoreactive component of L cell NGF had the same mobility in this solvent system. Thus, we conclude that the high-molecular-weight NGF secreted by L cells contains a noncovalently linked molecule that is chemically and immunologically similar to β-NGF from mouse salivary glands.

DISCUSSION

Several transformed as well as primary cells in culture have been shown to secrete a biologically active NGF that is im-

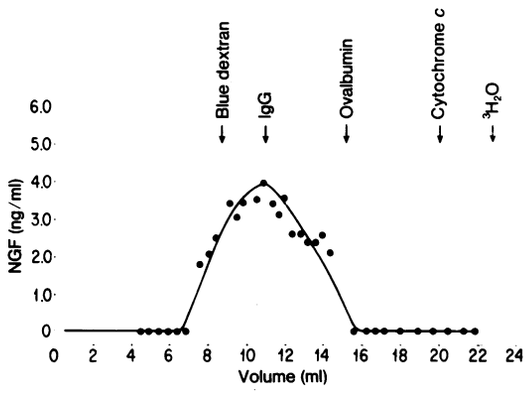

FIG. 4. Sephadex G-200 chromatographic profile of L cell NGF. Column dimensions, 1 × 26 cm; temperature 4°; solvent, 0.1 M potassium phosphate bovine serum albumin, 1 mg/ml; load, 200 μl of L cell NGF, 66 ng/ml. Dextran blue, ^3H_2O, human IgG (100 μl, 4.4 mg/ml), ovalbumin (100 μl, 40 mg/ml), and cytochrome *c* (100 μl, 20 mg/ml) were run separately. Concentrations of ovalbumin and IgG were measured by absorbance at 280 nm and cytochrome *c* at 412 nm.

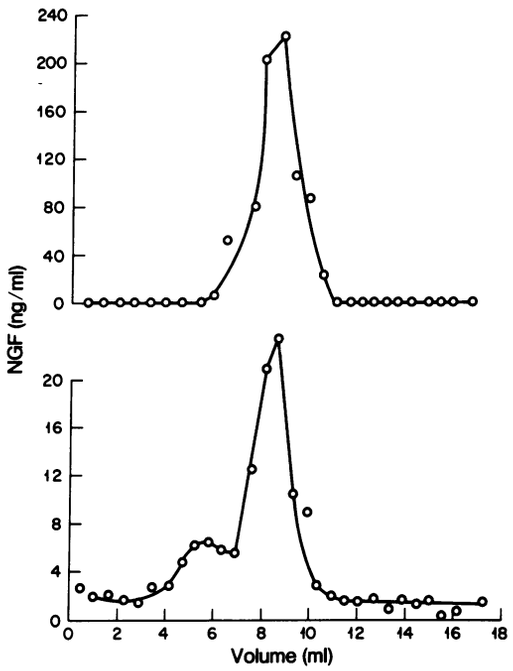

FIG. 5. Sephadex G-75 chromatography of β-NGF (*Upper*) and L cell NGF (*Lower*) in guanidine·HCl. Column, 1 × 23 cm; temperature, at 4°; solvent, 5 M guanidine·HCl/0.1 M potassium phosphate pH 7.0/bovine serum albumin, 1 mg/ml. *Upper*, 90 μl of β-NGF at 20 μg/ml. *Lower*, 90 μl of L cell NGF at 200 ng/ml. All fractions were dialyzed against 0.1 M potassium phosphate, pH 7.0, prior to radioimmunoassay.

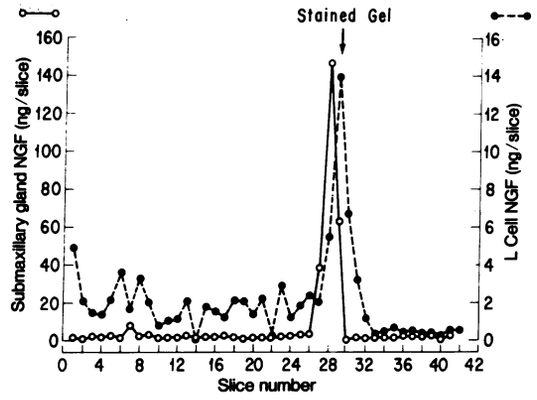

FIG. 6. Gel electrophoretic properties of L cell NGF and β-NGF. Polyacrylamide gels (15% cross-linked, 0.5 × 6 cm); solvent, 6 M urea/0.9 M acetic acid at 24°. Three separate gels were run. Two were loaded with 100 μl containing β-NGF at 25 μg/ml. One of these was stained with Coomassie blue (see arrow), and the other (○) was measured for NGF by radioimmunoassay after slicing the gel into 1.5-mm slices, eluting the protein with 1 ml of 0.01 M ammonium acetate bovine serum albumin, 1 mg/ml, lyophilizing, and redissolving in 0.1 M potassium phosphate, pH 7.0. A third gel (●) was loaded with 100 μl of L cell NGF at approximately 500 ng/ml and NGF in gel slices was measured by radioimmunoassay after processing as above.

munologically similar to the β-NGF isolated from male mouse submandibular glands. These include various different kinds of cells from several animals, including man (16); it has been suggested that secretion of NGF may be a property of many cells (15).

Until now, all physicochemical studies on NGF have utilized either β-NGF or its higher molecular weight form, 7S-NGF, both from mouse submandibular glands. The results of the present study reveal several chemical features of the NGF secreted by L cells that can be compared to those of the mouse salivary gland proteins.

1. L cell NGF in highly dilute aqueous solution is a stable, high-molecular-weight protein with molecular weight approximately 160,000. At comparable protein concentrations, β-NGF is almost completely dissociated into its two biologically active component polypeptide chains, each of molecular weight 13,259 (17).

2. As part of its structure 7S NGF [molecular weight, 140,000 (5)] contains β-NGF noncovalently linked to two other protein components. Yet, quite unlike L cell NGF under identical solvent conditions, the 7S complex is unstable in dilute solution. For example, at 7S-NGF concentrations on the order of 50 μg/ml, little, if any, 7S complex is present and at 1 μg/ml, the monomer of β-NGF (molecular weight, 13,259) is the predominant species (20). In contrast, at protein concentrations nearly 1000 times lower, L cell NGF remains stable as a high-molecular-weight complex.

3. L cell NGF contains, as part of its structure, a molecule that is closely similar to β-NGF in its size and electrophoretic properties.

4. The results illustrated in Fig. 2 indicate that the large L cell complex does not arise as a result of concentrating and processing of the culture medium. Rather, the protein has a high molecular weight and is stable as it is secreted.

5. Under the conditions presented in Fig. 3, an excess of mouse salivary gland β-NGF does not bind to the L cell protein. This result suggests that the L cell complex is a distinct species and that it does not arise from nonspecific binding of β-NGF to other L cell-secreted macromolecules. Moreover, it should be pointed out that L cell NGF is not a precursor (pro-NGF) in the sense in which that term is usually used, because L cell NGF is the form secreted naturally by the cells and there is no evidence for its enzymic conversion to a smaller form.

The reason why many different kinds of cells secrete NGF is not known. Nor is it clear why mouse submandibular glands contain such high concentrations of the factor. Removal of these glands has no apparent deleterious effects upon the animal (25) and serum NGF levels do not change (16). Thus, serum NGF must arise elsewhere than from the salivary gland and it has been suggested that the cell-secreted NGF is the source of the factor present in serum (10). Whatever may be the biologic function of gland NGF and cell-secreted NGF, the results of the present study reveal that the chemical properties of the two proteins are different. L cell NGF is highly stable in solution; 7S-NGF is not. This is particularly puzzling because, like gland NGF, the L cell factor is also of mouse origin.

There are at least two explanations that would account for these results. One is that L cell NGF and 7S-NGF are considerably different in their composition and structure, even though both proteins contain the nerve growth-promoting β-component. A second possibility is that mouse gland NGF undergoes enzymic modification during isolation and purification and that this process so alters the molecule that it becomes unstable in solution. It should be noted that this idea is supported by recent studies on the stability of NGF in fresh, unpurified submandibular gland homogenates. For example, the molecular size and stability of gland NGF are remarkably similar to those of the L cell-secreted factor when fresh gland homogenates are studied immediately after extraction (20). The fact that mouse submandibular glands are rich in proteases is also consistent with the hypothesis that considerable proteolytic modification may occur during the course of purification.

Two final points concerning L cell-secreted NGF should be mentioned. First, NGF is only one of many cell growth-pro-

moting factors that have been studied over the past several years (e.g., see ref. 26); one of the criteria used to distinguish mouse submandibular gland NGF from other growth factors is molecular size. Results of the present study indicate that comparison of molecular sizes of growth factors with NGF purified from mouse submandibular glands (e.g., β-NGF) might be misleading because the NGF secreted by L cells is quite different from purified gland NGF in size and stability. Second, in light of the observation that L cell NGF is different from NGF forms that have been described so far, the possibility cannot be excluded that this form of the molecule possesses biologic functions that are not shared by the NGF purified from mouse salivary glands.

We thank Drs. Richard A. Murphy and Judith D. Saide for many helpful discussions. N. J. P. was supported by National Institutes of Health Pre-Doctoral Training Grant GM-00451. The study was supported by Grants CA-17137 (M.Y.) and NINCDS-06021 (B.G.W.A.) from the National Institutes of Health.

1. Pantazis, N. J., Oger, J., Arnason, B. G. W. & Young, M. (1974) *Fed. Proc.* **33,** 1343, abstr.
2. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64,** 787–794.
3. Angeletti, R. H. Bradshaw, R. A. & Wade, R. D. (1971) *Biochemistry* **10,** 463–469.
4. Angeletti, R. H., Hermodson, M. A. & Bradshaw, R. A. (1973) *Biochemistry* **12,** 100–115.
5. Varon, S., Nomura, J. & Shooter, E. M. (1967) *Biochemistry* **6,** 2202–2209.
6. Burdman, J. A. & Goldstein, M. N. (1965) *J. Exp. Zool.* **160,** 183–188.
7. Levi-Montalcini, R. & Angeletti, P. U. (1968) *Physiol. Rev.* **48,** 534–539.
8. Ishii, D. N. & Shooter E. M. (1975) *J. Neurochem.* **25,** 843–851.
9. Berger, E. A. & Shooter, E. M. (1976) *Fed. Proc.* **35,** 1684, abstr.
10. Oger, J., Arnason, B. G. W., Pantazis, N., Lehrich, J. & Young, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1554–1558.
11. Young, M., Oger, J., Blanchard, M. H., Asdourian, H., Amos, H. & Arnason, B. G. W. (1975) *Science* **187,** 361–362.
12. Murphy, R. A., Pantazis, N. J., Arnason, B. G. W. & Young, M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1895–1898.
13. Murphy, R. A., Singer, R. H., Saide, J. D., Blanchard, M. H., Arnason, B. G. W. & Young, M. (1976) in *Neuroscience Abstracts* (Society for Neuroscience, Bethesda, MD), Vol. II, Part I, p. 587.
14. Murphy, R. A., Oger, J., Saide, J. D., Blanchard, M. H., Arnason, B. G. W., Hogan, C., Pantazis, N. J. & Young, M. (1977) *J. Cell Biol.* **72,** 769–773.
15. Bradshaw, R. A. & Young, M. (1976) *Biochem. Pharmacol.* **25,** 1445–1449.
16. Young, M., Murphy, R. A., Saide, J. D., Pantazis, N. J., Blanchard, M. H. & Arnason, B. G. W. (1976) in *Surface Membrane Receptors,* ed. Bradshaw, R. A. (Plenum Press, New York), pp. 247–267.
17. Young, M., Saide, J. D., Murphy, R. A. & Arnason, B. G. W. (1976) *J. Biol. Chem.* **251,** 459–464.
18. Varon, S., Nomura, J. & Shooter, E. M. (1967) *Proc. Natl. Acad. Sci. USA* **57,** 1782–1789.
19. Varon, S., Nomura, J. & Shooter, E. M. (1968) *Biochemistry* **7,** 1296–1303.
20. Pantazis, N. J., Murphy, R. A., Saide, J. D., Blanchard, M. H. & Young, M. (1977) *Biochemistry,* in press.
21. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130,** 337–346.
22. Tiselius, A., Pedersen, K. O. & Svedberg, T. (1937) *Nature* **140,** 848–849.
23. Yphantis, D. A. & Waugh, D. F. (1956) *J. Phys. Chem* **60,** 623–629.
24. Yphantis, D. A. & Waugh, D. F. (1956) *J. Phys. Chem* **60,** 630–635.
25. Levi-Montalcini, R. & Angeletti, P. U. (1968) in *Growth of the Nervous System,* eds. Wolstenholme, G. E. W. & O'Connor, M. (Little, Brown and Co., Boston), pp. 126–147.
26. Gospodarowicz, D. & Moran, J. S. (1976) *Annu. Rev. Biochem.* **45,** 531–558.