Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, 2672–2676, July 1977
Biochemistry

Molecular properties of the nerve growth factor secreted in mouse saliva

(submandibular gland/growth factors)

RICHARD A. MURPHY, JUDITH D. SAIDE, MURIEL H. BLANCHARD, AND MICHAEL YOUNG

Laboratory of Physical Biochemistry and Department of Medicine, Massachusetts General Hospital; and the Departments of Anatomy and Physiology, Harvard Medical School, Boston, Massachusetts, 02134

Communicated by C. B. Anfinsen, February 28, 1977

ABSTRACT Some molecular properties of the nerve growth factor (NGF) secreted in mouse saliva and that present in submandibular glands have been measured for comparison with previously studied forms of NGF. The results show that mouse saliva contains two biologically active NGF species. One has a molecular weight near 114,000, and the other, a molecular weight of 13,000. The larger form is being continuously degraded to yield the smaller one, probably as a result of a slow enzymatic process. Virtually identical results were obtained with crude submandibular gland extracts. The larger NGF is neither the well-known 7S NGF nor 2.5S NGF. Our results indicate that the larger salivary NGF is the naturally occurring form of NGF as it exists in the submandibular gland and as it is secreted in saliva. Its biological properties and its function in saliva, if any, remain to be elucidated.

In a preceding paper (1) we showed that the mouse submandibular gland is an exocrine, not endocrine, organ with respect to secretion of nerve growth factor (NGF). For example, serum levels of NGF do not change after submandibular gland removal and there are no sex differences in circulating levels of the factor, even though the male submandibular gland contains much more NGF than the female. Thus we suggested (1) that serum NGF probably arises by multifocal cellular secretion, since many different kinds of mammalian cells in culture produce the protein (2–7). The conclusion that the submandibular gland is an exocrine organ stems from the observation that extraordinarily high concentrations of NGF are present in submandibular gland saliva (1) (see also ref. 8). Further, male saliva contains higher concentrations of NGF than female saliva (1), and this feature reflects the sex difference in the amount of NGF in the gland. Since NGF is present in such high concentrations in saliva, it is likely that it serves some biological function in this secretion which is not yet understood. In the study presented below, we have examined some molecular properties of saliva NGF for comparison with previously studied forms of the protein.

At the present time, three different molecular forms of NGF have been recognized. Two of these have been isolated from male mouse submandibular glands and have been studied extensively. A third type of NGF is that secreted by mouse L cells in culture (2, 9). One submandibular gland form has been called 2.5S NGF (10). This species is a dimer (molecular weight 26,000) composed of two noncovalently linked, identical polypeptide chains whose primary structures are known (11). In dilute solution, the dimer dissociates into its monomeric subunits (molecular weight 13,000), which are the biologically active species in stimulating ganglionic neurite outgrowth *in vitro* (12). The other submandibular gland form has been named 7S NGF (13). This protein is isolated and purified from submandibular glands by a different procedure from that used to obtain 2.5S NGF. It has a molecular weight of 140,000 (13) and is composed of three different proteins termed α, β, and γ (14). The α and γ constituents have no known biological function, although the γ species displays arginine esterase activity (15). Only the β component of 7S NGF is biologically active in stimulating neurite outgrowth; by biological and immunological criteria, β-NGF is indistinguishable from the 2.5S NGF. These two species differ only in that during isolation of either one, limited and different proteolytic modifications occur, the extent of which depends upon the isolation conditions (11, 16). In what follows, we do not distinguish between 2.5S and β-NGF.

The NGF secreted by L cells is yet another species. This protein has a molecular weight close to 160,000 and, like 7S NGF, it contains 2.5S NGF as part of its structure (9). However, L-cell NGF differs from the 7S complex in at least one important respect. The 7S NGF structure is unstable in solution at neutral pH, and dissociates completely to yield a mixture of its components at concentrations (1 μg/ml) nearly 1000 times higher than those required to display biological activity (about 1 ng/ml) (17). Consequently, the biological activity of 7S NGF is due entirely to its 2.5S (β) component (17). In contrast to the marked instability of 7S NGF, L-cell NGF is completely stable in very dilute solution and does not dissociate even at concentrations as low as 1 ng/ml. This appreciable difference in stability between gland and L-cell NGF was surprising to us, since both proteins are of mouse origin. Yet the mouse submandibular gland is rich in proteases (18), and it seemed possible that proteolysis occurring during the process of purification of 7S NGF could account for the instability of the protein.

The results presented below demonstrate that NGF exists in saliva in a high- and a low-molecular-weight form. The larger NGF species is being continuously degraded as a function of time, and this process gives rise to the smaller NGF, which, by several criteria, we are unable to distinguish from 2.5S NGF. Chromatographic procedures are presented that effectively stop this degradation process. Further, the submandibular gland itself also contains these two forms of the molecule. The higher-molecular-weight species of NGF is clearly distinct from 7S NGF; it has not been recognized previously. It is probably the naturally occurring form of the protein in both the submandibular gland and in saliva.

MATERIALS AND METHODS

Reagents. Glass double-distilled water was used for all solutions, and all buffer salts were reagent grade. $Na^{125}I$ and ^3H_2O were obtained from New England Nuclear; Sephadex and blue dextran 2000 from Pharmacia; DE-52 (microgranular grade) from Whatman; bovine serum albumin (three times recrystallized) and horse heart ferricytochrome *c* from Sigma; human IgG and human serum albumin from Schwarz-Mann; Eagle's minimal essential medium and heat-inactivated fetal

Abbreviation: NGF, nerve growth factor.

calf serum from Gibco. The 2.5S NGF was isolated from adult male mouse submandibular glands by the procedure of Bocchini and Angeletti (10). All preparations of 2.5S NGF were shown to be electrophoretically homogeneous as described (2). Submandibular gland saliva was collected and stored as described (1).

Radioimmunoassay and Bioassay. Procedures for preparation and purification of ^{125}I-labeled NGF (specific activity 0.6 g-atom of I/mol), preparation of a monospecific antibody to 2.5S NGF, and the radioimmunoassay have been presented earlier (4). NGF biological activity was studied with dorsal root ganglia from 8-day chick embryos as described (4).

Molecular Weight Measurements. Molecular weights of saliva and submandibular gland NGF were determined by zonal gel filtration chromatography using Sephadex columns that were calibrated with blue dextran, ^3H_2O, and several proteins of known molecular weights. Weight-average partition coefficients (σ_w) were calculated from the relation

$$\sigma_w = (V_e - V_0)/(V_i - V_0)$$

where V_e is the macromolecule elution volume, V_0 is the void volume, and V_i is the elution volume of ^3H_2O. From plots of ln σ_w against ln (molecular weight) for the calibration proteins, apparent molecular weights of NGF were computed. Column fractions were collected in weighed plastic test tubes, and fraction volume was measured accurately to within 50 μl. Effluent NGF concentrations were measured by radioimmunoassay.

RESULTS

Properties of Submandibular Gland Saliva. Initial studies were carried out with saliva; Fig. 1 presents a series of gel filtration chromatographic patterns of saliva NGF under different conditions. Fig. 1*A* illustrates the Sephadex G-75 profile of a fresh sample of saliva taken from a single mouse. The immunoreactive NGF emerges as a single high-molecular-weight protein near the void volume. This behavior is completely different from that of 7S NGF, which, at comparable protein concentrations, is fully dissociated into its constituent components (17).

Of the seven different animals used for this study, only two gave results like that shown in Fig. 1*A*. In most cases, the pattern in Fig. 1*B* was observed, where two well-separated NGF peaks are seen. The higher-molecular-weight fraction emerges from the column at a position identical to that of the single high-molecular component shown in Fig. 1*A*. As will be seen later, the lower-molecular-weight fraction shown in Fig. 1*B* emerges at a position identical to that of 2.5S NGF. These results were fully reproducible; they did not arise from differences in the saliva collection procedures or methods of handling and chromatography (which did not vary). Rather, the results of Fig. 1 *A* and *B* reflect intrinsic properties of the two different saliva samples. Fig. 2 reveals that both immunoreactive fractions depicted in Fig. 1*B* are biologically active in producing neurite outgrowth in the sensory ganglion system.

To determine whether the high-molecular-weight component depicted in Fig. 1 *A* and *B* remained stable with time, we rechromatographed it as follows. A sample of saliva (which had been frozen since collection) was chromatographed, and a single high-molecular-weight NGF, such as that shown in Fig. 1*A*, was observed. The peak fraction was then allowed to remain at 4° for 48 hr, after which time it was again applied to a column of Sephadex G-75. The results (Fig. 1*C*) show that two NGF zones have appeared. Thus, some reaction must have occurred (as a function of time) to cause dissociation of the high-molecular-weight NGF component to yield the smaller one.

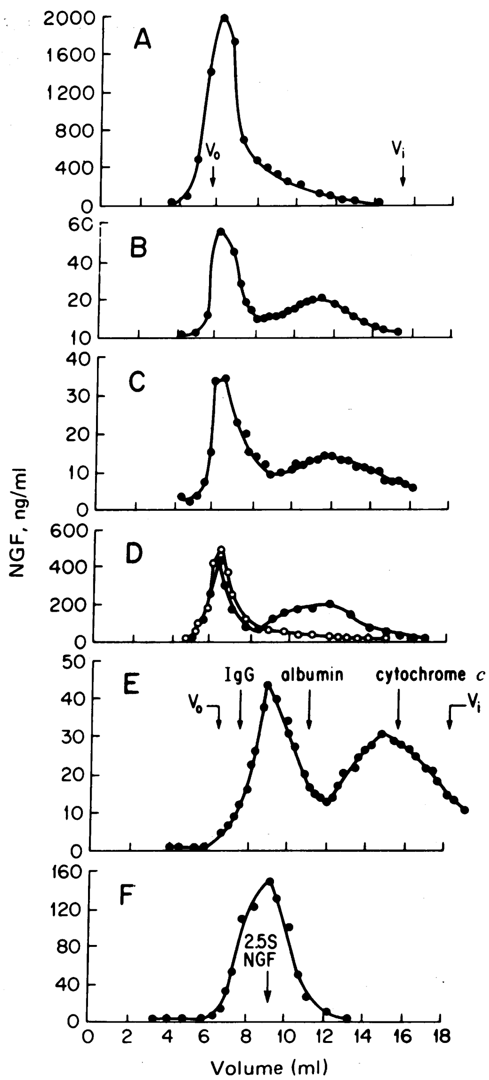

FIG. 1. Sephadex chromatographic properties of saliva NGF. (*A–D*) Sephadex G-75 column, 1 × 23 cm; solvent, 0.1 M potassium phosphate containing 1 mg/ml of bovine serum albumin at 4°. V_0 and V_i were determined with blue dextran and ^3H_2O, respectively. Flow rate, 9.8 ml/hr. Fractions (0.3 ml) were measured by radioimmunoassay. (*A*) Saliva (100 μl) containing 50 μg/ml of NGF was applied to the column; original saliva concentration, 504 μg/ml. (*B*) Saliva (100 μl) containing 2.3 μg/ml of NGF; original saliva concentration, 71 μg/ml. (*C*) Saliva (100 μl), obtained from the same mouse as that used for *A*, was chromatographed under conditions identical to those of *A*. Fractions of the single high-molecular-weight component that was observed were maintained for 48 hr at 4°. Then, 100 μl of the peak fraction was rechromatographed. (*D*) ○, Saliva (100 μl) containing 12 μg/ml of NGF; ●, 100 μl of the same saliva to which was added 2.5S NGF to a final concentration of 15 μg/ml. (*E*) Sephadex G-200, 1 × 23 cm; solvent as above at 4°; 100 μl of saliva containing 4 μg/ml of NGF was applied. The marker proteins, blue dextran, and ^3H_2O were run separately. (*F*) Sephadex G-75: solvent, 6 M guanidine·HCl at 4°. Saliva containing 3.5 μg/ml of NGF was dialyzed against 6 M guanidine·HCl, and 100 μl of this solution was applied. The 2.5S NGF was run separately. Before immunoassay, fractions were dialyzed against 0.1 M potassium phosphate, pH 7.0, to remove guanidine.

At this point, it seemed reasonable to think that perhaps we were observing an association-dissociation system in which the lower-molecular-weight NGF species shown in Fig. 1*B* was in equilibrium with the larger macromolecular form of NGF. To study this possibility, we generated a series of chromatographic

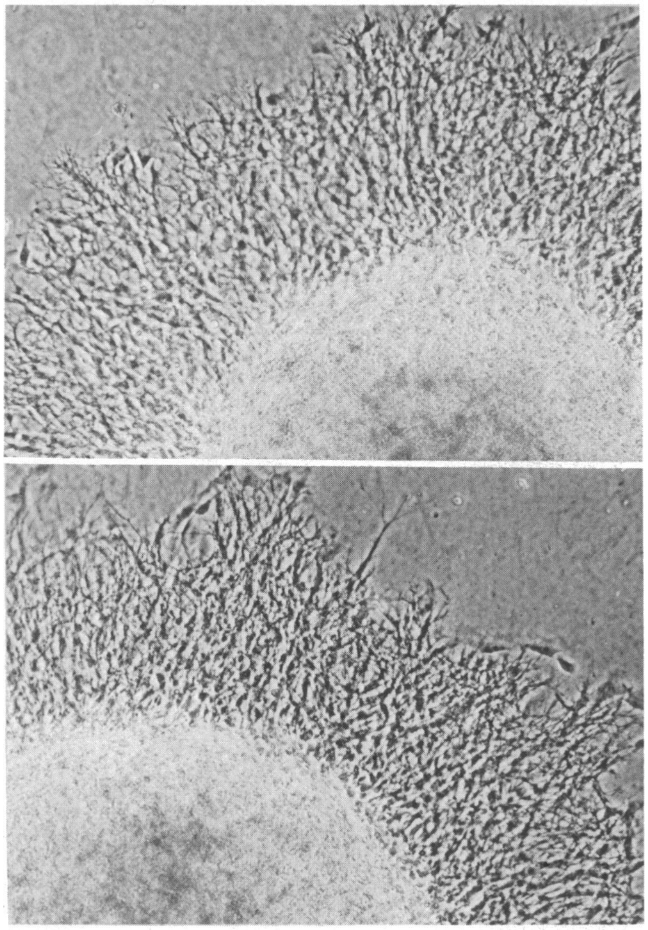

FIG. 2. Sensory ganglion bioassays of the two Sephadex G-75 fractions shown in Fig. 1B. The peak fractions were dialyzed against Eagle's minimal essential medium. Phase contrast photomicrographs. (×146.) (*Upper*) Higher-molecular-weight NGF. (*Lower*) Lower-molecular-weight NGF.

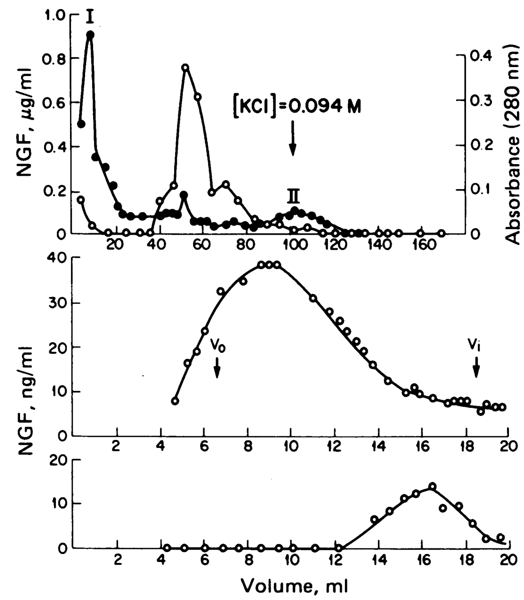

FIG. 3. Properties of saliva NGF fractions separated by ion-exchange chromatography. (*Top*) Column of DE-52 (1 × 6 cm), equilibrated with 25 mM Tris-HCl, pH 8.0. Saliva (0.3 ml, containing 150 μg/ml of NGF) was diluted with 1.0 ml of 25 mM Tris-HCl, pH 8.0, dialyzed against this solvent, and then applied to the column at 4°. A linear KCl gradient (total volume 200 ml) was begun to 0.2 M KCl at a flow rate of 8 ml/hr. ○, Absorbance (280 nm); ●, concentration of NGF measured by radioimmunoassay. (*Middle*) G-200 Sephadex column (1 × 23 cm). Solvent: 0.1 M potassium phosphate, pH 7.0, containing 1 mg/ml of bovine serum albumin, at 4°. Fractions comprising peak II from the DEAE-cellulose column above were pooled, desalted by dialysis, lyophilized, and dissolved in 0.5 ml of the column solvent; 200 μl of this solution was applied to the column. Blue dextran and ^3H_2O were run separately. (*Bottom*) Same conditions as above, except that pooled peak I from the DEAE-cellulose column was chromatographed on Sephadex G-200.

profiles such as that shown in Fig. 1B using several different saliva samples with widely varying initial NGF concentrations (0.38–24 μg/ml). In all cases, approximately equal amounts of NGF appeared in both peaks, independent of the initial concentration. These results are not compatible with the existence of an equilibrium reaction. Moreover, the results of Fig. 1D show that when 2.5S NGF is added to saliva, it does not bind to, nor participate in forming, the higher-molecular-weight NGF. A sample of saliva that had been frozen immediately after collection was thawed and promptly applied to Sephadex. Fig. 1D (open circles) reveals a single high-molecular-weight component. To an identical aliquot of the same saliva was then added an excess of pure 2.5S NGF and this solution was chromatographed upon the same column. Fig. 1D (closed circles) now shows that 2.5S NGF emerges close to the elution volume of ^3H_2O and, within experimental error of the radioimmunoassay, none of the 2.5S NGF became associated with the high-molecular-weight NGF. Thus, either the binding sites of the high-molecular-weight NGF for 2.5S NGF are saturated or, alternatively, 2.5S NGF is somehow altered during its purification such that it can no longer participate in forming the higher-molecular-weight protein.

A second possibility that would account for the presence of two NGF species is the occurrence of a slow degradation reaction leading to irreversible dissociation of the high-molecular-weight NGF. The following studies indicate that this is the case, since the reaction leading to dissociation of the large NGF species can be prevented by DEAE-cellulose chromatography. Fig. 3 *top* illustrates the DEAE-cellulose chromatographic profile of saliva; two NGF-immunoreactive components (marked I and II) can be separated. Fractions corresponding to each of these components were pooled separately and then chromatographed upon Sephadex G-200. Fig. 3 *middle* reveals that peak II corresponds to the high-molecular-weight NGF. No lower-molecular-weight NGF was detected. Moreover, the DEAE-cellulose fraction II was completely stable. No dissociation occurred even after 1 week's storage at 4°. Fig. 3 *bottom* shows that the lower-molecular-weight NGF is completely accounted for in the DEAE-cellulose fraction I. Taken together, the foregoing results indicate that NGF in saliva is unstable, that it is continuously undergoing degradation, and that this process can be prevented by ion-exchange chromatography. Furthermore, it is most likely that the slow transformation of high-molecular-weight NGF in saliva is enzymatically (and probably proteolytically) mediated and that the enzyme responsible is removed by the ion-exchange resin. Any nonenzymatic process, e.g., a slow oxidation reaction, would be expected to continue in spite of ion-exchange chromatography.

The apparent molecular sizes of the two NGF species, both before and after the DEAE-cellulose step, have been estimated by Sephadex G-200 gel filtration, using columns calibrated with proteins of known molecular weights. For this purpose, we have assumed that the gel filtration behavior of the two proteins is not anomalous and that they chromatograph like the globular calibration proteins used. Fig. 1E illustrates a typical elution

Table 1. Partition coefficients and apparent molecular weights of NGF in saliva and in submandibular gland homogenates

Source*	σ_w	Molecular weight
Saliva (Fig. 1*E*, Sephadex G-200)	0.21	111,000
	0.74	15,000
Saliva (Fig. 3, Sephadex G-200)	0.20	117,000
	0.82	11,000
Gland (Fig. 4, Sephadex G-200)	0.27	89,000
Gland (Fig. 4, Sephadex G-100)	0.63	13,000

* Refers to submandibular gland saliva or crude submandibular gland homogenates. Values for molecular weight were estimated from a plot of ln σ_w against ln (molecular weight) constructed from values of σ_w determined for IgG, serum albumin, and cytochrome *c*.

profile; Table 1 summarizes the weight-average partition coefficients (σ_w) and corresponding molecular weights. The mean value for the higher-molecular-weight component is 114,000, and for the lighter NGF species, 13,000. The latter number is virtually identical to the molecular weight of the two individual polypeptide chains of β- and 2.5S NGF (molecular weight 13,259) which have been shown to dissociate from one another at these low protein concentrations (12). Fig. 1*F* further shows that when saliva is subjected to gel filtration in the presence of a high concentration of guanidine·HCl, the high-molecular-weight NGF dissociates completely to yield a species whose molecular size is indistinguishable from that of 2.5S NGF. Thus, the 13,000-molecular-weight NGF is noncovalently linked to one or more other components to yield the 114,000-molecular-weight species present in saliva.

Properties of Submandibular Gland NGF. In light of the above results with saliva, we have examined the molecular properties of NGF in crude, fresh homogenates of whole submandibular glands. Two hundred freshly excised male submandibular glands were homogenized for 2 min on ice in the presence of 50 ml of 25 mM Tris·HCl, pH 8.0, with a high-speed Virtis homogenizer. After removal of insoluble material by centrifugation, the supernatant solution was dialyzed thoroughly against the same solvent and applied to a column of DEAE-cellulose as described in the legend to Fig. 4. The resulting gradient elution profile (Fig. 4 *top*), like that of saliva, reveals two distinct NGF components (I and II) which together comprise 80% of the NGF applied to the column as measured by radioimmunoassay. Fractions corresponding to peak II were pooled separately, desalted by dialysis, concentrated by lyophilization, and examined by gel filtration upon columns that were calibrated with proteins of known molecular weight. Fig. 4 *bottom* shows the elution profile of peak II, which emerges as a single, stable high-molecular-weight species; Fig. 4 *middle* shows the corresponding profile of the peak fraction of peak I. From values of σ_w, apparent molecular weights were calculated (Table 1). Within experimental error of the gel filtration method for estimating molecular weights, molecular weight values for peaks I and II are not significantly different from those of the two species of NGF secreted in saliva.

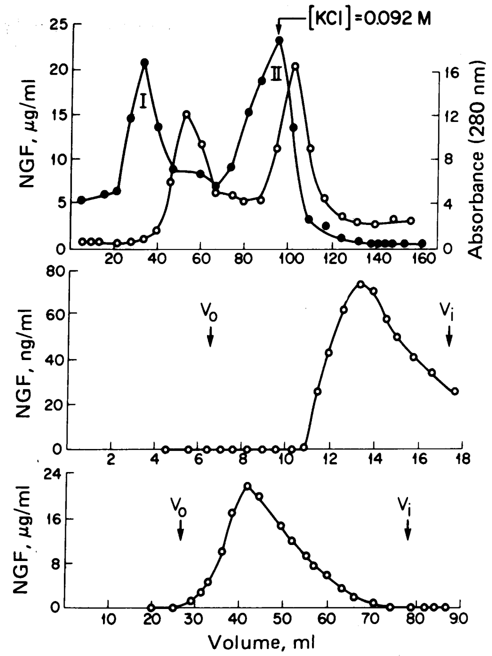

FIG. 4. Chromatographic properties of NGF in submandibular gland homogenates. (*Top*) Homogenate was prepared as described in the *text* and 40 ml (containing 66 μg/ml of NGF) was applied to a 1 × 20 cm column of DE-52. Elution conditions and gradient were identical to those described in the legend to Fig. 3. ○, Absorbance (280 nm); ●, concentration of NGF. (*Middle*) A 50-μl sample of the peak fraction from DE-52 peak I above was applied to 1 × 23 cm column of Sephadex G-100 equilibrated with 0.1 M potassium phosphate containing 1 mg/ml of bovine serum albumin. (*Bottom*) Fractions comprising peak II from DE-52 column above were pooled, desalted by dialysis, lyophilized, and dissolved in 3.0 ml of 0.1 M potassium phosphate, pH 7.0. The solution was applied to 1.5 × 46 cm column of Sephadex G-200 equilibrated with the same solvent at 4°. The solvent did not contain serum albumin. Blue dextran and ^3H_2O were run separately. Concentration of NGF was measured by radioimmunoassay.

DISCUSSION

The preceding results indicate that mouse submandibular gland saliva contains two biologically active molecular forms of NGF, and that the larger species is continuously being degraded, most likely enzymatically, to yield the smaller form. This process can be stopped by ion-exchange chromatography with DEAE-cellulose. Further, the partially purified higher-molecular-weight component is stable in aqueous solution at very low protein concentrations, and it contains as part of its structure a molecule that we have been unable to distinguish from the well-known 2.5S NGF.

The properties of NGF in fresh homogenates of submandibular glands are virtually identical to those of saliva NGF. Again, two components have been identified and both possess molecular sizes similar, and probably identical, to the two saliva species of NGF. Thus, we infer that the two NGF forms as they exist in mouse submandibular glands are the same molecular species that are secreted in saliva.

The physiological reason for the existence of two biologically highly active forms of NGF in saliva and glands is not clear to us. Nor do we understand the significance of the conversion of one form to the other. For example, mouse saliva can be obtained (Fig. 1*A*) that initially contains only the higher-molecular-weight NGF, and it may be that the slow degradation of this form is simply an unimportant side reaction that reflects the presence of proteases in saliva. Alternatively, the degradative process could serve to inactivate some as yet unknown biological property of the larger NGF.

It has been recognized for some time now that when either β-NGF or 2.5S NGF is isolated from submandibular glands, chemically minor (but perhaps biologically important) modifications occur at both the carboxyl and amino termini of the molecule (11, 16). It could be that these cleavage reactions are in part responsible for reducing the stability of the higher-molecular-weight NGF. On the other hand, it may be that more extensive, and as yet unrecognized, enzymatic modifications are required for dissociation to occur. Before this problem can be solved, knowledge of the subunit structure and amino acid sequence of the larger NGF species will be required.

Until now, it has been generally believed that the NGF form that exists in mouse salivary glands is 7S NGF (see, e.g., refs. 19 and 20). Several lines of evidence, as follow, suggest that this is not the case. (*i*) At relatively high-protein concentrations (e.g., 1 μg/ml), 7S NGF is highly unstable and virtually completely dissociated into its constituent polypeptide chains, including 2.5S NGF (17). Yet at concentrations much lower, the partially purified high-molecular-weight gland factor described here is completely stable (see Figs. 3 and 4). (*ii*) The 7S NGF has been reported to be in rapid, reversible equilibrium with its subunits (α, β, and γ) at relatively high protein concentrations (on the order of 0.2 mg/ml) (21, 22). In contrast, we have been unable to detect association–dissociation reactions of any kind which pertain to the high-molecular-weight saliva or gland NGF after the DEAE-cellulose chromatographic procedure depicted in Figs. 3 and 4, respectively. (*iii*) In their original paper on the purification of 7S NGF (13), Varon *et al.* noted that abnormally high flow rates (350 ml/hr) were required for DEAE-cellulose chromatography of 7S NGF and that when more normal flow rates were used, NGF appeared over several fractions and recovery was poor. Although this problem has apparently not received further study, Varon *et al.* (13) pointed out that it could have stemmed from dissociation of 7S NGF. Thus, all available evidence points to the conclusion that 7S NGF is not the molecular form of the protein that occurs naturally in either submandibular glands or in saliva. It is, however, possible that 7S NGF in some way is structurally related to the high-molecular-weight NGF described here. Alternatively, it could be that 7S NGF arises artificially during its purification. Whatever the origin and nature of 7S NGF may be, the present results, when taken together with earlier work (17), indicate that 7S NGF is sufficiently damaged during its isolation that it is no longer a stable complex.

The finding of a new, stable, high-molecular-weight form of NGF in glands and saliva suggests that this is the naturally occurring form of the protein that is synthesized by the glands and secreted in saliva; it may be that this protein has some function in saliva. Over the past several years, the chemical and biological properties of 2.5S and 7S NGF have been studied extensively. Yet, if the stable, high-molecular-weight NGF described here is the naturally occurring species, as the above results suggest, then it could be that many of the experiments and conclusions pertaining to the function of 7S or 2.5S NGF will need to be reexamined.

This work was supported by National Institutes of Health Grant CA 17137 to M.Y. and grants from the Muscular Dystrophy Association and the U.S. Public Health Service to R.A.M. J.D.S. was supported by a National Institutes of Health postdoctoral fellowship (NHLI).

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Murphy, R. A., Saide, J. D., Blanchard, M. H. & Young, M. (1977) *Proc. Natl. Acad. Sci. USA*, **74,** in press.
2. Oger, J., Arnason, B. G. W., Pantazis, N., Lehrich, J. & Young, M. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1554–1558.
3. Young, M., Oger, J., Blanchard, M. H., Asdourian, H., Amos, H. & Arnason, B. G. W. (1975) *Science* **187,** 361–362.
4. Murphy, R. A., Pantazis, N. J., Arnason, B. G. W. & Young, M. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1895–1898.
5. 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.
6. Murphy, R. A., Singer, R. H., Saide, J. D., Pantazis, N. J., Blanchard, M. H., byron, K. S., Arnason, B. G. W. & Young, M. (1977) *Proc. Nat. Acad. Sci. USA*, in press.
7. Young, M., Murphy, R. A., Saide, J. D., Pantazis, N. J., Blanchard, M. H. & Arnason, B. G. W. (1976) in *Surface Membrane Receptors*, eds. Bradshaw, R. A., Frazier, W. A., Merrell, R. C., Gottlieb, D. I. & Hogue-Angeletti, R. A. (Plenum Press, New York), pp. 247–267.
8. Wallace, L. J. & Partlow, L. M. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 4210–4214.
9. Pantazis, N. J., Blanchard, M. H., Arnason, B. G. W. & Young, M. (1977) *Proc. Natl. Acad. Sci. USA*, **74,** 1492–1496.
10. Bocchini, V. & Angeletti, P. U. (1969) *Proc. Natl. Acad. Sci. USA* **64,** 787–794.
11. Angeletti, R. H., Hermodson, M. H. & Bradshaw, R. A. (1973) *Biochemistry* **12,** 100–115.
12. Young, M., Saide, J. D., Murphy, R. A. & Arnason, B. G. W. (1976) *J. Biol. Chem.* **251,** 459–464.
13. Varon, S., Nomura, J. & Shooter, E. M. (1967) *Biochemistry* **6,** 2202–2209.
14. Varon, S., Nomura, J. & Shooter, E. M. (1968) *Biochemistry* **7,** 1296–1303.
15. Greene, L. A., Shooter, E. M. & Varon, S. (1968) *Proc. Natl. Acad. Sci. USA* **60,** 1383–1388.
16. Moore, J. B., Mobley, W. C. & Shooter, E. M. (1974) *Biochemistry* **13,** 833–840.
17. Pantazis, N. J., Murphy, R. A., Saide, J. D., Blanchard, M. H. & Young, M. (1977) *Biochemistry*, **16,** 1525–1530.
18. Sreebny, L. M. (1960) *Ann. N.Y. Acad. Sci.* **85,** 182–188.
19. Bamburg, J. R., Derby, M. H. & Shooter, E. M. (1971) *Neurobiology* **1,** 115–120.
20. Perez-Polo, J. R. & Shooter, E. M. (1975) *Neurobiology* **5,** 329–338.
21. Greene, L. A., Shooter, E. M. & Varon, S. (1969) *Biochemistry* **8,** 3735–3741.
22. Baker, M. E. (1975) *J. Biol. Chem.* **250,** 1714–1717.