The biosynthesis of β nerve growth factor (βNGF) was studied in isolated mouse submaxillary glands incubated with L-[35S]cysteine. Sodium dodecyl sulfate gels of anti-βNGF immunoprecipitates from labeled gland homogenates showed a single major peak of radioactivity, which co-migrated with purified βNGF. This species was nearly completely precipitated by the addition of equivalent amounts of anti-βNGF, but was absent from immunoprecipitates obtained by the addition of ferritin plus anti-ferritin. The cystine-containing tryptic peptides of the labeled species appeared identical with those of purified βNGF.

In submaxillary glands from adult male mice, labeling of βNGF represented approximately 0.2% of the trichloroacetic acid-precipitable radioactivity. Castration reduced this value to one-third, while testosterone treatment of castrated animals restored the relative βNGF synthesis to normal or more. No βNGF synthesis could be detected in glands from female animals.

Several tissues were examined for their ability to synthesize βNGF in culture. Only the submaxillary gland incorporated detectable amounts of radioactivity into βNGF. Labeling of βNGF could also be obtained by direct injection of isotope into the submaxillary gland in vivo. The results are discussed in terms of the integration of βNGF synthesis into neuronal development and maintenance.

The development of the nervous system requires strict temporal and spatial coordination of cell growth, differentiation, and survival. The discovery and isolation of nerve growth factor, a protein capable of modulating neuronal morphology and biochemistry, promised new insight into the molecular nature of such regulatory processes (1). NGF has now been detected in a variety of vertebrates (1, 2) and is generally recognized to play an important role in the development and maintenance of the sympathetic nervous system, and in the development of the sensory ganglia as well (1).

In the male mouse, the bulk of the NGF is found in the submaxillary gland (3) in the form of specific high molecular weight complex called 7 S NGF (4, 5). The complex can be dissociated into three classes of subunits (α, β, and γ) which have been purified (6). All the nerve growth-stimulating activity resides in the β subunit (βNGF), which is a dimer of two identical noncovalently associated chains (M, 13,200) (7-9) whose amino acid sequence has been determined (9). The γ subunit is a potent arginyl esterase (10) and it has been proposed that it may function as a cleaving enzyme in the processing of a βNGF precursor (9, 11). Support for this hypothesis has recently been obtained (12). No enzymatic activity has yet been found for the α subunit.

Purified βNGF will elicit profound biochemical and morphological effects from responsive neurons. The well known stimulation of axonal outgrowth from cultured ganglia has proven useful as a bioassay for the factor (13), and βNGF has also been reported to influence the directionality of fiber outgrowth (14-17). A variety of metabolic processes may be enhanced in the presence of βNGF, including cell enlargement and proliferation, glucose metabolism, RNA and protein synthesis, lipid synthesis (1), adenosine 3':5'-monophosphate production (Ref. 18, but also see Refs. 19 and 20), uptake of certain small molecules (21), and assembly of neurofilaments (22). Despite this plurality of effects, it remains unclear how these diverse functions are integrated into the overall pattern of neuronal development and maintenance. Our knowledge of βNGF synthesis is particularly deficient, and the role of this factor cannot be fully ascertained without a precise description of where and when it is produced and how its synthesis and processing are regulated. In this report, we show that isolated submaxillary glands synthesize βNGF de novo, and that the synthesis is regulated by steroid hormones. Preliminary versions of this work have been published (23, 24).

**EXPERIMENTAL PROCEDURES**

**Materials**

L-[35S]cysteine (20 to 60 Ci/mmol), Na[115]I (carrier-free), and iodo[14C]acetic acid (207 mCi/mmol) were purchased from New England Nuclear. Horse ferritin and rabbit anti-ferritin antisera were generous gifts from Marvin Wickens (Stanford University).
Methods

Purification and Labeling of βNGF—The subunit of nerve growth factor was isolated from 7 T 3 fibroblast by the method of Smith et al. (25). Purity was confirmed by isoelectric focusing in polyacrylamide gels (26) and by SDS gel electrophoresis (27). Concentrated solutions of βNGF were in 0.9% acetic acid at 26°C. Dilute solutions were stored at 4°C in Buffer A (0.05 M sodium acetate, pH 4.0, 0.5 mM sodium chloride, 0.2 mg/ml of sodium azide, and 1 mg/ml of bovine serum albumin).

βNGF (specific activity 2 to 3 x 10^6 cpm/μg) was prepared by the lactoperoxidase method as previously described (28). Solutions were stored in Buffer A.

Preparation of Antiserum to βNGF—Antiserum was prepared in female New Zealand white rabbits by the injection of purified βNGF. The antisum's adsorption of βNGF to histone and other serum proteins was determined. Serum samples were incubated for 1 h followed by SDS gel electrophoresis (27), centrifugation in 1 ml of Buffer C (Buffer A supplemented with 10 mM EDTA). Solutions were prepared by the lactoperoxidase method as previously described (29).

Preparation of Radioimmunoassay—βNGF was added to solutions of the gel slices on SDS gels of the immunoprecipitates. As discussed under "Results," Figs. 2 and 4, and Table I).

Analytical SDS Gel Electrophoresis—The washed immunoprecipitates were dissolved in 200 μl of the SDS sample buffer described by Laemmli (27), modified by the addition of 9 M urea which was required to completely dissociate the antigen—antibody complex. Following overnight incubation at 37°C, 160-μl aliquots were applied to discontinuous SDS gels (70 x 5 mm) (27) containing 15% (w/v) acrylamide and 0.4% (w/v) N,N'-methylenebisacrylamide. Electrophoresis was performed at 3.5 mA/tube until the bromphenol blue dye front approached the bottom of the gel. The gel slices were incubated overnight at 37°C in 10 ml of scintillation fluid containing per liter of toluene 4 g, 2,5-diphenyloxazole, 0.05 g of 1,4-bis(2-(5-phenyloxazolyl))benzene, 5 ml of Protosol (New England Nuclear), 0.5 ml of 0.1 M HCl, and 6 ml of H2O. Slices were counted on a liquid scintillation counter. For analysis of immunoprecipitates containing 125I-labeled βNGF, gel slices were counted on a well-type gamma counter. Aliquots (20 μl) of the gel samples were also counted to determine recovery of radioactivity after electrophoresis. After electrophoresis, the gel was dried at room temperature and counted.

Quantitation of βNGF Synthesis—βNGF synthesis was quantitated by counting the radioactivity at the βNGF position on SDS gels of washed immunoprecipitates obtained from labeled tissue extracts. Vision (25) devices were used. Electrophoresis was performed with an apparatus described by Furlong et al. (30) using a resolving gel (5 x 2.4 cm) at 14 mA. The elution chamber below the gel surface was continuously flushed with electrode buffer at 36 ml/h. Fractions (2.8 ml) were collected once the bromphenol blue dye front reached the bottom of the gel. Those containing the peak of radioactivity were pooled and purified βNGF was added to separate aliquots of tissue extracts. Net recoveries ranged between 71 and 83% and an average yield of 75% was used in all calculations (see "Results," Table I); (b) the fraction of the βNGF radioactivity which appeared in the βNGF peak gel slice was determined on SDS gels of the immunoprecipitates. As discussed under "Results" (Fig. 4B), this value was found to be 90%. βNGF synthesis was normalized relative to weight of tissue or to incorporation into total chloroacetic acid precipitable radioactivity.

Preparative SDS Gel Electrophoresis—Preparative SDS gel electrophoresis was used to isolate the major radioactive species observed in immunoprecipitates from labeled submaxillary gland extracts. The system of Laemmli (27) modified as described above was used. Electrophoresis was performed with an apparatus described by Furlong et al. (30) using a resolving gel (5 x 2.4 cm) at 14 mA. The elution chamber below the gel surface was continuously flushed with electrode buffer at 36 ml/h. Fractions (2.8 ml) were collected once the bromphenol blue dye front reached the bottom of the gel. Those containing the peak of radioactivity were pooled and lyophilized and suspended in the original volume with water. Then 250 μg of bovine serum albumin and the material was further concentrated by vacuum dialysis against 0.1 M Tris/HCl, pH 8, containing 8 M urea and 2 mM of EDTA.

Reduction and Carbamoylation of Proteins—For peptide map analysis, the carbamoylated peptides of the major 35S peak were compared with those of purified βNGF, alkylated with iodo[125I]acetic acid. The ∼25S-labeled material from the preparative SDS gel, in a volume of 1.6 ml, was reduced by the addition of dithiothreitol (5 mM final concentration). Recrystallized iodoacetic

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Acid was added to 20 mM, and alkylation was allowed to proceed for 15 min in the dark. The reaction was terminated by addition of β-mercaptoethanol to 200 mM.

Purified βNGF was alkylated with iodo[3H]acetic acid as follows: 3.25 mg of βNGF (1.5 μmol of total SH) in a volume of 1.1 ml were dialyzed against 0.5 M Tris-HCl, pH 8, containing 8 M urea and 2 mg/ml of EDTA. Then 30 μmol of dithiothreitol were added and the sample was continuously flushed with nitrogen for 4 h at room temperature. A mixture of 4.8 μmol of iodo[3H]acetic acid (1 mCi) plus 70 μmol of unlabeled recrystallized iodoacetic acid were added, and the sample was covered with aluminum foil and incubated 15 min at room temperature. The reaction was stopped by the addition of 700 μmol of β-mercaptoethanol, and the sample was dialyzed first against the original dialysis buffer and then against 0.2% acetic acid. Analysis indicated that the 3H-labeled alkylated βNGF contained 640 cpm/μg.

The tryptic map procedures are described in the legend to Fig. 5.

Protein Determination—Protein content was determined by the method of Lowry et al. (31).

RESULTS

Equivalence Point of βNGF Antiserum—The equivalence point of the anti-βNGF antiserum was determined by titrating increasing volumes of antiserum against a constant amount of purified βNGF containing tracer 125I-labeled βNGF. As shown in Fig. 1, the titration curves obtained by monitoring precipitation of either radioactivity or total protein were very similar. This finding agrees with a previous report demonstrating that 125I-labeled βNGF and unlabeled βNGF have similar avidities for this antiserum when tested in a solid state radioimmune assay (29). Identical titration curves were obtained at pH 7, although 0.5 M NaCl was not required (not shown). Using freshly prepared 125I-labeled βNGF, up to 90%...

![Fig. 1](http://www.jbc.org/content/806/18/806.f1)

**Fig. 1.** Titration curves of anti-βNGF antiserum. Samples containing 2 μg of βNGF plus 0.45 ng of 125I-labeled βNGF in 0.3 ml of Buffer A were titrated with the indicated volumes of antiserum. Following overnight incubation at 4°C, the immunoprecipitates were isolated by centrifugation and washed three times with Buffer A. The pellets were counted and their protein content determined.

![Fig. 2](http://www.jbc.org/content/806/18/806.f2)

**Fig. 2.** Comparison of wash procedures of labeled immunoprecipitates. Animals were treated and their submaxillary glands incubated with L-[35S]cystine (2 mCi/ml) as described under "Methods." Immunoprecipitates were obtained from 0.1 ml of gland supernatant and were washed by the indicated procedure. Washed immunoprecipitates were analyzed by SDS gel electrophoresis. In all SDS gel figures, the left-hand arrow represents the top of the resolving gel and the right-hand arrow represents the tracking dye. The βNGF position (solid bar) is determined from the mobility of purified βNGF run on a separate gel and stained with Coomassie blue. The wash procedures used were as follows: A, immunoprecipitate was collected in a nonsiliconized tube and washed three times with Buffer C by centrifugation in the same tube. B, the immunoprecipitate, collected in a nonsiliconized tube and washed once with Buffer C, was dissolved in 0.1 N NaOH. βNGF was reprecipitated by neutralization with 0.1 N HCl followed by the addition of Buffer D plus additional antiserum and an equivalent amount of unlabeled βNGF as described under "Methods." The resulting precipitate was washed once more with Buffer C in the same tube. C, same as B except siliconized tubes were employed, and the suspension of the immunoprecipitate was quantitatively transferred to a fresh tube before centrifugation during the final wash.
of the radioactivity could be immunoprecipitated at equivalence. (The residual 10% remained soluble even after the addition of more βNGF plus an equivalent volume of antiserum and thus probably represents βNGF molecules whose antigenicity was altered by the iodination procedure.) The extent of precipitation of tracer amounts of 125I-labeled βNGF is therefore a valid measure of the precipitation of total βNGF and can be used as a probe to determine the volume of antiserum required to quantitatively precipitate βNGF from tissue homogenates, and to calculate the recovery of βNGF on immunoprecipitation and washing.

Comparison of Washing Procedures for Immunoprecipitates Obtained from Labeled Tissue Extracts—Fig. 2A indicates that anti-βNGF antiserum added to a 35S-labeled submaxillary gland 105,000 × g supernatant precipitates a major peak of radioactivity which co-migrates on SDS gels with purified βNGF. There is, however, considerable radioactivity in other gel fractions, particularly in regions corresponding to the major labeled peaks in a trichloroacetic acid precipitate (Fig. 3). This radioactivity most likely represents nonspecific contamination by labeled submaxillary gland proteins, and its proximity to the βNGF position prevents accurate determination of the radioactivity in βNGF. As shown in Fig. 2 and Table I, the background can be markedly reduced by (a) dissolving the immunoprecipitate in NaOH and then reprecipitating by neutralization followed by the addition of buffer and additional carrier βNGF plus antiserum, and (b) the use of siliconized tubes and the transfer of the immunoprecipitate to a fresh tube prior to centrifugation during the final wash. The reduction in background contamination is accomplished with only a minor sacrifice of βNGF recovery (Table I), and this washing method was therefore employed in the experiments described below. It should be noted that even with this procedure, a considerable portion (30%) of the total radioactivity in the immunoprecipitate is nonspecific, and therefore accurate determination of βNGF synthesis requires analysis of the precipitated label by gel electrophoresis.

Specificity of Immunoprecipitation—The results shown in Fig. 4A provide further evidence that the labeled peak at the βNGF position is in fact βNGF. When an immunoprecipitate was obtained from the supernatant of the first precipitation by the addition of carrier βNGF plus equivalent antiserum, little radioactivity was observed at this position. The peak in the initial immunoprecipitate, therefore, represents a molecule which was nearly completely removed by the first addition of antiserum. This agrees with the calculated completeness of βNGF immunoprecipitation (90%) determined by the precipitation of tracer 125I-labeled βNGF immunoprecipitated from a separate aliquot of homogenate, and provides further confirmation of the accuracy of the equivalence point determined by immunotitration of tracer 125I-labeled βNGF added to gland homogenates. In a second control, addition of ferritin plus anti-ferritin in amounts sufficient to give a larger immunoprecipitate than that obtained with anti-βNGF did not precipitate the labeled peak at the βNGF position (Fig. 4A).

When tracer 125I-labeled βNGF was added to a separate aliquot of gland supernatant and immunoprecipitated in ex-

![Fig. 3. SDS gel of trichloroacetic acid precipitate from labeled submaxillary gland homogenate. Ten milliliters of ice cold 10% (w/v) trichloroacetic acid was added to 1 μl of the 35S-labeled gland supernatant described in Fig. 2, after the addition of 0.5 mg of bovine serum albumin as carrier. The precipitate was washed twice with 8 ml of trichloroacetic acid, then analyzed by SDS gel electrophoresis (see "Methods").](image)

![Fig. 4. SDS gel patterns of immunoprecipitates from submaxillary gland homogenates. Submaxillary glands were labeled with L-[35S]cystine, and immunoprecipitates were washed and analyzed as described under "Methods." A—○—○, immunoprecipitate obtained from 0.1 ml of gland supernatant using anti-βNGF; ○—○, immunoprecipitate obtained from the supernatant of the first anti-βNGF immunoprecipitation by the addition of unlabeled βNGF (3.5 μg) and an equivalent amount of anti-βNGF; A—△—△, immunoprecipitate obtained from 0.1 ml of gland supernatant by the addition of ferritin (5 μg) and an equivalent amount of anti-ferritin. B, 3 ng of 125I-labeled βNGF was added to 0.1 ml of gland supernatant, and the washed immunoprecipitate obtained using anti-βNGF.](image)
Biosynthesis of β Nerve Growth Factor

Fig. 5. Isoelectric focusing of tryptic peptides of 3H-labeled carboxymethylated βNGF and the carboxymethylated 35S-labeled. A portion of the carboxymethylated 35S-labeled peak from the preparative SDS gel (10,600 cpn containing 200 μg of carrier bovine serum albumin) was combined with purified βNGF which had been carboxymethylated with iodo[3H]acetic acid (84,500 cpm, 132 μg of βNGF). The mixture (total volume 0.16 ml) was dialyzed at 4°C against 0.1 M ammonium bicarbonate, pH 8.2, 40 μl of a 1 mg/ml solution of (tosyl+phenylalanine chloromethyl ketone)treated trypsin was added and the mixture was incubated for 1 h at 37°C. An additional 40 μl of trypsin solution was added and the incubation continued for 3 h. The mixture was lyophilized, dissolved in 0.3 ml of 8 M urea, and centrifuged to remove insoluble material. Then 0.12 ml of supernatant was added to 0.013 ml of 2 mM HCl containing 0.12 ml of supernatant was added to 0.013 ml of 2 mM HCl containing 10% (v/v) glycerol, 10% (v/v) Triton X-100, and 4 mg/ml of cytochrome c. The sample was applied to a gel (70 x 5 mm) containing 0.1% (w/v) acrylamide, 0.2% (w/v) N,N'-methylenebisacrylamide, 2% (w/v) ampholines (pH 3 to 6, LKB), and 8 M urea. Preliminary experiments indicated that all the 3H-labeled carboxymethylated peptides of purified βNGF focus within this pH range. The anode (upper reservoir) contained 3 mM HCl and the cathode contained 3 mM NaOH. After focusing for 4 h at 250 V, 2-mm gel slices were counted differentially for 35S and 3H. Counts were corrected for spillover and window settings. •—•, 35S; A-—A, 3H.

Table II

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Fraction of trichloroacetic acid-precipitable cpm which are in βNGF</th>
<th>Relative radioactivity in βNGF</th>
<th>Relative synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal male</td>
<td>0.22 100 645 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castrated male</td>
<td>0.07 31 223 35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castrated male + testosterone</td>
<td>0.25 114 666 103</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>&lt;0.01* &lt;4 &lt;19 &lt;3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table II shows the synthesis of βNGF in cultured submaxillary glands from animals under different hormonal states. Castration of adult male mice causes a marked reduction in relative βNGF synthesis, and testosterone treatment of castrated animals boosts the synthetic level to slightly above normal. βNGF synthesis is below detectable limits in submaxillary glands from adult females. βNGF Synthesis by Various Tissues—In order to test whether the βNGF synthesis observed in the submaxillary gland is merely a base-line level typical of all tissues or is truly a specialized function of the submaxillary gland, we measured synthesis in a variety of isolated tissues by the same methods. Of all the tissues examined, βNGF synthesis could only be detected in the submaxillary gland under the conditions employed (Table III).

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Fraction of trichloroacetic acid-precipitable cpm which are in βNGF</th>
<th>Relative radioactivity in βNGF</th>
<th>Relative synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal male</td>
<td>0.20 100 540 100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castrated male</td>
<td>0.06 31 197 31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castrated male + testosterone</td>
<td>0.27 134 1361 250</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>&lt;0.01* &lt;3 &lt;17 &lt;3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The values were determined by counting the radioactivity at the βNGF position on the SDS gels. Since no discrete βNGF peak was detected at this position with immunoprecipitates from female glands, the values presented for females are in fact overestimates of the true βNGF biosynthesis.

We conclude that isolated submaxillary glands are capable of de novo synthesis of βNGF. In a series of five separate experiments with submaxillary glands from castrated mice treated with testosterone (see "Methods"), the relative incorporation of label into βNGF was 0.25% ± 0.03% (S.E.) of the label incorporated into trichloroacetic acid-precipitable material. It should be noted that this may be an overestimate of the true relative rate of synthesis since βNGF is relatively rich in l-cystine (9), the amino acid used in these labeling studies.

βNGF in Culture Medium after Tissue Incubation—Following the incubation described in Fig. 4, bovine serum albumin was added to the culture medium (final concentration 1 mg/ml) which was then exhaustively dialyzed against Buffer A to

<table>
<thead>
<tr>
<th>Hormonal regulation of βNGF synthesis in mouse submaxillary glands</th>
</tr>
</thead>
</table>
| Normal males were sham operated. Castrated animals were used at least 21 days after operation. Testosterone administration is described under "Methods." Submaxillary glands were incubated in the presence of L-[35S]cystine (2 μCi/ml). The radioactivity in βNGF was determined by SDS gel electrophoresis of the washed immunoprecipitates from the gland homogenates (see "Methods").

The values were determined by counting the radioactivity at the βNGF position on the SDS gels. Since no discrete βNGF peak was detected at this position with immunoprecipitates from female glands, the values presented for females are in fact overestimates of the true βNGF biosynthesis.


<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction of trichloroacetic acid-precipitable cpm which are in βNGF</th>
<th>Radioactivity in βNGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Castrated mice treated with testosterone</td>
<td>0.233</td>
<td>588</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>&lt;0.005*</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Liver</td>
<td>&lt;0.001</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Kidney</td>
<td>&lt;0.003</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Spleen</td>
<td>&lt;0.003</td>
<td>&lt;13</td>
</tr>
<tr>
<td>Lung</td>
<td>&lt;0.005</td>
<td>&lt;19</td>
</tr>
<tr>
<td>Heart</td>
<td>&lt;0.002</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Adrenal*</td>
<td>&lt;0.007</td>
<td>&lt;24</td>
</tr>
<tr>
<td>Brain†</td>
<td>&lt;0.004</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Spinal cord*</td>
<td>&lt;0.007</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Sham-operated, untreated male mice</td>
<td>0.210</td>
<td>555</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>&lt;0.002</td>
<td>&lt;4</td>
</tr>
<tr>
<td>Testes*</td>
<td>&lt;0.009</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Vas deferens*</td>
<td>&lt;0.009</td>
<td>&lt;8</td>
</tr>
</tbody>
</table>

* Since only in the submaxillary gland immunoprecipitates were there discrete radioactive peaks at the βNGF position, the values presented for the other tissues represent overestimates of the true βNGF biosynthesis.
† The media were tested following the incubations by adding carrier βNGF plus antisem, and electrophoresing the washed immunoprecipitates. In no case was labeled βNGF detected in the medium.

βNGF Synthesis in Vivo—When L-[35S]cystine was injected directly into the submaxillary gland of a living mouse and the immunoprecipitate analyzed, radioactive βNGF was observed (data not shown). The label represented 0.46% of the trichloroacetic acid-precipitable radioactivity in the gland supernatant.

**DISCUSSION**

The submaxillary gland of the adult male mouse has long been recognized for its high NGF content (3), yet the source of this NGF has not been directly determined. Several lines of evidence argue against NGF uptake by the gland from the serum. (a) NGF injected directly into the circulation does not accumulate in the submaxillary gland (29, 34), (b) radioactive amino acids injected unilaterally into one lobe of the submaxillary gland preferentially label the NGF from the injected lobe (35), (c) the NGF activity in venous blood affluent to the gland exceeds that in arterial blood affluent to the gland (35), (d) daily injection of anti-NGF does not prevent the testosterone-induced rise in submaxillary gland NGF content in female or young male mice (34).

It is often assumed on the basis of these criteria that submaxillary gland NGF is produced *in situ*, but more direct evidence has thus far been lacking and the question is still controversial (36, 37). Levi-Montalcini and Angeletti (35) have reported that addition of anti-NGF antiserum to homogenates of submaxillary glands labeled in culture precipitates radioactivity, but neither analytical characterization of the labeled material nor controls for nonspecific precipitation were presented. In view of the relatively large amount of label precipitated (4 to 6% of the trichloroacetic acid-precipitable counts compared to our observations of approximately 0.2% for specific incorporation into βNGF), one is not convinced that the label in their immunoprecipitates represents NGF synthesized *in vitro*.

The findings presented herein provide the most conclusive evidence for *de novo* production of βNGF by mouse submaxillary glands, and the negligible levels of βNGF synthesis in other tissues examined (Table III) suggest that most if not all of the submaxillary gland βNGF is produced *in situ*. As anticipated from previous kinetic studies (29), the hormonal modulation of gland βNGF levels (3, 29, 32, 33) correlates with direct measurements of βNGF synthesis (Table II).

Still, the physiological function of the submaxillary gland βNGF remains unclear. The report of Hendry and Iverson (38) that sialectomy induces a transient drop in serum NGF levels suggested that the gland is the source of NGF for the circulation. However, Murphy et al. (39) were unable to detect this decline and proposed instead "that the serum factor arises from multifocal cellular secretion." In favor of this view is the observation that circulating NGF levels are eventually restored after sialectomy without concomitant regeneration of the gland (38), as well as the apparent ability of many primary (40-43) and transformed (43-47) cell types to produce NGF in culture. Clearly, the question of an endocrine role for the submaxillary gland in NGF production can be ascertained only when the effects of sialectomy on serum NGF levels are resolved. Alternatively, the possibility of an exocrine role for NGF secretion into the digestive tract has been considered (38) and given recent support by the finding of high NGF concentrations in mouse saliva (39, 48). It should be recalled that snake venom is another rich source of NGF (49), and that the venom gland is the phylogenetic homologue of the mammalian salivary gland.

Perhaps the most significant question concerning NGF synthesis is the mechanism by which the factor is delivered to those neurons which depend upon it for growth and survival. The problem is complicated not only by the multiplicity of potential synthesis sites, but also by the possibility of changing physiological sources at different stages of development. The suggestion of a subordinate role for circulating NGF has been put forth (21, 50), and alternate supply routes have been postulated. Mouse (40) and *humans* (51) neuroblastomas can apparently produce the factor, and several workers have emphasized the potential importance of glial cells in NGF production (21, 44, 47, 52), although this has been vigorously challenged (53). We were unable to detect βNGF synthesis in mouse brain or spinal cord (Table III) but have not yet examined synthesis by sensory or sympathetic ganglia. A provocative alternative is the potential involvement of target organs in NGF production. It is clear that NGF can be specifically taken up by both sympathetic (54, 55) and sensory (55) nerve terminals and transported retrogradely back to the cell body, suggesting the possibility of a direct NGF supply between effector organ and innervating neurons. Consistent with this, NGF production has been reported in mouse adrenals (42) and rat irides (40) *in vitro*, although our methods failed to detect synthesis in cultured mouse adrenals or vas deferens (organs which receive sympathetic innervation). The question becomes particularly intriguing in view of the apparent ability of NGF to attract growing sympathetic and sensory fibers both *in vivo* (14, 16) and *in vitro* (15, 17), and it has been proposed that NGF synthesis and release by an effector organ might ultimately determine that organ's density and...
A positive correlation would lend strong support to the concept of NGF as not only an essential growth and maintenance factor for responsive neurons, but also as a critical trophic messenger between target organ and growing axon.

Acknowledgment - We wish to thank Robert Nichols for his expert technical assistance.

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E A Berger and E M Shooter


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