# Nerve Growth Factor: Biosynthetic Products of the Mouse Salivary Glands. Characterization of Stable High Molecular Weight and 32 000-Dalton Nerve Growth Factors†

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ABSTRACT: Nerve growth factor (NGF) is a protein required for the growth and development of sensory and sympathetic neurons. The NGF is present in high concentrations in male mouse salivary glands, bovine seminal plasma, and snake venom. The physiological significance of NGF in these sources is not known: it might be a part of a high molecular weight (HMW) protein with possibly different biological function and be cleaved to the functional size by proteases. In an attempt to isolate a HMW protein containing as part of its structure the low molecular weight (LMW) NGF (2.5S), mouse salivary glands were homogenized in the presence of either 8 M urea or 6 M guanidine hydrochloride (Gdn·HCl) in order to denature proteases. This procedure revealed that the LMW NGF is a part of two HMW proteins that are biologically and immunologically homologous to the mouse 2.5S NGF. One of these HMW proteins ($M_r$ 32 000 NGF) was purified and shown to be biologically active in the NGF bioassay. Furthermore, this $M_r$ 32 000 NGF was cleaved by the $\gamma$ subunit of mouse HMW NGF to the 2.5S NGF. Evidence is also presented that there may be a HMW protein(s) with apparent molecular weights ranging from 94 000 to 200 000 and immunologically homologous to the three subunits ($\alpha$, $\beta$, $\gamma$) of 7S NGF. This HMW NGF is biologically active in the NGF bioassay, and its activity is inhibited by antibody to the $\beta$ subunit. Furthermore, in contrast to mouse 7S NGF, this HMW NGF does not dissociate in either 8 M urea or 6 M Gdn·HCl. These two HMW NGF may have biological functions in the mouse salivary glands other than those that have been attributed to the subunits of 7S NGF.

The nerve growth factor, a protein that regulates the development and normal function of sensory and sympathetic neurons, was originally discovered by Levi-Montalcini and her colleagues (Levi-Montalcini & Angeletti, 1968). It is present at high concentrations in male mouse salivary glands (Varon et al., 1967; Young et al., 1978a), guinea pig prostate glands (Harper et al., 1979), bovine seminal plasma (Harper et al., 1982), and snake venom (Hogue-Angeletti et al., 1976). Several cultured cell lines have been shown to synthesize and secrete NGF into the culture medium (Bradshaw & Young, 1976).

The physiological significance of nerve growth factor (NGF)[1] in nonneuronal sources is not known. The NGF in these sources might be a part of a HMW protein with different biological function(s). In the mouse salivary glands, the NGF is a part of a protein, molecular weight of 116 000–140 000 (7S NGF), that contains three different subunits, $\alpha$, $\beta$, and $\gamma$ (Varon et al., 1967). The 7S NGF is stable at pH 5–8 and at a high protein concentration (e.g., 1 mg/mL). Outside this pH range, and at low protein concentrations (e.g., 1 $\mu$g/mL), the protein is dissociated into its three subunits. The nerve-growth-promoting activity of mouse HMW NGF resides in the $\beta$ subunit, which consists of two identical chains of 118 amino acid residues ($M_r$ 13 259) that are noncovalently bound (Angeletti & Bradshaw, 1971). The $\gamma$ subunit, a serine protease with trypsin-like activity and an approximate molecular weight of 28 000 (233 amino acid residues), consists of two to three polypeptide chains bound by disulfide bridges (Thomas et al., 1981). The $\alpha$ subunit ($M_r$ 26 000), which does not have any known biological activity, is composed of two polypeptide chains bound by disulfide bridges (Stach et al., 1980). It has been suggested that the $\gamma$ subunit acts as a processing enzyme to cleave the precursor of $\beta$ (Pro-$\beta$) to the $\beta$ subunit and that the binary complex of $\gamma$ and $\beta$ subunits then binds to the $\alpha$ subunit to form 7S NGF (Berger & Shooter, 1977). Using in vitro translation of mouse salivary gland messenger RNA, these authors demonstrated that the Pro-$\beta$ had a molecular weight of 22 000 and the $\beta$ subunit was located at the amino-terminal of the Pro-$\beta$. Berger and Shooter also showed that other proteases such as trypsin and EGF-binding protein, may process the Pro-$\beta$ to the $\beta$ subunit. The $M_r$ 22 000 NGF has never been isolated from mouse salivary gland, and it is not known if Pro-$\beta$ is active in the NGF bioassay. Recently, Scott et al. (1983a) analyzed mouse salivary gland DNA and isolated a segment of DNA that coded for a protein ($M_r$ 30 000–32 000) which contained the entire sequence of the $\beta$ subunit. According to these authors, the $\beta$ subunit was located at the carboxy-terminal of the Pro-$\beta$.

The mouse salivary glands are rich in proteases that may be involved in the processing of any HMW protein in this

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[1] Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; HMW, high molecular weight; LMW, low molecular weight; Gdn·HCl, guanidine hydrochloride; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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source (Barka, 1980). It is known that these proteases are involved in the processing of both amino- and carboxy-terminal ends of the $\beta$ subunit when this subunit is not associated with other subunits in the form of 7S NGF (Moore et al., 1974; Mobley et al., 1976).

In order to isolate a naturally occurring HMW NGF, salivary glands were homogenized in the presence of either 8 M urea or 6 M Gdn·HCl, thereby denaturing most proteases, and the denatured samples were applied to gel filtration columns (Sepharose 6B) to separate the LMW proteases from the HMW proteins. By doing so, we were able to show that the $\beta$ subunit was a part of two HMW proteins. One of these HMW NGF, with the molecular weight of 32 000, was purified and shown to be immunologically and biologically homologous to the $\beta$ subunit. The other HMW NGF, which, in contrast to mouse 7S NGF, does not dissociate in either 8 M urea or 6 M Gdn·HCl, is immunologically and biologically homologous to the three subunits ($\alpha$, $\beta$, $\gamma$) of mouse NGF.

## Materials and Methods

Urea and SDS were from Pierce Chemical Co. Sepharose CL-6B, aldolase, ribonuclease A, ovalbumin, chymotrypsinogen A, and blue dextran 2000 were from Pharmacia Fine Chemicals. Carboxymethylcellulose was the product of Whatman. Purified collagen (Vitrogen 100), rabbit serum, heat-inactivated calf serum, and tissue culture media were from Flow Laboratories. All other reagents were of the highest purity commercially available. Unless otherwise specified, all protein manipulations were carried out at 4 °C. Protein concentrations were determined by measuring absorbance at 280 nm for protein with known extinction coefficient, or, for crude homogenate, by the method of Lowry et al. (1951).

*Animals.* All mice used in this study were adult male, CD-1 outbred albino, retired breeders (from Charles River Breeding Laboratories, Wilmington, MA).

*Isolation of HMW NGF and $\alpha$, $\beta$, and $\gamma$ Subunits from the Mouse Salivary Glands.* High molecular weight NGF was purified by the method of Young et al. (1978a). The $\alpha$, $\beta$, and $\gamma$ subunits were purified from HMW NGF by the method of Smith et al. (1968). If the HMW NGF and the subunits were judged to be pure by electrophoretic criteria (the isoelectric focusing gels, SDS–polyacrylamide gels, and 10% polyacrylamide gels at neutral pH), they were then divided into 100–200-$\mu$g aliquots and kept frozen until used.

*Homogenization of the Mouse Salivary Glands in the Presence of either 8 M Urea or 6 M Gdn·HCl.* All protein treatments with 8 M urea or 6 M Gdn·HCl were carried out at room temperature. The mouse salivary glands were homogenized in a motor-driven Teflon pestle tissue grinder in the presence of either 8 M urea or 6 M Gdn·HCl. The homogenate was centrifuged at 28000*g* for 30 min and the resulting supernatant was dialyzed against either 8 M urea or 6 M Gdn·HCl, centrifuged, and applied to a column of Sepharose 6B equilibrated with the same denaturant. Weight rather than volume was used to measure the elution position. Details are given in the legend of each figure. The column fractions to be renatured for radioimmunoassays were dialyzed against 1 L of 0.2 M sodium acetate, pH 5.0, containing 0.5 M NaCl, and then against 0.2 and 0.1 M potassium phosphate, pH 7.0, successively. The column protein peaks to be renatured and used for the next step of protein purification were dialyzed first against 1 L of 0.2M sodium acetate, pH 5.0, containing 0.5 M NaCl and then sequentially against 0.2 M, 0.05 M, and 1 mM Tris-HCl buffer, pH 7.5, before lyophilization.

*Gel Electrophoresis.* Electrophoresis in the polyacrylamide gels at neutral pH was according to Weber and Osborn (1969) with some modifications which were described in the previous report (Young et al., 1978a). The SDS–polyacrylamide gel electrophoresis was done by the method of Laemmli (1970). Molecular weight determination of the proteins, using SDS–polyacrylamide gels, was determined from a calibration curve, log molecular weight vs. $R_f$ (relative mobility), which was established by using "The LMW Calibration Kit" from Pharmacia. The isoelectric focusing gels were done by the method of O'Farrell (1975) and Perez-Polo and Shooter (1974) with some modifications. The lower reservoir buffer was 3 mM NaOH, and the upper reservoir buffer was 3 mM HCl. Gels of 30% acrylamide, 40% Bio-lyte 3/10, and stock riboflavin solution were cast in 5 × 100 mm glass tubes. Appropriate volumes of these solutions were mixed with powdered twice-crystallized urea and $H_2O$ to prepare gels that contained 7.5% acrylamide, 8 M urea, 2% Bio-Lyte 3/10, 0.000175% riboflavin, and 0.025% TEMED (*N,N,N'N'*-tetramethylethylenediamine). The proteins were dissolved in upper reservoir buffer containing 8 M urea. Electrophoresis was done with the negative electrode at the bottom of the gel at a constant voltage of 150 V for 1 h and subsequently 300 V for 8 h at 4 °C. After electrophoresis, the gels were fixed in 20% trichloroacetic acid for 1 h, stained overnight in staining solution [0.25% (w/v) Coomassie brilliant blue, 40% (v/v) methanol, and 10% (v/v) acetic acid], destained in 40% (v/v) methanol and 10% (v/v) acetic acid, and kept in 7.5% (v/v) acetic acid.

*Radioimmunoassay.* The radioimmunoassays used in these studies were for 2.5S[2] and HMW NGF, $\alpha$ and $\gamma$ subunits. Each of these samples was purified and labeled with $^{125}I$ by the method of Young et al. (1978b). Antibody to each of these proteins was prepared in rabbits as follows: 200 $\mu$g of each protein in 0.4 mL of complete Freund's adjuvant was injected subcutaneously at multiple sites. The animals were boosted 4 weeks later with 200 $\mu$g of protein, injected in the same fashion in incomplete Freund's adjuvant; this boost was repeated after 5 weeks, and the animals were bled the following week. The antisera prepared this way were specific and quantitatively bound $^{125}I$-NGF, $^{125}I$ $\alpha$ subunit, or $^{125}I$ $\gamma$ subunit at the final dilution of $1 \times 10^4$. The details of the radioimmunoassays are described by Young et al. (1978b). The protein samples for radioimmunoassays were previously dialyzed against 0.1 M $KH_2PO_4$, pH 7.0.

*NGF Bioassay.* The stimulation of nerve fiber outgrowth from explanted chick sensory and sympathetic ganglia forms the basis of the NGF bioassay. The bioassay followed the method of Murphy et al. (1975) with some modifications. Three to four dorsal root ganglia from a chick embryo (9-day gestation) were dissected and placed on collagen-coated coverslips in a small Petri dish. A nutrient culture medium (medium 199), supplemented with 10% heat-inactivated calf or rabbit serum, was added to the control ganglia. An identical solution containing different concentrations of NGF was added to the experimental ganglia. The preparations were evaluated microscopically after incubation for 18–24 h in a humidified atmosphere containing 5% $CO_2$ at 37 °C.

## Results

In order to prevent proteolytic cleavage, the mouse salivary glands were homogenized in the presence of either 8 M urea or 6 M Gdn·HCl. The NGF-containing extract was applied to a column of Sepharose 6B equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing either 8 M urea (urea–

[2] The 2.5S NGF differs from the $\beta$ subunit in that some proteolytic cleavages have occurred at both ends of the $\beta$ subunit to give rise to the 2.5S NGF.

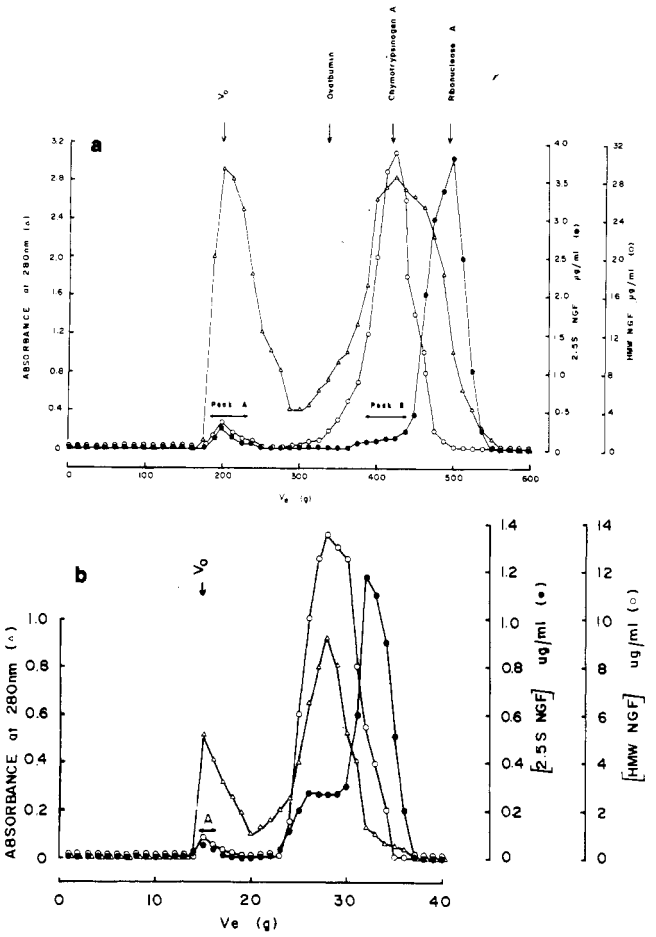


FIGURE 1: Sepharose 6B column chromatography of the mouse salivary glands homogenate. In panel a, the salivary glands from 20 mice were homogenized in 20 mL of 8 M urea in 50 mM sodium acetate buffer, pH 5.0, centrifuged at 28000*g* for 30 min, dialyzed against 50 mL of this buffer for 12 h, and recentrifuged, and the resulting supernatant was applied to a column of Sepharose 6B (2.5 × 88 cm) equilibrated with urea–acetate buffer and calibrated with the standard molecular weight proteins. The flow rate was 12 mL/h. The fraction volume was 2–3 mL. The column fractions were monitored by absorbance (280 nm) and 2.5S and HMW NGF radioimmunoassays. The arrows show the elution volumes of blue dextran 2000 and the standard molecular weight proteins (ovalbumin, ribonuclease A, and chymotrypsinogen A) eluted from this column. The two HMW NGF peaks, A and B, are marked in this figure. In panel b, the salivary glands from one mouse were homogenized in 1 mL of 6 M Gdn·HCl in 50 mM sodium acetate buffer, pH 5.0. The homogenate was centrifuged at 28000*g* for 30 min and dialyzed against 50 mL of guanidine–acetate buffer for 10 h. It was then diluted 1:5 with guanidine–acetate buffer, and 1 mL was applied to a column of Sepharose 6B (1 × 47 cm) equilibrated with this buffer. The flow rate was 6 mL/h. The fraction volume was approximately 1 mL. The arrow shows the elution volume of 1 mg/mL blue dextran, in equilibrating buffer, eluted from this column. NGF peak A eluted in the excluded volume of this column is marked in this figure.

acetate buffer) or 6 M Gdn·HCl and calibrated with the standard molecular weight proteins ovalbumin ($M_r$ 45 000), chymotrypsinogen A ($M_r$ 25 000), and ribonuclease A ($M_r$ 13 700) and with blue dextran 2000. The results of these experiments are shown in Figure 1a,b.

There are two factors in the equilibrating buffer of the columns in Figure 1: both the 8 M urea or 6 M Gdn·HCl as denaturants and the acidic pH, 5.0, which cause the 7S NGF to be dissociated into its subunits. The majority (85–90%) of the immunoreactive NGF was eluted as LMW proteins ($M_r$ 13 000–25 000). The remainder (10–15%) of the total NGF was eluted in two protein peaks which had higher molecular weights than the subunits of NGF. There was a NGF protein

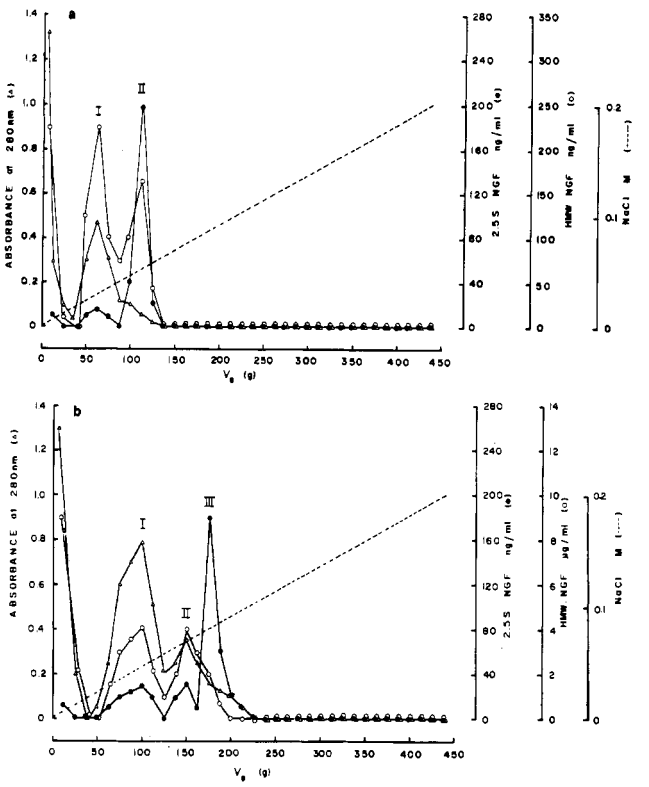


FIGURE 2: Carboxymethylcellulose column chromatography of NGF peaks A and B from the column of Sepharose 6B of Figure 1a. In panel a, NGF peak A of the column of Sepharose 6B ($V_e$ of 190–260 g) of Figure 1a was pooled, dialyzed against 500 mL of sodium acetate buffer, pH 5.0, containing 8 M urea, and centrifuged at 28000*g* for 30 min. The resulting supernatant was applied to a column of carboxymethylcellulose (1.5 × 42 cm) equilibrated with urea–acetate buffer. The flow rate was 10 mL/h. The fraction volume was 1–2 mL. After the column was loaded, a linear salt gradient of 0–0.2 M NaCl (200 × 200 mL) was begun, and the column fractions were monitored by absorbance (280 nm) and 2.5S and HMW NGF radioimmunoassays. In panel b, the fractions corresponding to NGF peak B of the column of Sepharose 6B ($V_e$ of 375–425 g) of Figure 1a were combined, dialyzed against 500 mL of urea–acetate buffer for 12 h, and analyzed as in panel a.

peak ("NGF peak A") eluted in the excluded volume of the column. The 2.5S NGF radioimmunoassay peak showed, in addition to the 2.5S NGF peak, a shoulder with a molecular weight of 30 000–32 000. This was called "NGF peak B". These two NGF peaks (NGF peak A and NGF peak B) were further analyzed in order to isolate HMW NGF.

*Analysis of HMW NGF in NGF Peak A of the Column in Figure 1a.* The fractions corresponding to NGF peak A from the column of Sepharose 6B in Figure 1a were pooled and applied to a column of carboxymethylcellulose equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 8 M urea. After the column was loaded, a linear salt gradient of 0–0.2 M NaCl was begun. There was one protein peak and two immunologically identified NGF peaks in the elution profile of this column (Figure 2a). The fractions corresponding to NGF peak I (which had higher protein concentration than peak II) in Figure 2a were pooled, renatured by dialysis against buffer without urea, lyophilized, and tested in the NGF bioassay and the radioimmunoassays for the $\alpha$, $\beta$, and $\gamma$ subunits and HMW NGF. The sample had nerve-growth-promoting activity in the NGF bioassay, and its activity was blocked by the anti-2.5S NGF (data not shown). The protein in this peak was immunologically reactive in the $\alpha$, $\beta$, and $\gamma$ subunits and the HMW NGF radioimmunoassays (Table I).

Analysis of NGF peak A of Figure 1a,b shows that it is

Table I: Immunoreactivity of HMW NGF in the NGF Peak I of the Column in Figure 2a in the HMW NGF and $\alpha$, $\beta$, and $\gamma$ Subunit Radioimmunoassay[a]

| radioimmunoassay | amounts (ng/mL) |
|---|---|
| HMW NGF | 237 |
| $\alpha$ subunit | 36 |
| $\beta$ subunit | 22 |
| $\gamma$ subunit | 39 |

[a] The NGF peak I of the carboxymethylcellulose column in Figure 2a (1 mL) was renatured by dialysis against buffer without urea. The sample (with the total protein concentration of approximately 100 $\mu$g/mL) was used for HMW NGF, $\alpha$, $\beta$, and $\gamma$ subunit radioimmunoassays.

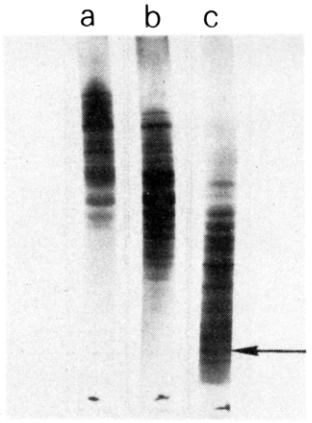


FIGURE 3: Isoelectric focusing gel pattern of NGF peaks I, II, and III from the column of carboxymethylcellulose in Figure 2b. The fractions corresponding to each of the three NGF peaks from the column of carboxymethylcellulose in Figure 2b were individually pooled, renatured by dialysis against buffer without urea, and lyophilized. The dry powder of each peak was dissolved in 5 mL of 50 mM Tris-HCl buffer, pH 7.5. One hundred micrograms of each protein peak was applied to the three (a, peak I; b, II; c, III) isoelectric focusing gels (7.5%). The pH increases from top to the bottom of the gels. The arrow shows the position of the protein band most reactive in the 2.5S NGF radioimmunoassay.

degraded to the subunits ($\alpha$, $\beta$, $\gamma$) of HMW NGF with the formation of an intermediate precursor ($M_r$ of ~70 000) when dialyzed against buffer without 8 M urea or 6 M Gdn·HCl (Saboori, 1984).

*Analysis of HMW NGF in NGF Peak B in the Column of Figure 1a.* The fractions corresponding to NGF peak B from the column of Figure 1a were pooled, dialyzed against urea–acetate buffer, and applied to a column of carboxymethylcellulose equilibrated with this buffer. This column resolved the sample into two protein and three immunoreactive NGF peaks, I, II, and III (Figure 2b). When these three NGF peaks were analyzed on isoelectric focusing gels, the NGF peaks I–III contained respectively mostly acidic, neutral, and basic proteins (Figure 3). Of these three NGF peaks, peak III was more reactive in the 2.5S NGF radioimmunoassay than peaks I and II, and therefore, it was pooled and renatured by dialysis against buffer without urea for further analysis. One hundred micrograms of the NGF peak III was applied to each of the two isoelectric focusing gels; after focusing, one gel was stained as a guide, and the other was sliced; individual slices were eluted and used for the 2.5S NGF radioimmunoassay. Of all the protein bands in Figure 3c, only the one marked with an arrow was reactive in the 2.5S NGF radioimmunoassay. In order to find out if the radioimmunoassayable protein band in the NGF peak III had the same isoelectric point as the 2.5S NGF, the latter protein (50 $\mu$g) was added to the NGF peak III (50 $\mu$g), and the isoelectric focusing experiment was repeated. When the individual gel slices were eluted and used for the 2.5S NGF radioimmunoassay, it was found that the NGF in peak III was more acidic than the 2.5S NGF (data not shown).

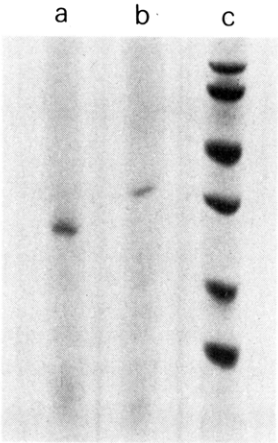


FIGURE 4: SDS–polyacrylamide gel pattern of the NGF protein band eluted from the isoelectric focusing gel in Figure 3c. NGF peak III of the column of carboxymethylcellulose in Figure 2b (100 $\mu$g) was loaded onto each of six isoelectric focusing gels (7.5%). After electrophoresis, one gel was stained as a guide. The other five gels were sliced, and the sections which contained NGF were combined and eluted. The eluted protein (20 $\mu$g) was denatured with SDS in the absence (a) and presence (b) of $\beta$-mercaptoethanol. These samples with the standard molecular weight proteins were loaded separately onto the SDS–polyacrylamide gels (11.25%). Column c shows the gel pattern of the standard molecular weight proteins (LMW Calibration Kit, from Pharmacia). Migration was from top to the bottom (anode) of the gels.

NGF peak III of column in Figure 2b was renatured by dialysis against buffer without urea and applied to another column of carboxymethylcellulose (in the absence of urea). The elution profile of this column showed that this NGF peak was eluted at 0.2 M NaCl (data not shown). When the 2.5S NGF was applied to this column and eluted with the same salt gradient as that used for NGF peak III in Figure 2b, 2.5S NGF was eluted at 0.5 M NaCl (data not shown). These experiments show that NGF peak III is eluted at a lower salt concentration (0.2 instead of 0.5 M NaCl) than the 2.5S NGF from the column of carboxymethylcellulose in the absence of 8 M urea. Thus, both ion-exchange chromatography and isoelectric focusing gel electrophoresis (Figure 3c) demonstrate that the NGF in peak III of Figure 2b is more acidic than 2.5S NGF.

The impurities in the main NGF peak of the column in Figure 2b (peak III) were not removed by repeating the chromatography on a carboxymethylcellulose column in the absence of urea. Hence, the NGF in this peak was eluted from the isoelectric focusing gels and used for the biochemical analysis. In order to determine the molecular weight of this protein, it was denatured with SDS in the presence and absence of $\beta$-mercaptoethanol and analyzed on SDS–polyacrylamide gels. The results are shown in Figure 4. When the sample was not reduced with $\beta$-mercaptoethanol, the gel pattern showed a single protein band with an approximate molecular weight of 25 000, but when reduced, two protein bands with approximate molecular weights of 32 000 and 22 000. This form of NGF is more acidic than the 2.5S NGF (see above) and has a higher molecular weight than the latter protein ($M_r$ 13 259).

To examine the possibility that the 2.5S NGF was generated by enzymatic cleavage of the $M_r$ 32 000 precursor in mouse salivary glands, it was decided to incubate $M_r$ 32 000 NGF with different concentrations of $\gamma$ subunit and at different incubation times at 37 °C. After incubation, the mixture of

the two proteins, with the nonincubated (control) sample, was applied separately to the isoelectric focusing gels to observe possible changes in the gel pattern of the incubated sample in comparison with that of the control. Incubation of the $M_r$ 32000 protein (at concentration of 120 $\mu$g/mL) with the catalytic amounts of the $\gamma$ subunit (4 $\mu$g/mL) for 2 h at 37 °C caused very little difference in the isoelectric focusing gel pattern of the incubated sample from that of the control. When equal amounts of both proteins (M/M) were used, two protein bands (marked a and b), which were absent in the control sample (Figure 5a, gel A), appeared in the gel pattern of the incubated one (Figure 5a, gel B). The appearance of these two new protein bands coincides with a decrease in the intensity of the stained band of the control sample (which is marked c in Figure 5a, gel A). These changes were not observed when the $\gamma$ subunit was incubated alone at the same concentration and incubation time (Figure 5a, gel C). The protein band a had the same p*I* as the $\alpha$ subunit of 7S NGF, and band b had the same p*I* as the $\beta$ subunit.

The enzymatic effect of the $\gamma$ subunit on the $M_r$ 32000 NGF was confirmed when $M_r$ 32000 NGF was labeled with $^{125}$I and the experiment of Figure 5a was repeated. The results of the control and incubated samples are shown in Figure 5b. After 2 h of incubation at 37 °C, approximately 50% of the labeled protein was cleaved specifically by the $\gamma$ subunit to two new protein bands. These two new protein bands were analyzed on SDS–polyacrylamide gels, the gels were sliced, and $^{125}$I counts in the gel slices were determined. These protein bands have approximate molecular weights of 19000 and 13000 (data not shown).

In order to determine whether the effect of $\gamma$ subunit on the $M_r$ 32000 NGF was specific, trypsin and EGF-binding protein were used instead of the $\gamma$ subunit in the experiment of Figure 5b. The EGF-binding protein is a part of HMW EGF, isolated from mouse salivary glands (Carpenter & Cohen, 1979). After incubation of the $M_r$ 32000 NGF for 2 h at 37 °C, the labeled protein was completely degraded by trypsin into at least five peptides and was partially (50%) degraded by the EGF-binding protein into at least seven peptides (data not shown). Thus, in contrast to the effect of the $\gamma$ subunit on the $M_r$ 32000 NGF, trypsin and EGF-binding protein cleaved the $M_r$ 32000 NGF into at least five peptides and did not generate the two specific bands.

Protein bands a and b from Figure 5a were eluted from an isoelectric focusing gel and examined in the NGF bioassay and the 2.5S NGF and $\alpha$ subunit radioimmunoassays. Protein band a was not reactive in any of the radioimmunoassays and did not promote nerve fiber outgrowth in the NGF bioassay. Protein bands b and c were both positive in the NGF bioassay, and their effects in this assay were blocked by anti-2.5S NGF (Figure 6). Compared to 2.5S NGF, protein band c was 10 times less reactive in the NGF bioassay and 100 times less reactive in the 2.5S NGF radioimmunoassay.

*Amino Acid Sequence of $M_r$ 32000 NGF.* To further characterize the $M_r$ 32000 NGF, the amino acid sequence at the amino-terminal end of this protein was determined by the method of Chang et al. (1978). The sequence was found to be $NH_2$-Ala-Val-Gln-Gly-Ala-.

## Discussion

Mouse salivary glands contain an unusually high concentration of a number of growth factors, including NGF and EGF (Barka, 1980). These growth factors may be parts of larger proteins with other possible physiological functions. The HMW precursors of NGF and EGF have been detected by analysis of cloned mouse salivary gland DNA (Scott et al., 1983a,b; Ullrich et al., 1983; Gray et al., 1983) and in vitro translation of mouse salivary gland mRNA (Berger & Shooter, 1977; Frey et al., 1979). However, the quantity of the growth factor precursors which can be obtained from transcription and translation of cloned DNA may not be sufficient for biochemical analysis unless the corresponding genes are first purified and then inserted into plasmid vectors for amplification of the gene(s).

We devised a biochemical method in which the precursor of these growth factors, especially of NGF, could be isolated in large quantities. In this method, the mouse salivary gland proteases, which might be involved in the processing of large proteins into LMW growth factors, were denatured with either

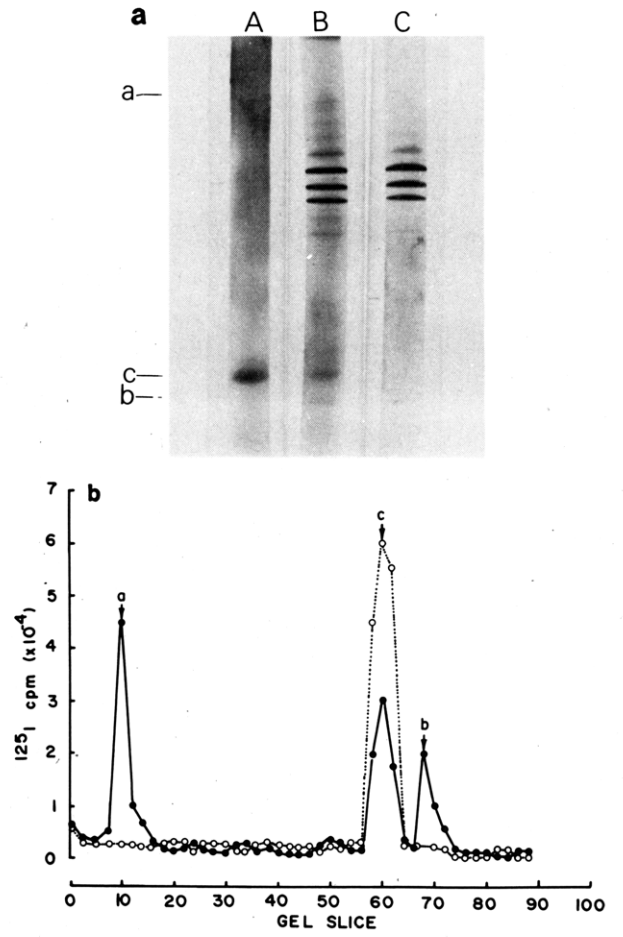


FIGURE 5: Isoelectric focusing gel pattern of the control $M_r$ 32000 NGF, $\gamma$ subunit incubated with $M_r$ 32000 NGF, and $\gamma$ subunit alone. In panel a, $M_r$ 32000 NGF was eluted from the isoelectric focusing gels, and 30 $\mu$g of this protein without (gel A) or with (gel B) the $\gamma$ subunit (30 $\mu$g) of 7S NGF was added to 250 $\mu$L of 50 mM Tris-HCl buffer, pH 7.5, and incubated at 37 °C for 2 h. In gel C, 30 $\mu$g of $\gamma$ subunit was added to 250 $\mu$L of Tris buffer and incubated with the above samples. After incubation, each sample was lyophilized, redissolved in 100 $\mu$L of 3 mM HCl containing 8 M urea, and applied to the isoelectric focusing gels (7.5%). The pH increases from top to the bottom of the gels. In panel b, the $M_r$ 32000 NGF was eluted from isoelectric focusing gels, and 30 $\mu$g of protein was labeled with $^{125}$I by the method of Young et al. (1978b). The labeled $M_r$ 32000 NGF was diluted 1:100 with unlabeled protein before use. The diluted protein (60 $\mu$g) was added to 60 $\mu$g of the $\gamma$ subunit in 500 $\mu$L of 50 mM Tris-HCl buffer, pH 7.5. Two hundred fifty microliters of the sample was kept at −20 °C as a control, and the other 250 $\mu$L was incubated at 37 °C for 2 h. After incubation, both samples were lyophilized, redissolved in 100 $\mu$L of 3 mM HCl containing 8 M urea, and applied to an isoelectric focusing gel. After electrophoresis, each gel was sliced, and $^{125}$I counts in the gel slices of the control (○) and incubated (●) samples were determined. The radioactive peaks a–c have the same isoelectric point as that of protein bands a–c in panel a. The pH increases from left to the right of the graph.

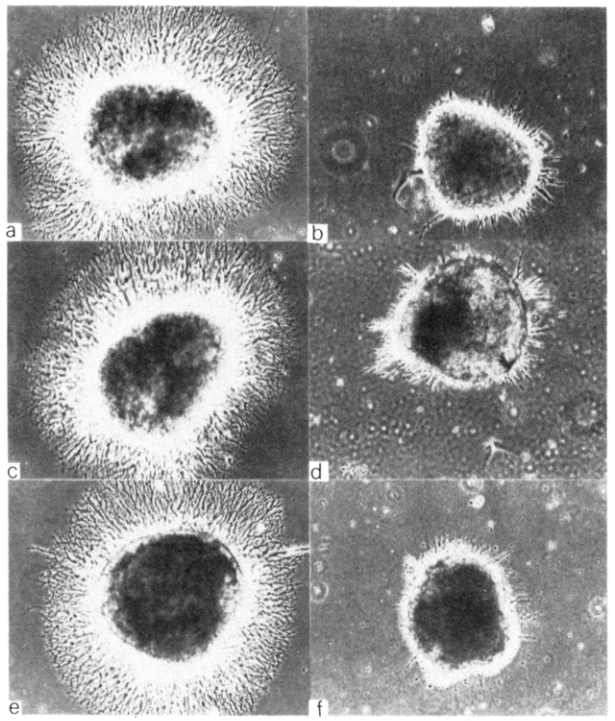


FIGURE 6: Nerve-growth-promoting activities of 2.5S NGF and the eluted protein bands b and c in Figure 5a. The nerve-growth-promoting effects of the standard (2.5S NGF) and the eluted protein bands b and c from the isoelectric focusing gel in Figure 5a on 9-day-old chick embryo ganglia were determined. Part a shows the response of the chick ganglion to 10 ng/mL 2.5S NGF as a standard; part b, control (no NGF is added); part c, 100 ng/mL NGF (as determined by the 2.5S NGF radioimmunoassay) of the eluted protein band c; part d, the same as part c but with added anti-2.5S NGF; part e, 15 ng/mL eluted protein band b; part f, the same as part e but with added anti-2.5S NGF. In parts d and f, 10% rabbit anti-2.5S NGF was added to the culture medium instead of the normal rabbit serum in other samples.

8 M urea or 6 M Gdn·HCl in 50 mM sodium acetate buffer, pH 5.0. These denatured proteases were then separated from HMW proteins by gel filtration columns equilibrated with either 8 M urea or 6 M Gdn·HCl. In the case of NGF, the other advantage of the equilibrating buffer of the column (8 M urea or 6 M Gdn·HCl and pH 5.0) is to dissociate 7S NGF into its LMW subunits ($\alpha$, $\beta$, and $\gamma$).

The experimental data presented indicated that the subunits of 7S NGF are components of a HMW protein with a molecular weight in the range 94000–200000. This HMW NGF, in contrast to the mouse 7S NGF, does not dissociate in either 8 M urea or 6 M Gdn·HCl (Figure 1a,b). It is reactive in the HMW NGF, $\alpha$, $\beta$, and $\gamma$ subunit radioimmunoassay (Table I). It is also active in the NGF bioassay, and its activity is blocked by the 2.5S NGF antibody. Analysis of NGF peak A of Figure 1a,b shows that it is degraded to the subunits ($\alpha$, $\beta$, and $\gamma$) of HMW NGF with the formation of an intermediate precursor ($M_r$ ~70000) when dialyzed against buffer without denaturant. Most proteases are known to be denatured or inactivated by 6 M Gdn·HCl, and these proteases might have become activated when the sample was dialyzed against buffer without this denaturant.

Although the NGF from NGF peak A of Figure 1a,b has not been purified, the experimental data presented in this report show that it is possible the subunits of 7S NGF ($\alpha$, $\beta$, and $\gamma$) are cleaved enzymatically from this HMW protein during purification processes.

A $M_r$ 32000 NGF is isolated from NGF peak B of Figure 1a which is reactive in the 2.5S NGF radioimmunoassay. This form of NGF is more acidic than 2.5S NGF (Figure 3) and can be cleaved by the $\gamma$ subunit of HMW NGF into 2.5S NGF (Figure 5a,b). $M_r$ 32000 NGF was cleaved nonspecifically into at least five peptides by trypsin and EGF-binding protein. The current studies have physically isolated a $M_r$ 32000 NGF. This protein has the biochemical properties of the Pro-NGF predicted from the nucleotide sequencing of Scott et al. (1983a).

The amino acid sequence at the amino-terminal end of the Pro-NGF isolated in this report was determined and found to be $NH_2$-Ala-Val-Gln-Gly-Ala. The amino acid sequence of Pro-NGF (deduced from DNA sequence) presented by Scott et al. (1983a) also contains this sequence between $Arg^{27}$ and $Gly^{33}$. The pro-NGF isolated in this report contains 27 amino acid residues less than the one reported by Scott et al. (1983a). Most secretory proteins contain a signal peptide of approximately 30 amino acids at their amino-terminal end (Steiner et al., 1980). The NGF may be considered a secretory protein, and the 27 amino acid residues at the amino-terminal end may be the signal peptide proteolytically cleaved during NGF secretion.

The $\gamma$ subunit, which cleaved the $M_r$ 32000 NGF in the experiments of Figure 5a,b, was at a very high concentration (1 mol of $\gamma$ subunit/mol of $M_r$ 32000 NGF was used in these experiments). Because of this, another conclusion from the data might be that the $\gamma$ subunit was not acting as a processing enzyme. In support of this contention is the fact that most processing enzymes are membrane-bound (Kenny, 1977), whereas the $\gamma$ subunit is not. If this conclusion is correct, the question would be, what is the role of the two subunits ($\gamma$ and $\beta$) in the HMW NGF?

The physiological significance of NGF in the male mouse salivary glands and other sources which contain a high concentration of this protein (such as snake venom and bovine seminal plasma) is not known. The experimental data presented in this report show that the LMW NGF is a part of HMW protein(s) in the male mouse salivary glands. This may be true for other sources of NGF which do not play a major role in the nervous system, and the LMW NGF in these sources may be cleaved enzymatically from a larger protein.

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**Registry No.** NGF, 9061-61-4; pro-NGF, 86924-00-7.

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# Stereochemical Course of Thiophosphoryl Transfer Catalyzed by Cytosolic Phosphoenolpyruvate Carboxykinase†

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ABSTRACT: Rat liver cytosolic phosphoenolpyruvate carboxykinase (PEPCK) utilizes inosine 5′-(3-thiotriphosphate) (ITPγS) as an excellent substrate, with $K_m$ and $V$ values of 0.08 mM and 37 μmol min$^{-1}$ (mg of protein)$^{-1}$, respectively, compared with the corresponding values of 0.168 mM and 76 μmol min$^{-1}$ (mg of protein)$^{-1}$ for ITP. Thus, the $V/K_m$ values for the two substrates are the same. Reaction of ($R_P$)-[γ-$^{18}O_2$]ITPγS with oxalacetate catalyzed by cytosolic PEPCK produces ($S_P$)-thio[$^{18}$O]phosphoenolpyruvate. Therefore, thiophosphoryl transfer catalyzed by this enzyme proceeds with overall inversion of configuration at P. The reaction mechanism involves an uneven number of phosphotransfer steps, most likely a single step transfer between bound substrates. The results do not support the involvement of a phosphoryl enzyme intermediate in the mechanism.

Mammalian liver phosphoenolpyruvate carboxykinase (PEPCK)[1] is found in two cellular compartments, the mitochondria and the cytosol. Notwithstanding similarities between them, the cytosolic and mitochondrial forms are known to be different enzymes (Tilghman et al., 1976). The two forms are immunochemically distinct (Ballard & Hanson, 1969) and are encorded by different mRNA species transcribed from different genes (Hod et al., 1982).

The stereochemical course of phosphoryl transfer catalyzed by guinea pig liver mitochondrial PEPCK was previously investigated by Sheu et al. (1984) and found to proceed with inversion of configuration. This was interpreted to indicate that the phosphoryl group is probably transferred directly between the substrates by a single step, nucleophilic displacement mechanism. As for the cytosolic enzyme, it has been reported that rat liver cytosolic PEPCK catalyzes the

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[1] Abbreviations: PEPCK, phosphoenolpyruvate carboxykinase; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; IMP, inosine 5′-phosphate; ITPγS, inosine 5′-(3-thiotriphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); ADPβS, adenosine 5′-(2-thiodiphosphate); ADPαS, adenosine 5′-(1-thiodiphosphate); GTP, guanosine 5′-triphosphate; ITP, inosine 5′-triphosphate; NADH, reduced nicotinamide adenine dinucleotide; IDP, inosine 5′-diphosphate; 2′,3′-(methoxymethylidene)-IMP, 2′,3′-(methoxymethylidene)inosine 5′-phosphate; 2′,3′-(methoxymethylidene)-AMP, 2′,3′-(methoxymethylidene)adenosine 5′-phosphate; GC/MS, gas chromatography/mass spectrometry.

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