THE PREPARATION OF NERVE GROWTH FACTOR FROM THE PROSTATE OF THE GUINEA-PIG AND ISOLATION OF IMMUNOGENICALLY PURE MATERIAL FROM THE MOUSE SUBMANDIBULAR GLAND

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1. Introduction

It was recently discovered [1] in these laboratories that the prostate of the guinea-pig is (like the submandibular gland of the adult male mouse) a rich source of nerve growth factor (NGF). We now report, by a modification of the method in [2], the total purification of the factor from this source and describe some of its properties. A similar purification method can also be used to prepare the β-subunit of NGF from mouse submandibular gland free of immunogenic contaminants. We have shown [3] that such contaminants are extremely widespread and may seriously interfere with the interpretation of biochemical and biological experiments.

2. Materials and methods

2.1. *Isolation of immunogenically pure NGF from mouse submandibular gland*

The initial steps were carried out as in [2]. The procedure for the final chromatographic step (CM-cellulose, column dimensions 2.5 X 25 cm) was, however, different: protein was eluted (flow rate 55 ml/h) from the column using Tris–HCl buffer (0.05 M, pH 9.0) then a linear gradient of NaCl ($\leqslant$0.5 M, total vol. 1 litre) was applied. This procedure removed remaining contaminating proteins and immunogenically pure NGF was eluted as the major peak.

2.2. *Isolation of NGF from guinea-pig prostate*

The prostate–coagulating gland complexes were dissected from the animals (Porton Albinos) and stored frozen until used. Samples of tissue (~40 g from 60 guinea-pigs) were thawed as required and homogenised for 1 min in 150 ml of cold distilled water using a Waring Blender. Debris was removed by centrifugation (17 000 X g, 1 h). The supernatant was dialysed twice against sodium phosphate buffer (0.02 M, pH 6.8, 4 litres) over 12 h at 4°C (all subsequent steps were carried out at the same temperature).

The dialysed supernatant was loaded onto a column (2.5 X 40 cm) of CM-cellulose and the column was eluted with the sodium phosphate buffer (flow rate 50 ml/h) until the absorbance of the eluate fell to <0.5. The eluate was dialysed twice against very dilute sodium phosphate buffer (0.25 mM, pH 6.8, 4 litres) over 24 h. Sufficient sodium citrate buffer (1.0 M, pH 3.0) was added to give final buffer conc. 0.1 M. This dissociates the aggregate of proteins containing NGF (see section 4). Solid NaCl was then added to 0.4 M. The solution was allowed to stand for 5 min and precipitated material removed by centrifugation (5000 X g, 15 min).

The supernatant was then applied to a second column (2.5 X 25 cm), of CM-cellulose equilibrated with sodium citrate buffer (0.1 M, pH 3.0) containing 0.4 M NaCl. Elution (flow rate 55 ml/h) was carried out with this buffer and the eluate discarded. The column was then eluted with Tris–HCl buffer

(0.05 M, pH 9.0) until no more absorbing material appeared in the eluate. The final step was the application of a linear gradient of NaCl (≤0.5 M, total vol. 1 litre) using the same Tris–HCl buffer. One major peak was obtained and this represented pure NGF.

2.3. *Electrophoresis in polyacrylamide gels*

Isoelectric focussing and disc electrophoresis (at pH 3.8 and pH 5.2) were carried out as in [4].

The method of Laemmli [5] was used for SDS–electrophoresis and 15% crosslinked gels were employed. Samples of ~5 μg NGF were applied to the gels together with 60 μg of a standard mixture (Sigma) containing egg-white lysozyme, bovine-milk β-lactoglobulin, bovine trypsinogen (treated with PMSF), porcine pepsin, ovalbumin, bovine serum albumin and bromophenol blue marker.

2.4. *N-terminal analysis*

Reaction was carried out as in [6]. The dansyl derivatives were identified by thin-layer chromatography as in [7].

2.5. *Immunodiffusion*

Double immunodiffusion was carried out using agar gels as in [3].

2.6. *Assay for NGF*

The standard tissue culture procedure [8] as modified [9] was used. All dilutions were carried out in apparatus made of polystyrene in order to minimise losses due to surface adsorption [9].

2.7. *Preparation of antisera*

Antiserum to pure NGF from guinea-pig prostates was raised in rabbits by the method in [10]. Horse antiserum to mouse-NGF was a gift from the Wellcome Foundation (batch 8849/46).

3. Results and conclusions

We had found [3] that the preparation of the β-subunit to mouse-NGF as described [11,12] does not give an immunogenically pure product. We identified the contaminant as (so-called) γ-globulin [3] and found that it could be readily removed by passage through a column of Sephadex G-75. The new (rapid) method described [2] is considerably more convenient than the earlier procedure but also gives a product containing immunogenic contaminants [3]. We attempted to remove these by passage through Sephadex G-75 but failed to do so. The immunodiffusion plate in fig.1a shows clearly the presence of an impurity. Whatever the substance is, it differs from that found in previous preparations of NGF [3]. We succeeded in removing it, however, by the use of a linear salt gradient in the final chromatographic step (as above). The result of immunodiffusion of the final product is shown in fig.1b. There is no trace of a contaminant and the precipitin band shows the same (unusual) characteristics reported [3] for immunogenically pure mouse-NGF.

Preliminary experiments with NGF from guinea-pig prostate using gel filtration showed that it eluted as a high molecular weight complex (details not given here). This behaviour is the same as that originally

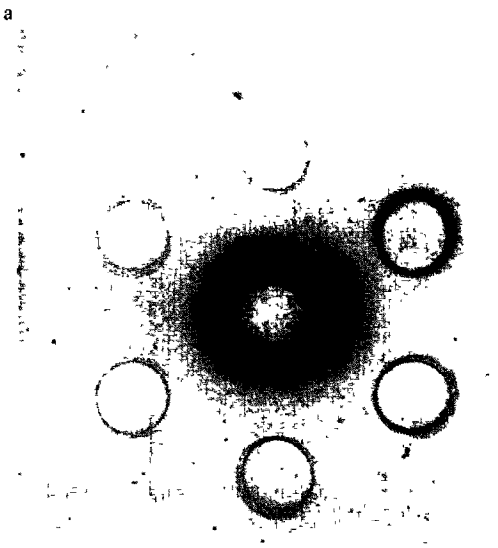
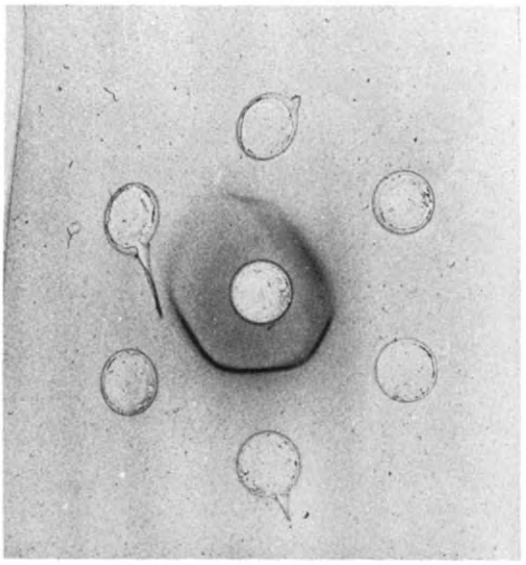


Fig.1. Immunodiffusion studies on nerve growth factors from different sources. In all cases, antiserum (3 μl) was placed in the central well and antigen (3 μl) in the outer wells. Concentration of antigen ranges from 5–0.15 mg/ml or 1–0.03 mg/ml in doubling dilutions with the highest concentrations in the 'two o'clock' position.

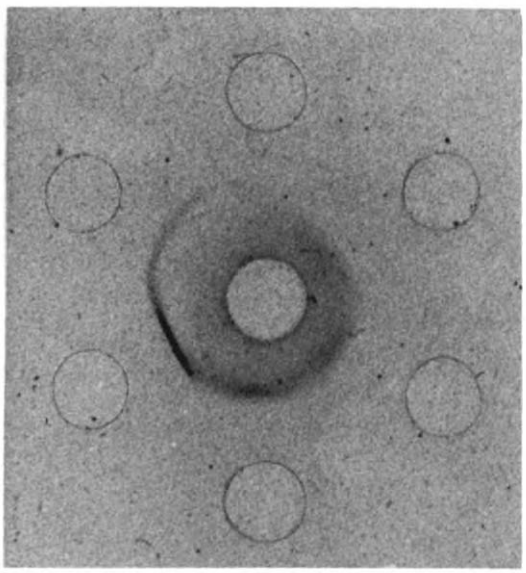
(a) Antigen: NGF prepared from mouse submandibular gland by the method in [2] and passed through a Sephadex G-75 gel filtration column. Antigen: 5–0.15 mg/ml. Antibody: horse antiserum to NGF (Wellcome, batch 8849/46).

b



(b) Antigen: NGF prepared from mouse submandibular gland by the new modified method described here. Antigen: 1–0.03 mg/ml. Antiserum as for (a). At higher concentrations (5–0.15 mg/ml) of this antigen, the precipitin bands become faint and diffuse. No trace of the contaminant seen in (a) was noted.

c



(c) Antigen: NGF prepared from guinea-pig prostate. Antibody: homologous rabbit antiserum. Concentrations as in (b). At higher concentrations, the pattern showed the same characteristics as described in (b).

reported for the mouse-factor [11]. They showed that the complex (from the mouse) consisted of three proteins, one of which is NGF and one of which is an esterase. Consistently, we find that the complex from the guinea-pig also shows esterase activity. However, the complex from the guinea-pig is more stable than that from the mouse and requires more acidic conditions for dissociation (pH 3.0 instead of pH 4.0).

With the above facts in mind we devised a variant of the procedure [2] in which we used:

(a) Sodium citrate buffer at pH 3.0 to achieve dissociation of the complex;
(b) A linear salt gradient in the final chromatographic step to remove immunogenic contaminants.

The yield of purified material was ~3 mg from 40 g glands and the overall recovery was ~35% of the activity in the starting homogenate. The NGF was maximally active at 10^{-7}–10^{-8} g/ml. The product obtained was immunogenically pure: immunodiffusion against rabbit antiserum showed only one precipitin band (fig.1c) with the same unusual characteristics described [3] for the mouse-factor.

The material migrated as a single band on gel electrophoresis both at pH 3.8 and at pH 5.2. The band was, however, somewhat diffuse and this may be due to an adsorption effect similar to that reported for the snake-factor [4].

Gel electrophoresis in polyacrylamide containing SDS gave mol. wt 14 500 ± 500. This value is very close to the known value (by sequence analysis [13]) of the molecular weight for the β-subunit of mouse-NGF. Obviously the two molecules are very similar in size and the preliminary results of amino acid analysis show that they are also very similar in composition.

N-terminal analysis showed serine as the only detectable amino acid residue. This is of interest since the N-terminal residue of native mouse-NGF (uncleaved) is also serine. Isoelectric focussing using a gradient between pH 3 and pH 10 showed the presence of three closely spaced bands with values of pI corresponding to 8.5 ± 0.5. These bands occurred at a slightly less alkaline pH than those observed with the mouse-factor but probably arise in the same way, i.e., by partial cleavage from the C-terminus [2]. A further detailed comparison of the proteins from the two sources is at present in progress and will be of obvious interest.

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