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Properties of the β Nerve Growth Factor Receptor of Avian Dorsal Root Ganglia

(iodination/membrane)

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ABSTRACT **Iodination of the β nerve growth factor protein up to 4.1 iodines per mole of nerve growth factor was achieved with no loss of biological activity. At this level of incorporation no native or monoiodo nerve growth factor remained, the major species being the tri- and tetraiodo derivatives. An ^{125}I-labeled β nerve growth factor of high specific activity containing 0.5 iodine per mole of β nerve growth factor was used to determine the specific binding of nerve growth factor to dorsal root ganglion cells of 8-day-old embryonic chicks. The specific binding of ^{125}I-labeled nerve growth factor reached saturation at 30–50 ng/ml and half-saturation at 7–8 ng/ml (0.26 nM). The number of receptors per responsive medio-dorsal cell was calculated to be about 2×10^4. The pattern of displacement of bound ^{125}I-labeled β nerve growth factor by native β nerve growth factor showed that the two proteins had identical affinities for the receptor. The specificity of the binding for β nerve growth factor was demonstrated by the fact that only native β nerve growth factor displaced the bound ^{125}I-labeled form. Partially inactivated derivatives of β nerve growth factor retained the same fraction of their specific-binding capacity as of their biological activity. The specificity of the binding for cell type was shown by the lack of any specific component of ^{125}I-labeled β nerve growth factor binding to liver or brain cells. The rate constant for association of β nerve growth factor with its receptor, k_1, was 1.0×10^7 mol^{-1} sec^{-1} and the rate constant for dissociation, k_{-1}, 1.2×10^{-3} sec^{-1}.**

Nerve growth factor (NGF) enhances the growth and differentiation of both sensory and sympathetic ganglia (1, 2). Two potent sources for NGF have been discovered, namely snake venom and the mouse submaxillary gland (2), and it was from the first source that Cohen (3, 4) isolated a partially purified NGF preparation and demonstration that NGF was a protein. That NGF plays a role in the normal development of sympathetic ganglia was apparent from the observation (5, 6) that injection of NGF antiserum into newborn animals led to the virtual destruction of the developing sympathetic ganglia.

More recent studies (7) have shown that NGF activity in the submaxillary gland is associated with a large protein complex, 7S NGF. The β subunit (βNGF), which is the subunit possessing nerve growth activity, is made up of two identical peptide chains (8). When isolated from purified 7S NGF, these two chains retain their COOH-terminal arginine residue and NH_2-terminal octapeptide sequences, which are missing from a proportion of the peptide chains in 2.5S NGF (9). The amino-acid sequences of both chains in 2.5S NGF have recently been reported (10).

Although it is known that NGF stimulates most anabolic processes of the responsive cells (2, 11, 12) and promotes neurite outgrowth in the absence of RNA synthesis (13), little is known of how NGF brings about these pleotrophic responses. A possible clue lies in the recent demonstration that peptide hormones such as insulin and glucagon elicit their effects on responsive cells by a mechanism that involves interaction with a specific membrane receptor (14). It seemed reasonable, therefore, to look for NGF receptors in the responsive sensory ganglia. This report describes a method for determining the specific binding of NGF to responsive ganglionic cells as well as some of the characteristics of the βNGF receptor. A brief account of this work has been given (15).

Abbreviations: GBS, Gey's balanced salts solution; PBG, phosphate-buffered Gey's solution; βNGF, β subunit of 7S nerve growth factor.

MATERIALS AND METHODS

Nerve Growth Factor and Other Proteins. The protein species used in all of these studies was the β subunit of 7S NGF isolated by the methods described by Varon, Nomura and Shooter (7) and Smith, Varon, and Shooter (16). Lactoperoxidase, a generous gift of Dr. Donna Arndt, was stored at a concentration of 55 μM at −20°. Collagen was extracted from rat-tail tendons and used to coat 22 × 22-mm glass coverslips by a modified form of the method of Ehrman and Gey (17) as adapted by Bornstein (18). Proinsulin was a gift of Dr. Donald Steiner.

Chemicals and Buffers. Radiolabeled iodine (^{125}I) was obtained from New England Nuclear Corp. Gey's balanced salt solution (GBS) was prepared according to the published formula (19). The incubation buffer for binding assays consisted of a 25 mM phosphate-buffered modification of this formula with NaCl reduced to keep the ionic strength constant. This buffer is referred to as phosphate-buffered Gey's (PBG). Ampholytes were obtained from LKB-Producter AB, Sweden.

Iodination of βNGF. The iodination procedure was an adaptation of that of Marchalonis (20). To obtain iodinated βNGF for chemical studies, the ^{125}I isotope was diluted 1:500 with nonradioactive ^{127}I isotope as the potassium salt. The reaction was done in phosphate buffer (pH 7.4), I = 0.1. To 50 μg of βNGF were added 0.25 μg of lactoperoxidase (final enzyme-to-protein ratio of 1:200), various amounts of the Na^{125}I, K^{127}I mixture (mole ratios of iodine to βNGF from

0 to 5), and H_2O_2 in sufficient phosphate buffer to bring the final H_2O_2 concentration to 400 μM and the final volume to 0.24 ml. The reaction was done at 4°. The extent of iodination was measured by the percentage of trichloroacetic acid-precipitable counts. Reaction efficiencies were 80–90%.

The average number of iodines bound per molecule of βNGF was calculated by multiplying the number of moles of iodine present per mol of βNGF by the reaction efficiency. Iodination of tyrosine [the predominant reactive residue (21)] lowers the pK of the hydroxyl proton. Since βNGF is a basic protein (pI = 9.3), each additional bound iodine results in a lowering of its isoelectric point and permits the various iodo-derivatives and the native protein to be separated by isoelectric focusing in acrylamide gel (22).

[^{125}I]βNGF of high specific activity was made as follows: Aliquots of 1.0 mCi of Na^{125}I in 10 μl or less were obtained from New England Nuclear Corp. in a "V-vial." To these were added 10–12 μg of βNGF, 50–60 ng of lactoperoxidase, and H_2O_2 in sufficient phosphate buffer to bring the final volume to 50 μl and the final H_2O_2 concentration to 400 μM; other conditions were as described above. Reaction efficiencies were 20–40%. Complete reaction represents the addition of 1.17 mol of ^{125}I per mol of βNGF. The [^{125}I]βNGF was used within 2 weeks of preparation.

Bioassays for NGF Activity. Dorsal root ganglia were removed from 8-day-old chick embryos (White leghorn, Kimber Farms, Fremont, Calif.) and pooled in chilled GBS. In the original bioassay procedures, the ganglia were cultured in the presence of NGF either in semisolid plasma clots (23) or on a collagen surface in a richly supplemented medium (e.g., ref. 24). It has now been observed that ganglia respond to NGF in an identical manner when they are grown on a collagen surface with only GBS as medium (K. Herrup and J. C. Gordon, unpublished data). For βNGF, maximum response was obtained at approximately 10 ng/ml.

Binding Assay. All assays were run in PBG. Cellular material for these assays was, unless otherwise noted, obtained from dorsal root ganglia of 8-day-old chick embryos. The ganglia were dissociated by gently drawing them in and out of a 5.0-ml pipet for 2 min. Any remaining clumps were removed by centrifugation at 500 rpm for 5 sec in an IEC clinical centrifuge. Concentrations of 1 to 3 × 10^6 cells per ml were used. Both liver and whole brain samples from 8-day chick embryos were dissociated in a similar manner. Cell concentrations were 3 × 10^7 cells per ml for liver and 5 × 10^8 cells per ml for brain. In the assay, cells were incubated with various amounts of [^{125}I]βNGF, with or without other proteins, for 60–120 min. To separate the bound from free [^{125}I]βNGF, the cells were sedimented by centrifugation through a two-step sucrose gradient in a Beckman microfuge tube. The bottom layer consisted of 75 μl of 0.3 M sucrose in PBG. On to this was overlayered 100 μl of 0.15 M sucrose in PBG. Finally, 100 μl of the sample to be assayed was layered on top. The tubes were centrifuged for 10 min in a Beckman Microfuge (model 152, rotor speed about 10,000 rpm). After spinning, the bottom 5–6 mm of the tube, containing the pelleted cells and the bound [^{125}I]βNGF, was cut off. Both top and bottom were counted in a Nuclear Chicago well type γ-scintillation counter for sufficient time to obtain at least 1000 counts. In control experiments where cells were omitted, only about 0.01% of the overlayered [^{125}I]βNGF sedimented to the bottom of the tube. The amount of [^{125}I]βNGF bound to the cells was calculated as:

$$\frac{\text{cpm bottom}}{\text{cpm bottom} + \text{cpm top}} \times \text{ng of } [^{125}\text{I}]\beta\text{NGF in each tube}$$

All assays were done in triplicate to quintuplicate. Standard deviations were around 5%. Specific and nonspecific binding was distinguished as described in *Results*.

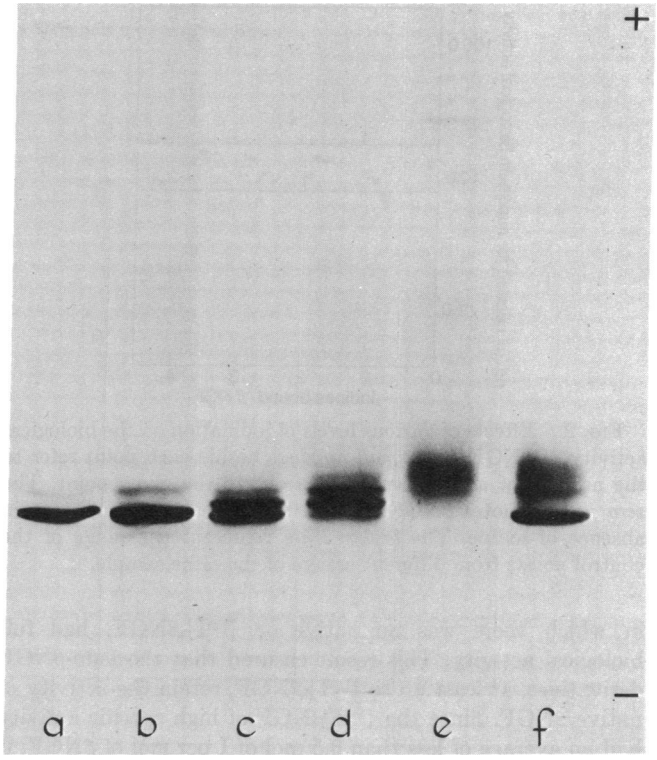

FIG. 1. Isoelectric focusing gels of various iodinated βNGF derivatives. Analyses in 8-cm 7½% polyacrylamide gels with 4% pH 3–10 ampholines. (*a*) native βNGF; (*b*) βNGF exposed to reaction conditions in the absence of iodine; (*c*) average of one iodine bound per βNGF; (*d*) two iodines per βNGF; (*e*) four iodines per βNGF; (*f*) coelectrophoresis of *a* and *e*.

RESULTS

Iodination of βNGF. With increasing iodination, βNGF species of decreasing isoelectric points were observed (Fig. 1). It is clear from these results that any given level of iodination is achieved by contributions from several different iodo-derivatives of βNGF. The contribution of each iodo-derivative to the total level of iodination was estimated from peak areas after each gel was scanned. The best fit of the data occurred when it was assumed that each succeeding species of lower isoelectric point than native βNGF represented the addition of one I per βNGF molecule. At the average iodination level of 4 I per mol of βNGF, therefore, both native βNGF and the species [^{125}I]$_1$βNGF were absent; [^{125}I]$_2$βNGF accounted for about 10%, [^{125}I]$_3$βNGF for 30%, [^{125}I]$_4$βNGF for 35%, and [^{125}I]$_5$βNGF for 25% of the total protein.

The effects of iodination on the NGF activity were examined in the bioassay. As can be seen in Fig. 2, the amounts of the various iodo-βNGF preparations needed for maximum response remained constant and equal to that for native βNGF. In particular, the mixture with 4 I per mol of βNGF,

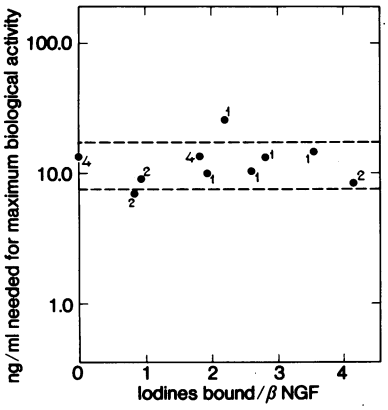

FIG. 2. Effects of various levels of iodination on the biological activity of βNGF. The *small numbers* beside each point refer to the number of assays averaged to obtain the given point. The *zero point* denotes βNGF exposed to the reaction conditions in absence of iodine. The *broken lines* represent the range of the control points from different assays of the same sample.

in which there was no native or $[^{125}I]_1\beta$NGF, had full biological activity. This result ensured that the iodo-βNGF derivatives, at least up to $[^{125}I]_3\beta$NGF, retain the activity of native βNGF. Since the [125I]βNGF of high specific activity had an average of less than 0.5 mol of I per mol of βNGF, it could be successfully used as a probe for the NGF receptor.

Binding of [125I]βNGF to Embryonic Sensory Ganglion Cells. The total binding to dissociated dorsal root ganglion cells was measured at various concentrations of [125I]βNGF of high specific activity. This binding displayed two components: a high-affinity component which saturated at concentrations in the ng/ml range and a low-affinity component which had a much larger capacity and increased linearly at least up to concentrations as high as 10 μg/ml. This second component is hereafter referred to as the nonspecific binding, in keeping with previous work with other proteins (14). It was measured for any given concentration of [125I]βNGF by addition of a large excess (5–10 μg/ml) of nonradioactive βNGF to a duplicate reaction mixture of cells and [125I]βNGF. For a given concentration of [125I]βNGF, therefore, the value for the specific (i.e., high-affinity) binding is calculated by subtracting the value for the nonspecific from that for the total binding.

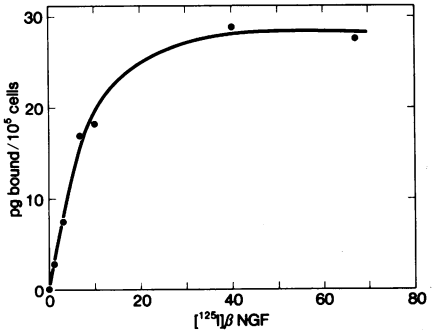

FIG. 3. Specific binding of [125I]βNGF as a function of [125I]-βNGF concentration (ng/ml). The cells, at a concentration of 1.6 × 10^6 cells per ml, were incubated in triplicate with various [125I]NGF concentrations for 120 min at 24°. The results, expressed as specific picograms sedimented per 10^5 cells, are the difference of total minus nonspecific binding (see *text*).

Saturation Curve of Specific Binding. Fig. 3 shows the plot of the specific binding of [125I]βNGF to ganglion cells as a function of the concentration of [125I]βNGF. The binding curve reached saturation at about 30–50 ng/ml of [125I]βNGF with half saturation (at 24°) at about 7–8 ng/ml. The latter values correspond to the concentration that produces maximum fiber outgrowth in the bioassay at 37°, suggesting that the specific binding of [125I]βNGF correlates well with its biological activity. The data in Fig. 3 also permit the determination of the number of specific binding sites per responsive cell. Saturation of the receptors occurred at about 28 pg/10^5 cells. If 29% of the dorsal root ganglion cells are neurons (25) and roughly half of these are the responsive medio-dorsal type (26), then there are approximately 21,000 receptors per responsive cell. This figure may be compared to the estimate of 11,000 insulin receptors per fat cell (14).

Specificity of the Receptor–βNGF Interaction. The specificity of the binding was demonstrated by the fact that other proteins, even at very high concentrations, failed to displace bound [125I]βNGF from the ganglion cells. No loss in nonspecific binding is observed due to the large excess of nonspecific binding sites. In contrast, native βNGF, alone or in 7S NGF, was effective (Table 1). It is of interest that proinsulin did not displace [125I]βNGF.

The binding of native and [125I]βNGF was compared in detail. If both species have the same affinity for the receptor, the amount of [125I]βNGF specifically bound to the cells, expressed as the percent of the total specific binding, should be a linear function of the percent of the total βNGF that is [125I]βNGF. The data for this experiment are shown in Fig. 4, and the linearity of this displacement plot confirmed that the receptor binds native and [125I]βNGF with equal affinity. This result in the binding assay parallels that found in the bioassay, where the two proteins elicited maximum fiber outgrowth at the same concentration (Fig. 2).

The correlation of specific binding to biological activity was further tested with partially inactivated βNGF preparations. The latter were prepared by exposure of βNGF to 66% acetic acid. Although incubation in 66% acetic acid for 7 days at room temperature results in no change in the molecular weight (W. C. Mobley, personal communication) or isoelectric point of βNGF, its specific biological activity is

TABLE 1. *Displacement of bound [125I]βNGF by various proteins*

Additive	Concentration	% of total bound cpm displaced*
None	—	0
βNGF	10 μg/ml	61
7S NGF	3 μg/ml	58
αNGF subunit	3 μg/ml	0
Cytochrome *c*	5 μg/ml	0
Bovine-serum albumin	5 μg/ml	0
Proinsulin	30 ng/ml	0
Proinsulin	300 ng/ml	0
Proinsulin	3 μg/ml	0
Proinsulin	30 μg/ml	0

* Values are the pg/10^5 cells displaced by the additive divided by the total pg bound in the presence of 30 ng/ml of [125I]βNGF times 100.

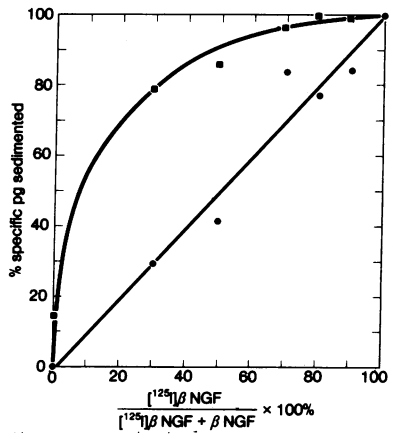

FIG. 4. Displacement of bound [125I]βNGF by βNGF and a partially inactive derivative. [125I]βNGF and different amounts of either native βNGF (●) or dialyzed NGF (■) were incubated with 1.4 and 5 × 10⁶ cells per ml, respectively, for 120 min at 24°. Assays were done in quintuplicate.

reduced to 10% of the normal value. The ability of this protein to displace [125I]βNGF was tested in binding assays with various proportions of the inactivated and [125I]βNGF (Fig. 4). The initial slope of the curve in Fig. 4 can be shown to be the ratio of the affinities of [125I]βNGF and the inactivated βNGF for the receptor. The actual value of this initial slope showed that the modified protein had approximately an 8-fold lower affinity for the receptor than native βNGF, a result which correlates well with the lowered biological activity in the bioassay.

The binding of [125I]βNGF varied linearly with the number of ganglion cells in the assay (data not shown). That the binding showed specificity with respect to cell type was borne out by the finding that neither dissociated liver nor whole brain cells from 8-day-old chick embryos displayed specific binding.

Kinetics of the Receptor–βNGF Interaction. At 24° the rate of specific binding was rapid (Fig. 5*a*). Saturation was achieved after 10 min and half-saturation within 1 min after addition of the cells to the reaction mixture. The data fit a second-order rate equation which gives a rate constant, k_1, of 1.0×10^7 mol^{-1} sec^{-1}, assuming βNGF to have a molecular weight of 26,500 (P. Pignatti, personal communication).

The rate of dissociation was determined by displacement of [125I]βNGF with a 200-fold excess of native βNGF from ganglion cells presaturated with [125I]βNGF. The rate of dissociation at 24° was first order (Fig. 5*b*) with a half-life for the receptor–βNGF complex of 10 min. The rate constant for the dissociation, k_{-1}, calculated from these data is 1.2×10^{-3} sec^{-1}.

The dissociation constant for the receptor–βNGF interaction calculated from the ratio of the rate constants, k_{-1}/k_1, is 1.2×10^{-10} M. The value of this constant determined from the half-saturation of the receptor (Fig. 3) is 2.6×10^{-10} M, which is in reasonably close agreement with the figure calculated from the rate constants.

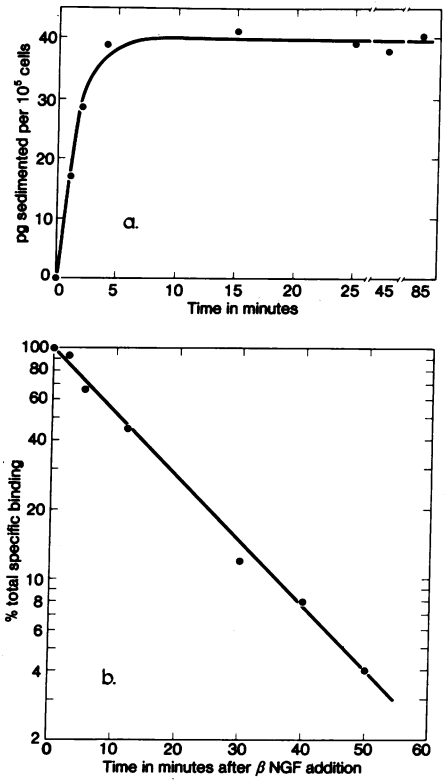

FIG. 5. Kinetics of the interaction of [125I]βNGF and its receptor. (*a*) Rate of association. Aliquots of cells were added to [125I]βNGF alone or to [125I]βNGF plus βNGF so that the final concentrations were 30 ng/ml of [125I]βNGF, 10 μg/ml of βNGF, and 1.4 × 10⁶ cells per ml. Samples were incubated at 25° and overlayered. Centrifugation was begun at the time indicated. Except for the 2-min point, total and nonspecific binding were assayed at the same time. All assays were done in triplicate. (*b*) Rate of dissociation. Cells (3 × 10⁶ cells per ml) were equilibrated with 50 ng/ml of [125I]βNGF for 120 min at 25°. Displacement was begun by the addition of a 200-fold excess of βNGF in a small volume. This mixture was incubated at 25° and overlayered on gradients. At the times shown, centrifugation was begun. Nonspecific binding did not change during the course of the assay.

DISCUSSION

The specific binding of [125I]βNGF to cells of embryonic dorsal root ganglia displays saturation (within a range of concentrations that is physiologically relevant), specificity for the protein (in that only native βNGF displaces the bound [125I]βNGF), and specificity for the responsive ganglion cells. Thus, the concentration of [125I]βNGF at which half-saturation occurs at 24° (7–8 μg/ml) is very close to the concentrations required for optimal fiber outgrowth in the bioassay and to the levels of NGF in mouse serum (27). These data suggest that the specific binding is a reflection of the interaction of βNGF with its physiologic receptor. Supporting evidence is found in the correlation between the results of the binding assays and those of the bioassay. For example, βNGF modified by dialysis against acetic acid, though less active in the bioassay, retains the same fraction of its specific-binding capacity as it does of its biological activity.

In the present study, the ganglia were dissociated mechanically by gentle aspiration rather than by the use of trypsin in order to preserve, as much as possible, the surface proteins of the cells. Cuatrecasas has already shown (28) that substantial loss of insulin-receptor binding is observed after fat cells are exposed to relatively low concentrations of trypsin.

TABLE 2. *Comparison of kinetics of insulin and βNGF interaction with their receptors*

Constant	βNGF	Insulin*
k_1	1×10^7 mol^{-1} sec^{-1}	1.5×10^7 mol^{-1} sec^{-1}
k_{-1}	1.2×10^{-3} sec^{-1}	7.4×10^{-4} sec^{-1}
Half-saturation of receptor	2.6×10^{-10} M	8.1×10^{-11}M

* All insulin data from ref. 14.

Mechanical dispersion, therefore, should allow the more accurate measurement of total receptor binding.

Assuming that βNGF receptors are a property of the mediodorsal neurons of the dorsal root ganglia, the number of receptors per cell is of the same order of magnitude as the number of insulin receptors per fat cell (14). Other properties of the βNGF receptor are also like those of the insulin receptor system. In particular, the kinetics of the receptor–protein interactions are similar (Table 2). In spite of these similarities and the fact that proinsulin will displace insulin from its receptor (29), proinsulin has no effect on the specific binding of βNGF to its receptor. It would appear that if βNGF and proinsulin evolved from a common gene, as indicated by homology in their amino-acid sequences (30), this evolution was paralleled by a divergent evolution of their receptors.

The kinetic data for the βNGF receptor are consistent with the idea that the receptor is on the outer surface of the cell membrane. It seems unlikely that the observed association and dissociation rates could both be achieved by mechanisms that transport βNGF in and out of the cell. In particular, the dissociation results would require the pre-equilibrated [^{125}I]βNGF to be pumped out against a 200-fold excess of unlabeled βNGF outside the cells.

The present experiments do not distinguish between specific binding to neuronal or non-neuronal cells, and in view of the report (25) that the non-neuronal cells may substitute for NGF, it will be of considerable interest to examine purified individual cell types for specific binding sites.

Little is known about the events that follow the interaction of βNGF with its receptor. Other protein trophic factors have been described which act through a receptor-linked adenylate cyclase to alter intracellular levels of cyclic AMP (31). Although Roisen *et al.* (24) have shown that externally applied 1.0 mM dibutyryl cAMP will stimulate fiber outgrowth from embryonic dorsal root ganglia, it is not known if the physiological response is mediated by increases in intracellular cAMP. Whether or not neurotubule protein syntheses or assembly is stimulated by NGF interacting with responsive cells is also not clear (32, 33).

1. Bueker, E. D. (1948) *Anat. Rec.* **102**, 369–390.
2. Levi-Montalcini, R. & Angeletti, P. U. (1968) *Phys. Rev.* **48**, 534–569.
3. Cohen, S. & Levi-Montalcini, R. (1956) *Proc. Nat. Acad. Sci. USA* **42**, 571–574.
4. Cohen, S. (1959) *J. Biol. Chem.* **234**, 1129–1137.
5. Levi-Montalcini, R. & Booker, B. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 384–391.
6. Cohen, S. (1960) *Proc. Nat. Acad. Sci. USA* **46**, 302–311.
7. Varon, S., Nomura, J. & Shooter, E. M. (1967) *Biochemistry* **6**, 2202–2209.
8. Greene, L. A., Varon, S., Piltch, A. & Shooter, E. M. (1971) *Neurobiology* **1**, 37–48.
9. Angeletti, R. H. & Bradshaw, R. A. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2417–2420.
10. Angeletti, R. H., Mercanti, D. & Bradshaw, R. A. (1973) *Biochemistry* **12**, 90–100 and 100–115.
11. Schenkein, I. (1971) in *Handbook of Neurochemistry*, ed. Lajtha, A. (Plenum Press, New York), chap. 16, pp. 503–524.
12. Jacobson, M. (1970) *Developmental Neurobiology* (Holt Rinehart & Winston, Inc., New York), p. 221.
13. Partlow, L. M. & Larabee, M. G. (1971) *J. Neurochem.* **18**, 2101–2118.
14. Cuatrecasas, P. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1264–1268.
15. Herrup, K. & Shooter, E. M. (1973) *Trans. Amer. Soc. Neurochem.* **4**, 83.
16. Smith, A. P., Varon, S. & Shooter, E. M. (1968) *Biochemistry* **7**, 3259–3268.
17. Ehrman, R. L. & Gey, G. O. (1956) *J. Nat. Cancer Inst.* **16**, 1375–1404.
18. Bornstein, M. B. (1958) *Lab. Invest.* **7**, 134–137.
19. Gey, G. O. & Gey, M. K. (1936) *Amer. J. Cancer* **27**, 45–76.
20. Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–305.
21. Means, G. E. & Feeney, R. E. (1971) *Chemical Modification of Proteins* (Holden-Day, Inc., San Francisco), chap. 9.
22. Smith, A. P., Greene, L. A., Fisk, H. R., Varon, S. & Shooter, E. M. *Biochemistry* **8**, 4918–4926.
23. Levi-Montalcini, R., Meyer, H. & Hamburger, V. (1954) *Cancer Res.* **14**, 49–57.
24. Roisen, F. J., Murphy, R. A., Pichichero, M. E. & Braden, W. G. (1972) *Science* **175**, 73–74.
25. Burnham, P., Raiborn, C. & Varon, S. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3556–3560.
26. Levi-Montalcini, R. & Hamburger, V. (1953) *J. Exp. Zool.* **123**, 233–388.
27. Hendry, I. A. (1972) *Biochem. J.* **128**, 1265–1272.
28. Cuatrecasas, P. (1971) *J. Biol. Chem.* **246**, 6522–6531.
29. Gavin, J. R., Gorden, P., Roth, J., Archer, J. A. & Buell, D. N. (1973) *J. Biol. Chem.* **248**, 2202–2207.
30. Frazier, W. A., Angeletti, R. H. & Bradshaw, R. A. (1972) *Science* **176**, 482–488.
31. Birnbaumer, L., Pohl, S. L., Krans, M. J. & Rodbel, M. (1970) in *Role of Cyclic AMP in Cell Function*, eds. Greengard, P. & Costa, E. (Biochemical Psychopharmacology, Raven Press, New York), Vol. 3, pp. 185–208.
32. Yamada, K. A. & Wessels, N. K. (1971) *Exp. Cell Res.* **66**, 346–352.
33. Hier, D. B., Arnason, B. G. W. & Young, M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2268–2272.