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Interaction of Nerve Growth Factor with Surface Membranes: Biological Competence of Insolubilized Nerve Growth Factor

(nerve growth factor–Sepharose/sensory ganglia/neurite outgrowth/neuron preservation/nerve growth factor receptor)

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ABSTRACT **Nerve growth factor was insolubilized by covalent attachment to Sepharose beads. Nerve growth factor–Sepharose was biologically active in both the neurite outgrowth assay for nerve growth factor and in preserving responsive neurons *in vitro*. Modification of the bioassay to detect solubilized activity of nerve growth factor and histological examination of ganglia treated with nerve growth factor–Sepharose revealed that nerve growth factor–Sepharose prepared by reaction in 6 M guanidine hydrochloride released negligible amounts of solubilized nerve growth factor activity. These observations extend the previously noted correlations on the structure and function of nerve growth factor and insulin to include the primary action of these two proteins. Thus nerve growth factor, like insulin, appears to express its biological activity by first binding to a receptor on the surface membrane of responsive cells.**

Nerve growth factor (NGF) is a protein of wide species distribution which appears to be essential to embryonic development and continued maintenance of the sympathetic nervous system (1). While the best-known effect of NGF is its ability to stimulate production of a halo of nerve fibers by embryonic sensory or sympathetic ganglia *in vitro*, the protein also preserves these ganglionic cells, which degenerate and die in the absence of NGF (1, 2). The striking similarities between the NGF metabolic response and the pleiotypic response evoked by insulin in nonneuronal cells *in vitro*, along with structural relationships between the two proteins, suggested that NGF and insulin have evolved from a common ancestral gene (3). A molecular basis for the common functional properties of these two proteins was suggested by the results of topographical mapping and solution conformation studies of 2.5S NGF which were consistent with the hypothesis that NGF and insulin may share regions of similar three-dimensional conformation (4). It is thus of interest to determine whether these structurally related hormones accomplish their similar functions through a common mechanism of action.

Using insulin–Sepharose derivatives, Cuatrecasas has shown that the primary action of insulin in initiating a response in its target cells is combination with surface-membrane structures (5). If NGF and insulin have evolved from a common ancestral hormone, NGF might be expected to act on its target neurons in a manner analogous with insulin action. To test this possibility, NGF–Sepharose derivatives were prepared and tested for activity in the NGF biological assay, which depends on nerve-fiber outgrowth from embryonic sympathetic or sensory ganglia (6). The derivatives were also examined for the ability to preserve cells of these ganglia cultured *in vitro*, a well-known effect of soluble NGF (2). The biological assay was modified to detect solubilized NGF activity in amounts as low as 7.5×10^{-5} pmol; we found that generation of solubilized NGF activity followed a predictable time course. NGF–Sepharose derivatives displayed considerable biological activity under conditions in which there was no detectable soluble NGF activity.

Abbreviation: NGF, nerve growth factor.

EXPERIMENTAL

2.5S Mouse nerve growth factor (NGF) was prepared from submaxillary glands of adult male mice by the procedure of Bocchini and Angeletti (7). Sepharose 4-B was from Sigma Chemical Co., cyanogen bromide was from Baker, thrombin (topical) was obtained from Parke–Davis, Eagle's medium with Earle's salts was from GIBCO, and reagents for amino-acid analysis were from Pierce Chemical Co. Fresh chicken plasma was obtained by cardiac puncture.

NGF was coupled directly to cyanogen bromide-activated Sepharose 4-B (8) either at pH 6.4 in 0.2 M citrate or at pH 7.0 in 0.2 M phosphate–6 M guanidine hydrochloride. The NGF had been incubated in 6 M guanidine hydrochloride buffer for at least 24 hr before coupling, which proceeded for 16 hr at room temperature. The NGF–Sepharose (10 ml) was then transferred to a coarse sintered glass funnel and washed with 200 ml of 6 M guanidine hydrochloride–0.2 M phosphate (pH 7.0); 4 liters of 8 M urea in 0.5 N acetic acid; 4 liters of 0.1 N acetic acid; and then 20 liters of 0.05 M sodium acetate (pH 5.0), plus 0.1 M sodium chloride. This washing procedure required over 24 hr. Immediately before bioassay, NGF–Sepharose was washed with 1 liter of 0.2 M phosphate (pH 7.0) followed by 1 liter of sterile physiological saline. Control Sepharose was prepared in an identical manner except that lysine was coupled instead of NGF. The amount of NGF bound in the washed Sepharose derivatives was determined by amino-acid analysis (Spinco 120C) by the method of Moore and Stein (9) after hydrolysis in 6 N HCl at 110° for 24 hr under reduced pressure.

Biological assays were performed with 8-day chick sensory ganglia or 11- to 13-day sympathetic ganglia in plasma clot cultures in paraffin-sealed 35-mm plastic petri dishes by a modification of the procedures of Levi-Montalcini *et al.* (6). For detection of solubilized NGF activity, the assay ganglia were placed in a small clot in the bottom of the dish, which

was then surrounded by a clot of 10-times the volume containing NGF–Sepharose at 5-times the concentration (total amount 50 times) used in direct assay. Alternatively, the two clots were placed side by side. Both these methods detect solubilized NGF, which diffuses from the clot containing NGF–Sepharose into the clot that has the assay ganglia. Direct assays of NGF–Sepharose, diffusion assays to detect solubilized activity, soluble NGF standards, and negative controls without NGF were always performed together, with the same reagents. Ganglia were prepared for histological examination by standard techniques and stained with toluidine blue after 10-μm serial sections were cut.

RESULTS

Properties of NGF-Sepharose Derivatives. NGF could be coupled readily to cyanogen bromide-activated Sepharose at pH 5–9. Coupling at pH 6.4 gave greater than 90% attachment of NGF to the polymer, resulting in derivatives containing as much as 2 mg (0.15 μmol) of NGF per ml of Sepharose. Amino-acid analysis of these preparations revealed the loss of about one residue of lysine, indicating that most molecules of NGF were attached to the Sepharose at a single site. A specific lysyl residue could not be identified, however, and thus there are probably several possible residues through

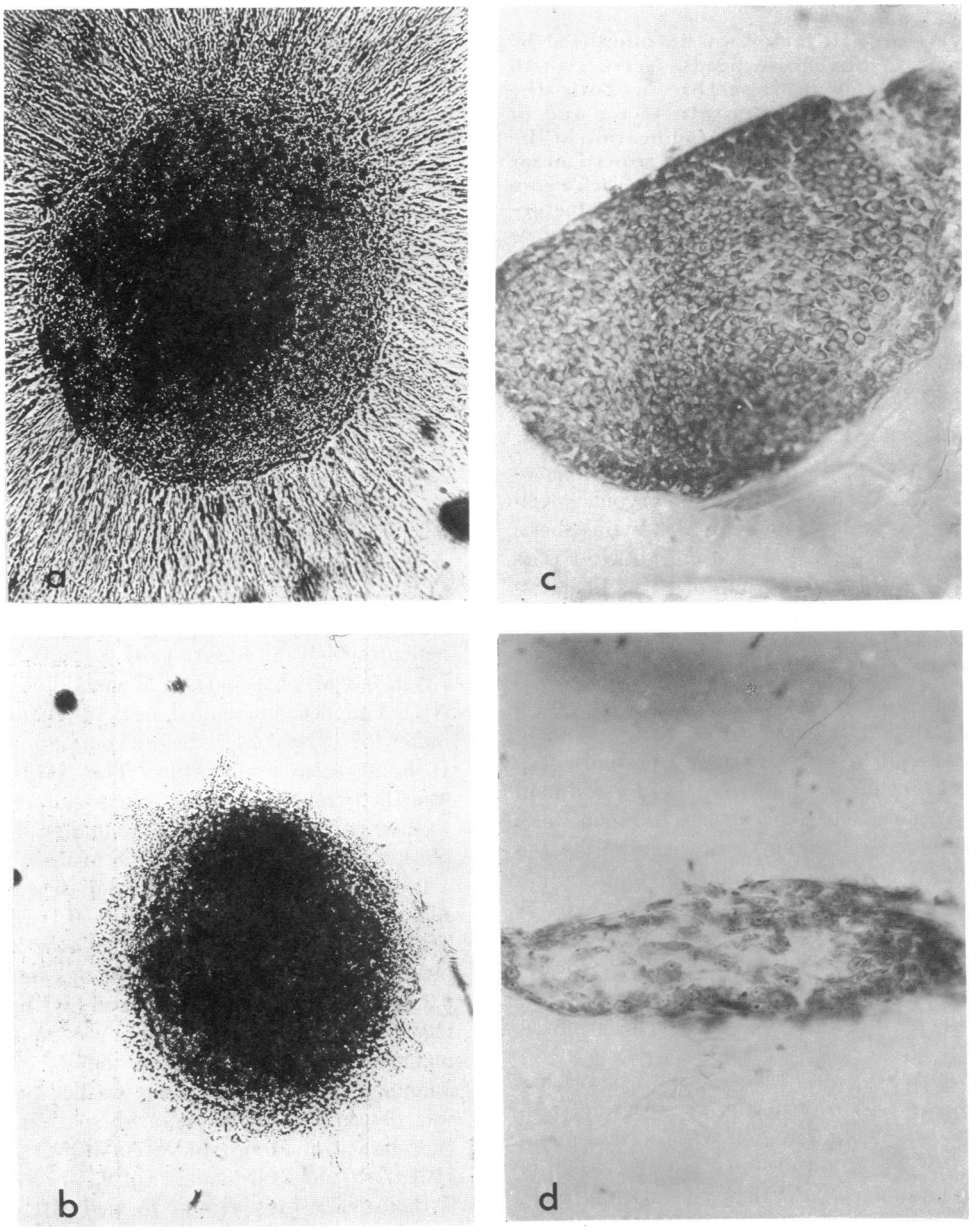

FIG. 1. (*a*) A micrograph of an 8-day chick sensory ganglion that was incubated with an optimal concentration (1 ng in 75 μl, or 10^{-9} M) of soluble NGF for 18 hr (×66). This is the smallest amount of NGF that will produce a maximal (5+) fiber response. (*c*) A representative 10-μm section of such a ganglion showing the preservation of large numbers of viable cells (×165). (*b*) A ganglion incubated without NGF (×66). (*d*) Extensive cell degeneration that occurs in such ganglia (×165). Note the small number of cells and the apparently empty areas of unstained dead cells in this ganglion.

which attachment can occur. Such derivatives were used to adsorb NGF antibodies from immune serum, indicating the immunological integrity of the bound NGF. Even though no biological activity could be detected in 1000-fold concentrates of the wash solutions of these derivatives, more sensitive tests for solubilized NGF revealed leakage of prohibitive amounts of NGF activity (>1 pg of NGF per 25 μl of beads in culture).

Coupling at pH 7.0 (0.2 M phosphate) in 6 M guanidine hydrochloride resulted in less highly substituted derivatives containing 500–4000 pmol of NGF per ml of Sepharose. These preparations were more suitable for direct biological assay.

Biological Activity of NGF–Sepharose. Embryonic sensory and sympathetic ganglia respond to optimal concentrations of soluble NGF *in vitro* with the well-known halo of neurites, which grow from the ganglia in 12–18 hr (2) (Fig. 1*a*). About 7.5×10^{-2} pmol of NGF (1 ng) in a culture 75 μl in volume (10^{-9} M) was required to give this response. When stained sections of these ganglia are examined with a light microscope, the vast majority of the cells are well preserved (Fig. 1*c*), whereas many cells of ganglia incubated without NGF (Fig. 1*b*) have degenerated to the point where cells are no longer recognizable (Fig. 1*d*). Both fiber outgrowth and cell preservation were used as criteria for activity of NGF–Sepharose.

When 2.5 μl of Sepharose beads containing 10 pmol of NGF were incubated in plasma clot culture with either sensory or sympathetic ganglia, the Sepharose beads appeared to penetrate the mass of cells and, after several hours, many beads were found inside the ganglia. In contrast, control Sepharose beads seldom were seen in contact with ganglia and were never seen to penetrate the mass of cells. The degree of fiber outgrowth observed depended on the number of NGF–Sepharose beads that packed around and into the ganglia. In most cases, large numbers of beads established contact with the ganglia, and the extent of fiber outgrowth approached that elicited by soluble NGF. However, when occasionally only a few beads were seen associated with a ganglion, only a small number of fibers were seen and these originated at the sites where NGF–Sepharose beads touched the ganglia. When 2.5 μl of a less highly substituted preparation of NGF–Sepharose beads containing 1.3 pmol of NGF were assayed, the fiber outgrowth appeared somewhat less, even though large numbers of beads packed around the ganglia. Control Sepharose did not elicit fiber growth even when in contact with the surface of ganglia.

These results are summarized for a large number of bioassay cultures in Table 1. The fiber outgrowth is expressed as a fiber index (10), which is obtained by summing the score for the extent of outgrowth on a 0–5 scale (least to most), determined by comparison with soluble NGF controls, for each observation, and dividing by the number of observations. Fiber indexes obtained during several hundred series of soluble NGF controls are given for comparison. Clearly, the preparation of NGF–Sepharose that contains 4000 pmol of NGF per ml is nearly as active in promoting fiber outgrowth as soluble NGF, while the preparation containing 500 pmol of NGF per ml displays somewhat less activity. The total amount of NGF present in the NGF–Sepharose aliquot is about 100-times the amount of soluble NGF required to give a comparable fiber index. Antiserum to NGF completely eliminated fiber outgrowth stimulated by NGF–Sepharose. Micrographs of the fiber response elicited by the more highly substituted NGF–Sepharose (preparation *A*, Table 1) are shown in Fig. 2*a* and *b*. Note that in the culture with NGF–Sepharose, the beads have packed into and around the ganglion.

TABLE 1. *Biological assay of NGF–Sepharose**

Preparation of NGF–Sepharose	pmol of NGF per ml of Sepharose	Beads assayed (μl)	NGF (pmol)	Fiber index†	
				Direct	Diffusion
A	4000	2.5	10.0	4.6 (96)	0.1 (61)
		25.0	100.0	—	0.5 (86)
B	500	2.5	1.25	3.5 (84)	0.1 (28)
		25.0	12.5	—	0.4 (80)
Control	0	25.0	—	0 (20)	—

* These preparations of NGF–Sepharose were coupled in and washed with 6 M guanidine hydrochloride.

† Soluble NGF. No. of observations is given in parentheses.

Controls:	*pg*	*amol*	*Fiber index*
	0.1	0.75	0
	0.1	7.5	0.1
	1	75	0.6
	10	750	1.6
	100	7,500	2.5
	1,000	75,000	5.0

The degree of cell preservation revealed by examination of serial 10-μm sections of ganglia treated with NGF–Sepharose was equivalent to that produced by soluble NGF. The preserved cells were, however, localized to the regions immediately adjacent to the NGF–Sepharose beads, many of which lay entirely within the boundary of the ganglia (Fig. 2*c* and *d*). This observation suggests not only that NGF–Sepharose is effective in preserving ganglion cells, but also, that the amount of solubilized NGF present in these cultures is indeed negligible. If this were not the case, even cells within the ganglion distant from the NGF–Sepharose beads would have been preserved. Antiserum to NGF completely eliminated the localized cell preservation of NGF–Sepharose and the more generalized cell preservation produced by soluble NGF.

Detection of Solubilized NGF Activity. By placing assay ganglia in a plasma clot adjacent to a clot containing NGF–Sepharose, it was possible to detect diffusible activity in the range of 1 pg (75×10^{-6} pmol) of NGF, which corresponds to about 1/100,000 of the total bound NGF in the aliquot used in the direct assay. This was demonstrated both by the inclusion of known amounts of soluble NGF in the clot adjacent to the assay ganglia and by the activity observed when preparations of NGF–Sepharose not coupled in 6 M guanidine hydrochloride were assayed. The degree of activity observed in the ganglia clot was characteristic of an amount of NGF $^1/_{10}$ of that placed in the adjacent clot. Thus, by including 10-times the number of NGF–Sepharose beads in a diffusion experiment, as in a direct assay experiment, it is possible to determine the amounts of solubilized NGF present in the direct assay. A more sensitive geometry of the diffusion assay is to place the assay ganglia in a small clot in the center of the dish and surround this clot with another much larger one containing the NGF–Sepharose.

By both methods of assay, the NGF–Sepharose prepared in 6 M guanidine hydrochloride had negligible solubilized

activity on day 1 or 2 after the initial washing procedures. On days 3 and 4, small amounts of solubilized activity appeared (fiber index 0–0.5) while by day 7, solubilized activity gave a fiber index of 1–2. Since a bioassay can be performed in 18 hr, this time course of generation of solubilized NGF activity is sufficiently slow to allow several assays to be performed when negligible solubilized activity is present in NGF–Sepharose preparations. Incubation of NGF-Sepharose beads in 6 M guanidine hydrochloride for 24 hr and rewashing with the same procedure used immediately after preparation of the derivative eliminated the solubilized activity, which reappeared with the same time course described above. In practice, results of direct assays were discarded unless the fiber index of the diffusion control ganglia was less than 0.5 (Table 1), and positive soluble NGF controls gave the fiber indexes reported in Table 1. Histological examination of these assay ganglia, which had a fiber index of less than 0.5, revealed large numbers of necrotic cells, comparable to controls incubated in the complete absence of NGF (Fig. 1*b* and *d*). Direct biological assay of the supernatant of a 1:1 slurry of NGF–Sepharose that had been incubated 1–4 days after it was washed, revealed no solubilized activity.

The tests for solubilized NGF activity exclude the possibility that free NGF exists in the preparations of NGF–Sepharose at the time of assay or is being generated during the 18-hr incubation in the plasma clot. They do not, however, rule out the possibility that the ganglia themselves might liberate solubilized NGF activity either by proteolytic

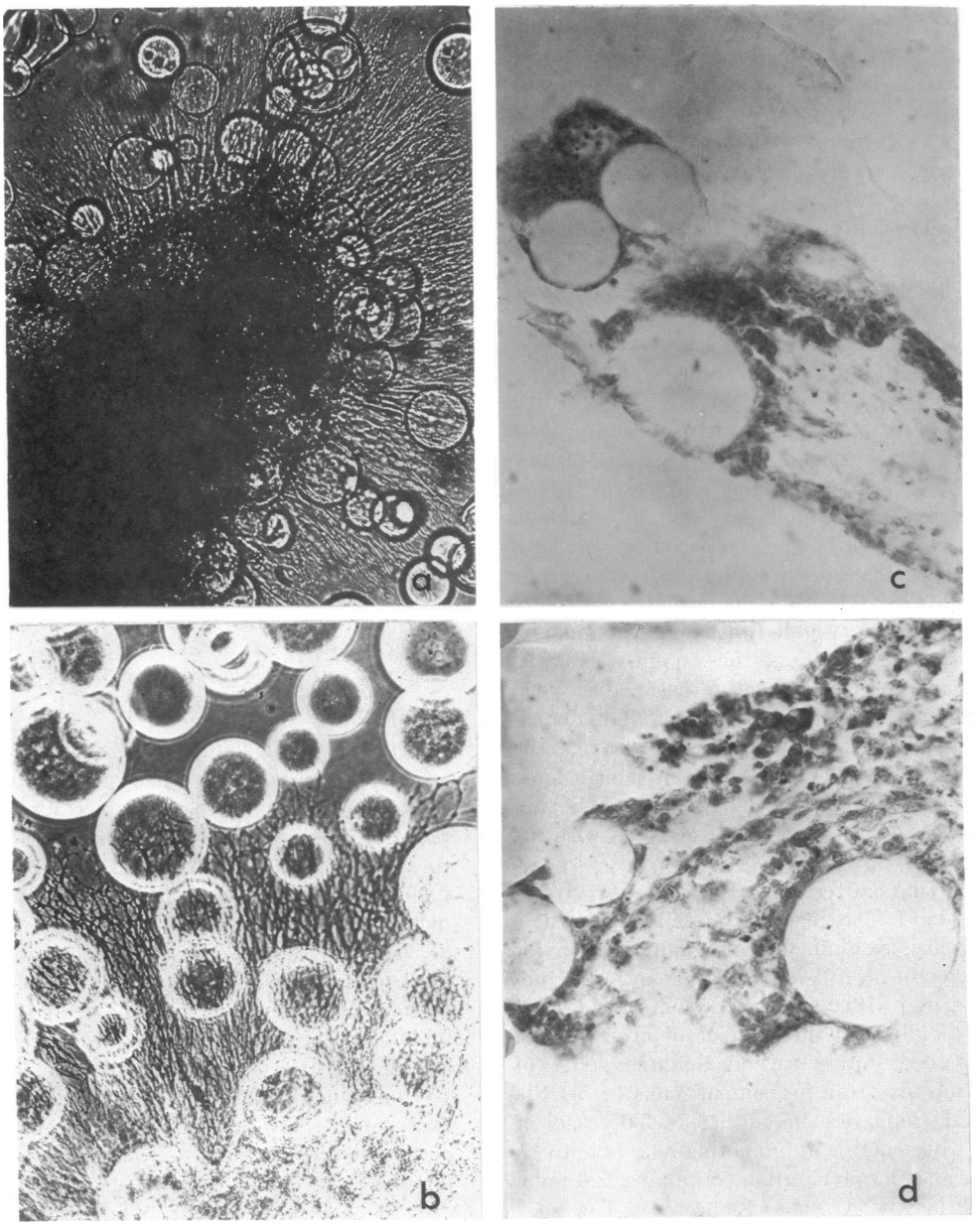

FIG. 2. (*a*) Micrograph of an 8-day chick sensory ganglion that was incubated for 18 hr with 2.5 μl of NGF–Sepharose beads (preparation *A*, Table 1; ×66). (*b*) A higher magnification (×165) of the periphery of the ganglion in *a*. (*c* and *d*) Micrographs of 10-μm sections of ganglia treated with NGF–Sepharose showing Sepharose beads that have infiltrated the ganglia and the monolayer of well-preserved cells which surround them (compare to Fig. 1*c* and *d*).

release of active fragments of NGF or by destruction of the Sepharose matrix to which the NGF is covalently attached. Generation of NGF activity by this means can be readily tested in a diffusion experiment by including large numbers of ganglia in the same clot with the NGF–Sepharose beads. If the interaction of these beads with ganglia had liberated solubilized NGF activity, it would have been detected by fiber growth from the assay ganglia in the adjacent clot. While ganglia in the same clot with the NGF–Sepharose were always heavily infiltrated with beads and responded with a fiber index of 4–5, assay ganglia in the adjacent clot always gave a fiber index of less than 0.2 (50 observations). Thus, no significant NGF activity is solubilized by the interaction of NGF–Sepharose with the ganglia.

DISCUSSION

The demonstration of the biological competence of an insolubilized hormone depends on two types of evidence. First, the insolubilized protein must elicit the same biological response as the free hormone, with appropriate allowances being made for alterations in the geometry of the interaction of the hormone with its target cells. Second, the possibility that the activity of the insolubilized material may be due to leakage of the bound hormone must be rigorously excluded.

Since stimulation of nerve-fiber outgrowth by NGF–Sepharose is readily demonstrable (Fig. 2 and Table 1), the major point of consideration is whether or not the activity is due to solubilized NGF leaking from the derivative. Four kinds of experiments indicate that the activity observed in the direct assay of NGF–Sepharose is not due to activity of solubilized NGF activity. (*1*) In ganglia that touch only a few beads, only localized tufts of nerve fibers are seen, even though in the same culture other ganglia, which are extensively infiltrated by NGF–Sepharose beads, produce extensive fiber growth. (*2*) Histological examination reveals that, even in the presence of extensive fiber outgrowth, neurons that are not in contact with NGF-Sepharose beads appear necrotic (Fig. 2*c* and *d*). (*3*) In diffusion experiments containing 10-times the amount of NGF–Sepharose used in direct experiments, no solubilized NGF activity was detected, even under conditions where soluble controls indicated that significant activity would have been easily detectable. (*4*) No NGF activity could be detected in the supernatant of a concentrated slurry of NGF-Sepharose. Furthermore, the possibility that the interaction of ganglia with NGF–Sepharose might lead to release of NGF activity was excluded by diffusion experiments involving incubation of large numbers of ganglia in the clots containing NGF–Sepharose. It would thus appear that release of significant levels of NGF activity from the NGF–Sepharose has been ruled out.

Not only do these experiments show that the nerve-growth and cell-preservation activity of NGF is mediated through its combination with the cell surface, they also indicate that the active species in this interaction is the NGF monomer. Since the derivatives were prepared in 6 M guanidine hydrochloride after incubation of the NGF for times shown to completely dissociate the 2.5S dimer (4, 11), the only molecular species available for coupling was the NGF monomer. Even though NGF is a dimer (11) at concentrations of about 10^{-5} M, it may well exist as a monomer at physiological concentrations four to six orders of magnitude lower.

This demonstration that NGF can exert its effects on neurons by interacting with external membrane structures, the low concentrations (10^{-11}–10^{-9} M) at which it is active, and the structural and functional similarities previously noted with insulin (3) all suggest that the interaction of NGF with surface membranes involves a receptor molecule, as suggested by Bradshaw *et al.* (12). The probable nature of this receptor is suggested by the inference that the structural genes for NGF and insulin have evolved from an ancestral gene coding for a primitive ancestral hormone. In this regard, Banerjee *et al.* (13) have reported the specific binding of [^{125}I]NGF in microsomal fractions of rabbit superior cervical (sympathetic) ganglia, which is similar to that noted with the interaction of insulin and its surface receptor. However, since these experiments were performed with a microsomal fraction, it is not possible to conclude that the NGF was bound to the plasma membrane. The biological activity of NGF–Sepharose reported here does show that the binding activity is localized at the surface membrane, presumably at the NGF receptor.

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